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## Bi-allelic hydroxymethylbilane synthase inactivation defines a homogenous clinico-molecular subtype of hepatocellular carcinoma

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Authors’ contributions

Conception and design: LM, JZR, EL; data acquisition: JZ, GA, GENTHEP Consortium, JFB, JC, XM, data analysis: LM, JZ, ET, QB, interpretation of the data: LM, JZ, XM, JZR; writing of article: LM, JZR, EL.

Conflict of interest

The authors declare that they have no competing interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2022.05.018>.

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## Abstract

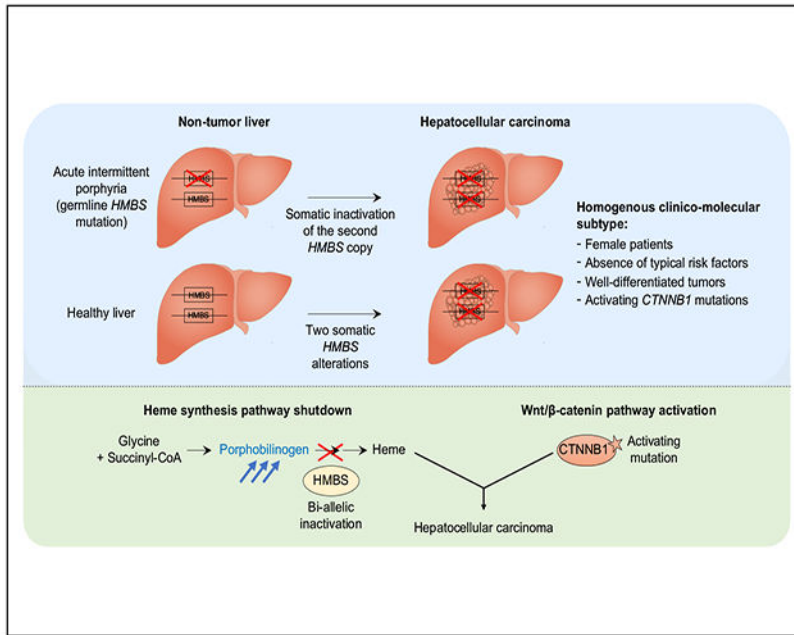
**Background & Aims:** Acute intermittent porphyria (AIP), caused by heterozygous germline mutations of the heme synthesis pathway enzyme HMBS (hydroxymethylbilane synthase), confers a high risk of hepatocellular carcinoma (HCC) development. Yet, the role of HMBS in liver tumorigenesis remains unclear.

**Methods:** Herein, we explore HMBS alterations in a large series of 758 HCC cases, including 4 patients with AIP. We quantify the impact of *HMBS* mutations on heme biosynthesis pathway intermediates and we investigate the molecular and clinical features of *HMBS*-mutated tumors.

**Results:** We identify recurrent bi-allelic HMBS inactivation, both in patients with AIP acquiring a second somatic *HMBS* mutation and in sporadic HCC with 2 somatic hits. *HMBS* alterations are enriched in truncating mutations, in particular in splice regions, leading to abnormal transcript structures. Bi-allelic HMBS inactivation results in a massive accumulation of its toxic substrate porphobilinogen and synergizes with *CTNNB1*-activating mutations, leading to the development of well-differentiated tumors with a transcriptomic signature of Wnt/ $\beta$ -catenin pathway activation and a DNA methylation signature related to ageing. HMBS-inactivated HCC mostly affects females, in the absence of fibrosis and classical HCC risk factors.

**Conclusions:** These data identify *HMBS* as a tumor suppressor gene whose bi-allelic inactivation defines a homogenous clinical and molecular HCC subtype.

## Graphical abstract



### Lay summary:

Heme (the precursor to hemoglobin, which plays a key role in oxygen transport around the body) synthesis occurs in the liver and involves several enzymes including hydroxymethylbilane synthase (HMBS). *HMBS* mutations cause acute intermittent porphyria, a disease caused by the accumulation of toxic porphyrin precursors. Herein, we show that HMBS inactivation is also involved in the development of liver cancers with distinct clinical and molecular characteristics.

