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Homozygosity Mapping Identifies a Bile Acid Biosynthetic Defect in an Adult with Cirrhosis of Unknown Etiology

Vered Molho-Pessach[1], **Jonathan J. Rios**[1], **Chao Xing**[1], **Kenneth D.R. Setchell**[4], **Jonathan C. Cohen**[2], and **Helen H. Hobbs**[1,2,3]

[1]McDermott Center for Human Growth and Development, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[2]Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[3]Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[4]Department of Pathology and Laboratory Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229 and the and Department of Pediatrics of the University of Cincinnati College of Medicine

Abstract

The most common inborn error of bile acid metabolism is 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (3β-HSD) deficiency, a disorder that usually presents in early childhood with hepatic dysfunction. Timely diagnosis of this disorder is crucial since it can be effectively treated with primary bile acid replacement. Here we describe a 24-year-old woman from Iran with cirrhosis of unknown etiology. Her sister and a first cousin died of cirrhosis (ages 19 and 6 years) and another 32-year old first cousin had a self-limited liver disorder in childhood that resolved at age 9 years. The family history was consistent with the notion that affected family members were homozygous for a mutant allele inherited identical-by-descent. A genome-wide analysis of 2.5 million single nucleotide polymorphisms (SNP) was performed to identify regions of homozygosity that were present in the proband and the 32-year old first cousin, but not in a healthy relative. One of these regions contained the gene encoding 3β-HSD (*HSD3B7*). Sequence analysis of *HSD3B7* revealed that the proband and her 32-year old cousin were homozygous for a frame shift mutation (c.45_46del AG, p.T15Tfsx27) in exon 1. The diagnosis of 3β-HSD deficiency was confirmed by documenting high levels of 3β-hydroxy-Δ^5 bile acids in the serum of the first cousin using mass spectrometry. To our knowledge, the 32-year old relative in this family represents the oldest asymptomatic patient with this disorder. *Conclusion*: This study highlights the clinical utility of homozygosity mapping in diagnosing autosomal recessive metabolic disorders. This family illustrates the wide variation in expressivity that occurs in 3β-HSD deficiency and underscores the need to consider a bile acid synthetic defect as a possible cause of liver disease in adults.

Keywords

HSD3B7; consanguinity; cholestasis; inborn error; cholic acid

Contact information: Helen H. Hobbs, Department of Molecular Genetics, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9046, USA. helen.hobbs@utsouthwestern.edu.

Bile acids are synthesized in the liver from cholesterol by a complex series of enzymatic reactions (1). The rate-limiting step in the pathway is the addition of a hydroxyl group to the carbon at the 7 position (C7) of the sterol ring, a reaction catalyzed by the enzyme cholesterol 7α-hydroxylase (CYP7A1). This is followed by isomerization of the Δ^5 bond to the Δ^4 position and oxidation of the 3β-hydroxyl group to a 3-oxo moiety. Both of these reactions are catalyzed by 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (3β-HSD), a 369 amino acid membrane-bound protein in the endoplasmic reticulum that is encoded by *HSD3B7* (2).

Mutations in *HSD3B7* cause 3β-HSD deficiency (#OMIM 607765) (3), a rare autosomal recessive disorder that represents the most common inborn error of bile acid synthesis (2, 4). The disorder was originally described by Clayton *et al.* (5) in 1987. The diagnosis was established by measuring bile acids in the urine using mass spectrometry in a consanguineous Saudi Arabian family in which several family members presented as neonates with giant cell hepatitis and bridging cirrhosis (5). Over 50 patients with this disorder from at least 40 unrelated families have subsequently been reported (3, 6–16). *HSD3B7* was cloned by Schwarz *et al.* in 2000 (17) and 21 different mutations have been described that cause 3β-HSD deficiency (3, 6, 7, 12, 13).

