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Research Letter

Screening for Mutations Causing ACOX2 Deficiency-associated Hypertransaminasemia in Patients with Cryptogenic Liver Injury

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Elevation of serum transaminases is a frequent cause of consultation in hepatology departments.[1] The most common causes of hypertransaminasemia are alcoholic and metabolic dysfunction–associated steatotic liver disease, viral infections, autoimmune liver disease, celiac disease, iron overload, Wilson's disease, and exposure to toxic compounds, including drugs.[2] Although serum biochemistry, serological methods, functional approaches, imaging techniques, and liver biopsy can help to identify the etiology of liver damage, up to 15% of hypertransaminasemia cases in adults[3] and 13% in children[4] remain idiopathic.

Since the first case of acyl-CoA oxidase 2 (ACOX2) deficiency-associated hypertransaminasemia (ADAH) was reported at the International Liver Congress™ – EASL (April 2016; Barcelona, Spain),[5] several cases caused by different mutations in the *ACOX2* gene have been identified by our group[5–8] and others.[9,10] This has led to the speculation that some cryptogenic liver damage may be related to ADAH.

In this context, in recent years, a relatively high number of cases of severe pediatric idiopathic acute hepatitis (PIAH) have been described in children under 14 years of age in different European countries, requiring hospital admission and, in some patients, liver transplantation. As the etiology was not defined, several causes were considered to explain these conditions. This included infection by adenoviruses, as well as by specific strains of SARS-CoV-2. However, despite some clues have been reported,[11,12] the exact etiology of this condition remains unclear.[13]

The hypothesis of this study was that mutations in the *ACOX2* gene leading to peroxisomal dysfunction in cholesterol side-chain shortening could favor the accumulation of toxic intermediate metabolites of bile acid synthesis and act as a predisposing factor. This would induce mild liver damage on its own but markedly increase liver vulnerability to factors such as adenovirus infections or other infectious agents capable of triggering more serious liver injury. Accordingly, the aim was to determine the presence of mutations in the *ACOX2* gene in a relatively large number of samples from patients with PIAH. For comparison, we also included in this screening potential cases of ADAH from two small cohorts with different pathological conditions associated with liver injury that mainly affect adults and whose etiology has long been under investigation, namely, hepatocellular carcinoma (HCC) arising in non-cirrhotic liver[14] and drug-induced liver injury (DILI). DILI cases can be classified as intrinsic, which is predictable and associated with known dose-dependent drug toxicity, and idiosyncratic, in which the damage is unexpected for the administered drug and implies enhanced liver susceptibility.[15,16] All cases included in this study were idiosyncratic DILI. Patients were classified at the time of diagnosis using the updated Roussel were classified at the time of diagnosis using the updated Roussel Uclaf Causality Assessment Method (RUCAM).[17] However, for inclusion in this study, we did not differentiate between hepatocellular, cholestatic, or mixed types of idiosyncratic DILI.[18]

Plasma and white blood cell samples were collected from adults with HCC or DILI at the Hospital Universitario de Navarra (Spain) and from children with PIAH reported at the Centro Nacional de Microbiología (Majadahonda, Madrid, Spain). The research protocol adhered to the ethical guidelines of the 2024 Declaration of Helsinki. The use of genetic information was approved by the Human Ethics Committees of the participating institutions. Blood samples were collected