### Keywords

Hepatocellular carcinoma; Acute intermittent porphyria; Heme biosynthesis pathway; Cancer Genomics

### Introduction

Hepatocellular carcinoma (HCC) is a diverse, heterogeneous disease with an annual global incidence of almost 1 million. HCC often arises on a background of chronic liver disease which may be caused by viral hepatitis infection (HBV, HCV), alcohol, non-alcoholic steatohepatitis associated with metabolic syndrome, or environmental exposure to toxins such as aflatoxin B and aristolochic acid.<sup>1</sup> HCC may also arise in the absence of cirrhosis due to the activation of oncogenes by viral insertions or structural rearrangements, or from the malignant transformation of benign hepatocellular adenomas.<sup>1-3</sup> However, ~10% of HCCs cannot be attributed to typical risk factors. At the molecular level, whole-exome/genome sequencing (WES/WGS) studies have uncovered tens of driver genes and 11 cellular pathways recurrently altered in HCC.<sup>4-6</sup> These include *TERT* alterations resulting in telomerase activation, activation of CTNNB1/WNT pathway, or inactivation of TP53, along with alterations of chromatin remodeling (*ARID1A*), mTOR (mammalian target of

rapamycin) signaling, and regulators of oxidative stress.<sup>7</sup> However, power estimations show that additional sequencing efforts are needed to unravel rare driver genes involved in small subsets of patients.<sup>8</sup> Thus, novel genetic and risk factor associations contributing to HCC tumorigenesis remain to be identified.

Various clinical studies have identified an increased risk of HCC among patients with acute intermittent porphyria (AIP).<sup>9–15</sup> AIP is a rare genetic disease caused by germline inactivating mutations in 1 allele of *HMBS* (hydroxymethylbilane synthase), encoding the enzyme that converts porphobilinogen to hydroxymethylbilane in the heme biosynthesis pathway.<sup>16</sup> AIP symptoms include painful neurovisceral attacks with severe abdominal pain, vomiting, tachycardia and hypertension.<sup>17,18</sup> Although AIP is an autosomal dominant disease, its penetrance is very low and only ~1% of *HMBS* mutation carriers will experience acute attacks.<sup>19,20</sup> These attacks are triggered by numerous factors (such as alcohol, stress, increased progesterone, and various prescription drugs) which increase demand for heme and expression and/or activity of ALAS1, the enzyme preceding *HMBS* in the heme synthesis pathway.<sup>17,18,21</sup> This results in saturation of the available functional *HMBS* and causes accumulation of toxic intermediates such as delta-aminolevulinic acid (ALA) and porphobilinogen (PBG).<sup>22,23</sup> Several retrospective and prospective cohort studies have identified an increased incidence of HCC among both symptomatic and asymptomatic carriers of germline *HMBS*-inactivating mutations, with standardized risk ratios ranging from 2 to 38, independent of the traditional HCC risk factors mentioned above.<sup>9–12,15,24,25</sup> These tumors often arise in livers without background fibrosis or cirrhosis, although underlying chronic injury cannot be ruled out. It has been proposed that insufficient *HMBS* activity and accumulation of ALA and PBG could lead to increased levels of oxidative stress and mitochondrial damage, promoting mutagenesis and increasing cancer risk.<sup>14,21</sup> However, no definitive mechanism has yet been identified. Interestingly, a case report revealed that a patient with AIP, with 1 defective *HMBS* allele, developed an HCC tumor which carried an acquired somatic mutation in the second *HMBS* allele, resulting in complete loss of *HMBS* activity in the tumor.<sup>26</sup> Although isolated, this report raises the question of whether *HMBS* could act as a tumor suppressor, beyond its role in the pathogenesis of AIP.

To better understand the role of *HMBS* in HCC development, we herein conducted a large-scale analysis of *HMBS* mutations in 784 HCC cases from in-house (LICA-FR) and The Cancer Genome Atlas (TCGA) data sets. We identify a subset of HCC with bi-allelic inactivation of *HMBS*, both in patients with and without AIP. We explore the functional consequences of *HMBS* mutations on heme pathway metabolites, and we characterize the clinical and molecular characteristics of *HMBS*-inactivated tumors.

## Materials and methods

### Description of the cohort

We analyzed WES/WGS data from a total of 758 HCC cases. WGS of 4 HCC (and matched non-tumor liver) cases that developed in 3 patients with AIP was performed for this study. Previously published WGS (n = 60) and WES (n = 347) data from our group (LICA-FR series<sup>3,4,49–51</sup>) were also reanalyzed for *HMBS* mutations. HCC samples and

their non-tumor counterparts were collected from patients surgically treated in 4 French hospitals located in the Bordeaux and Paris region. The study was approved by institutional review board committees (CCPPRB Paris Saint-Louis IRB00003835). Written informed consent was obtained in accordance with French legislation. All samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Clinical features of the 411 HCC cases from the LICA-FR series are summarized in Table S1. We also analyzed WES data from TCGA-LIHC project.<sup>6</sup> Cholangiocarcinomas and mixed forms of HCC were discarded from TCGA series to keep only pure HCC samples (n = 347).

