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Life with too much polyprenol: polyprenol reductase deficiency

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ABSTRACT

Congenital disorders of glycosylation (CDG) are caused by a dysfunction of glycosylation, an essential step in the manufacturing process of glycoproteins. This paper focuses on a 6-year-old patient with a new type of CDG-I caused by a defect of the steroid 5α reductase type 3 gene (SRD5A3). The clinical features were psychomotor retardation, pathological nystagmus, slight muscular hypotonia and microcephaly. SRD5A3 was recently identified encoding the polyprenol reductase, an enzyme catalyzing the final step of the biosynthesis of dolichol, which is required for the assembly of the glycans needed for N-glycosylation.
Although an early homozygous stop-codon (c.57G>A [W19X]) with no functional protein was found in the patient, about 70% of transferrin (Tf) was correctly glycosylated. Quantification of dolichol and unreduced polyprenol in the patient's fibroblasts demonstrated a high polyprenol/dolichol ratio with normal amounts of dolichol, indicating that high polyprenol levels might compete with dolichol for the initiation of N-glycan assembly but without supporting normal glycosylation and that there must be an alternative pathway for dolichol biosynthesis.

1. Introduction

1.1. CDG

Congenital disorders of glycosylation (CDG) are severe diseases with multiple systemic features that often remain undiagnosed. They are caused by a dysfunction in an important process of protein manufacturing called glycosylation, which is localized in the endoplasmatic reticulum (ER) and the Golgi compartment.

In the first biosynthetic step of the glycan moiety used for N-glycosylation, several monosaccharides are assembled on dolichyl phosphate, a lipid anchor in the ER membrane, generating a lipid-linked oligosaccharide (LLO) consisting of 14 saccharide units ($Glc_3Man_9GlcNAc_2$). Four Man and all Glc residues are transferred to the growing LLO structure from dolichol-phosphate-mannose (Dol-P-Man) or Dol-P-Glc intermediates, respectively. The oligosaccharyl transferase (OST) subsequently transfers this structure en bloc to an asparagine within a so-called glycosylation consensus sequence of a nascent protein. Afterwards, the N-linked glycan is trimmed and elongated in a series of reactions ending in the late Golgi compartment.

Depending on where the process of glycosylation is disrupted, the corresponding disorders were called CDG-I or -II. CDG-I involved all defects concerning the biosynthetic reactions up to and including OST, whereas CDG-II disorders were localized within the subsequent modifications of the protein-linked oligosaccharide [1]. A new nomenclature was recently proposed [2,3]. It includes defects of protein N-glycosylation and O-glycosylation, defects of glycosphingolipid and glycosylphosphatidylinositol anchor glycosylation, and defects in multiple glycosylation and other pathways, including SRD5A3-CDG.

Abbreviations: CDG, congenital disorders of glycosylation; SRD5A3, steroid 5α reductase type 3; Tf, transferrin; ER, endoplasmatic reticulum; LLO, lipid-linked oligosaccharide; Glc, glucose; Man, mannose; GlcNAc, N-acetylglucosamine; Dol-P-Man, dolichol-phosphate-mannose; OST, oligosaccharyl transferase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzym A; ATP, adenosine triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; CTP, cytidine triphosphate; IEF, isoelectric focusing; HPLC, high performance liquid chromatography; IMPP, immunoprecipitation; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism; FBS, Fetal Bovine Serum; EDTA, Ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; LC-MRM, liquid chromatography-coupled multiple reaction monitoring; TSH, thyroid-stimulating hormone; fT3, free triiodothyronine; fT4, free tetraiodothyronine; LDH, lactate dehydrogenase; CK-MB, creatine kinase muscle brain; gamma-GT, gamma glutamyl transpeptidase; AP, alkaline phosphatase; GDP, guanosine diphosphate; SLOS, Smith–Lemli–Opitz syndrome; PERK, protein kinase R-like ER kinase; eIEF2α, eukaryotic initiation factor 2α.

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Major features of CDG syndrome include psychomotor retardation, cardiomyopathy, decreased antithrombin III activity, abnormal eye movements or failure to thrive [4,5].

1.2. Dolichol phosphate biosynthesis and SRD5A3

Dolichol belongs to the family of long-chain polyisoprenoids with one terminal saturated isoprene unit. It was discovered and extracted from pig liver and human kidney in 1960 [6]. Seven years later, Behrens and Leloir [7] described dolichol phosphate as a membrane bound polyisoprenyl lipid-anchor needed for N-glycosylation. Its biosynthetic pathway was proposed, though incomplete by reason of several unknown enzymes [8].

The first part of dolichol biosynthesis in mammals is the mevalonate pathway. Two acetyl-CoA molecules condense to acetoacetyl-CoA with the release of CoA-SH. 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) synthase adds another acetyl-CoA creating HMG-CoA which is reduced to mevalonate by HMG-CoA reductase.

During the second phase, several ATP-depending phosphorylation reactions, a decarboxylation and a final dephosphorylation step result in isopentenyl diphosphate. Isopentenyl diphosphate and its isomere, dimethylallyl diphosphate, react to form geranyl diphosphate releasing a diphosphate. A following prenyltransferase catalyzes in a similar way the condensation of geranyl diphosphate with isopentenyl diphosphate creating farnesyl diphosphate. Farnesyl diphosphate is the basic intermediate for the biosynthesis of cholesterol, ubiquinone and dolichol.

The unique part of dolichol biosynthesis starts with a specific cis-prenyltransferase which successively adds isopentenyl diphosphate units to generate dehydrodolichol diphosphate (syn. polyprenyl diphosphate). In the current model, a polyprenyl diphosphate phosphatase removes both phosphate residues generating polyprenol (dehydrodolichol), although this phosphatase still needs to be verified [9,10]. At this point, polyprenol reductase reduces the polyprenol's alpha isoprene unit using nicotinamide adenine dinucleotide phosphate (NADPH) and generating dolichol [8,11] (Fig. 1). Eventually, a dolichol kinase transfers phosphate from cytidine triphosphate (CTP) to dolichol [11,12].

2. Methods

2.1. Isoelectric focusing (IEF) and high performance liquid chromatography (HPLC)

For agarose-gel preparation, 22 mg agarose and 2.2 ml water were cooked for 20–30 min before 110 µl ampholine (pH5.0–7.0; Amersham Biosciences) was added. The gel was fixed on Gel Fix for agarose (Pharmacia Biotech).

The volume of 10 µl serum, 66.6 µl 0.9% NaCl and 2.3 µl 10 mM Fe(III)-citrat (0.0245 g/10 ml water) was incubated for 10 min at room temperature and completed with 2.3 µl 0.1 M $NaHCO_3$ (0.084 g/10 ml water). The IEF ran on a Pharmacia Phast System.

