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CASE REPORT

A novel mutation in the biliverdin reductase-A gene combined with liver cirrhosis results in hyperbiliverdinaemia (green jaundice)

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Keywords
biliverdin – biliverdin reductase A – green jaundice – hyperbiliverdinaemia – liver cirrhosis

Abbreviations
BVR, biliverdin reductase; HO, haeme oxygenase; LC-MS, liquid chromatography coupled to mass spectrometry; PCR, polymerase chain reaction.

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Abstract

Background: Hyperbiliverdinaemia is a poorly defined clinical sign that has been infrequently reported in cases of liver cirrhosis or liver carcinoma, usually indicating a poor long-term prognosis. *Aims:* To clarify the pathogenesis of hyperbiliverdinaemia in an extended case report. *Methods:* A 64-year-old man with alcoholic cirrhosis was admitted to hospital with severe bleeding from oesophageal varices. Ultrasonography showed ascites, but no dilatation of the biliary tree. The skin, sclerae, plasma, urine and ascites of the patient showed a greenish appearance. Bilirubin levels were normal, and there were no signs of haemolysis. Biliverdin was analysed in plasma and urine with liquid chromatography coupled to mass spectrometry. The seven exonic regions of the biliverdin reductase-A (BVR-A) gene was amplified by polymerase chain reaction and sequenced. *Results:* Biliverdin was present in plasma and urine. In nucleotide 52 of exon I of the DNA isolated from the hyperbiliverdinaemic patient, we discovered a novel heterozygous C → T nonsense mutation converting an arginine (CGA) in position 18 into a stop codon (TGA) (R18Stop) predicted to truncate the protein N-terminally to the active site Tyr97. Two children of the proband were heterozygous for the identical mutation in the BVR-A gene, but had no clinical signs of liver disease and had normal levels of biliverdin. The BVR-A gene mutation was not found in 200 healthy volunteers or nine patients with end-stage liver cirrhosis. *Conclusion:* Hyperbiliverdinaemia (green jaundice) with green plasma and urine may be caused by a genetic defect in the BVR-A gene in conjunction with decompensated liver cirrhosis.

Jaundice is a common clinical sign characterized by increased bilirubin levels, which may be caused by hepatic failure, blockage of biliary outflow or increased degradation of haemoglobin (haemolysis, ineffective erythropoesis) (1). More than 60 years ago, an uncommon variant described as 'green jaundice' was observed (2). Clinical observations and analytical procedures suggested that this condition, characterized by a green coloration of plasma and urine, was related to hyperbiliverdinaemia (3–5). The clinical diagnoses associated with acquired forms of hyperbiliverdinaemia were heterogeneous, such as liver failure, intoxication with indomethacin (6), *Pseudomonas* septicaemia (7) and poor nutritional status (8).

In healthy individuals, plasma levels of biliverdin are low, because biliverdin reductases (BVR) in peripheral organs efficiently reduce biliverdin to bilirubin (9). The formation of bilirubin starts with degradation of a haeme group by haeme oxygenases (HO) 1 and 2, resulting in the production of biliverdin and iron under the release of carbon monoxide. In the next step, BVRs in peripheral organs reduce biliverdin to bilirubin (9). BVR exists in two forms, the adult A-form and the fetal B-form, both of which are encoded by separate genes (5, 10). BVR-A is evolutionarily conserved and expressed in adults in various tissues, catalysing the reduction of biliverdin IXα (11). BVR has been proposed to act as a cytoprotectant by mediating the formation of the antioxidant bilirubin (12, 13). BVR is located mainly in the cytoplasm; however, recently it was detected in the nucleus in animals exposed to oxidative stress, acting as a transcriptor factor and a dual-specificity kinase (11, 14). Although acquired deficiencies in BVR have been suggested, no genetic defects have previously been described in either the A- or the B-form (15, 16).

Within the haeme degradation pathway, only one case with another genetic defect has been described, in which

*Deceased.

both the HO-1 alleles were mutated, leading to a total lack of the regulated form of HO (17). This 2-year-old child had growth retardation, renal damage, hepatomegaly and haemolytic anaemia.

We describe for the first time a novel mutation in the BVR-A gene, which in a patient with decompensated liver cirrhosis led to the development of green jaundice with increased concentrations of biliverdin in plasma and urine.

Patients and methods

Case report

A 63-year-old male presented in May 2002 with fatigue, weight loss and nausea. He was a smoker and had previously undergone cholecystectomy. There was no history of drug abuse. He had received blood transfusions following a car accident during the 1970s. He denied alcohol overconsumption. At presentation, serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were twice the upper normal limit; aspartate aminotransferase (AST) was increased three-fold, and γ-glutamyltransferase (GT) 15-fold. Prothrombin complex ratio [international normalized ratio (INR)] was 1.3, and serum albumin 29 g/L. Haemoglobin, thrombocyte count, serum creatinine and bilirubin were within the normal range. Serologies were negative for hepatitis A, B and C. There were no autoantibodies suggesting autoimmune liver disease. Ferritin was increased to 780 μg/L, but transferrin saturation was 17% and HFE mutation analysis was normal regarding the C282Y and H63D mutations. Computed tomography scan revealed a small amount of ascitic fluid and an irregular attenuation of the liver parenchyma, but without focal lesions, and there was no dilatation of the biliary tree. Endoscopy demonstrated large oesophageal varices and a mosaic pattern of the gastric mucosa, suggesting portal hypertensive gastropathy. Propranolol was introduced (20 mg b.i.d.).