### Patients with AIP

Three patients with HCC diagnosed in a context of acute intermittent porphyria (AIP) were included in this study. Patient #1606 was a 77-year-old female who developed an HCC more than 20 years after AIP diagnosis. Two years after the initial surgical resection, the tumor relapsed with 2 nodules (Edmonson grade 3), one of which (FR-1606T) was sampled and sequenced in this study. Subsequent treatments included radiotherapy, chemoembolization and sorafenib. The patient died from HCC progression 10 years after the initial diagnosis. Patient #4027 (male) was diagnosed with asymptomatic AIP at 33 years-old following a porphyria episode in his sister. He developed a bifocal HCC at age 64 with macrotrabecular massive histology.<sup>52</sup> Both nodules were surgically removed and analyzed in this study. This patient relapsed 11 years later and was treated with radiofrequency ablation. Patient #4029 was a 77-year-old female with AIP who developed a well-differentiated HCC. Following surgical resection, no relapse was detected until the patient died 7 years later from another cause. In all cases we also sampled and sequenced adjacent non-tumor liver.

### Whole-genome and RNA-sequencing

We performed WGS of 4 HCC cases (and matched non-tumor liver samples) that developed in 3 patients with AIP. We performed RNA-sequencing (RNA-seq) for 3 of them. Sequencing data generation and bioinformatic analyses are described extensively in the supplementary methods.

### Mutational signature analysis

We used Palimpsest<sup>53</sup> to extract single base substitution (SBS) signatures in the LICA-FR WGS data set. We first performed a *de novo* extraction to identify potentially new signatures. This analysis revealed 5 signatures, all corresponding to COSMIC signatures known to be operative in liver cancers (Fig. S1). In absence of a new signature, we then estimated the exposures of the 10 known liver cancer signatures (SBS1, 4, 5, 6, 12, 16, 17, 22, 23 and 24) in our series, and we compared their prevalence in HCC with or without HMBS inactivation.

### Heme pathway intermediates quantification in frozen liver samples

Three heme biosynthesis pathway intermediates were quantified in frozen HCC and matched non-tumor liver samples: porphobilinogen, protoporphyrin IX (PPIX) and hemin. Heme is not stable *in vitro* and undergoes spontaneous oxidation to hemin. The level of hemin should be the same as heme. Liver samples were homogenized in water (50

mg tissue in 300  $\mu$ l water). Then 300  $\mu$ l of methanol:acetonitrile (v/v, 1:1) was added to a 100  $\mu$ l aliquot of liver homogenate. After centrifugation, the supernatant was injected into ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC-QTOFMS) for the analyses of Hemin and PPIX following previously reported methods.<sup>54,55</sup> For PBG analysis, we used acetonitrile to precipitate proteins in liver homogenates, and the porphobilinogen in the supernatant was further derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate before detection by UPLC-QTOFMS. For UPLC separation, we used the Acquity UPLC BEH C18 column (2.1  $\times$  100 mm, 1.7  $\mu$ m) with acetonitrile/water containing 0.1% formic acid as the mobile phase, at a temperature of 50°C. The QTOFMS system was operated in positive mode with electrospray ionization. The detailed mass parameters were published in a previous report.<sup>56</sup>

### Multiomic characterization of *HMBS*-mutated HCC

To characterize the multiomic features of *HMBS*-mutated HCC, we analyzed RNA-seq (285 HCC and 29 non-tumor liver samples) and methylation array data (Illumina Infinium HumanMethylation450 BeadChip, 194 HCC and 35 non-tumor liver samples) available for part of the LICA-FR cohort. Except RNA-seq data for 3 HCCs that developed in patients with AIP, all data were previously published.<sup>3,29,50,51</sup> T-stochastic neighbor embedding (tSNE) was used to project the expression and methylation data sets into 2 dimensions using the Rtsne package (<https://github.com/jkrijthe/Rtsne>). Gene expression (G1-G6) and DNA methylation subgroups (M1-M7) were defined as previously described.<sup>29,57</sup> Gene expression signatures<sup>58,59</sup> and DNA methylation components<sup>29</sup> representing coordinated transcriptomic and epigenetic processes recurrently altered in HCC were quantified in each sample and compared between *HMBS*-mutated and other HCCs using Wilcoxon rank-sum tests.

