Mutations in \textit{SRD5B1} (\textit{AKR1D1}), the gene encoding \(\Delta^4\)-3-oxosteroid 5\(\beta\)-reductase, in hepatitis and liver failure in infancy

H A Lemonde, E J Custard, J Bouquet, M Duran, H Overmars, P J Scambler, P T Clayton

\textbf{Background:} A substantial group of patients with cholestatic liver disease in infancy excrete, as the major urinary bile acids, the glycine and taurine conjugates of \(7\alpha\)-hydroxy-3-oxo-4-cholenoic acid and \(7\alpha,12\alpha\)-dihydroxy-3-oxo-4-cholenoic acid. It has been proposed that some (but not all) of these have mutations in the gene encoding \(\Delta^4\)-3-oxosteroid 5\(\beta\)-reductase (\textit{SRD5B1}; \textit{AKR1D1}, OMIM 604741).

\textbf{Aims:} Our aim was to identify mutations in the \textit{SRD5B1} gene in patients in whom chenodeoxycholic acid and cholic acid were absent or present at low concentrations in plasma and urine, as these seemed strong candidates for genetic 5\(\beta\)-reductase deficiency.

\textbf{Patients and subjects:} We studied three patients with neonatal onset cholestatic liver disease and normal \(\gamma\)-glutamyl transpeptidase in whom 3-oxo-\(\Delta^4\) bile acids were the major bile acids in urine and plasma and saturated bile acids were at low concentration or undetectable. Any base changes detected in \textit{SRD5B1} were sought in the parents and siblings and in 50 ethnically matched control subjects.

\textbf{Methods:} DNA was extracted from blood and the nine exons of \textit{SRD5B1} were amplified and sequenced. Restriction enzymes were used to screen the DNA of parents, siblings, and controls.

\textbf{Results:} Mutations in the \textit{SRD5B1} gene were identified in all three children. Patient MS was homozygous for a missense mutation (662 C>T) causing a Pro198Leu amino acid substitution; patient BH was homozygous for a single base deletion (511 delT) causing a frame shift and a premature stop codon in exon 5; and patient RM was homozygous for a missense mutation (385 C>T) causing a Leu106Phe amino acid substitution. All had liver biopsies showing a giant cell hepatitis; in two, prominent extramedullary haemopoiesis was noted. MS was cured by treatment with chenodeoxycholic acid and cholic acid; BH showed initial improvement but then deteriorated and required liver transplantation; RM had advanced liver disease when treatment was started and also progressed to liver failure.

\textbf{Conclusions:} Analysis of blood samples for \textit{SRD5B1} mutations can be used to diagnose genetic 5\(\beta\)-reductase deficiency and distinguish these patients from those who have another cause of 3-oxo-\(\Delta^4\) bile aciduria, for example, severe liver damage. Patients with genetic 5\(\beta\)-reductase deficiency may respond well to treatment with chenodeoxycholic acid and cholic acid if liver disease is not too advanced.
Patient MS
This is the Sardinian girl described previously.6 She was the second child of healthy parents who were not knowingly consanguineous. She presented at three weeks with hyperbilirubinaemia (316 µM, conjugated 145), raised transaminases (aspartate aminotransferase (AST) 2279 U/l; alanine aminotransferase (ALT) 1123 U/l), and a prolonged prothrombin time (15.4 seconds; control 12). Cholestasis persisted and was associated with steatorrhoea, failure to thrive, and rickets. A liver biopsy at three months showed lobular disarray resulting from extensive giant cell transformation and necrotic foci with granulocyte accumulation. Hepatocytes contained fat and bile pigment. Failure to thrive, steatorrhoea, and fat soluble vitamin malabsorption continued despite ursodeoxycholic acid treatment. At eight months her bilirubin was still elevated (88 µM; conjugated 35 µM), and she was transfused with blood. Tests started to deteriorate and this continued despite bile duct proliferation but no cholangiocarcinoma bile ducts, and no signs of copper or iron accumulation. Increased extramedullary haematopoiesis was noted.