Two months later, he fell ill with haematemesis, endoscopy showing bleeding oesophageal varices, which were treated with ligation therapy. At this time, he admitted present and previous alcohol overconsumption. He then refrained from alcohol, underwent repeated ligation procedures and improved biochemically with decreasing aminotransferases and increasing serum albumin. On one occasion, he described the urine being dark green in the morning; however, it turned into a normal colour during the day. Nine months later, he again presented at the emergency ward with haematemesis and dark stools. It was now noted that the skin and the sclerae were tainted greenish. Haemoglobin was 81 g/L, creatinine 170 μg/L, prothrombin complex ratio (INR) 2.5 and serum albumin 23 g/L. Bilirubin was normal. The plasma, urine and stools were green. He received two units of blood, plasma, and was treated with terlipressin and i.v. antibiotics. Despite treatment, the bleeding continued and a Sengstaken tube was introduced. Endoscopy showed a cardiac ulcer and small oesophageal varices. Large amounts of green bile were seen in the duodenum. Ultrasonography showed ascites and a reversed portal flow. The biliary tree was not dilated and the hepatic veins had a normal width. Two days later, the bleeding relapsed and he received two more units of blood. Paracentesis of greenish-coloured ascites was performed. He developed encephalopathy grade 3. The patient died in a recurrent bleeding episode. Although prothrombin time and serum albumin levels were abnormal, the plasma bilirubin stayed low-to-normal (2–25 μmol/L) during the whole course of events. A normal haptoglobin level (0.5 g/L) throughout the clinical course indicated absence of significant haemolysis. Autopsy showed micronodular cirrhosis, varices and ulcerations in the distal oesophagus, dilatation of the heart with hypertrophy of the left chamber and pulmonary oedema. The cause of death was bleeding from ruptured oesophageal varices. Liver histology displayed micronodular liver cirrhosis with a low-to-moderate accumulation of iron in the hepatocytes (Fig. 1). There was a slight steatosis, but no signs of portal inflammation. There was no dilation of sinusoids or the central venules.

Total bilirubin concentration in plasma

Lithium heparin plasma was collected and total bilirubin concentration was analysed in a Modular auto analyzer (Roche Diagnostics Scandinavia, Bromma, Sweden) using the BIL-T bilirubin DPD 2,5-dichlorophenyldiazonium salt (DPD) method (Roche catalogue number 2144336), having a precision of 1.5% expressed as total CV at the level of 16 μM.

Identification of the green moiety in plasma

Blood was collected in Vacutainer tubes (Becton Dickinson 606480, Franklin Lakes, NJ, USA) containing lithium heparin, and the plasma was separated by centrifugation at 1550 *g*. The green-coloured plasma of the proband was subject to ultrafiltration, which showed that the green moiety was strongly bound to plasma proteins. The unidentified green moiety could be extracted from plasma by the following procedure: to 300 μl of sample, 300 μL of 0.5 M ammonia was added and the mixture was drawn by vacuum through an Oasis HLB 1 cm^3 SPE column (Waters, Milford, MA, USA), pretreated with 1 ml methanol and 1 ml water. The column was washed with 1 ml water and 1 ml 70% methanol in 25 mM acetic acid. The green moiety was eluted with 1 ml of 70% methanol in 25 mM ammonia. Five microlitres of the eluate was injected on the liquid chromatography coupled to mass spectrometry (LC-MS), which was run in SCAN mode with the fragmentor voltage set to 70–230 V (data not shown). Samples were then run in SIM-mode on $m/z = 297.1$ (150 V) and $m/z = 583.2$ (200 V) (Fig. 3).