3β-HSD deficiency usually presents in the neonatal period or in early childhood with cholestatic jaundice, hepatosplenomegaly, steatorrhea, rickets, bleeding and failure to thrive (2). The disease invariably progresses to cirrhosis. In a few cases of late-onset 3β-HSD deficiency, the disease presents in the second or third decades of life (3, 6, 18). Typical clinical features that distinguish 3β-HSD deficiency from other causes of early-onset liver disease include the absence of pruritus, a normal serum level of gamma glutamyl transpeptidase (GGT), and normal or low levels of total serum primary bile acids when measured by routine methods, despite the presence of cholestasis (2, 7, 19, 20). The diagnosis is established by finding high levels of sulfated 3β,7α-dihydroxy-5-cholenoic and 3β,7α-trihydroxy-5-cholenoic acids in the urine or serum (2, 5, 20).

Here, we describe an Arab-Iranian family in which several individuals developed cirrhosis of unknown etiology. Homozygosity mapping was used to identify homozygous regions of genomic DNA that were shared by 2 affected cousins but not by an unaffected family member. The two affected individuals in the family that were available for sampling were homozygous for an inactivating mutation in *HSD3B7*. The family is remarkable for the variability in age of onset and clinical severity of the disease among affected members.

PATIENTS AND METHODS

Human Subjects

The study protocol was approved by the UT Southwestern Institutional Review Board. Fasting blood samples were collected after written informed consent was obtained. Plasma and serum were isolated, aliquoted and stored at −80°C. Fasting serum levels of glucose, lipids/lipoproteins and liver enzymes were measured using an automated analyzer and genomic DNA was extracted from blood using an AutopureLS DNA Extractor (Qiagen, Germantown, MD).

Homozygosity Mapping and Mutation Detection

To detect candidate genomic regions of extended homozygosity, DNA from the proband (III.14), an affected first cousin (III.5) and an unaffected first cousin (III.6) was assayed for 2.4 million SNPs using the HumanOmni 2.5BeadChip microarray (Illumina, San Diego, CA). Briefly, genomic DNA was denatured and amplified overnight at 37°C. The amplified DNA was enzymatically fragmented and then incubated overnight at 48°C with the

BeadChip containing locus-specific 50-mer probes. The array was then washed and a single-base extension reaction was performed using labeled nucleotides to extend the captured DNA template. The BeadChip was imaged using the iScan system and visualized using GenomeStudio software (v2010.2).

The genotypes from the microarrays were exported from GenomeStudio and analyzed using Partek Genomics Suite software (Partek, St. Louis, MO). All samples were successfully genotyped for >99.4 % of all SNPs. Genotypes were analyzed using a Hidden Markov Model to identify extended regions of homozygosity. Homozygosity was compared between the samples using custom Perl scripts.

Sanger DNA Sequencing of HSD3B7

The 9 exons of *HSD3B7* were amplified by PCR from genomic DNA of the proband using flanking oligonucleotides exactly as described (3).

Fast Atom Bombardment Mass Spectrometry

Negative ion fast atom bombardment mass spectrometry was used to analyze the bile acids in the serum exactly as previously described (21).

Results

Cirrhosis of Unknown Etiology

A 24-year-old Arab woman (III.14) from the southwestern region of Iran (Khuzestan) presented with cirrhosis of unknown etiology complicated by portal hypertension, varices, ascites and hypersplenism. The patient had a history of jaundice, abdominal swelling and recurrent episodes of mucocutaneous bleeding since age 6. Her younger sister (III.16) developed liver disease in her early teens and died of cirrhosis at age 19 (Fig. 1). A first cousin (III.1) died of liver disease at age 6 and her sister, a 32 year old reportedly healthy woman (III.5), had self-limited jaundice and abdominal swelling as a child that fully resolved by age 9.