Afterwards 80 µl Tf antibody (Dako A0061) was added for visualization, washed with water, stained with Coomassie blue R250 and discolored with 300 ml Destain (350 ml ethanol + 650 ml water + 100 ml acetic acid).

HPLC of carbohydrate deficient Tf (CDT) was done as described [13].

2.2. Immunoprecipitation (IMPP) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-Page)

IMPP and SDS-Page was done as described [14].

2.3. LLO analysis

LLO analysis was done as described [15].

2.4. Homozygosity mapping by SNP-array

The patient's genomic DNA was isolated from peripheral blood lymphocytes by the salting out procedure as described previously [16]. DNA was prepared and hybridized to an Affymetrix Genome-

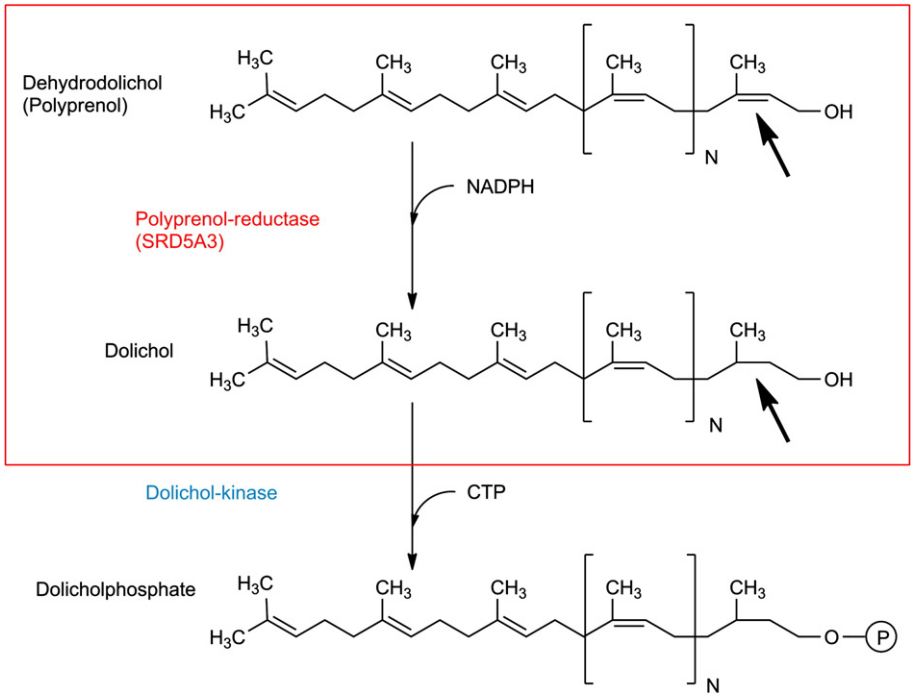

Fig. 1. Last steps of the current dolichol-P biosynthesis pathway model. Steroid 5α reductase type 3 (SRD5A3) acts as polyprenol reductase by reducing the terminal alpha isoprene unit of the polyprenol creating dolichol.

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Wide Human SNP Array 6.0 (Affymetrix, High Wycombe, UK) according to the manufacturer's protocol and further analyzed with an Affymetrix GeneChip Scanner 3000 7 G. Bioinformatic data processing was performed using the Affymetrix Genotyping Console 4.0 with default settings. The call rate exceeded 97%. Analysis regarding regions of homozygosity in the index patient was carried out with the Chromosome Analysis Suite 1.1 Software (Annotation Files Version NA30). Chromosomal segments were regarded as homozygous if they contained at least10 homozygous SNPs in series, minimum segment size was set as 1 Mb.

2.5. Cell culture

Fibroblasts were cultured in MEM (Minimal Essential Medium; PAA Laboratories (Company)-GE Healthcare) with 2 mM L-glutamine, 100 μg/ml penicillin and streptomycin, and 10% FBS (Fetal Bovine Serum; Invitrogen). For dolichol- and polyprenol-analysis, cells were incubated for 20 h in FBS-free MEM supplement. Afterwards fibroblasts were washed with Dulbecco's Phosphate Buffered Saline (PAA Laboratories), trypsinized with trypsin/EDTA solution (Biochrom AG), pelleted and stored at −80 °C.

2.6. Mutation analysis of SRD5A3

According to the manufacturer's protocol, RNA-preparation from patient's fibroblasts was done using the "RNeasy" Mini Kit (QIAGEN) followed by cDNA-synthesis with SuperScript™ III Reverse Transcriptase (Invitrogen/Life technologies Corp.).

With QIAmp DNA mini kit (QIAGEN) genomic DNA was isolated from the patient's EDTA blood sample after the manufacturer's protocol.

For amplification the following primers synthesized by Invitrogen were used: SRD5A3 IF(13U): (5′ to 3′) TGT AAA ACG ACG GCC AGT AGG CTG AGA CCG GTG CGC CG; SRD5A3 IR(13R): (5′ to 3′) CAG GAA ACA GCT ATG ACC GCC ATC CAT TGG CAC TTG GC; SRD5A3 IIF (13U): (5′ to 3′) TGT AAA ACG ACG GCC AGT CCT GCT TTG GTG CCT TAC TC; SRD5A3 IIR(13R): (5′ to 3′) CAG GAA ACA GCT ATG ACC ACT GCC ATG CTC ATT CAG TG.

Q-solution was added to the master-mix before PCR cycling was performed using the following conditions: 94 °C for 4 min; 35 cycles (94 °C for 1 min; 68 °C for 1.5 min; 72 °C for 1.5 min); 72 °C for 10 min.

Afterwards PCR-products were treated with the USB PCR Product Pre-Sequencing Kit from Affymetrix/USB and with the BigDye Terminator Kit 3.1 (Applied Biosystems) under the terms of the manufacturer's protocol. Cycling conditions were: 96 °C for 2 min and 25× (94 °C for 10 s, 50 °C for 5 s, 60 °C for 2 min). Purification was performed with Sephadex/Millipore System (Pharmacia; Millipore; Applied Biosystems) and sequencing was done on an ABI Prism 3730.

The genomic DNA was amplified with primer SRD5A3 IF(13U): (5′ to 3′) TGT AAA ACG ACG GCC AGT AGG CTG AGA CCG GTG CGC CG; and primer SRD5A3 ex1R2(13R): (5′to 3′) CAG GAA ACA GCT ATG ACC CTC GGC GTC CGC GGA CAC C. PCR-Conditions with Q-solution: 94 °C for 4 min; 35 cycles (94 °C for 1 min; 65 °C for 1.5 min; 72 °C for 1.5 min); 72 °C for 5 min.