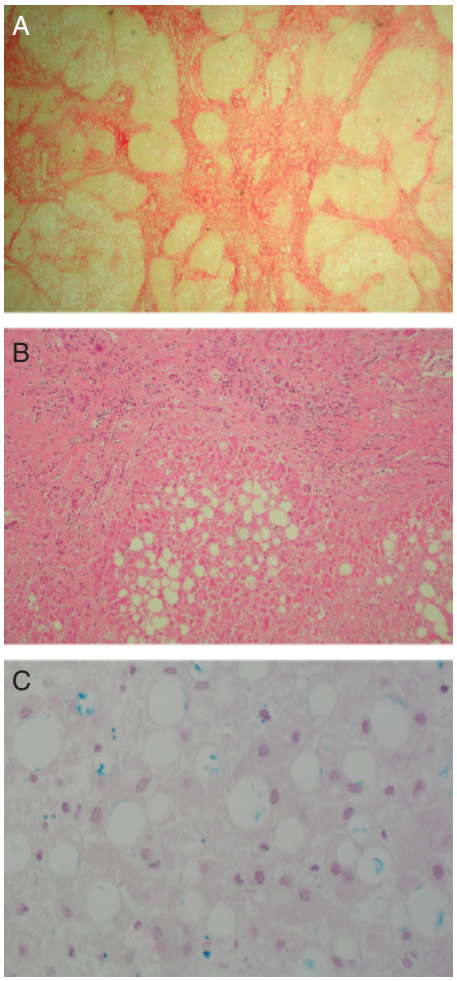

Fig. 1. Histological postmortem specimen of liver tissue from the proband. (A) Sirius (collagen) staining demonstrating cirrhotic liver, displaying a nodular pattern with circular fibrous septa (× 4 lens, Olympus BX45 microscope, Olympus, Solna, Sweden). (B) Haematoxylin-eosin staining demonstrating cirrhotic liver nodules with macrovesicular steatosis (× 10 lens). (C) Perls' blue staining demonstrating scarce iron deposits in Kupffer cells and fat-laden hepatocytes (× 40 lens).

Fluorescence spectrophotometry of plasma

Fluorescence spectrophotometry was performed on plasma obtained from the patient using a Hitachi F-2000 spectrophotometer (Hitachi High-Technologies, Krefeld, Germany). As a control, normal human plasma and plasma from a patient with porphyria cutanea tarda was used. Excitation was performed at 401 nm and emission spectra between 400 and 800 nm were recorded.

Verification and quantification of biliverdin in plasma and urine

Biliverdin was analysed in plasma and in urine by LC-MS. Biliverdin (ICN Biomedicals, catalogue number 194886, OH, USA) was used as the standard. An 1100 LC-MS system (Agilent; Avondale, PA, USA) equipped with a Luna C18 100 × 2.0 mm column with 3 µm particles (Phenomenex; Torrance, CA, USA) was used. The single-quadropole mass spectrometer had an atmospheric pressure electrospray interface and used positive-ionization mode with the following spray chamber settings: nebulizer pressure 20 psig, capillary voltage 2000 V, drying gas temperature 350 °C and drying gas flow rate 10 L/min. The chromatographic conditions were as follows: eluent flow rate 0.3 ml/min, column temperature 40 °C, a gradient eluent formed from eluent A (25 mmol/L formic acid with 2% acetonitrile) and eluent B (25 mmol/L formic acid with 70% acetonitrile) increasing from 0% B to 100% B in 4.5 min. The gradient delay time was approximately 2.8 min. Measurement of biliverdin concentration was performed on solid-phase-extracted serum by the following procedure: to 200 µL of serum, 300 µL of 0.2 M NaOH was added and the mixture was absorbed on a solid-phase Oasis HLB column (Waters). The column was washed with 1 ml water and then 1 ml 70% methanol in 25 mM acetic acid+5 mM NaOH. Biliverdin was eluted with 1 ml of 70% methanol in 25 mM phosphate buffer (pH 7.0). Substance recovery was 98.25% in the eluate. The eluate was analysed by high-pressure liquid chromatography (HPLC) using a UV-spectrophotometric detector at 370 nm. The eluent was 50% acetonitrile in 25 mM acetic acid and 5 mM NaOH. The analysis was performed at 50 °C. The limit of detection was 24 nM and the linearity range was 24–10 000 nM. Biliverdin was used as an external standard in a concentration of 10.285 mM. Samples above the linearity range were diluted 10-fold before assay.

Sequencing of the biliverdin reductase-A gene

Preparation of DNA from whole blood sampled with EDTA addition was performed using the Blood and Cell culture DNA Midi Kit (Qiagen, Hilden, Germany, catalogue number 13343). Polymerase chain reaction (PCR) amplification of exonic sequence of the BVR-A gene of the proband was performed using standard reagents and 400 nM (final concentration) of each primer. Annealing temperatures ranged between 50 and 56 °C. Primers used for PCR amplification are listed in Table 1. Primers were positioned to include the 20–25 bases within the splice donor and acceptor regions. The PCR products were displayed by agarose gel electrophoresis. Exonic PCR products were purified by excision of specific bands from agarose gels, followed by elution of DNA on a Gene-Elute column (Sigma Co., St. Louis, MO, USA). Direct sequencing of purified PCR products was performed on an ABI sequencer using the Big-Dye Terminator chemistry (version 2), and the respective upstream and downstream PCR primers were used for priming the sequencing reaction.