On physical examination the proband had jaundice, multiple echymoses, splenomegaly and mild pedal edema. Laboratory evaluation revealed mildly elevated levels of aspartate aminotransferase (AST), (67 IU/L, normal range 13 – 40 IU/L), alanine aminotransferase (ALT) (50 IU/L, normal range, 10 – 40 IU/L), alkaline phosphatase (ALKP) (153 IU/L, normal range 38 – 126 IU/L), and normal GGT levels (14 IU/L, normal range 4 – 63 IU/L). Her serum bilirubin was 1.8 mg/dL (normal range, 0.2 – 1.3 mg/dL) with a direct bilirubin of 1.3 mg/dL (normal range, 0.0 – 0.3 mg/dL). Her prothrombin international normalized ratio was increased (INR 2.0, normal range 0.8–1.2) and serum albumin level was reduced (3 g/dL, normal range 3.4–5.4 g/dL). Abdominal computerized tomography showed a small nodular liver, numerous splenic and gastroesophageal varices and marked splenomegaly (spleen span of 24 cm). Liver biopsy revealed extensive bridging fibrosis with abnormal ducts encircling parenchymal nodules. Laboratory evaluation was negative for Wilson disease, hemochromatosis and α_1 anti-trypsin deficiency as well as for viral or autoimmune hepatitis. She denied any history of alcohol abuse.

Blood samples were collected from the 13 family members who were available for study (Fig. 1). The proband's parents (II.10 and II.11) were first cousins and two of her paternal uncles (II.2 and II.4) married first cousins. Two brothers (II.4 and II.10) had married two sisters (II.5 and II.11) in the family. The 32-year old offspring of a paternal uncle (III.5) had been diagnosed with liver disease in childhood but was subsequently asymptomatic and had

normal serum levels of hepatic enzymes (AST=21 IU/L, ALT=30 IU/L, ALKP=67 IU/L) and bilirubin (total, 0.9 mg/dL; direct, 0.3 mg/dL) at the time of this study.

Homozygosity Mapping and Mutation Detection

The inheritance pattern of liver disease in the family was most consistent with an autosomal recessive disorder. Given the high level of consanguinity in the family, we hypothesized that the affected family members were homozygous for a mutation inherited identical-by-descent from a common ancestor.

Genotype analysis revealed extensive homozygosity in all three family members, including single regions encompassing 63% and 78% of chromosomes 10 and 19, respectively, in the affected first cousin (III.5). We focused on those runs of homozygosity (ROH) that were > 3 Mb since regions of this length are uncommon in the general population (22) (Fig. 2). Candidate regions were further refined by identifying those ROH that were shared by both affected patients but not by the unaffected family member. The resulting candidate regions totaled 36.5 Mb or 1.2% of the genome (Fig. 2).

A 6.3 Mb region of homozygosity on chromosome 16 (25,073063–31,378235) that was shared by the proband and her affected cousin but not with the unaffected cousin harbored an excellent candidate gene, *HSD3B7*. We PCR amplified and sequenced the coding region of *HSD3B7* in the proband using flanking oligonucleotides to amplify each of the 9 exons of the gene (3). The proband and her affected first cousin (III.5) were both homozygous for a 2-basepair deletion in exon 1 of *HSD3B7* (c.45_46del AG, p.T15Tfsx27) that was not present in the unaffected cousin. Exon 1 of *HSD3B7* was then sequenced in the other family members. Both parents of the affected first cousin (III.5) were heterozygous for the mutation. The proband's parents were not available for sampling but four of their siblings were heterozygous for the mutation (II.2, II.4, II.7 and II.14) (Fig. 1).

Biochemical Confirmation of a Genetic Defect in Bile Acid Synthesis

We confirmed the diagnosis of 3β-HSD deficiency by using negative ion fast atom bombardment mass spectrometry (21) to analyze the bile acids in the serum of family member III.5. The results definitively established a defect in bile acid synthesis consistent with a deficiency in the activity of 3β -HSD (formally called 3β -hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase). The negative ion mass spectrum of the serum (Fig. 3) was remarkable in revealing the presence of ions consistent with an array of atypical bile acids not normally detected in serum by FAB-MS. The triplets of ions at *m/z* 453, 469 and 485 (sulfate conjugates) and m/z 510, 526 and 542 (glyco-sulfate conjugates) are characteristic of monohydroxy-, dihydroxy- and trihydroxy- bile acids, respectively, with a structural feature of a 3β -hydroxy-Δ^5 structure (i.e. unsaturated C_{24} bile acids), respectively, and these are signature metabolites for this genetic defect in bile acid synthesis (2, 21). There was a complete absence of the glycine and taurine conjugates of the primary bile acids of cholic (m/z 498 and 514) and chenodeoxycholic acids (*m/z* 448 and 464), typically observed in patients with cholestasis when bile acid synthesis is intact.