Sequencing was done as described earlier.

2.7. Lipid extraction and liquid chromatography-coupled multiple reaction monitoring (LC-MRM) analysis of polyprenol and dolichol in human fibroblasts

Lipid extraction was performed according to Bligh and Dyer [17]. Analysis of dolichol and polyprenol in fibroblast cells were performed by LC-MRM using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps and a SCL-10A system controller) coupled to a 4000 Q-Trap hybrid triple quadrupole linear ion-trap mass spectrometer equipped with a Turbo V ion source (Applied Biosystems Inc, Foster City, CA). LC was operated at a flow rate of 200 μl/min with a linear gradient as follows: 100% of mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 μm, 2.1×50 mm) was obtained from Agilent.

MRM was performed in the negative ion mode with mass spectrometry (MS) settings as follows: CUR = 20 psi, GS1 = 20 psi, GS2 = 30 psi, IS = −4500 V, TEM = 350 °C, ihe = ON, DP = −40 V, EP = −10 V and CXP = −5 V. The voltage used for collision-induced dissociation was −40 V. The MRM pairs are as follows: 1302.2/59 for polyprenol-18; 1370.2/59 for polyprenol-19; 1438.2/59 for polyprenol-20; 1304.2/59 for dolichol-18; 1372.2/59 for dolichol-19; 1440.2/59 for dolichol-20. In these MRM pairs, the precursor ions are the $[M + \text{acetate}]^-$ adduct ions, and the product ions are the acetate ions (m/z 59).

3. Results

3.1. Case report

The patient is a Pakistani boy whose parents are cousins once removed and with further consanguinity in the paternal line (Fig. 2). The two other siblings are unaffected. He was born premature after 35 weeks of gestation. A neuropediatric examination at the age of 4 months revealed opisthotonus, muscle hypertonia, psychomotor retardation, downbeat-nystagmus and failure of fixation.

No cerebral seizures occurred, but psychomotor development was delayed. The boy started to walk at 22 months.

At the age of 3 years, the boy was in a good general state of health. His speech development was retarded and limited to a few single words (mom, dad). He was attentious, cooperative, showed normal reflex responses with a slight hypotonic muscle tone. His height was 97 cm (10–15th percentile) and his head circumference 49 cm (3–10th percentile). Blood pressure and heart rate were normal.

Laboratory examinations showed normal thyroid function (TSH, fT3, fT4), normal lactate concentration (1.5 mmol/l) as well as reference values for LDH, transaminases, CK, CKMB, bilirubin, gamma-GT and AP. Carbohydrate-deficient Tf was increased to 16.7% (diasialo-Tf) in 2008, indicating a congenital disorder of glycosylation. To exclude a virus-induced hepatitis, the serum was checked for HAV, HBV and HCV—all with negative results.

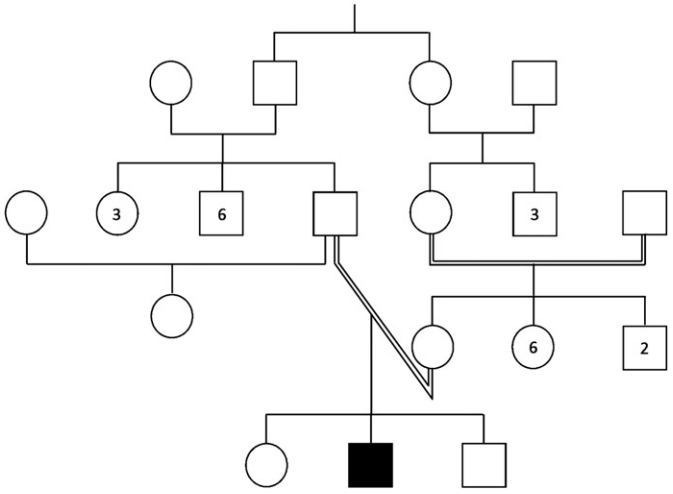

Fig. 2. Family tree. The patient's parents are cousins once removed. Only one child is affected. Digits represent the amount of people (male or female).

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An analysis of long-chain fatty acids revealed normal values.

Cranial MRI at the age of 3 9/12 years showed no pathological findings.

3.2. CDG diagnostics: IEF, IMPP, SDS-Page, HPLC

The standard CDG diagnostic screening is the IEF of serum Tf [18], a protein from the liver consisting of 679 amino acids with two bi-antennary oligosaccharides each with two negative sialic acid residues. Hypoglycosylation of this protein leads to missing saccharide-units or missing branches and, due to the loss of sialic acids with their electric charge, to a different isoelectric point. The major fraction in physiologic Tf is tetrasialo-Tf.

For detailed analyses of the patient's serum we started an IEF, an IMPP and a SDS-PAGE, separating the Tf by charge and molecular size. These tests showed an absence of a whole glycan chain on the protein, leading to the assumption of an early disruption of glycosylation pathway. In case of CDG-I, en bloc transfer of the glycan moiety is reduced, leading to some Tf-molecules with only one or not any bi-antennary glycan moiety. Thus, tetrasialo-Tf is reduced whereas di- and asialo-Tf are increased. The results clearly identified our patient as CDG type I (Figs. 3 and 4). A HPLC analysis in 2009 confirmed this report: 0.25% asialo-Tf, monosialo-Tf was below level of detection, 18.89% disialo-Tf, 3.44% trisialo-Tf, 73.90% tetrasialo-Tf and 3.53% pentasialo-Tf. The physiological reference values are: asialo-Tf and monosialo-Tf: below level of detection, disialo-Tf: 1.10 +/− 0.72, trisialo-Tf: 3.76 +/− 2.60, tetrasialo-Tf: 89.84 +/− 4.16, pentasialo-Tf: 6.4 +/− 3.80.

3.3. LLO analysis

Analysis of LLO derived from fibroblasts by HPLC gave variable results. Whereas the normal $Glc_3Man_9GlcNAc_2$ peak was found in some experiments, an accumulation of $Man_5GlcNAc_2$ was found in others, indicating that the amount of Dol-P-Glc might be limiting.

3.4. Homozygosity mapping and DNA sequencing

Since the parents were related, homozygosity mapping was used under the assumption of an autosomal recessive inheritance in order to determine candidate genes involved in glycosylation present in the homozygous genomic regions of the patient. Autosomal homozygous regions over 5 Mbp were: Chromosome 3 (p21.31–p21.1; 5211 kbp), chromosome 4 (p14–p12; 10654 kbp), (q11- q21.23; 32735 kbp) and chromosome 19 (p13.12- p12; 8136 kbp).