## Results

### Molecular history of HCC developed in AIP patients

We sequenced the whole genomes of 4 HCC samples from 3 patients (2 females, 1 male) with diagnosed AIP. We identified, in each patient, a germline *HMBS* mutation (1 splice, 2 missense) that was documented as pathogenically associated with AIP in the Human Gene Mutation Database<sup>27</sup>. These mutations were found in both the tumor and non-tumor liver tissues (Fig. 1). The 4 HCC cases had acquired a median of 11,335 somatic point mutations, 673 indels and 26 structural variants, comparable with HCC developed in patients without AIP. Mutational signature analysis revealed a combination of COSMIC signatures 4, 5 and 16, ubiquitous in HCC (Fig. S1). We did not identify any new signature that would be in favor of a genotoxic role of heme pathway intermediates. Interestingly, all 4 tumors had acquired pathogenic somatic mutations of *HMBS* (1 splice, 1 frameshift, 2 missense), resulting in bi-allelic inactivation of the gene. These mutations were found only in tumor tissues. Other recurrent driver alterations included *TERT* promoter mutations (n = 4), activating mutations in *CTNNB1* exon 3 (n = 3) and *FGA* frame-shift indels (n = 2). Private mutations were also found in *CDKN2A*, *RPS6KA3*, *ZNRF3* and *KRAS* (hotspot mutation G12D). Of note, 2 synchronous HCC nodules were analyzed for patient #4027. These tumors displayed completely different mutation catalogues and thus result from independent tumor initiation events. Each nodule had acquired *TERT* promoter mutations

and a distinct set of mutations in the same driver genes *HMBS*, *CTNNB1* and *FGA*. Thus, HCC development in patients with AIP systematically involves inactivation of the second *HMBS* allele and follows a conserved evolutionary path with mutations activating *TERT* and *CTNNB1* oncogenes.

### Bi-allelic *HMBS* inactivation in sporadic HCC

To identify sporadic HCC potentially related to *HMBS* deficiency, we next screened *HMBS* mutations in 754 HCC cases from inhouse (LICA-FR, n = 60 genomes and 347 exomes, Table S1) and TCGA series (n = 347 exomes<sup>6</sup>). This data set includes patients with diverse fibrosis stages (39% F0-F1, 17% F2-F3, 44% F4) and diverse risk factors including alcohol intake (34%), HBV (33%) or HCV (18%) infection, and metabolic syndrome (12%). 21% of patients had no identified etiology. We identified 9 somatic *HMBS* mutations in 7 patients (4 in LICA-FR, 3 in TCGA series, Table 1), a low frequency yet significantly higher than expected by chance given gene size and mutation rate ( $p = 1.7 \times 10^{-4}$ , MutSigCV algorithm<sup>27</sup>), indicating a positive selection of *HMBS* mutations in HCC. Consistent with a tumor suppressive role, somatic *HMBS* mutations were mostly truncating, with an enrichment of mutations in splice regions (Fig. 2A). Using matched RNA-seq data, we confirmed the functional impact of splice mutations which all lead to abnormal transcript structures with varying degrees of intron retention or exon shrinkage (Fig. 2B). Of note, 7 of the 9 somatic *HMBS* mutations were documented as pathogenic mutations associated with AIP in the Human Gene Mutation Database.<sup>28</sup> Also consistent with a tumor suppressive role, 5/7 patients with somatic *HMBS* mutations displayed evidence of bi-allelic inactivation. Two patients (TCGA-EP-A26S and TCGA-ZP-A9D4) harbored rare pathogenic germline mutations known to cause AIP; they may correspond to porphyria cases not annotated as such in TCGA database or healthy carriers of AIP-predisposing variants. Two patients (FR-1731T and FR-429T) harbored 2 different somatic mutations, and FR-1598T displayed a somatic mutation and a focal copy-neutral loss of heterozygosity, leading to the loss of the wild-type copy (Fig. S2). Overall, these data identify a rare subset of HCC with biallelic *HMBS* inactivation. Although we cannot formally exclude that 2 *HMBS* alterations may affect the same allele, the co-occurrence of 2 *HMBS* alterations in 5/7 patients is highly unlikely to occur by chance ( $p = 1.0 \times 10^{-11}$ , Fisher's exact test) and strongly suggests a bi-allelic inactivation of the enzyme in these tumors.