Family member III.5 was referred to a hepatologist to commence treatment with bile acids.

Discussion

Here we report the identification of a family with 3β-HSD deficiency in which affected individuals show striking phenotypic variability. The proband had chronic liver disease since childhood, but survived without medical care into her early 20s and then died at age 24. Her paternal first cousin (III.1) died at age 6 years of liver disease. The sister of III.1 (III. 5) had an apparently self-limited liver disorder in childhood that was severe enough to

require multiple hospitalizations and yet she has been asymptomatic for the last 22 years. We confirmed that she was homozygous for a null allele of *HSD3B7*, yet her liver function tests were normal at age 32 years. The lack of 3β-HSD activity was biochemically confirmed by FAB-MS analysis of the serum. This 32 year old family member represents the oldest patient yet diagnosed with 3β-HSD deficiency and the only adult with the disorder who does not have symptoms. The disorder can present in older children and teenagers, typically with mild elevations in transaminases, fat-soluble vitamin malabsorption and sometimes with rickets that resolves with vitamin supplementation; the disease then presents later with hepatosplenomegaly (2). To our knowledge, only one other patient with 3β-HSD deficiency was diagnosed as an adult; he had neonatal cholestasis and rickets in childhood and presented again at age 26 with cholestasis (23).

The clinical features of 3β-HSD deficiency are not easy to distinguish from those of other inherited disorders of bile acid synthesis or transport (2).. Currently, the diagnosis is dependent on the measurement of bile acids in the urine using mass spectrometry (21). Patients with 3β-HSD deficiency accumulate 3β-hydroxy-Δ^5 bile acids that are reduced in mass by two Daltons from normal saturated bile acids, indicating the presence of a double-bond. Most of these abnormal bile acids are preferentially sulfated at the 3β-hydroxy group and are conjugated in the side-chain with glycine, but not with taurine (5). In the absence of a urine sample from individual III.5, we confirmed the deficiency in 3β-HSD activity by analyzing an extract of her serum. It is usually difficult to detect bile acids by FAB-MS of serum unless a patient has significant cholestasis (24), so the presence of atypical 3β-hydroxy-Δ^5-C_{24}- bile acids in serum not only established definitively this genetic defect in bile acid synthesis but also the presence of cholestasis, despite the patient having normal serum liver function tests. These atypical bile acids are cholestatic and hepatotoxic (25) and in the absence of normal primary bile acids their continued synthesis leads to progressive cholestatic liver disease (2).

Liver injury in 3β-HSD deficiency is the result of a lack of normal primary bile acids that are required to stimulate bile flow, combined with the presence of increased production of 3β-hydroxy-Δ^5- bile acids that accumulate due to the enzyme defect (26). Replacement therapy with oral administration of the primary bile acid, cholic acid reduces the levels of 3β-hydroxy-Δ^5- bile acids through negative feedback inhibition on endogenous bile acid synthesis and this leads to a normalization of the clinical symptoms, liver function tests, and liver histology if initiated prior to development of significant cirrhosis (3, 7, 10, 14, 18, 19). Prolonged treatment with cholic acid (>15 years) is both safe and efficacious (7, 18, 21).

3β-HSD deficiency shows variable expressivity and pleiotropy, but the absence of symptoms in a 32 year old is remarkable. Other clinically asymptomatic individuals have been identified in the course of screening families of patients with 3β-HSD deficiency, but all were significantly younger than this patient. In one family, mass spectrometry of urinary bile acids revealed asymptomatic 3β-HSD deficiency in two of the probands siblings, ages 2 months and 3 years; both siblings had normal serum levels of liver enzymes, although one was vitamin D deficient (15).

