Case Report

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Biallelic inactivation of protoporphyrinogen oxidase and hydroxymethylbilane synthase is associated with liver cancer in acute porphyrias

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Abstract

Variegate porphyria (VP) and acute intermittent porphyria (AIP), the two most common types of acute porphyrias (AHPs), result from a partial deficiency of protoporphyrinogen oxidase (PPOX) and hydroxymethylbilane synthase (HMBS), respectively. A rare but serious complication in the AHPs is hepatocellular carcinoma (HCC). However, the underlying pathomechanisms are yet unknown. We performed DNA sequence analysis in cancerous and non-cancerous liver tissue of a VP and an AIP patient, both with HCC. In samples of both cancerous and non-cancerous liver tissues from the patients, we identified the underlying *PPOX* and *HMBS* germline mutations, c.1082dupC and p.G111R, respectively. Additionally, we detected a second somatic mutation, only in the cancer tissue i.e., p.L416X in the *PPOX* gene of the VP patient and p.L220X in the *HMBS* gene of the AIP patient, both located *in trans* to the respective germline mutations. Both somatic mutations were not detected in 10 non-porphyria-associated HCCs. Our data demonstrate that in the hepatic cancer tissue of AHP patients, somatic second-hit mutations result in nearly complete inactivation of the enzymes catalyzing major steps in the heme biosynthetic pathway. Both PPOX and HMBS, which might act as tumor suppressors, play a crucial role in the development of HCC in these individuals.

Introduction

Porphyrias are rare metabolic disorders caused by a specific, predominantly inherited dysfunction of one of the eight enzymes along the heme biosynthetic pathway [1]. These enzymatic defects are associated with a pathological accumulation of porphyrins and/or porphyrin precursors in urine, stool and blood. All intermediates of the heme biosynthetic pathway are potentially cytotoxic and their accumulation can cause cutaneous photosensitivity and acute neurovisceral symptoms [1]. These neurovisceral symptoms are the clinical hallmark of the autosomal dominant acute hepatic porphyrias (AHPs), which comprise acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), and variegate porphyria (VP). VP (OMIM 176200) results from a partial deficiency of protoporphyrinogen oxidase (PPOX; EC 1.3.3.4), the penultimate enzyme in the heme biosynthetic pathway. AIP (OMIM 176000) is caused by a likewise partial deficiency of hydroxymethylbilane synthase (HMBS; EC 2.5.1.61), the

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Abbreviations: VP, variegate porphyria; AIP, acute intermittent porphyria; HCP, hereditary coproporphyria; AHP, acute hepatic porphyrias; PPOX, protoporphyrinogen oxidase; HMBS, hydroxymethylbilane synthase; HCC, hepatocellular carcinoma; MRI, magnetic resonance imaging; PCR, polymerase chain reaction.

third enzyme in heme biosynthesis [1]. In the AHPs, the enzyme deficiencies are predominantly expressed in the liver [1]. A serious complication in these porphyrias is the development of hepatocellular carcinoma (HCC) [2–5]. Aminolevulinic acid, increased in all acute porphyrias, has been shown to have a genotoxic potential [6].

HCC is the most frequent type of primary liver cancer and one of the most common neoplasms in the world [7]. The prognosis of HCC is poor, with mortality being almost as high as its incidence [7]. To date, several well-defined risk factors have been described [7]. In 80–90% of the affected patients, liver cirrhosis plays a pivotal pathogenetic role [8]. In Asia and Africa, aflatoxin B1 intake through contaminated food and hepatitis B virus infection are the main causes of cirrhosis. By contrast, in Japan and Europe, cirrhosis is mainly attributable to hepatitis C virus infection, alcohol ingestion and exposure to chemical carcinogens [8]. HCC also occurs in certain monogenetic diseases, including hemochromatosis, Wilson disease, and the AHPs. In an effort to elucidate possible genetic mechanisms contributing to hepatocellular carcinogenesis, we studied two patients with VP and AIP, respectively, both of whom developed HCC [3].

Materials and methods

Clinical material and DNA extraction

In patient 1, we collected post-mortem hepatic tissues from different regions (3 cancerous and 10 non-cancerous regions), as well as cardiac tissue. In patient 2, we obtained surgically removed tissues of both HCC and non-cancerous liver. In addition, cancerous liver tissues of 10 unrelated non-porphyric individuals with HCC of identical histological subtype, were used as controls. DNA was extracted from all samples.

Loss of heterozygosity (LOH) studies

In patient 1, we analyzed four microsatellite markers flanking the *PPOX* gene on 1q22-23 in both centromeric and telomeric directions (cen-D1S398, D1S484, D1S2705, D1S1677-tel) [9].

Somatic mutation analysis

We sequenced the coding regions and adjacent splice sites of the *PPOX* gene in the paraffin-embedded cancerous and non-cancerous liver samples, the heart samples of patient 1, as well as the cancerous liver sections of 10 unrelated HCC patients according to a previously described method [9]. In patient 2, we performed automated sequencing of all coding exons and exon-intron boundaries of the *HMBS* gene, in DNA samples derived from both cancerous and non-cancerous liver tissue samples [10].