Two genes involved in early N-glycan assembly steps were identified in the homozygous regions. The gene for GDP-mannose pyrophosphorylase B was found in the homozygous region on chromosome 3 but did not show mutations. In the 32 Mbp region on chromosome 4 (Fig. 5) (q11–q21.23) 196 genes were present (based on Ensembl Genomic Browser). Among these we found the steroid 5α reductase type 3 (SRD5A3, OMIM ID: 612379), a gene with 318 amino acids, which was recently described [19]. Sequencing the amplified cDNA we found a homozygous mutation in exon 1 (c.57G>A) generating a stop codon with a protein truncation after 19 amino acids (Fig. 6) suggesting a complete loss of function of SRD5A3. The controls showed no mutation. Genomic DNA of the patient and his parents were sequenced for confirmation. The parents were heterozygous and the patient was homozygous for the SRD5A3-mutation (c.57G>A; A/A) (Fig. 7).

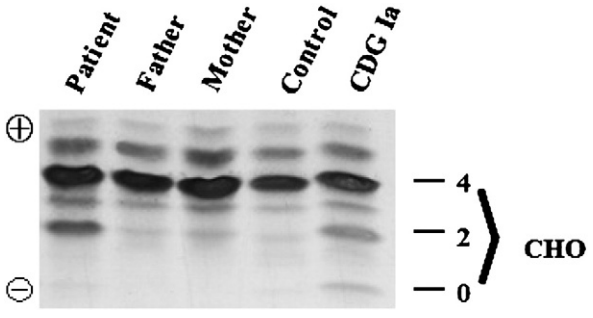

Fig. 3. Isoelectric focusing of serum transferrin. The control shows two bi-antennary oligosaccharides each with two negative sialic acid residues; the major fraction is tetrasialo-Tf. Additional bands are seen in CDGs due to missing monosaccharide units and missing branches. In the patient, tetrasialo-Tf is reduced whereas di- and asialo-Tf are increased. CHO = carbohydrate side chains.

3.5. LC-MRM analysis of polyprenol and dolichol in human fibroblasts

Since clearly more than 70% of patient's Tf was normally glycosylated, we analyzed his fibroblasts and the fibroblasts of two healthy controls with LC-MRM, wondering whether N-glycan assembly in this case occurred on polyprenol or whether there is an alternative pathway for the biosynthesis of dolichol.

Residual amounts of polyprenols were detectable, but levels of polyprenols in the patient were much higher than in the healthy controls (Figs. 8 a and b).

Surprisingly, dolichol levels in the patient's fibroblasts were comparable to the healthy controls (Figs. 9 a and b) leading to about 3-folds higher polyprenol/dolichol ratios in the patient in comparison to controls (Fig. 10).

MS data were not normalized to an internal or exogenous standard. Figures show the raw MS ion signal data.

These results suggest an alternative pathway for dolichol biosynthesis.

4. Discussion

The stepwise assembly of N-linked glycans requires dolichol, a polyisoprenoid. In the last step of dolichol biosynthesis, the terminal alpha isoprene unit of the polyprenol is reduced.

SRD5A3 was initially presumed to be a 5α-steroid reductase. It was identified by a genome-wide expression profile analysis in human prostate cancer cells [20]. It consists of 318 amino acids. Recent studies idenified SRD5A3 as the polyprenol reductase involved in the final step of dolichol biosynthesis [19].

The clinical features of SRD5A3 deficiency were first described in a consanguineous Emirati family with four affected children [21]. The children had dysmorphic features, coloboma of the iris, retina or optic disc, short stature, feeding difficulties, hypertrichosis and hyperkeratosis of the skin. Brain MRI revealed frontal polymicrogyria. One of the children had a transposition of the great arteries. Five out of seven patients had cerebellar vermis hypoplasia [19]. Genome-wide linkage analysis revealed a homozygous region on chromosome 4 and the SRD5A3 gene was identified by mutation analysis of candidate genes within this region [19]. Since abnormal Tf glycosylation was found in these children, CDG-Ix patients were screened for SRD5A3 mutations revealing a total of 11 patients with mutations in this gene [19]. Nystagmus was reported in nearly all of them.

Morava et al. reported 12 patients from nine families with SRD5A3-CDG [22]. Two of the affected children were from two different consanguineous Turkish families and were originally reported by Prietsch [23] and Assman [24]. They had the same mutation as our patient (c.57G>A [W19X]) and showed a similar picture of clinical symptoms: Muscle hypertonia, psychomotor retardation and nystagmus. By reason of the geographic distance a potential founder effect between the two Turkish children and our patient is unlikely. Morava et al. also compared the clinical symptoms and biochemical findings of these 12 SRD5A3-CDG-patients and found no genotype–phenotype correlation [22].

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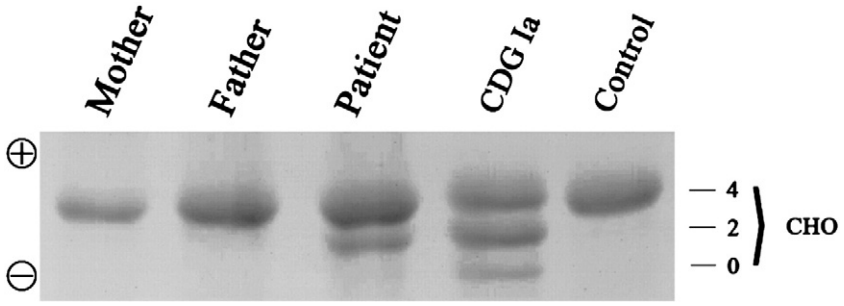

Fig. 4. IMPP and SDS-Page. The SDS-Page separates the Tf by molecular size. Thus, hypoglycosylated Tf migrates faster, as it is shown in patient and positive control. Tetrasialo-Tf is reduced whereas di- and asialo-Tf are increased. CHO = carbohydrate side chains.

SRD5A3 deficiency was also identified as the cause of Kahrizi syndrome [25,26]. The syndrome was described in three adult Iranian siblings with severe mental retardation. The patients were unable to speak. Two of the three patients had iris coloboma. The patients had short stature, cataracts, kyphosis and joint contractures developed later in life. No seizures occurred. Homozygosity mapping revealed a single interval of homozygosity that was unique to the patients and was localized to the pericentrometric region of chromosome 4.

SRD5A3 is the human ortholog of the yeast DFG10 gene. The phenotype of the yeast DFG10 mutant can be rescued by human SRD5A3 [19]. Disruption of the SRD5A3 gene in mice is lethal at embryonic day 12.5 [19].