Patients with 3β-HSD deficiency differ in presentation. Some patients present with signs of liver disease (jaundice, hepatosplenomegaly), others with fat soluble vitamin deficiencies (hypocalcemia, rickets, coagulopathy) or fat malabsorption as a result of cholestasis, or a combination of these features (2, 3, 6–16, 18). The proband in our family did not have clinical evidence of cholestasis at presentation, although her bilirubin level was mildly elevated. Although she did not report symptoms consistent with fat malabsorption, she had a history of recurrent mucocutaneous bleeding since childhood which was likely caused by vitamin K deficiency due to cholestasis.

The mechanism responsible for the phenotypic variability in 3β-HSD deficiency remains unknown. One possibility is functional redundancy, such that another enzyme compensates for the loss of 3β-HSD activity. Differences in the ability to metabolize the hepatotoxic and cholestatic bile acids, possibly by intestinal bacterial flora or by other endogenous pathways, could also contribute to the wide variability in expression of this disorder. Finally, individuals may differ in the rate of excretion of the toxic bile acids due to differences in the rate of secretion or efficiency of reabsorption of bile acids that enter the biliary enterohepatic circulation. None of these possibilities explain the mild phenotype in our patient since she had no detectable primary bile acids and the levels of abnormal 3β-hydroxy-Δ^5 bile acids in her serum were comparable to those seen in other patients with clinically severe disease.

The c.45–46del AG mutation in *HSD3B7* identified in this family was previously found in two unrelated families of British and Canadian origin (3) and in a French-Senegalese patient with 3β-HSD deficiency (7). No haplotype data are available to determine if the mutation is a new or recurrent mutation, but the presence of the same mutation in patients of diverse ethnicities implies that this may be a mutational hot spot. Patients carrying this mutation do not show any distinguishing phenotypic features and the age at presentation varies from a few months to 13.5 years. Genotype-phenotype correlation has not been demonstrated for any of the other 20 mutations reported in *HSD3B7*.

It is essential to establish the diagnosis of 3β-HSD deficiency since this is a treatable disorder. Patient III.5 is an ideal candidate for oral cholic acid therapy, which can be expected to lead to a resolution of cholestasis, a suppression of the atypical bile acids by feedback inhibition on hepatic bile acids synthesis, and a concomitant clinical improvement; initiation of oral cholic acid therapy in most cases results in a striking reversal of the histological hallmarks of the disease, even at relatively advanced stages (7–9). Dramatic responses to treatment were seen even among patients with severe cholestasis and marked lobular and septal portal fibrosis on liver biopsy (7).

Here we used homozygosity mapping with single nucleotide polymorphism (SNP) microarray genotyping as an initial genetic test to pinpoint the causative mutation in this family. With the increasing use of SNP microarrays for whole genome scanning, homozygosity mapping has become easy and rapid. This approach is particularly powerful in situations where there is an increased likelihood of inheriting two alleles identical-by-descent, such as consanguinity or inbreeding. Although the likelihood of homozygosity is smaller in outbred populations, homozygosity mapping has become easy and rapid and widely available through the use of SNP microarrays for routine cytogenetic analysis.

The extent of homozygosity found in this family confirmed a high degree of consanguinity. Offspring of first cousins are expected to be homozygous for ~ 6% (or 1/16th) of their genome. However, in populations with a history of consanguineous matings, the proportion of the genome that is homozygous can reach 11% when considering only homozygous regions that are associated with recessive disease, that is, greater than 3 cM (27). In this family, individuals III.5, III.6 and III.14, whose DNA was used for homozygosity mapping, were offspring of first cousins. Their genome homozygosity associated with recessive disease was estimated to be 21%, 9.5% and 10%, respectively, indicating that consanguinity was practiced for generations in this family.

An alternative genetic approach that could have been used to identify the causative gene in this family is whole exome (or whole genome) sequencing (28). This approach has the potential added advantage of revealing modifier genes that contribute to the phenotypic variability of this disorder. Unfortunately, it is unlikely that exome sequencing would have

been helpful in identifying modifier genes contributing to the phenotypic variability in this family, given the small number of affected individuals (n=2) available for study and the large number of sequence variations found in genomes. A large scale sequencing study that includes large numbers of carefully phenotyped patients with 3β-HSD deficiency may provide the opportunity to identify modifier genes for this disorder.