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Table 1. Design of oligonucleotides used for polymerase chain reaction amplification of the exons in the human biliverdin reductase-A gene*

Exon number	Size of PCR product (bp)	Oligonucleotides used in PCR amplifications of exonic sequence
1	121	Forward primer: 5′-CAGTGACCGAAGGAAGAGACC-3′ Reverse primer: 5′-AAAGGGAATGGAGACTCT-3′
2	258	Forward primer: 5′-CTGCTCGATGCCTACAGTGT-3′ Reverse primer: 5′-ATCTCCATGTCCTGCAGCTA-3′
3	253	Forward primer: 5′-CCTGTCGGGAGGAGGGGAGA-3′ Reverse primer: 5′-TTGCAGTACTGCACTGCTGT-3′
4	208	Forward primer: 5′-CAGTGAAGCTTATTCGAAGT-3′ Reverse primer: 5′-CATAAAGATGATATCACAGA-3′
5	251	Forward primer: 5′-CTCTGGGATGCACACCTAGACA-3′ Reverse primer: 5′-CCCATCCCAGAGAGCCAGGA-3′
6	296	Forward primer: 5′-GTCCTGCTTTCCACTTGGCA-3′ Reverse primer: 5′-ATACCTGCAAATGTACTAGGA-3′
7	482	Forward primer: 5′-AGGCGGTCTGGTGCCAGCAA-3′ Reverse primer: 5′-AACCCCAAATATCCCAACAT-3′

*The sequence of the human biliverdin reductase-A (BVR-A) gene was obtained from a chromosomal contig (GenBank accession number NT_007819).

Control groups

A population of healthy consenting volunteers recruited from students and hospital staff ($n = 30$) were assayed for the levels of biliverdin and bilirubin in lithium heparin plasma. In order to assess gene frequency in the general population, another cohort of healthy volunteers recruited in a similar way ($n = 200$) were tested for the presence of the novel R18Stop mutation in the BVR-A gene. Patients diagnosed with end-stage liver cirrhosis but lacking the BVR-A R18Stop mutation ($n = 9$) were analysed for plasma levels of biliverdin and bilirubin.

Mutation analysis of the biliverdin reductase-A R18Stop variant

A restriction fragment length polymorphism (RFLP) assay was developed to detect the novel R18Stop mutation in the human BVR-A gene using genomic DNA from the patient's relatives, a cohort of nine patients with liver insufficiency and a cohort of 200 healthy controls. The following primers were used 5′-GACTCCACCTTGGT CCCTTGT-3′ (forward) and 5′-AGCCACCTCGACAC GAAGCCA-3′ (reverse) to amplify a 155 bp fragment of exon II encompassing the region containing the mutation. PCR amplification was performed in a final volume of 25 µl, using 12.5 pmol of each primer, for a total of 30 cycles. The annealing temperature was optimized to 56 °C. An aliquot of the PCR product was digested with restriction endonuclease *Hae*III (Promega, Biotech, Nacka, Sweden) in order to detect the R18Stop variant. The digestion conditions were according to the manufacturer's recommendations. The digested PCR product was electrophoresed on a 2% agarose gel. The R18Stop mutation removes the *Hae*III cleavage site in the 155 bp amplicon, leading to the following possible cleavage patterns: 88+67 bp (*normal*), 155+88+67 bp (*R18Stop heterozygosity*) and 155 bp (*R18Stop homozygosity*). A DNA sample from the proband was included on each gel as a positive control for R18Stop heterozygosity.

Northern blot

A commercial multiple-tissue Northern blot (Clontech, Mountain View, CA, USA, catalogue number 636818), encompassing mRNA extracted from a panel of different human tissues, was probed using a ^{32}P-dCTP-labelled BVR-A cDNA probe obtained by PCR amplification of human brain cDNA. For amplification, the proofreading enzyme *Thermus termophilus* DNA polymerase was used to amplify the entire BVR-A peptide-coding region, using the oligonucleotide 5′-CAGTGACCGAAGGAAGA GACC-3′ as the forward primer and 5′-TCGCTCTTCCA AAGTGGCAGA-3′ as the reverse primer. The PCR product was subcloned into pCR2.1 (Invitrogen, Paisley, UK) and sequenced. An *Eco*RI fragment was used as a hybridization probe using techniques described previously (18).

Statistics

Correlations between plasma biliverdin and plasma bilirubin levels were made with simple regression analysis, which was used for calculating the correlation coefficient. A P-value < 0.05 was considered statistically significant.

Ethical approval

The study was approved by the ethical committee at Karolinska University Hospital, Huddinge, and was performed in accordance with the Helsinki Declaration of 1975.