In SRD5A3 fibroblasts we found dolichol concentrations that were comparable to healthy controls suggesting that there must be an alternative pathway for dolichol biosynthesis. In addition, polyprenol could serve as the acceptor molecule for the biosynthesis of N-glycans working with the lower efficiency.

Although the amount of dolichol was not reduced in SRD5A3 fibroblasts, hypoglycosylation occurred in the patient. Several explanations seem possible:

1.) Accumulation of intermediate products of the dolichol pathway might be toxic and inhibit glycosylation reactions. A change of isoprenoid levels could influence the cell's metabolism as it has

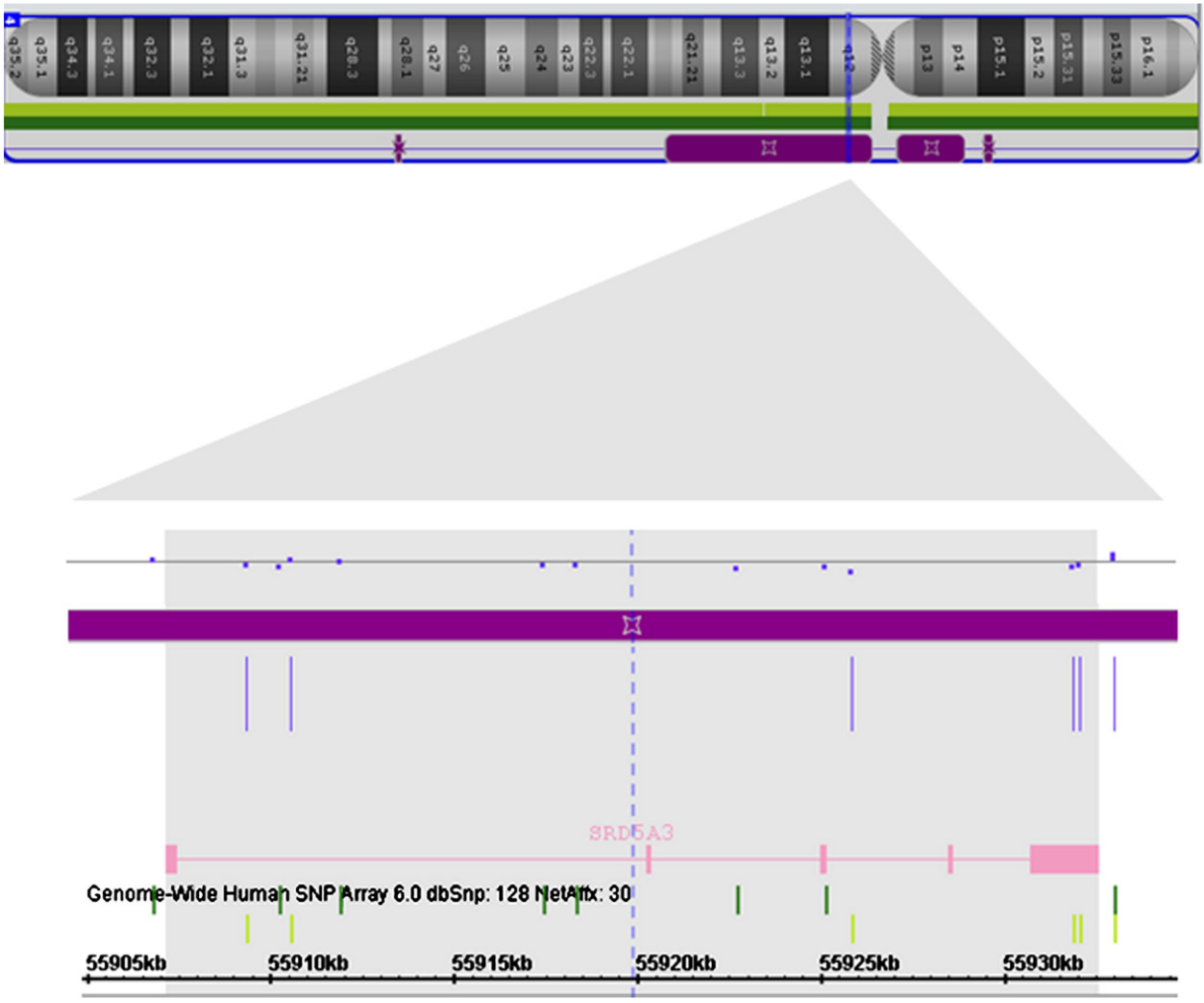

Fig. 5. Copy number neutral 32, 7 Mb LOH region on chromosome 4 detected by SNP array analysis.

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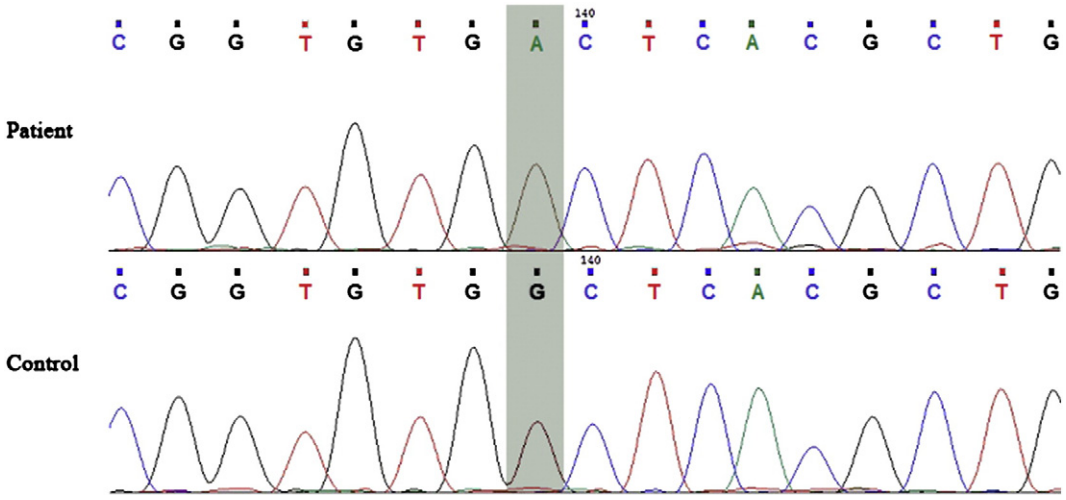

Fig. 6. Mutation analysis—cDNA. SRD5A3 mutation c.57G > A [W19X] in patient's cDNA.

been associated with Alzheimer's disease [27–30] and neuronal ceroid lipofuscinosis [31]. Recently a mutation in DHDDS (OMIM ID: 613861), encoding dehydrodolichol diphosphate synthase, was discovered in an ethnic group called Ashkenazi Jews [32]. DHDDS (syn. cis-prenyltransferase) is located two steps earlier in dolichol de novo biosynthesis than the polyprenol reductase, creating polyprenol by cis-prenyl elongation of farnesyl pyrophosphate (FPP) [32]. Interestingly, symptoms were not as severe as in our patient and restricted to retinitis pigmentosa. No other organs were affected. Referring to toxic accumulation, FPP might have a less fatal impact than polyprenol.