By the age of six weeks, liver function had deteriorated significantly (international normalised ratio 2.0, APTT >190 seconds, bilirubin 365 µmol/l, ALT 785). She was treated with ursodeoxycholic acid (30 mg twice daily). Bilirubin fell slightly, to 207 µmol/l at 10 weeks. From 10 weeks, RM was treated with chenodeoxycholic acid (10 mg three times daily). There was no improvement in liver function tests and the development of liver failure led to referral for transplantation at 19 weeks. One day after transplantation the patient developed severe cerebral oedema with absence of all reflexes and one day later she died.

Plasma and urine bile acid analyses
Urine samples from MS and BH were analysed by liquid secondary ionisation mass spectrometry (LSIMS), as described previously.6 7 A urine and plasma sample from RM were analysed by electrospray ionisation tandem mass spectrometry (ESI-MS/MS) using a “parents of m/z 74” scan for glycan conjugates and a “parents of m/z 80” scan for taurine conjugates.6 7 Analyses of (non-sulphated) plasma and urine bile acids by gas chromatography-mass spectrometry (GC-MS) were undertaken using established methods.6 7 9

Analysis of the 5β-reductase gene (SRD5B1)
Initial genetic studies of 5β-reductase focused on the characterisation of cDNA from liver biopsy samples.10 Subsequent to the determination of the genomic sequence of SRD5B1,11 studies concentrated on genomic DNA: the nine exons of the gene from each patient were amplified using a single round of PCR utilising a Gene Amp PCR System 9700 machine with Bioline Taq polymerase. The temperature program included an initial denaturing step of 94°C for four minutes followed by 28 cycles of denaturing at 94°C for one minute, annealing at 60°C for one minute 30 seconds, and extension at 72°C for two minutes 15 seconds. A final extension step of 72°C for seven minutes was also employed. Details of the primer pairs used are given in table 1. Direct sequencing of exon amplimers was achieved using an ABI Prism 377 automated DNA sequencer with ABI Prism Dye Terminator Cycle Sequencing reactions. Once putative mutations were found, parents and control populations were screened for these mutations using restriction enzyme digests. BanI was used to screen the exon 4 amplimer for
the mutation found in BH, while DelT was used to screen the exon 3 amplifier for the mutation found in RM. FokI was used to screen a novel amplifier (see table 1) for the mutation found in MS.

RESULTS

Plasma and urine bile acid analyses

Analysis of urine by LSIMS or ESI-MS/MS showed that, in all three patients, the largest bile acid peaks were consistent with a monohydroxy-oxo-cholenoic acid present as the glycine conjugate (m/z 444) and a dihydroxy-oxo-cholenoic acid present as the glycine conjugate (m/z 460) and the taurine conjugate (m/z 510) (fig 1). Peaks attributable to the glycine and taurine conjugates of chenodeoxycholic acid (448, 464, 498, and 514) were very small or undetectable above background. The sample from BH showed an additional prominent peak of mass/charge ratio 552, consistent with a taurine conjugated dihydroxy-oxo-cholenoic acid (fig 1). Analysis of plasma and urine samples from MS and BH by GC-MS following treatment with cholyglycine hydrolase confirmed that the major bile acids were 7α-hydroxy-3-oxo-4-cholenoic acid and 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (table 2). Chenodeoxycholic acid was undetectable in both plasma samples; cholic acid was absent from the plasma of MS and present in a trace amount in the plasma of BH. Analysis of plasma from MR by ESI-MS/MS indicated that mono- and di-hydroxy-oxocholenoic acids were the major bile acids; chenodeoxycholic acid and cholic acid concentrations were normal/low. (The true concentrations may have been lower than those shown in table 2; there is the potential for isomeric compounds such as allo bile acids to interfere with analysis of chenodeoxycholic acid and cholic acid by ESI-MS/MS.)