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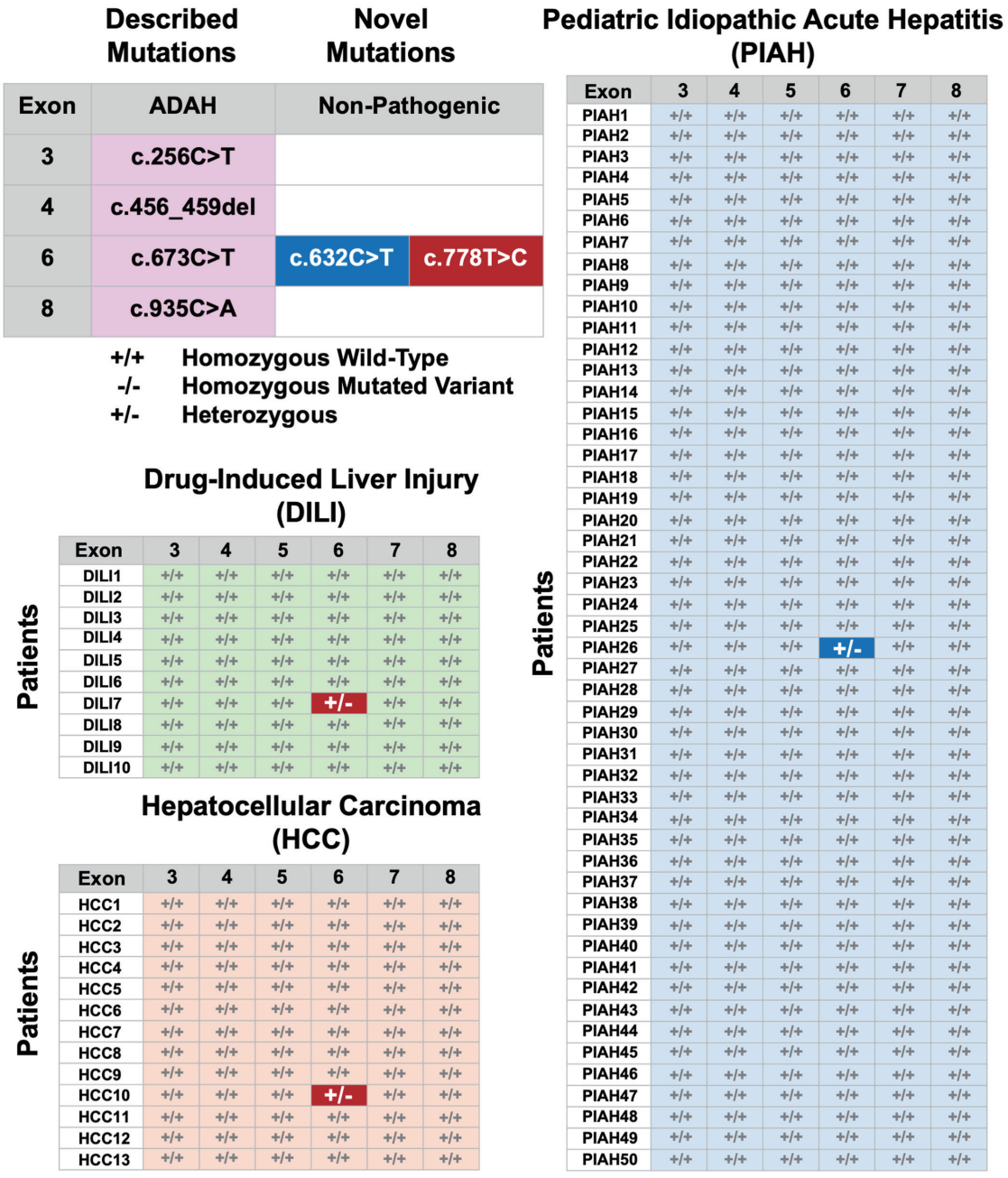

Fig. 1. Scheme of previously described pathogenic mutations and novel mutations in the *ACOX2* mRNA region from exon 3 to exon 8. The table depicts the results of the screening of these mutations in children with pediatric idiopathic acute hepatitis (PIAH, n = 50) and adult patients with idiosyncratic drug-induced liver injury (DILI, n = 10; patients with intrinsic DILI were excluded) or hepatocellular carcinoma (HCC) developed in the absence of liver cirrhosis (n = 13).

after overnight fasting. Bile acid concentrations in plasma were measured by HPLC-MS/MS. All patients included in the study had hypertransaminasemia together with other abnormal liver function parameters, as determined using standard clinical automatic analyzers. Reference normal serum ranges for adults were: alanine aminotransferase 7–55 U/L, aspartate aminotransferase 8–48 U/L, alkaline phosphatase 40–129 U/L, albumin 3.5–5.0 g/dL, total protein 6.3–7.9 g/dL, total bilirubin 0.1–1.2 mg/dL, direct bilirubin <0.3 mg/dL, gamma-glutamyl transferase 8–61 U/L, lactate dehydrogenase 122–222 U/L, and prothrombin time 9.4–12.5 s. These ranges were adapted to pediatric values depending on gender and age (0 to 14 years).[19] Patients with acute liver failure were excluded. DNA was obtained from total blood samples (DNeasy Blood & Tissue Kit, Qiagen) to amplify the coding sequence of *ACOX2* by high-fidelity Polymerase Chain Reaction (PCR).[6] The amplified fragments containing both the exons and the exon-intron boundaries from at least two PCR reactions per exon were purified by agarose gel electrophoresis. Amplicons were then sequenced in both directions using forward and reverse primers as previously described.[6] The genetic region analyzed included exons where pathogenic

mutations in the coding sequence of *ACOX2* have been identified,[5–10] i.e., exon 3 to exon 8 (Fig. 1).