2.) The 3-fold increase of polyprenol found in SRD5A3 fibroblasts could compete with dolichol as the lipid-anchor for N-glycosylation. In the first step of N-glycosylation, dolichol kinase transfers phosphate from CTP to dolichol (Fig. 1) [11,12]. Substrate specificity of dolichol kinase was tested in rat liver and

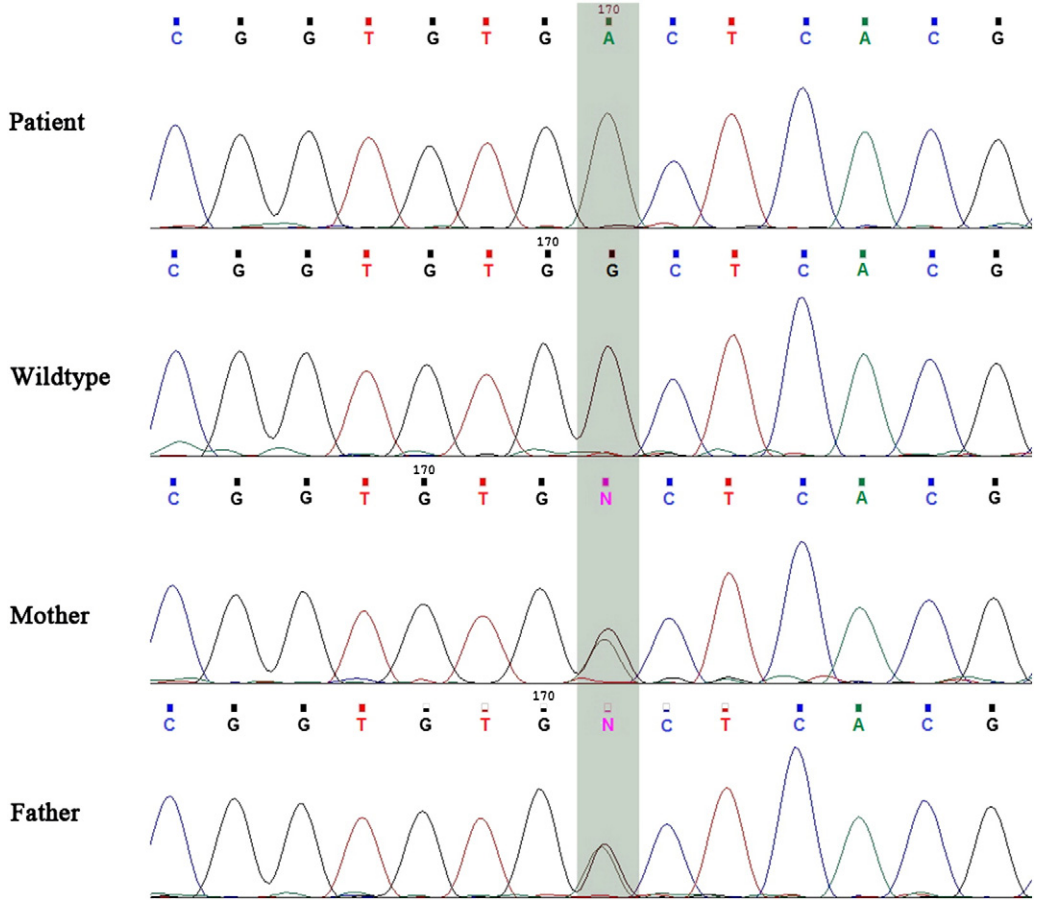

Fig. 7. Mutation analysis—gDNA. Heterozygousity for SRD5A3 (Exon 1 c.57G/A) is shown in the parent's gDNA. The patient's gDNA contains the SRD5A3-mutation (Exon 1 c.57G > A; A/A homozygosity). Wildtype-control: Exon 1 c.57G > A; G/G.

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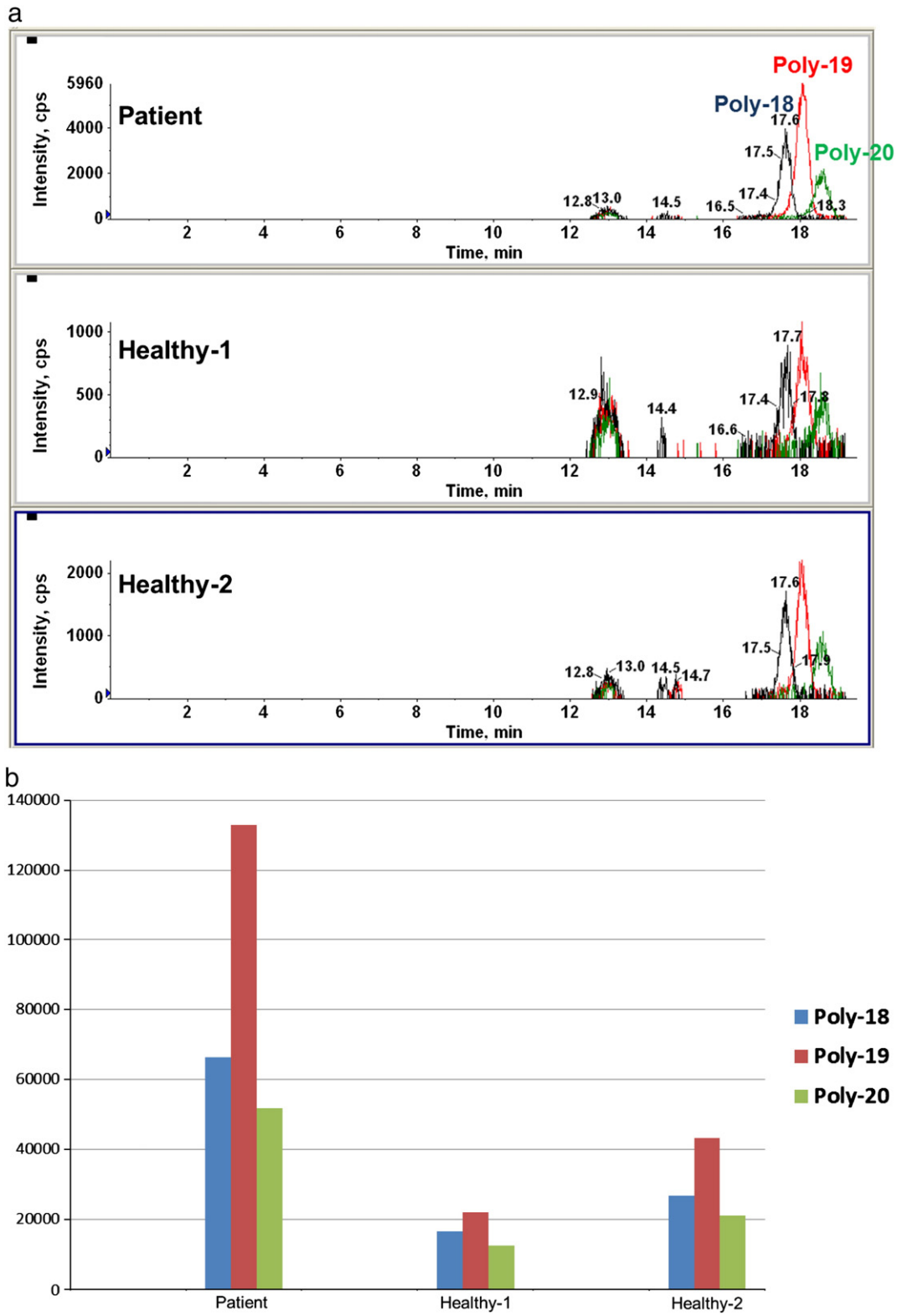