In conclusion, we present here a highly consanguineous Arab-Iranian pedigree with four individuals suffering from 3β-HSD deficiency, caused by a recurrent mutation. The clinical presentation was extremely variable with both prolonged asymptomatic and fatal course occurring in the different affected family members. Increased awareness of possible 3β-HSD deficiency in clinical evaluation of cirrhosis in young adults, as well as in children, is essential, since this condition has an excellent prognosis with primary bile acid treatment.

Acknowledgments

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Abbreviations

ALT	alanine aminotransferase
ALKP	alkaline phosphatase
AST	aspartate aminotransferase
CT	computerized tomography
FAB-MS	fast atom bombardment ionization mass spectrometry
GGT	gamma glutamyl transferase

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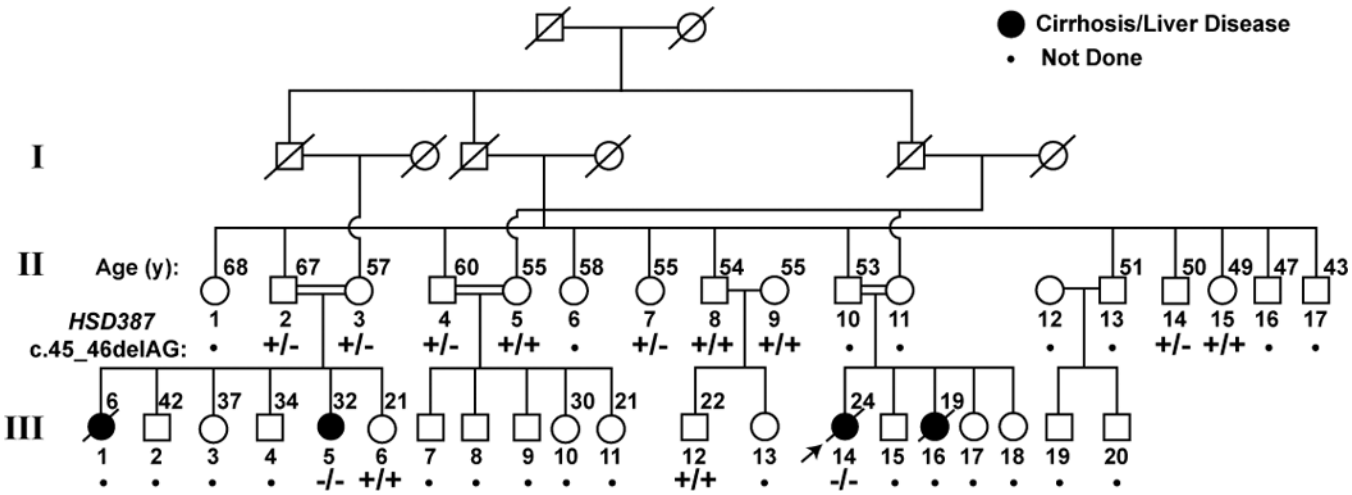

Fig. 1.

Pedigree of Arab-Iranian family with 3β-HSD deficiency. Generations are designated by Roman numerals and each individual within a generation is designated by a number. The arrow indicates the proband. A point denotes individuals for whom we were unable to obtain DNA. Known ages (in years) are at the upper right of each individual.