Analysis of the 5β-reductase gene (SRD5B1)

A point mutation or single base deletion was found in all three patients. The results of the sequencing and restriction enzyme digests are summarised in table 3. All three patients were homozygous for their mutation and all parents were heterozygotes, as was an unaffected sibling of BH. The point mutation found in MS (662 C>T) was not found in a control population of 100 chromosomes; 38 of these were from the Sardinian population and the remaining 62 were unspecified Caucasian samples. The mutations found in patients BH (511 delT) and RM (385 C>T) and were not found in 100 chromosomes from individuals from the Indian subcontinent.

DISCUSSION

Analysis of bile acids and alcohols in plasma and urine has become an essential part of the investigation of infants with cholestatic jaundice. Several characteristic cholanoid profiles have led to the discovery of mutations in genes encoding enzymes in the bile acid synthesis pathways. These include inborn errors affecting 3β-hydroxy-Δ5-4-3-oxosteroid 5β-reductase deficiency. Analysis of the human 5β-reductase cDNA sequence allowed Sumazaki et al to show that, in at least one infant with liver failure, the 5β-reductase mRNA had a normal sequence. This confirmed the suspicion that some children who excrete 3-oxo-Δ4 bile acids as the major urinary bile acids do not have genetic 5β-reductase deficiency. Patient MS had cholanoid profiles that showed almost complete absence of chenodeoxycholic acid and cholic acid. Her liver disease resolved completely on treatment with chenodeoxycholic acid plus cholic acid having failed to respond to ursodeoxycholic acid. This suggested strongly that she had mutations in the 5β-reductase gene. Sequencing of her genomic DNA showed that she was homozygous for a single base substitution 662 C>T. The base substitution abolishes the 5 base recognition sequence for the restriction enzyme FokI. Restriction fragment length analyses showed that the patient’s parents were heterozygous for the mutation and that it could not be detected in 100 ethnically matched genomic DNA samples, excluding the possibility that it was a

Table 1 Primer pairs for each exon of the 5β-reductase gene and the primer used to generate an amplimer suitable for restriction enzyme digest in patient MS

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>1</td>
<td>ggtggttaaatccctttcctcc</td>
<td>ggtggttaaatccctttcctcc</td>
</tr>
<tr>
<td>2</td>
<td>aagaaacaaacacgccagcaaggg</td>
<td>aagaaacaaacacgccagcaaggg</td>
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<td>3</td>
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<td>ggtggttaaatccctttcctcc</td>
</tr>
<tr>
<td>9</td>
<td>ggtggttaaatccctttcctcc</td>
<td>ggtggttaaatccctttcctcc</td>
</tr>
<tr>
<td>MS RE product</td>
<td>tgtttcttatcctcccttccc</td>
<td>tgtttcttatcctcccttccc</td>
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Figure 1 Negative ion liquid secondary ionisation mass spectrometry (LSIMS) analysis of a urine sample from patient BH. Identities of peaks (confirmed by gas chromatography-mass spectrometry): m/z 444 and 460, glycine conjugates of 7α-hydroxy-3-oxo-4-cholenoic acid and 7α,12α-dihydroxy-3-oxo-4-cholenoic acid; m/z 494, 510, and 552, taurine conjugates of 7α-hydroxy-3-oxo-4-cholenoic acid, 7α,12α-dihydroxy-3-oxo-4-cholenoic acid, and 7α,12α-dihydroxy-3-oxo-4-cholenoic acid.
Mutations in SRD5B1

common polymorphism in Caucasians. The 662 C>T mutation is a missense mutation that leads to the amino acid substitution Pro198Leu in the 5β-reductase protein. Comparison of the predicted secondary structure of 5β-reductase with two other NADPH binding proteins, glutathione reductase and NADPH-cytochrome P-450 reductase, suggests that proline 198 is in the NADPH binding domain of the 5β-reductase protein. Substitution of a leucine residue for the proline residue can be predicted to alter the secondary structure of this domain. Thus even if the protein is translated normally and escapes quality control processes, the mutant enzyme is unlikely to bind NADPH normally and is therefore likely to be catalytically inactive.