Fig. 8. a. Polyprenol in fibroblasts. Analysis of polyprenol in fibroblasts cells was performed by liquid chromatography-coupled multiple reaction monitoring (LC-MRM). b. Polyprenol in fibroblasts. The results showed a higher level of polyprenols in the patient than in the healthy controls.

demonstrated a preference for the saturation of the alpha unit with 2.5-fold more activity concerning dolichol-16 and -19 compared to the corresponding polyprenols [33]. Furthermore, for the transfer of mannosyl residues from GDP-mannose in rats, polyprenol was identified as a weak acceptor while dolichol showed good results [34]. In contrast to bacteria, glycosylation with polyprenol in human seems to be reduced if not impossible. Nevertheless, polyprenol might compete with dolichol in early N-glycan biosynthesis reactions thereby reducing the amount of dolichol-linked oligsaccharides used for protein glycosylation.

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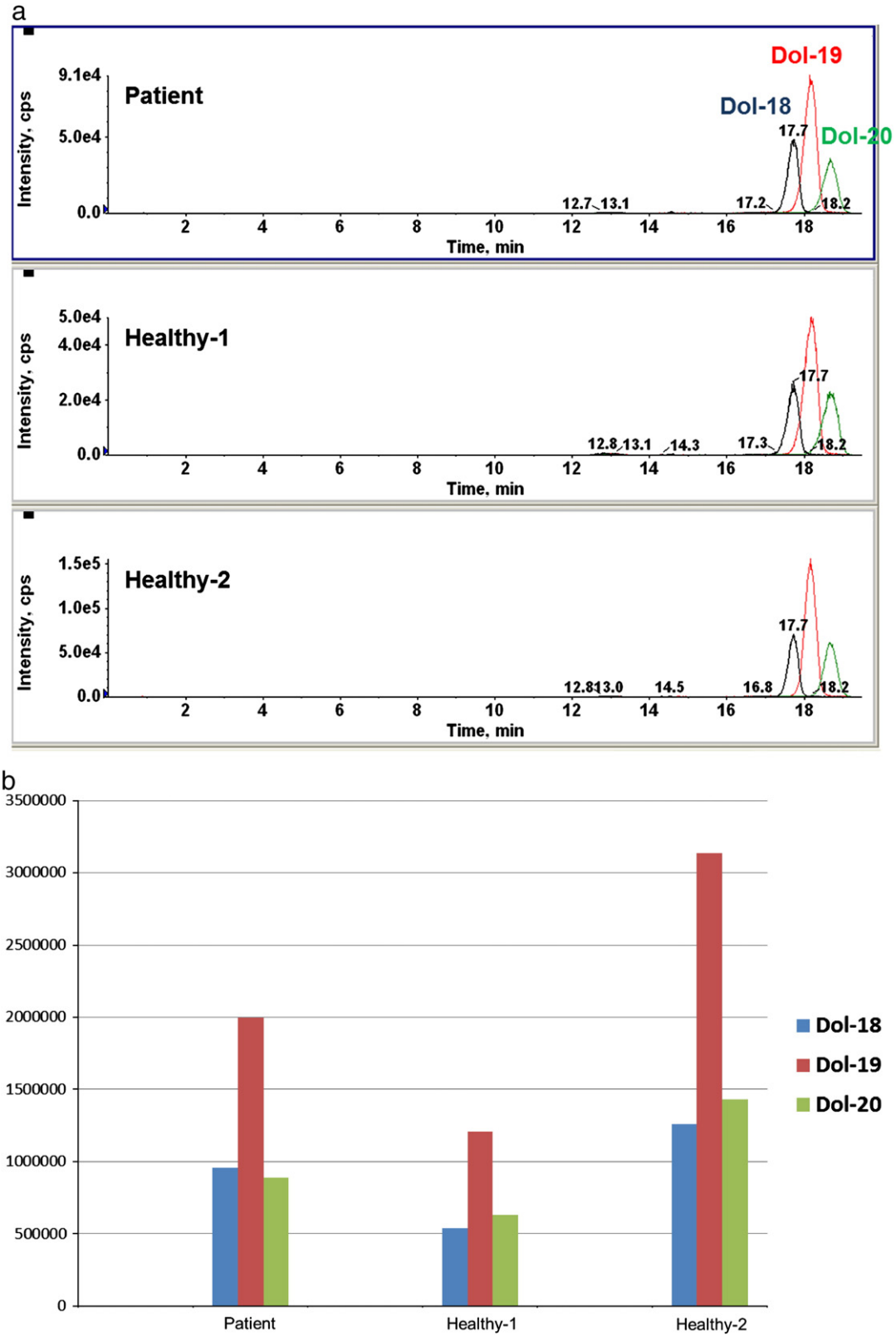

Fig. 9. a. Dolichol in fibroblasts. Analysis of dolichol in fibroblast cells were also performed by liquid chromatography-coupled multiple reaction monitoring (LC-MRM).b: Dolichol in fibroblasts. Dolichol content in the patient's fibroblasts was comparable to the healthy controls.

