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

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\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\(\rightarrow\)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\(\rightarrow\)T) causing a Leu129Phe 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.

In 1988, Clayton \textit{et al} and Setchell \textit{et al} reported that, in some infants with liver disease, the major urinary bile acids were 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.\(^1\)\(^2\) This suggested reduced activity of the enzyme responsible for saturating the \(\Delta^4\) double bond during the synthesis of bile acids from cholesterol (that is, \(\Delta^4\)-3-oxosteroid 5\(\beta\)-reductase deficiency). However, it was not clear which, if any, of these patients had defects in the gene encoding the 5\(\beta\)-reductase enzyme. Some clearly had a recognised cause of liver disease such as tyrosinaemia or hepatitis B,\(^3\) suggesting that their “5\(\beta\)-reductase deficiency” was secondary to liver damage rather than a primary genetic defect. However, Shneider \textit{et al} described excretion of 3-oxo-\(\Delta^4\) bile acids as the major urinary bile acids in two unrelated infants with features of neonatal haemochromatosis and they suggested that 5\(\beta\)-reductase deficiency was the cause of neonatal haemochromatosis.\(^3\)

In 1994, Kondo \textit{et al} reported the cDNA sequence for human 5\(\beta\)-reductase.\(^4\) Sumazaki \textit{et al} used this information to investigate the possibility of 5\(\beta\)-reductase deficiency in a Japanese neonate with liver failure and 3-oxo-\(\Delta^4\) bile aciduria.\(^5\) Using liver tissue, they sequenced the entire cDNA for the enzyme and showed that it was normal, effectively excluding genetic 5\(\beta\)-reductase deficiency. Meanwhile, Clayton \textit{et al} described a cholestatic infant in whom the presence of large amounts of 3-oxo-\(\Delta^4\) bile acids in plasma and urine was associated with low concentrations of chenodeoxycholic acid and cholic acid.\(^6\) This infant had failed to improve on ursodeoxycholic acid therapy but liver function returned to normal on replacement of the natural primary bile acids, chenodeoxycholic acid and cholic acid. It was argued that this girl probably did have a primary genetic 5\(\beta\)-reductase deficiency. This paper describes the identification of a homozygous missense mutation in her 5\(\beta\)-reductase gene and identification of different homozygous mutations in the 5\(\beta\)-reductase genes of two other infants, both of whom had liver disease severe enough to require transplantation.

\textbf{PATIENTS AND METHODS}

All studies were undertaken with permission from the Research Ethics Committee of Great Ormond Street Hospital/Institute of Child Health.
Patient MS
This is the Sardinian girl described previously. 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 steatorrhea, 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, steatorrhea, and fat soluble vitamin malabsorption continued despite ursodeoxycholic acid treatment. At eight months her bilirubin was still elevated (88 μM; conjugated 35 μM), as were her transaminases (AST 511 U/l, ALT 252 U/l) but γ-glutamyl transpeptidase (γ-GT) was normal (36 U/l). Clotting times were normal but vitamin E was low at 4.8 μM (normal 11.5–35) despite oral supplements. The liver still showed a giant cell hepatitis with steatisis. Treatment with 8 mg/kg/day of chenodeoxycholic acid and 8 mg/kg/day of cholic acid led to normalisation of liver function within three months and she remains well with normal liver function tests at the age of nine years (on bile acid replacement therapy).

Patient BH
BH is the first child of healthy Pakistani parents who are first cousins. On the first day of life he became jaundiced with dark urine and yellow stools. Cholestatic jaundice persisted and at the age of eight weeks he was found to have a liver edge palpable 3 cm from the right costal margin and a spleen tip palpable 1 cm from the left costal margin. Total bilirubin was 160 μmol/l (conjugated 82 μmol/l); ALT 333 U/l (normal 5–45); γ-GT normal; alkaline phosphatase (ALP) 842 U/l (65–265); albumin 35 g/l; cholesterol 4.6 mM; vitamin A 97 μg/l (200–430); 25-hydroxy-vitamin D3 15 nmol/l (15–100); vitamin E 8.1 μmol/l (11.5–35); prothrombin time 28 seconds (12–16); activated partial thromboplastin time (APTT) 75 seconds (29–40); and thrombin time 14.1 seconds (8–12). Abdominal ultrasound showed normal hepatic echotexture and a spleen of 6.4 cm (<6.0); the gall bladder could not be seen. A HIDA scan showed no excretion of isotope into the gut. A liver biopsy showed preserved architecture. Portal tracts showed bile duct proliferation but no cholangiolar bile plugging. The parenchymal cells showed marked giant cell transformation with intracellular bile pigment accumulation and occasional single necrotic liver cells. The appearances were of a giant cell hepatitis but there was also marked extramedullary haemopoiesis. Investigations for known causes of hepatitis of infancy were negative except for the bile acid abnormalities described below. Ultrasound of the abdomen showed a normal sized liver and spleen, a small gall bladder, and some irregularity in liver density. A liver biopsy revealed a giant cell hepatitis, no increase in bile ducts, and no signs of copper or iron accumulation. Increased extramedullary haemopoiesis was noted.

By the age of six weeks, liver function had deteriorated significantly (international normalised ratio INR 8.0, 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. 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 glycine conjugates and a “parents of m/z 80” scan for taurine conjugates. Analyses of (non-sulphated) plasma and urine bile acids by gas chromatography-mass spectrometry (GC-MS) were undertaken using established methods.