Results

High levels of unconjugated biliverdin in the green plasma of the proband (Figs 2 and 3)

The proband presented with a clinical picture including green plasma (Fig. 2) and a greenish tinge of the urine. As porphyrias connected with haeme synthesis defects have previously been connected with colouring of both plasma and urine, scanning of heparin plasma from the patient

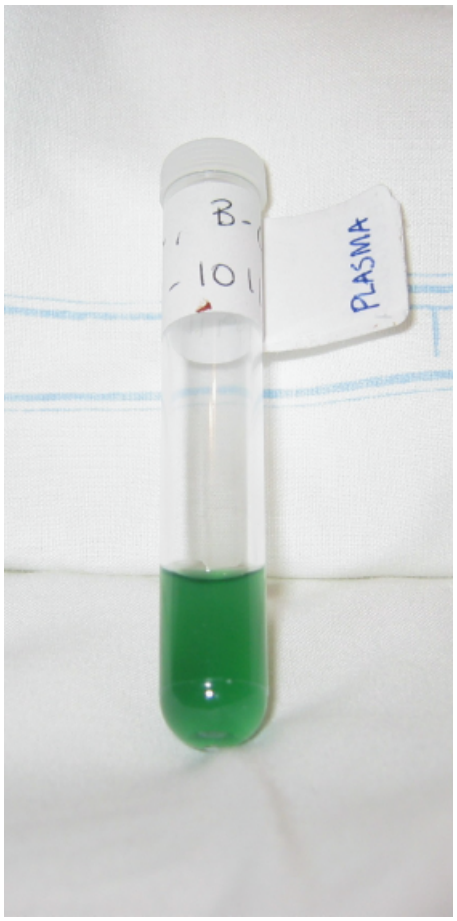

Fig. 2. Visual inspection of the plasma of the proband revealed a greenish tinge deviating from that seen in normal plasma.

was performed in a fluorescence spectrophotometer within the emission spectrum interval between 400 and 800 nm. A small maximum around 620 nm was detected, similar to plasma from a patient with porphyria cutanea tarda (data not shown). Total plasma porphyrin concentration in the plasma of the proband was slightly increased to 32 nM (reference value < 10 nM), and HPLC analysis indicated a dominance of copro (III) porphyrins. As the patient had no history of a cutaneous porphyria, the increase in porphyrin levels was considered indicative of nonspecific liver damage as may be seen in alcohol cirrhosis (19). Furthermore, no indications of accumulation of other substances present in porphyria could be detected in the plasma of the proband. Therefore, a novel method for identification of the green moiety was developed, as described in "Patients and methods", and the eluate was injected on the LC-MS. A peak with a retention time of 6.8 min was found, which had two ion masses: 297.1 and 583.2 (Fig. 3A and B). No other significant peaks were found. The identity of this peak as unconjugated biliverdin was confirmed by analysing a plasma sample to which unconjugated biliverdin was added and the sample was analysed in selective ion mode at both ions (Fig. 3C and D). The sample of the proband contained 72 μM biliverdin. Biliverdin was also found in a urine sample from the proband and in a green precipitate from urine. Biliverdin glucuronide was not identified in plasma or urine because pretreatment of the samples with β-glucuronidase failed to increase the concentration of biliverdin.

Identification of a novel mutation in the biliverdin reductase A gene (Figs 4–6)

The increase in unconjugated biliverdin in plasma of the proband indicated a possible block in the further metabolism of biliverdin to bilirubin. Therefore, the coding region of the BVR-A gene was amplified by PCR (for primer sequences, see Table 1) and sequenced using DNA from the proband. The sequence obtained from exon I showed a homozygous G → A conversion in the seventh base, leading to a change in the third amino acid from alanine (GCA) to threonine (ACA). This sense mutation is a polymorphism because it was found on both alleles of BVR-A cDNA cloned from normal human brain. The presence of threonine as the third amino acid residue has previously been reported in the rat (20). However, in nucleotide 52 of exon II, a heterozygous C → T nonsense mutation converting an arginine (CGA) into a stop codon (TGA) (R18Stop) was detected in the proband (Fig. 4a). No mutations or polymorphisms were detected in the other five exonic regions sequenced. All exons showed splicing according to the GT–AG rule, and no sequence alterations that could interfere with the splicing mechanism were found in the proband. To assess allele frequency of the novel BVR-A R18Stop mutation, 200 healthy controls were screened by RFLP (Fig. 4b). All the controls were homozygous for the C allele and the allelic frequency of the T allele was therefore $< 0.75\%$ in a mid-Swedish population.

We then asked whether the novel R18Stop mutation could be detected among relatives of the proband. Altogether, five relatives were asked to participate in the study (Fig. 5). The two children tested, one female aged 38 years and one male aged 31 years, showed heterozygosity for the R18Stop variant while one of the proband's sisters did not carry the mutation.

Using a multiple-tissue Northern blot, human BVR-A mRNA was shown to be ubiquitously expressed, with the highest expression detected in brain, heart, skeletal muscle, spleen and kidney and low expression in the liver (Fig. 6).

