Autosomal recessive HEM/greenberg skeletal dysplasia is caused by 3 beta-hydroxysterol Delta(14)-reductase deficiency due to mutations in the lamin B receptor gene


Published in:
American Journal of Human Genetics

DOI:
10.1086/373938

Citation for published version (APA):
Autosomal Recessive HEM/Greenberg Skeletal Dysplasia Is Caused by 3β-Hydroxysterol Δ14-Reductase Deficiency Due to Mutations in the Lamin B Receptor Gene

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Hydrops-ectopic calcification—“moth-eaten” (HEM) or Greenberg skeletal dysplasia is an autosomal recessive chondrodystrophy with a lethal course, characterized by fetal hydrops, short limbs, and abnormal chondro-osseous calcification. We found elevated levels of cholesta-8,14-dien-3β-ol in cultured skin fibroblasts of an 18-wk-old fetus with HEM, compatible with a deficiency of the cholesterol biosynthetic enzyme 3β-hydroxysterol Δ14-reductase. Sequence analysis of two candidate genes encoding putative human sterol Δ14-reductases (TM7SF2 and LBR) identified a homozygous 1599–1605TCTTCTA→CTAGAAG substitution in exon 13 of the LBR gene encoding the lamin B receptor, which results in a truncated protein. Functional complementation of the HEM cells by transfection with control LBR cDNA confirmed that LBR encoded the defective sterol Δ14-reductase. Mutations in LBR recently have been reported also to cause Pelger-Huet anomaly, an autosomal dominant trait characterized by hypolobulated nuclei and abnormal chromatin structure in granulocytes. The fact that the healthy mother of the fetus showed hypolobulated nuclei in 60% of her granulocytes confirms that classic Pelger-Huet anomaly represents the heterozygous state of 3β-hydroxysterol Δ14-reductase deficiency.
guineous parents (F=1/16) were both healthy, and the family history was noncontributory. Since sterol analysis elsewhere of tissues and cells of six fetuses with HEM obtained from a skeletal dysplasia repository suggested a deficiency of 3β-hydroxysterol Δ14-reductase (R. I. Kelley, unpublished data; Kelley and Herman 2001), we analyzed the sterol content in cultured primary skin fibroblasts obtained from the fetus with HEM. To this end, the cells were cultured in lipoprotein-depleted medium for 4 d, after which the sterols were extracted and analyzed by gas chromatography/mass spectrometry (GC/MS), as described elsewhere (Waterham et al. 2001). This revealed elevated levels of cholesta-8,14-dien-3β-ol (fig. 1A), a cholesterol precursor that was not detected in fibroblasts from healthy control subjects or in chorionic villi and amniotic fluid cells from fetuses of similar age. In analogy with sterol Δ14-reductase-deficient erg2Δ mutants of yeast, which accumulate ergosta-8,14-dien-3β-ol (Crowley et al. 1996; Silve et al. 1998), this finding pointed to a deficiency of the enzyme 3β-hydroxysterol Δ14-reductase, which catalyzes the reduction of the Δ14-double bond in early sterol intermediates (fig. 1B).

Two different genes have been reported to encode human sterol Δ14-reductases: LBR (GenBank accession number L25932–L25941) at 1q42 and TM7SF2 (GenBank accession number AF096303) at 11q13. LBR encodes the lamin B receptor, a 70.4-kDa protein of the inner nuclear membrane that contains an N-terminal lamin B/DNA-binding domain of ~200 amino acids followed by a C-terminal sterol reductase-like domain of ~450 amino acids (Holmer et al. 1998), which exhibits sterol Δ14-reductase activity when expressed in yeast (Silve et al. 1998). TM7SF2 encodes a sterol reductase of 46.4 kDa located in the endoplasmic reticulum (ER) membrane (Holmer et al. 1998), which exhibits sterol Δ14-reductase activity when overexpressed in COS-7 cells (Roberti et al. 2002). On the basis of the highest sequence similarity of the encoded protein with fungal sterol Δ14-reductases and its localization in the ER membrane (as are most other sterol reductases known to date), the TM7SF2 gene was considered the best candidate to encode the defective 3β-hydroxysterol Δ14-reductase. However, sequencing, by means of −21M13 and M13rev fluorescent primers (Waterham et al. 2001), of all exons and flanking intron sequences of TM7SF2 amplified by PCR (TM7SF2-specific primer sequences available upon request) did not identify mutations. Subsequent sequencing of PCR-amplified exons and flanking intron sequences of the LBR gene (table I), by means of −21M13 and M13rev fluorescent primers (Waterham et al. 2001), identified a homozygous substitution of seven nucleotides in exon 13 (fig. 2). Homozygosity was confirmed by finding the same mutation in heterozygous form in genomic DNA of the mother; no DNA from the father was available. The same homozygous substitution was also observed in LBR cDNA (GenBank accession number L25931) prepared from total RNA isolated from the fetus’s cells, which indicates that the mutation neither affects transcription nor is part of a nontranscribed pseudogene. The 1599–1605TCTTCTA→CTAGAAG substitution introduces a stop codon (underlined) that leads to the production of a truncated protein lacking the C-terminal 82 amino acids. Since the C-terminus is highly conserved among the various sterol reductases (Holmer et al. 1998; Waterham et al. 1998), it is very likely that no functional sterol Δ14-reductase is produced.