The detailed analysis of pathogenic mutations in the ORF of *ACOX2* was performed in the cohort of children with PIAH previously reported by Martinez-Laso *et al.*[11] Although the global genetic analysis of these patients revealed a relationship between this liver pathology and the presence of specific human leukocyte antigen variants, suggesting an autoimmune predisposition to injury caused by various viruses,[11] the pathogenesis of this condition has not yet been fully clarified.[12] Unfortunately, the results of the present screening do not provide a clue to unravel this intriguing question. Among the fifty patients analyzed, only one individual carried a mutation in the analyzed region of the *ACOX2* gene (Fig. 1). This was a previously unreported change, c.632C>T in exon 6, which leads to p.S211L. This is a missense mutation resulting in the substitution of serine, a small, polar amino acid capable of hydrogen bonding and often involved in active sites or phosphorylation, with leucine, a larger, non-polar, hydrophobic amino acid that typically contributes to the hydrophobic core of proteins. Although this substitution may have functional consequences, the mutation was found in a heterozygous state. In our experience, even in cases of pathogenic variants, when they appear as a single heterozygous mutation in individuals who are close relatives of ADAH patients, these mutations do not have a phenotypic impact.[5–8]

Regarding the cohorts of patients with idiosyncratic DILI or HCC developed in non-cirrhotic liver, owing to the limited number of samples, bile acid levels were measured only in patients with DILI. These values showed high variability, ranging from normal (total bile acid concentration below 10 μM, in four patients) to mild elevation (more than 10 μM but below 20 μM, three patients) or even marked hypercholanemia (71–385 μM, three patients). None of the previously described pathogenic mutations (c.256C>T, c.456_459del, c.673C>T, c.935C>A) were found in these cohorts (Fig. 1). In contrast, a novel mutation was identified in one individual from each cohort. This change (c.778T>C, p.F271L) was also located in exon 6 of the *ACOX2* gene. The substitution of phenylalanine with leucine represents a missense mutation involving two hydrophobic amino acids. Despite both being nonpolar, phenylalanine contains an aromatic ring, while leucine has an aliphatic side chain. This structural difference might have functional consequences, which could be relevant in the homozygous state. However, in both patients carrying this mutation, it was present only in one allele. As discussed above, no phenotypic repercussions are expected in the heterozygous state of these mutations.

In conclusion, although this study was conducted with a limited data set and the findings should be regarded as preliminary, ADAH was not found to be a predisposing condition for PIAH, nor was it present in our cohorts of idiosyncratic DILI or non-cirrhotic HCC. These findings indicate that ADAH is a rare entity scarcely represented in these groups of patients. However, since ADAH patients respond well to treatment with ursodeoxycholic acid (UDCA),[8] it is advisable to perform serum bile acid profiling by HPLC-MS/MS followed by confirmation through mutational analysis of the *ACOX2* gene in patients with hypertransaminasemia of unknown origin, because if they are positive for ADAH, an effective and safe therapy can be recommended.

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Conflict of interest

The authors have no conflict of interest related to this publication.

Author contributions

Conceptualization (JJGM, MJM, EH, JP, AA, LB), funding acquisition (JJGM, AA), investigation, data interpretation (JJGM, EH, MJM, MAP, MMC), methodology (EH, MJM, MAP), supervision (JJGM), writing - original draft (JJGM, EH, MJM), and review & editing (JJGM, EH, MJM, MAP, MMC, JP, AA, LB). All authors read and critically revised the manuscript and approved the final version.

Ethical statement

The research protocol adhered to the ethical guidelines outlined in the 2024 Declaration of Helsinki. The use of genetic information was approved by the Human Ethics Committees of the involved institutions (CEIC: PI81106 and CEI PI 49_2023). Informed consent was obtained from patients entering this study or from their legal representatives.

Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may be subject to ethical or legal restrictions.

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