Plasma levels of biliverdin and bilirubin in the proband, relatives, cirrhosis patients and controls (Figs 7 and 8)

Using the newly developed LC-MS assay, levels of biliverdin were measured in the plasma of 30 healthy individuals. Levels of biliverdin were 0.9–6.5 μM in normal plasma. In addition, total bilirubin was

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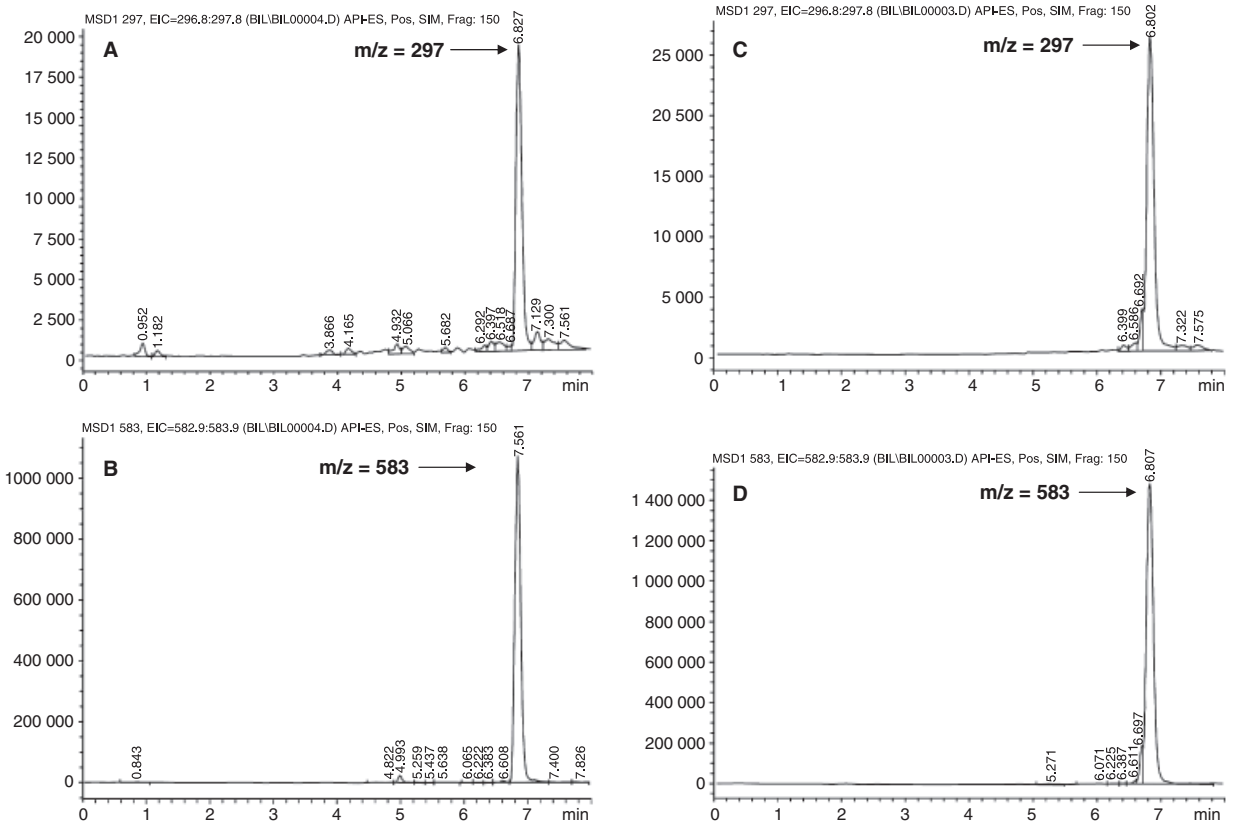

Fig. 3. LC-MS chromatogram obtained from (A and B) patient plasma and (C and D) 100 μM pure unconjugated biliverdin added to a plasma pool obtained from healthy volunteers. Biliverdin was positively identified as ions of *m/z* 297 (A and C) and 583 (B and D).

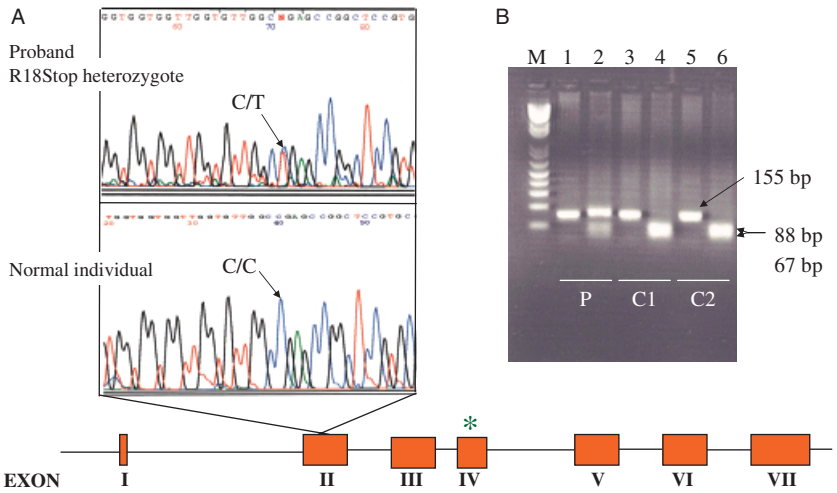

Fig. 4. (A) DNA sequence obtained from exon II of the biliverdin reductase-A gene of the proband with hyperbiliverdinaemia (upper panel) and a healthy person (lower panel), demonstrating a heterozygous C → T nonsense mutation converting an arginine (CGA) in position 18 into a stop codon (TGA). (B) Polymerase chain reaction amplification before restriction (lanes 1, 3 and 5) and RFLP for *Hae*III (lanes 2, 4 and 6) obtained for the patient (P) and the healthy controls (C1–C2). *Note that the stop codon in exon II truncates the C-terminal part of the protein containing the active site serine in exon IV.

determined in these individuals. Levels of biliverdin and total bilirubin were strongly correlated ($r^2 = 0.91$) in healthy individuals (Fig. 7).