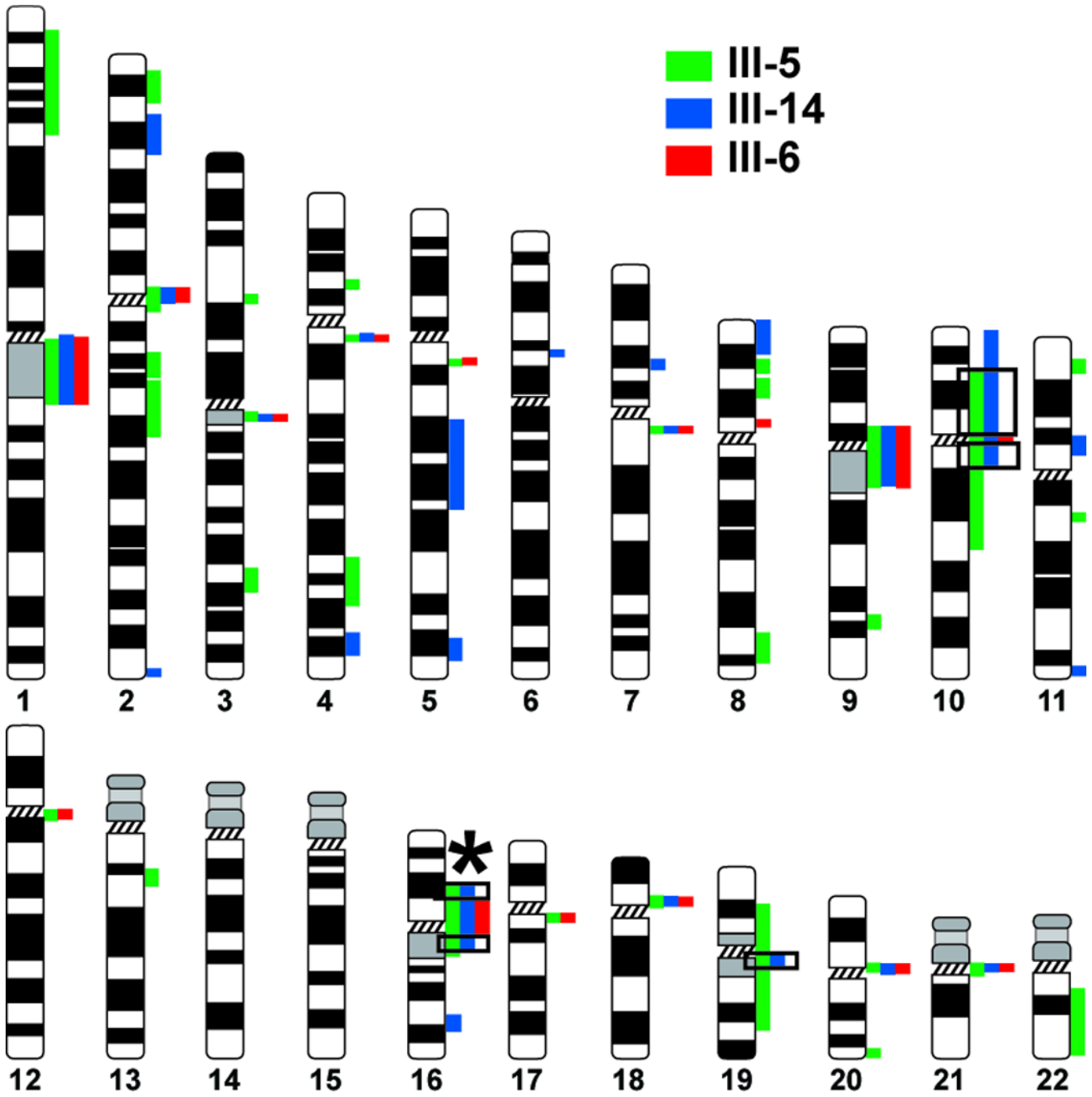

Fig. 2.

Runs of homozygosity >3 Mb in two affected and one unaffected family member. A total of 2.4 million SNPs were genotyped in the proband (III.14), an affected first cousin (III.5) and an unaffected first cousin (III.6). Regions of homozygosity >3 Mb are shown. The regions that were present in both affected family members that were not homozygous in an unaffected family member are shown in boxes. The location of the run of homozygosity that contained the causative mutation in *HSD3B7* is shown by an asterisk.