To confirm that LBR indeed encodes the defective sterol Δ14-reductase, the patient’s fibroblasts were transfected transiently with control LBR cDNA by means of retroviral transduction, using the Phoenix retroviral system (Kinsella and Nolan 1996; Michiels et al. 2000). To this end, the coding region of LBR cDNA was amplified by PCR from control cDNA using a BamHI-tagged LBR cDNA-specific forward primer (5′-TTAAGGATCCCTGTGTCACCGCCGGAACC-3′) and an LBR cDNA-specific reverse primer (5′-AAGTACGACCTGTACG-CGGAGACCC-3′). The amplified LBR cDNA was cloned first in the pGEM-T vector (Clontech) and sequenced to exclude PCR-introduced errors. Subsequently, the LBR cDNA was released from pGEM-T with BamHI and NotI and

Figure 1 Sterol synthesis in cells from a control and the fetus with HEM. A, GC/MS sterol analysis in primary skin fibroblasts cultured for 4 d in lipoprotein-depleted medium revealed elevated levels of cholesta-8,14-dien-3β-ol (peak 2) in cells of the fetus with HEM, whereas, in control cells, only cholesterol (peak 1) was detected. B, The accumulation of cholesta-8,14-dien-3β-ol in HEM cells points to a deficiency of the enzyme 3β-hydroxysterol Δ14-reductase, which catalyzes the reduction of the C14,15-double bond of cholesta-8,14-dien-3β-ol to produce cholesta-8-en-3β-ol.
ligated into the BamHI and NotI sites of the pLZRS-IRES-ZEO/pBR plasmid (Michiels et al. 2000). The resulting pLZRS-LBR-IRES-ZEO/pBR plasmid and, as a control, empty pLZRS-IRES-ZEO/pBR plasmid were transfected into amphotrophic Phoenix FNX-A packaging cells (gift from Dr. G. P. Nolan) using lipofectamine-plus (Invitrogen), and helper-free recombinant retrovirus was produced as described elsewhere (Michiels et al. 2000). LBR-containing and, as a control, non-LBR-containing viral supernatants (7 ml) were added to patient’s fibroblasts, seeded 1 d prior to the experiment at 40% confluence in T75 culture dishes. After 24 h of incubation, the viral supernatants were replaced by lipoprotein-depleted Dulbecco modified Eagle medium. After 4 d of culturing in the lipoprotein-depleted medium, cells were harvested, and sterols were extracted and analyzed by GC/MS, as described elsewhere (Waterham et al. 2001). The levels of cholesta-8,14-dien-3β-ol were corrected for total protein and recovery of the internal sterol standard epicoprostanol, which was added prior to the sterol extractions. In two independent experiments, we observed a marked reduction of 41% and 49.5% of cholesta-8,14-dien-3β-ol in the patient’s cells that were transduced with the LBR-containing retroviral supernatant, when compared with the identically treated cells transduced with the non-LBR-containing retroviral supernatant.

When this work was near completion, Hoffmann et al. (2002) reported that heterozygous mutations in LBR are responsible for Pelger-Huët anomaly (PHA [MIM 169400]). This is a benign autosomal dominant disorder of leukocyte development characterized by hypolobulated nuclei and abnormal chromatin structure in granulocytes of heterozygotes but without evident clinical symptoms (Pelger 1928; Huët 1932). Presumed PHA homozygotes present with ovoid granulocyte nuclei; may have developmental delay; display variable skeletal abnormalities, such as polydactyly and metacarpal shortening (Aznar and Vaya 1981; Von Siegert et al. 1983; Hoffmann et al. 2002); and may reach adult age (Hoffmann et al. 2002). Moreover, PHA homozygosity leads to short-limbed chondrodysplasia in rabbits (Nachtsheim 1950) and cats (Latimer et al. 1988). These findings combined with ours imply that HEM/Greenberg skeletal dysplasia and homozygous PHA are allelic disorders that display a wide clinical spectrum, with nonviable fetuses with HEM representing the severe end and minor limb defects representing the mild end of the spectrum. It is unfortunate that no sterol analysis to further substantiate this was performed in the single individual with homozygous PHA reported by Hoffmann et al. (2002). However, the mother of the fetus showed hypolobulated