Patients diagnosed with decompensated liver cirrhosis (Child–Pugh B and C) but lacking the BVR-A R18Stop mutation ($n = 9$) were analysed for plasma levels of

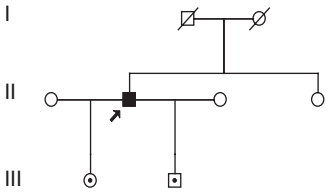

Fig. 5. Pedigree showing the second-generation heterozygous proband (arrow) whose clinical picture of hyperbiliverdinaemia was elicited by liver decompensation, and his two heterozygous children.

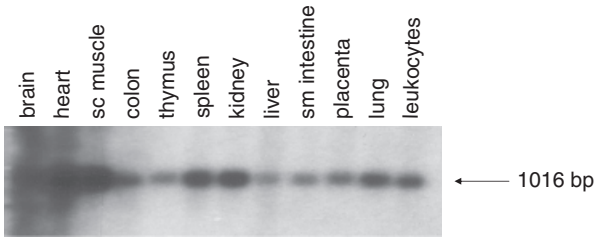

Fig. 6. Northern tissue blot demonstrating the ubiquitous expression of human biliverdin reductase-A mRNA.

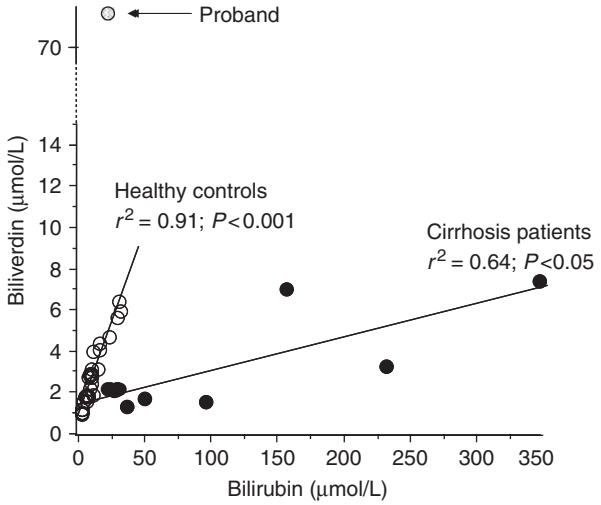

Fig. 7. Correlation of plasma biliverdin vs bilirubin in healthy controls and patients with liver cirrhosis in relation to the hyperbiliverdinaemic proband. There was a strong and significant correlation between these two variables in healthy individuals. In cirrhosis patients, the correlation was weaker but significant.

biliverdin and bilirubin. A significant correlation between biliverdin and bilirubin levels was seen in this cohort of patients (Fig. 7). No significant increase in plasma biliverdin was recorded as compared with healthy individuals, while their plasma bilirubin was increased (Fig. 8).

Relatives of the proband participating in the study were also assayed for levels of biliverdin and total bilirubin in their plasma. One sister lacking the R18Stop mutation was healthy and she had normal levels of biliverdin and bilirubin in plasma. The proband's son was obese (BMI = 50) and had slightly increased ALT and GT levels. There were no laboratory or clinical signs indicating liver cirrhosis [prothrombin complex ratio (INR), albumin, bilirubin, haemoglobin and thrombocyte count were normal]. The proband's daughter was healthy, having normal ALT, AST, GT, ALP, bilirubin, blood counts, albumin and prothrombin complex ratio (INR). Both the heterozygous children had normal levels of biliverdin in their plasma (Fig. 8).

Discussion

The present paper is to our knowledge the first description of a mutation in the BVR-A gene, and presents novel aspects on the role of the liver in regard to haeme degradation. In our proband, the macroscopic green tinge of the urine was first observed by him 6 months before death, at a stage when the liver function was already compromised. This indicates that the presence of this condition was acquired late during progressive liver decompensation. On admission for bleeding oesophageal varices, the urine and plasma was clearly green, as demonstrated in Figure 2. Plasma bilirubin levels were normal in spite of liver decompensation with ascites, abnormal prothrombin complex ratio (INR) and albumin.