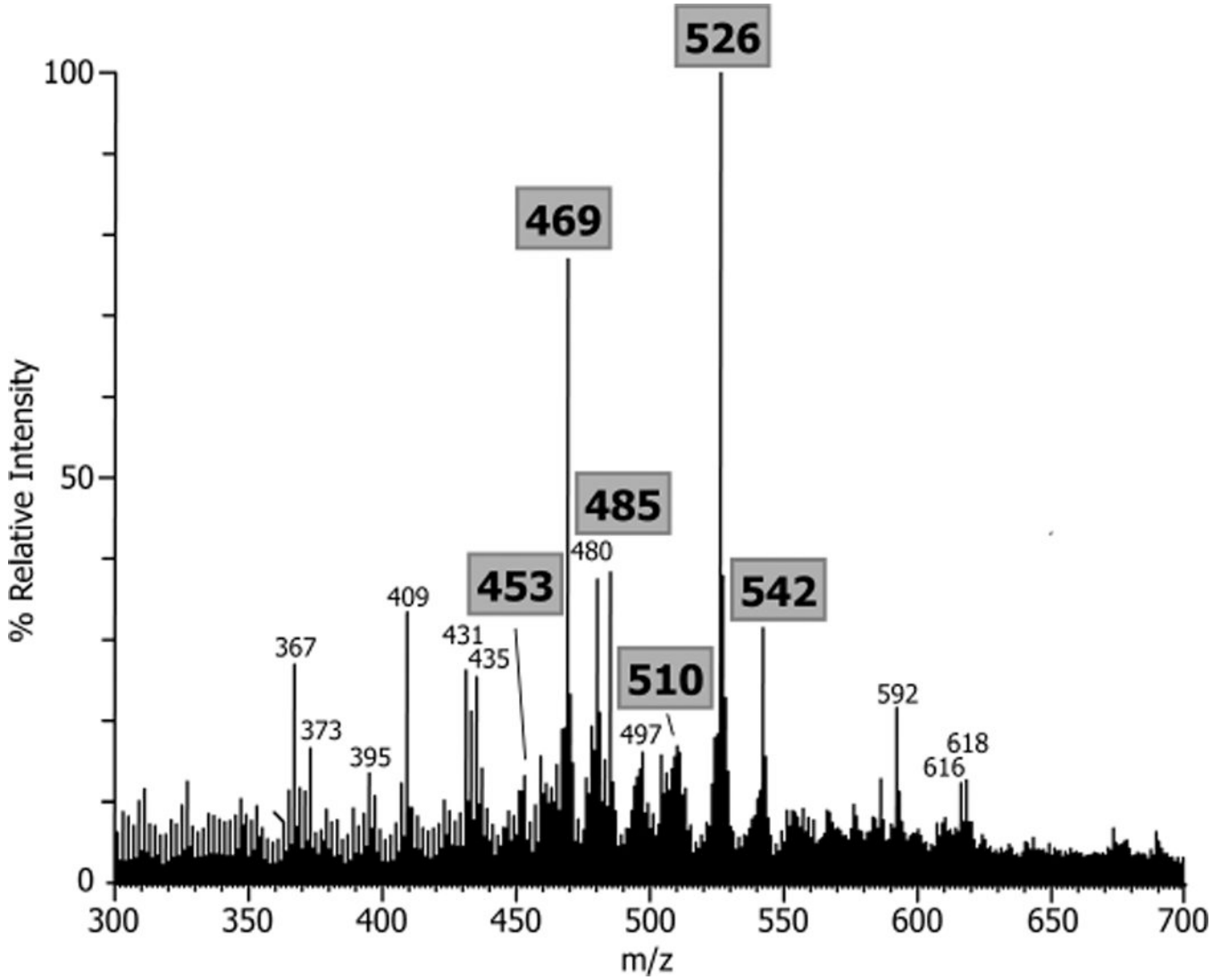

Fig. 3.

The negative ion FAB-MS mass spectrum of a serum extract of a family member III.5 with a defect in bile acid synthesis caused by a deficiency in the activity of 3b-hydroxy-C_{27}-steroid oxidoreductase established by the identification of homozygosity for a mutation in the *HSD3B7* gene. The atypical bile acids with a 3β-hydroxy-Δ^5- structure (highlighted by the boxes) that are the signature metabolites for this defect established biochemical confirmation of the lack of activity of the enzyme.