Patient BH, like MS, had cholanoid profiles that showed almost undetectable amounts of chenodeoxycholic acid and cholic acid. He also showed an initial response to treatment with chenodeoxycholic acid and cholic acid, although a drop in chenodeoxycholic acid and cholic acid that were very low for an infant with cholestasis; mutations showed amounts of chenodeoxycholic acid and cholic acid were apparently higher than expected. Failure to synthesise chenodeoxycholic acid and cholic acid are seen in other defects of bile acid synthesis (for example, progressive familial intrahepatic cholestasis type II due to mutations in the gene encoding the canaliculi there is reduced production of the soluble form lining the biliary system. Thus if bile acids are not entering the canaliculi and exert a detergent effect on the membranes released from the microvilli when bile acids are secreted into hepatocytes and bile duct epithelial cells. It is probably only normally located on microvilli at the canalicular surface of hepatocytes and bile duct epithelial cells.

Table 2  Plasma concentrations of chenodeoxycholic acid and cholic acid and their 3-oxo-Δ4 analogues in the plasma of children with mutations in the SRD5B1 gene causing Δ5-3-oxosteroid 5β-reductase deficiency

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>Plasma concentration (μmol/l)</th>
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<tbody>
<tr>
<td></td>
<td>Patient MS* (age 8 months)</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>0.26</td>
</tr>
<tr>
<td>7α-hydroxy-3-oxo-4-choleenoic acid</td>
<td>1.9</td>
</tr>
<tr>
<td>7α,12β-dihydroxy-3-oxo-4-choleenoic acid</td>
<td>2.1</td>
</tr>
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Table 3  Summary of the mutations found on DNA sequencing of the 5β-reductase gene and subsequent restriction digest of parental/sibling DNA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sequencing</th>
<th>Restriction enzyme digest</th>
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<tbody>
<tr>
<td>MS</td>
<td>662 C&gt;T missense mutation causing a Pro198Leu amino acid substitution</td>
<td>Parents heterozygous for the 662 C&gt;T missense mutation</td>
</tr>
<tr>
<td>BH</td>
<td>511T deletion causing frame shift and a premature stop codon in exon 3</td>
<td>Parents and unaffected sibling heterozygous for the 511T deletion</td>
</tr>
<tr>
<td>RM</td>
<td>385 C&gt;T missense mutation causing a Leu106Phe amino acid substitution</td>
<td>Parents heterozygous for the 385 C&gt;T missense mutation</td>
</tr>
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</table>
concentrations of these bile acids with consequent failure to activate the farnesoid X receptor (FXR) and hence switch on the bile acid export pump (BSEP) and the canicular conjugated bilirubin transporter (MRP2). Failure to activate MRP2 may contribute to the jaundice in these patients and also to poor excretion of HIDA.

There are some significant differences between the biochemical findings in these three patients and other putative cases of 5β-reductase deficiency. Thus the cases reported by Setchell and colleagues had elevated plasma concentrations of cheno-deoxycholic acid and a high ratio of cheno-deoxycholic acid to cholic acid. Other cases reported by Kummer et al had increased concentrations of total plasma bile acids (method of assay and cheno-deoxycholic acid/cholic acid ratio not specified). Our own experience is that many infants with severe liver disease (and some infants with milder disease) show this pattern of high plasma cheno-deoxycholic acid, high cheno/cholic ratio, and moderate 3-oxo-4 bile aciduria—it is much commoner than the profiles described above for patients with proven SRD5B1 mutations. There are four possible explanations for a cholanoïd profile characterised by increased excretion of 3-oxo-Δ4 bile acids in urine and a high plasma cheno-deoxycholic acid concentration.

These patients have SRD5B1 mutations that leave some residual 5β-reductase activity. Some cheno-deoxycholic acid is synthesised and inhibition of BSEP by 3-oxo-Δ4 bile acids causes reflux of bile acids into the plasma leading to elevated plasma concentrations.