The green plasma and urine indicated either pharmacological intoxication or pooling of some green-coloured metabolite. Therefore, pharmacological intoxications had to be excluded. Propofol intoxication may cause greenish coloration of urine (21), but this compound was not detected. An alternative compound is dipyridamol, however, giving the urine a slightly more yellow coloration (22). The possible accumulation of an internally produced substance was therefore obvious. Based on the high degree of protein binding, its behaviour during extraction and its molecular weight as determined by LC-MS on normal plasma and plasma from the proband, it was suspected that the green moiety would represent biliverdin. By pretreatment of normal plasma from the proband with glucuronidase, together with analysis of samples in which normal plasma was spiked with commercially obtained biliverdin, the green moiety was identified as unconjugated biliverdin. Hence, we suspected that the proband had a mutated BVR-A gene. The exons, including the splice junctions, were amplified from the proband by PCR and sequenced. We show for the first time a heterozygosity for a C → T nonsense mutation converting an arginine (CGA) in position 18 (17) into a stop codon (TGA) in the second exon in the DNA from the proband. Because the active site of BVR-A is predicted to be positioned in Tyr97 and the site of hydride transfer from NAD(P)H that drives catalysis in Glu96, Glu123 and Glu126 (23, 24), a loss of at least half the enzyme activity is predicted to occur.

However, this predicted loss of activity was not enough to induce hyperbiliverdinaemia in the children carrying the same mutation but having a normal liver function. Unfortunately, further biochemical sampling, which

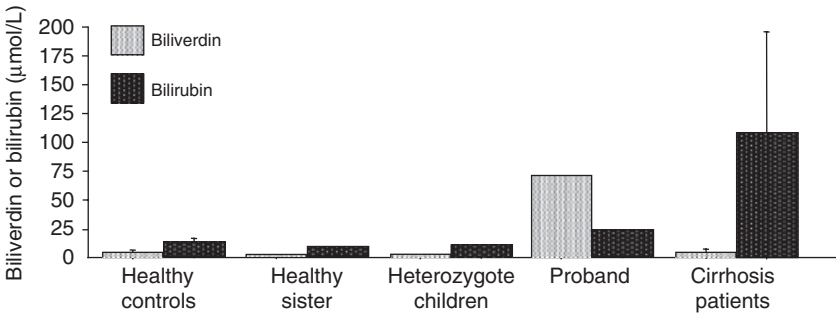

Fig. 8. Mean values of biliverdin and total bilirubin concentrations in healthy controls ($n = 30$) and mutation-free patients with end-stage liver cirrhosis ($n = 9$) lacking the biliverdin reductase R18Stop mutation compared with the hyperbiliverdinaemic proband, his sister lacking the mutation, and his two heterozygous children. Error bars represent the 95th percentile confidence interval.

would allow us to measure BVR activity in blood cells, from the two children was not possible because they both rejected repeated blood testing.

In the proband, it appears unlikely that the other allele of the BVR-A gene also was mutated outside the coding region, because the patient first noted green urine at the time of liver decompensation. Saturation of BVR-A may have contributed to the high level of biliverdin relative to bilirubin in the proband. BVR-A activity may also be regulated by phosphorylation–dephosphorylation (25). This was not possible to assess because adequate post-mortem samples could not be secured. Taken together, our study indicates that the mutation of the BVR-A gene, leading to a possible loss of enzyme activity, in combination with decompensated liver cirrhosis, leads to the development of hyperbiliverdinaemia.

In the normal state, biliverdin is efficiently metabolized to bilirubin, leading to plasma levels of biliverdin being only 25% of those of bilirubin (Fig. 8). This is also true in cirrhotic patients who lacked the novel BVR-A R18Stop mutation, having biliverdin levels similar to healthy controls, although plasma levels of bilirubin were elevated (Figs 7 and 8). Among healthy controls, there was a strong correlation between plasma biliverdin and bilirubin concentrations.

In spite of a widespread expression of BVR-A in extrahepatic organs, our patient with the mutated BVR-A and decompensated liver cirrhosis developed hyperbiliverdinaemia. Therefore, a decreased clearance of biliverdin from the circulation could also play a role, as a result of porto-systemic shunting and impaired bile formation. BVR-B predominates in the human fetus and is primarily confined to liver and muscle tissue (10). A compensatory upregulation of BVR-B in our patient seems unlikely because the A isoform is predominant in the adult liver (5).

The prevalence of the novel BVR-A mutation in the general Swedish population is low, and the mutation must therefore be characterized as familial, as we found an allelic frequency of $< 0.75\%$. The increase in biliverdin is not likely to cause the liver disease, because adult children carrying the identical mutation of the BVR-A gene were healthy, and high levels of biliverdin are not harmful in other species such as fishes, birds and lizards. In fact, these species use biliverdin as the main final product in haeme degradation (26). No human case with total BVR-A deficiency and no mouse strain with targeted disruption of BVR-A has so far been described that could illustrate the possible biochemical and phenodevelopmental consequences of a lack of BVR-A activity.

To conclude, we have demonstrated that a novel familial mutation in the BVR-A gene, together with decompensated liver cirrhosis, may result in hyperbiliverdinaemia (green jaundice), indicating that an increased concentration of plasma biliverdin signals insufficient haeme degradation together with an impaired liver function.

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