Analysis of the 5β-reductase gene (SRD5B1)
Initial genetic studies of 5β-reductase focused on the characterisation of cDNA from liver biopsy samples. Subsequent to the determination of the genomic sequence of SRD5B1, 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 amplifier for
and BH by GC-MS following treatment with cholyglycine hydroxylase 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). Chenoxycholic 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; chenoxycholic 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 alfa bile acids to interfere with analysis of chenoxycholic acid and cholic acid by ESI-MS/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 448) and the taurine conjugate (m/z 464) and a dihydroxy-oxo-cholenoic acid as the glycine conjugate (m/z 494) and the taurine conjugate (m/z 510) (fig 1). Peaks attributable to the glycine and taurine conjugates of chenodeoxycholic acid and cholic acid (m/z 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 hydroxylase 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). Chenoxycholic 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; chenoxycholic 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 alfa bile acids to interfere with analysis of chenoxycholic acid and cholic acid by ESI-MS/MS.)

**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-Δ5-7-27-sterol dehydrogenase/isormerase,12 13 oxysterol 7α-hydroxylase,14 sterol 27-hydroxylase,15 16 and α-methyl-acyl-CoA racemase.17 18 Cholanoid analyses have also helped to define gene defects that affect peroxisome biogenesis7 9 or peroxisomal β-oxidation enzymes;19–21 in most of these disorders neurological symptoms dominate the clinical picture.22 23

To date, it has proved difficult to establish that infants with cholestatic liver disease and a cholanoid profile characterised by excretion of 3-oxo-Δ4 bile acids have a genetic deficiency of Δ5-3-oxosteroid 5β-reductase. In some infants, the liver biopsy showed absence of 5β-reductase mRNA in a labile protein or a truncated protein and this was taken as evidence of a genetic deficiency.1 However, the possibility of a labile mRNA or protein could not be excluded.

Determination of the human 5β-reductase cDNA sequence4 allowed Sumazaki et al to show that, in at least one infant with liver failure, the 5β-reductase mRNA had a normal sequence.5 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 chenoxycholic acid and cholic acid. Her liver disease resolved completely on treatment with chenoxycholic 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

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**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

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**Figure 1** Negative ion liquid secondary ionisation mass spectrometry (LSIMS) analyses 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.7 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 time when transaminases were considerably elevated. This phenomenon, as well as evidence of malabsorption of fat and fat soluble vitamins, is seen in other defects of bile acid synthesis (for example, progressive familial intrahepatic cholestasis type II) due to mutations in the gene encoding the bile acid export pump (BSEP). The reason for the low γ-GT in these patients is not certain. It is known that γ-GT is normally located on microvilli at the canalicular surface of hepatocytes and bile duct epithelial cells. It is probably only released from the microvilli when bile acids are secreted into the canalicular and exert a detergent effect on the membranes lining the biliary system. Thus if bile acids are not entering the canalicular there is reduced production of the soluble form of γ-GT that normally accumulates in the circulation in response to damage to bile ducts and hepatocytes.

The bile acid profiles in all three patients with SRD5B1 mutations showed amounts of chenodeoxycholic acid and cholic acid that were very low for an infant with cholestasis; this is consistent with the notion that Δ3,3-oxosteroiA 5β-reductase is required for all biosynthetic pathways for the major primary bile acids. Failure to synthesise chenodeoxycholic acid and cholic acid may lead to low hepatocyte

<table>
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<th>Patient</th>
<th>Sequencing</th>
<th>Restriction enzyme digest</th>
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<td>MS 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>
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<tr>
<td>BH 511T deletion causing frame shift and a premature stop codon in exon 5</td>
<td>Parents and unaffected sibling heterozygous for the 511T deletion</td>
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<td>RM 385 C&gt;T missense mutation causing a Leu106Phf amino acid substitution</td>
<td>Parents heterozygous for the 385 C&gt;T missense mutation</td>
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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 canalicular 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 and Kimura and colleagues had elevated plasma concentrations of chenodeoxycholic acid and a high ratio of chenodeoxycholic acid to cholic acid. Other cases reported by Kimura et al had increased concentrations of total plasma bile acids (method of assay and chenodeoxycholic 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 chenodeoxycholic acid, high cheno/cholic ratio, and moderate 3-oxo-Δ4 bile aciduria—it is much commoner than the profiles described above for patients with proven SRD5B1mutations. There are four possible explanations for a cholanoid profile characterised by increased excretion of 3-oxo-Δ4 bile acids in urine and a high plasma chenodeoxycholic acid concentration.

- These patients have SRD5B1 mutations that leave some residual 5β-reductase activity. Some chenodeoxycholic 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 chenodeoxycholic 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 chenodeoxycholic 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 chenodeoxycholic 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 chenodeoxycholic 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 chenodeoxycholic 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 chenodeoxycholic acid and cholic acid was extremely effective in one of our patients with SRD5B1 mutations (the only one for whom treatment was started when bilirubin was <200 μM, ALT was <600 U/l, and the post vitamin K prothrombin time was normal). In patients with high plasma chenodeoxycholic 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.

**ACKNOWLEDGEMENTS**

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