We also sequenced the following genes in DNA samples isolated from the cancerous liver tissue of both patients: all coding exons of the tumor suppressor p53 (*TP53*) gene; exons 2 and 3 of the catenin beta 1 (*CTNNB1*) gene; codons 12, 13, 61, and 146 of the *KRAS* gene; and codons 600 and 601 of the *BRAF* gene. The sensitivity of our sequencing method is approximately 10%.

Allelic discrimination assay

In patient 1, we amplified a 463 bp-segment of the *PPOX* gene containing both exons 10 (location of c.1082dupC) and 11 (location of p.K416X) from cancerous liver material, using the following primer set: 5′-AAAAAATGGGAAGGAGAGAC-3′ and 5′-GGAGAGCTGAGGGAAGTTTATC-3′. The PCR product was directly cloned into the TOPO cloning system (Invitrogen® Life Technologies, Karlsruhe, Germany).

In patient 2, we performed a PCR reaction, with genomic DNA isolated from surgically excised fresh cancerous liver tissue, to amplify a segment of the *HMBS* gene containing exons 7 to 12 using the following primer set: 5′-GCTTCC-TGAACTGCCCTAG-3′ and 5′-ACATCACTGAAAAGGCAAC-3′. The PCR product of 3122 bp was directly cloned into the TOPO cloning system. Clones containing the respective inserts were sequenced to determine the presence of mutations.

Immunohistochemistry

In patient 1, tissue sections of 3 μm were deparaffinized and rehydrated. For PPOX immunostaining, epitopes were retrieved by heating the slides in EnVision™ Flex target retrieval solution, high pH, for 1 min at 97 °C, and cooling to 6 °C before blocking endogenous peroxidases with EnVision™ Flex peroxidase blocking solution (DAKO, Hamburg, Germany). Sections were then blocked with serum free protein block for 20 min at room temperature, followed by an overnight incubation at 4 °C, with a 1/100 dilution of mouse anti-PPOX antibody (Clone 2F10, Sigma-Aldrich), in EnVision™ Flex antibody diluent. Slides were then exposed to EnVision™ Flex/HRP for 20 min at room temperature, followed by detection of specifically bound antibodies, with AEC+ high sensitivity substrate chromogen for 20 min, resulting in a red precipitate. Non-immune mouse serum IgG was used as an isotype-specific negative control.

For HePar-1 and glypican-3 immunostaining, the pre-treated sections were incubated with a monoclonal mouse anti-HePar antibody in a 1/200 dilution and a mouse anti-glypican-3 antibody in a 1/100 dilution in the antibody diluent, for 20 min at room temperature, followed by incubation with EnVision™ Flex/HRP for 20 min and visualization with EnVision™ Flex DAB+ substrate solution for 10 min. In patient 2, the reticulin staining was performed according to a standard protocol.

Case report

Patient 1

An 83-year old Swiss female VP patient was diagnosed with HCC in 2006 [3]. Her medical history did not reveal any environmental or viral risk factors. Since the tumor was inoperable at the time of diagnosis, she received palliative care and died 20 months thereafter. An autopsy revealed multiple and partially necrotic nodules in the liver. Microscopic examination of the tumor showed a multifocal, moderately differentiated HCC surrounded by partially cirrhotic liver tissue [3].

Patient 2

In 2003, a 68-year old Swiss female was diagnosed as an asymptomatic carrier of germline mutation p.G111R in the *HMBS* gene that was known in her family with AIP. She had never experienced an acute porphyric attack and her urinary PBG concentrations were only slightly above the normal range (2.0 μmol/mmol creatinine; normal <1.25 μmol/mmol creatinine). In 2010, two solid lesions of 1.7 cm and 2.7 cm, highly suspicious of HCC, were detected in liver segment IV by ultrasonography and subsequent magnetic resonance imaging (MRI). Ultrasound-guided liver biopsies from both lesions only showed steatotic liver tissue but did not reveal any malignancy. Six months later, the larger of the two lesions was no longer detectable by ultrasonography, whereas the smaller lesion had increased in size to 2.1 cm. Eighteen months after the initial detection, an MRI showed that this lesion had further increased to 3.9 × 3.5 cm. In addition, two novel lesions of less than 1 cm were detectable in the same liver segment. Serum alpha-fetoprotein and liver enzyme levels were normal. No signs of liver cirrhosis were found. Because of the fast growth of the first lesion, it was decided to surgically remove the tumor. Following removal by segmental liver resection in December 2011, histopathological examination showed a well-encapsulated and well-differentiated HCC of 6 cm in the non-cirrhotic liver. Two years after surgery the patient is doing well and has not yet shown any signs of tumor recurrence.

Germline mutations

In patient 1, we detected a heterozygous germline mutation of a single nucleotide insertion, c.1082dupC in exon 10 of the *PPOX*