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### Table 1

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Forward Primer (5′→3′)</th>
<th>Reverse Primer (5′→3′)</th>
<th>PCR Program</th>
</tr>
</thead>
</table>

* −21M13: tgt aaa acg acg gcc agt.

* M13rev: cag gaa aca gct atg acc.

* The amplification by PCR of genomic DNA, by means of a “standard” or a “touchdown” PCR amplification program, was performed as described elsewhere (Houten et al. 2001), using the indicated LBR-specific primers.
nuclei in 60% of her granulocytes (fig. 3), which demonstrates that classic PHA represents the heterozygous state of 3β-hydroxysterol Δ14-reductase deficiency.

HEM/Greenberg skeletal dysplasia is the sixth inherited disorder of cholesterol biosynthesis for which the molecular basis now has been resolved. Most of these disorders represent previously reported clinical entities of unknown etiology that could be attributed to different enzyme defects in the postsqualene segment of the cholesterol biosynthetic pathway after the finding of abnormally increased levels of intermediate sterol metabolites in patients, followed by the demonstration of disease-causing mutations in genes encoding the implicated enzymes (Kelley and Herman 2001; Porter 2002; Waterham 2002). Apart from autosomal recessive HEM/Greenberg skeletal dysplasia, these include the three autosomal recessive disorders Smith-Lemli-Opitz syndrome (MIM 270400), which is caused by 3β-hydroxysterol Δ7-reductase deficiency (Fitzky et al. 1998; Wassif et al. 1998; Waterham et al. 1998); desmosterolosis (MIM 602398), which is caused by 3β-hydroxysterol Δ24-reductase deficiency (Waterham et al. 2001); and lathosterolosis (MIM 607330), which is caused by 3β-hydroxysterol Δ5-desaturase deficiency (Brunetti-Pierri et al. 2002). In addition, the molecular basis of two X-linked dominant inherited disorders of cholesterol biosynthesis has been resolved: Conradi-Hunermann-Happle syndrome (MIM 302960) and CHILD syndrome (MIM 308050) are caused by deficiencies of sterol Δ5-Δ7-isomerase (Bra- 2001; Waterham 1998) and a sterol C-4 demethylase (Konig et al. 2000), respectively. Patients afflicted with these inborn errors of cholesterol biosynthesis are characterized, in general, by multiple morphogenetic and congenital anomalies, including skeletal, internal organ, and/or skin abnormalities (Kelley and Herman 2001; Porter 2002; Waterham 2002).

Figure 3  Blood smear of the mother of the present fetus with HEM showing the two characteristics of PHA: granulocytes with the typical pince-nez appearance of the bilobed nuclei and coarse clumping of chromatin.

The occurrence of an abnormal granulocyte chromatine structure in the hetero- and homozygous individuals with PHA and the presence of (severe) skeletal abnormalities in individuals with homozygous HEM/Greenberg skeletal dysplasia indicate that the lamin B receptor has two different physiological functions, namely, preserving chromatin structure by promoting heterochromatin binding to the inner nuclear membrane (Ye and Worman 1994) and functioning as the primary sterol Δ14-reductase in human cholesterol biosynthesis. The latter was somewhat unexpected, since all enzymes involved in the postsqualene cholesterol biosynthesis pathway have been localized to the ER membrane, whereas the lamin B receptor is present (predominantly) in the inner nuclear membrane. In this respect, the gene product of TM7SF2 a priori seemed a better candidate, since it is localized in the ER membrane (Holmer et al. 1998) and also exhibits sterol Δ14-reductase activity (Roberti et al. 2002). Now that TM7SF2 is not the primary sterol Δ14-reductase involved in human cholesterol synthesis, its physiological function remains to be discovered.

Acknowledgments

We thank Gerrit-Jan Romeijn for technical assistance in the initial stages of this study. H.R.W. is supported by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for TM7SF2 gene [accession number AF096303], LBR gene [accession numbers L25932–L25941], and LBR cDNA [accession number L25931])


References