3.) The operating alternative pathway cannot create the necessary amount of dolichol at the right location. One dolichol phosphate is required as the carrier while seven others import glucose and mannose into the ER [4]. Therefore, a locally decreased pool of dolichol can lead to reduced glycosylation. A dolichol-pyrophosphate-phosphatase in yeast (CWH8) and mammalian cells (DolPP1) is known to recycle Dol-P-P after OST has transferred $Glc_3Man_9GlcNAc_2$ to the protein, keeping up a steady

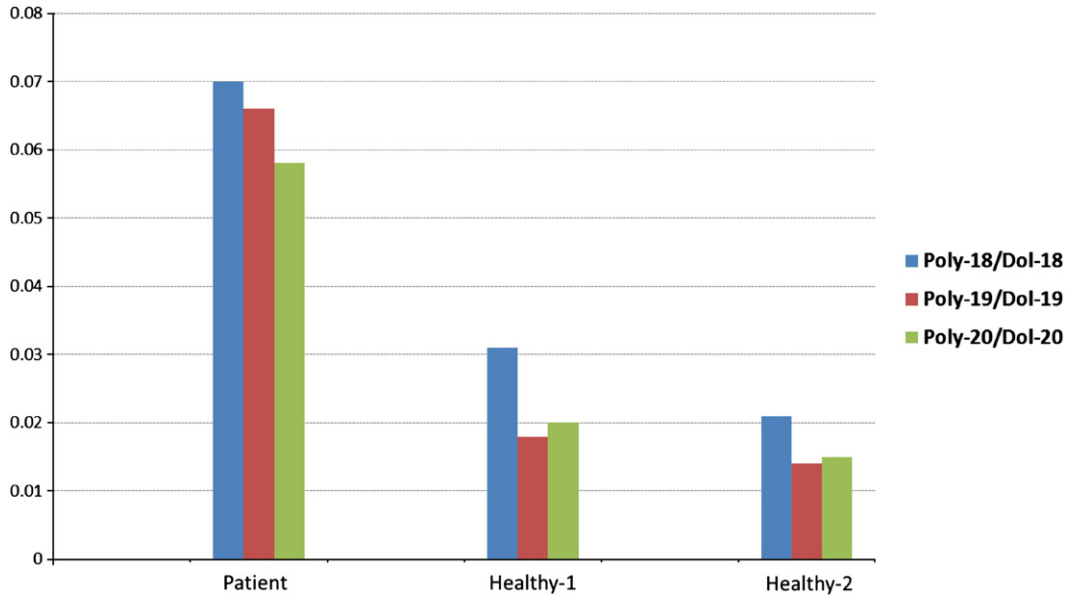

Fig. 10. Polyprenol/dolichol ratios in fibroblasts. Due to increased levels of polyprenol, the patient's polyprenol/dolichol ratio is about 3-folds higher than in healthy fibroblasts.

pool of dolichol for glycosylation [35]. Whether this is enough in patients with SRD5A3-mutations or whether this dolichol is at the right location still needs to be verified.

4.) The amount of dolichol-linked oligosaccharides might be sufficient for glycosylation in SRD5A3 fibroblasts but might be insufficient in cells with higher rates of N-glycan biosynthesis like liver cells.

It is intriguing to speculate that improvement of the dolichol/polyprenol ratio could be the key for a successful therapy for SRD5A3-deficient patients. Very little is known about the dolichol contents of food. Polyprenols are the dominant polyisoprenoids in plant photosynthetic tissues accompanied by the traces of dolichols. Only plant roots, yeast or the animal tissues could be considered as a possible source of dolichols [36]. However, the dolichol-amount absorbed by nutrition is known to be insignificant in rats [37]. Further research on dolichol content in plants or food and its absorption in human will be necessary.

Pharmacological interventions might also be possible. In CDG fibroblasts with a dolichol-phosphate-mannose synthase deficiency (CDG-Ie) the amount of dolichol-phosphate-mannose was increased by using the squalene synthase inhibitor zaragosic acid A (ZGA) [38].

In contrast to statins, squalene synthase inhibitors inhibit cholesterol biosynthesis later-on in the pathway, i.e. after the step where dolichol biosynthesis is separated from the cholesterol biosynthesis pathway. In SRD5A3, directing the metabolic flux towards polyisoprenoid biosynthesis might have contrary effects and might worsen the patient's symptoms, e.g. if hypothesis #2 is correct. Alternatively it might be useful if e.g. hypothesis #4 is correct.

On the other hand, statins inhibit the cholesterol and dolichol biosynthesis by interfering with mevalonate biosynthesis at the level of HMG-CoA reductase and are used for treatment of patients at risk for secondary cardiovascular events [39]. The mevalonate pathway is the early common part of the dolichol and cholesterol biosynthesis. Its interruption would therefore reduce not only cholesterol but polyisoprenoids as well. If accumulation of polyprenol was deleterious as discussed, this might improve the patient's symptoms. Otherwise, hypoglycosylation of proteins might increase due to reduced dolichol production [40].

Statins have been used in Smith–Lemli–Opitz syndrome (SLOS) treatment. SLOS is caused by a defect of sterol-Δ^7-reductase that leads to an accumulation of the cholesterol precursor 7-dehydrocholesterol [41]. Its production can be inhibited by statin treatment [42]. Pappu, Connor et al. [43] described a 7-fold higher urinary excretion rate of dolichol and ubiquinone in SLOS children than in control children. This again shows a shift in the mevalonate/cholesterol pathway towards other products such as nonsterol isoprenoids. Furthermore Pappu, Connor et al. used a high-cholesterol diet, decreasing the urinary excretion rate of dolichol by 70% and ubiquinone by 67%, possibly due to a negative feedback mechanism. In patients with polyprenol reductase deficiency a cholesterol supplementation might have similar effects on the polyprenol excretion and possibly on the polyprenol content in cells.

Metazoans protect themselves from potential toxic substrate accumulation in cells with a mechanism called translation attenuation. High ER stress rates, for example due to improperly glycosylated proteins, induce the protein kinase R-like ER kinase (PERK). This kinase then phosphorylates the eukaryotic initiation factor 2α (eIEF2α), which reduces the number of translated proteins in the ER. Hence, fewer proteins are glycosylated but regularly [44]. Translation balancing still needs to be verified in human cells. Effects on fibroblasts from CDG-Ia patients showed variable results [44] but hold promise for a future therapeutic option.

5. Conclusion

This study emphasizes the possibility of an alternative dolichol pathway, demonstrated by normal dolichol levels in the described patient. In addition, the cell biological basis for SRD5A3-CDG pathology is discussed and potential treatment options are demonstrated. Further research on the alternative dolichol pathway and, along with that, on therapeutic options for SRD5A3-CDG and other CDG is required.

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This paper is dedicated to Chris Raetz, who passed away on August 16, 2011.

We feel honored and are deeply grateful for his cooperation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ymgme.2011.12.017.

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