These patients do not have a genetic defect in the SRD5B1 gene but rather secondary changes in the liver lead to inactivation of the 5β-reductase enzyme. The 5β-reductase protein may be unstable; certainly early attempts at purification proved problematical. It may be prone to chemical attack. Zhu et al showed that it can form adducts with acetaldehyde in vivo.

There are no mutations in the SRD5B1 gene but the amount of mRNA in liver cells is reduced. mRNA may be unstable. Kondo et al showed that the 3′ untranslated region of the 5β-reductase mRNA contained multiple AU rich elements. Such AUREs are associated with rapidly degrading mRNAs such as those encoding cytokines and lymphokines. It is also possible that expression of SRD5B1 is regulated by transcription factors as is the case with other key enzymes in oxysterol metabolism and bile acid synthesis (see below).

These patients have persistence of, or reversion to, the fetal pattern of bile acid metabolism. The major pathway of bile acid synthesis in the fetus probably starts with the production of 27-hydroxycholesterol and produces mainly cheno-deoxycholic acid. This is the predominant bile acid in the fetus; however, significant amounts of 7α-hydroxy-3-oxo-4-cholenoic acid are also produced. BSEP is probably inactive in fetal life and it is therefore likely that a substantial proportion of the cheno-deoxycholic acid (and 7α-hydroxy-3-oxo-4-cholenoic acid) synthesised in the hepatocyte enter the blood stream. Increased cholic acid synthesis and activation of the BSEP normally occur at the time of birth; if this activation does not occur and the fetal pattern of bile acid metabolism persists, one would predict high plasma cheno-deoxycholic acid, high cheno/cholic ratio, and 7α-hydroxy-3-oxo-4-cholenoic acid in plasma. It is becoming clear that regulation of bile acid synthesis and secretion is controlled by nuclear receptors such as FXR, and to a lesser extent by the liver X receptors and the pregnane X receptor/steroid and xenobiotic receptor. Recent studies suggest that full activation of the BSEP might require the natural analogue of guggulsterone as well as cheno-deoxycholic acid (a known agonist at the FXR receptor). It is possible therefore that persistence of a fetal pattern of bile acid synthesis and secretion is caused by failure of activation of transcription factors (including FXR) by the natural analogue of guggulsterone.

Further work is required to establish whether patients with 3-oxo-Δ4 bile aciduria and elevated plasma cheno-deoxycholic acid concentrations have mutations in the SRD5B1 gene or whether they have other gene defects or non-genetic liver diseases that lead to secondary reduction in 5β-reductase enzyme activity. This is of more than academic interest. Treatment with cheno-deoxycholic acid and cholic acid was extremely effective in one of our patients with high plasma cheno-deoxycholic acid concentrations, the combination of urso-deoxycholic acid and cholic acid appeared to be more effective. In the future, nuclear receptor agonists may have a role in carefully defined patients. Based on our current knowledge of 5β-reductase mutations, a suggested algorithm for diagnosis of genetic 5β-reductase deficiency is shown in fig 2.

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**Figure 2** Suggested algorithm for distinguishing between primary genetic 5β-reductase deficiency and other cause of 3-oxo-Δ4 bile aciduria. ESI-MS/MS, electrospray ionisation tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry.

**Table 1** Cholestatic liver disease in 1st year of life

- Urine analysis by FAB-MS or ESI-MS/MS
  - nvl 444>448, 460>464, 494>498, and 510>514
  - Y N Look for other causes of liver damage
- Plasma bile acid analysis by GC-MS
  - Major bile acids are 7α-OH-3-oxo-4-cholanic acid and 7α,12α-diol-3-oxo-4-cholanic acid
  - Chenodeoxycholic acid <2 μM
  - Y N Look for other causes of liver damage
- Base changes detected, polymorphisms excluded
- Sequence SRD5B1
  - Y N Look for other causes of liver damage
- Primary genetic 5β-reductase deficiency
Mutations in SRD5B1

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