### *HMBS* inactivation results in a massive accumulation of PBG in HCC

Next, we investigated the functional consequences of *HMBS* mutations on the heme biosynthesis pathway. We used UPLC-QTOFMS to quantify the levels of 3 heme synthesis intermediates: PBG (the substrate of *HMBS*), PPIX and heme (the oxidative product of heme) (Fig. 3 and Table S2). We analyzed HCC and matched non-tumor liver tissues from 10 patients, including 1 HCC developed in a context of AIP (#1606), 3 sporadic HCCs with bi-allelic somatic inactivation of *HMBS* (#428, #1598 and #1731), and 6 sporadic HCCs without *HMBS* inactivation for control comparison. PBG was barely detectable in the non-tumor liver tissues of sporadic HCCs and in HCC samples without *HMBS* inactivation (median <50 ng per g of tissue, range 0-1,415 ng/g). In contrast, germline *HMBS* mutation resulted in PBG accumulation in the non-tumor liver of the patient with AIP #1606 (8,022 ng/g), with a striking increase upon inactivation of the second *HMBS* allele in the tumor



(32,547 ng/g). Similarly, the 3 tumors with bi-allelic somatic *HMBS* inactivation displayed a massive accumulation of PBG (median = 36,223 ng/g, range 25,575-55,289 ng/g). Overall, HCCs with bi-allelic *HMBS* inactivation displayed a significant increase of PBG compared to both non-tumor liver tissue and tumors without *HMBS* inactivation ( $p = 0.0010$ , Wilcoxon rank-sum test). The late-stage intermediates PPIX and hemin were detected at low levels in all the samples (median = 193 ng/g and 426 ng/g respectively), with a trend towards decreased PPIX levels in *HMBS*-inactivated HCC with respect to other HCC and non-tumor liver tissues (median = 143 ng/g vs. 237 ng/g,  $p = 0.10$ , Wilcoxon rank-sum test). Thus, biallelic *HMBS* inactivation in HCC results in a blockage of the heme synthesis pathway with major accumulation of its toxic precursor PBG.

### **HMBS inactivation defines a homogenous clinical and molecular HCC subgroup**

Patients with *HMBS* inactivation displayed homogenous clinical characteristics (Fig. 4A), with an enrichment of females (70%,  $p = 0.0035$ , Fisher's exact test), low fibrosis with F0-F1 Metavir fibrosis scores (90%,  $p = 0.0019$ , Fisher's exact test) and an absence of classical HCC risk factors (70%,  $p = 0.00094$ , Fisher's exact test). There was no significant difference in patient age, Edmonson differentiation grade, or tumor size. At the molecular level, *HMBS* mutations frequently co-occurred with *CTNNB1*-activating mutations (82%,  $p = 0.00018$ , Fisher's exact test) and to a lesser extent *ARID2* (27%,  $p = 0.026$ ) and *CDKN2A* mutations (18%,  $p = 0.048$ ), indicating a synergistic effect on tumorigenesis (Fig. 4B). *TERT* promoter mutations were also frequent (71%) although not significantly enriched. By contrast, *HMBS* mutations were exclusive with *TP53* mutations (0%,  $p = 0.040$ ). *HMBS*-inactivated tumors displayed homogenous gene expression and DNA methylation profiles, evidenced by their tight clustering in t-distributed stochastic neighbor embedding visualizations (Fig. 4C). At the transcriptomic level, *HMBS*-inactivated HCC mostly belonged to the G5 and G6 subgroups associated with Wnt/ $\beta$ -catenin pathway activation,<sup>40,41</sup> displayed high expression of differentiation markers and low expression of proliferation markers compared with other HCCs (Fig. 4D). At the epigenetic level, *HMBS*-inactivated tumors clustered within methylation subgroup M6 (enriched in well-differentiated *CTNNB1*-mutated tumors). Compared to other HCC, they displayed higher contribution of age-related signatures of hyper-(MC1) and hypomethylation (MC12) and lower contribution of methylation signatures related to male sex (MC3) and immune infiltration (MC6) (Fig. 4E). This methylation pattern is consistent with the enrichment in female sex and generally advanced age of this tumor subgroup, and with the low levels of immune infiltration in *CTNNB1*-mutated HCC.<sup>29,30</sup> Overall, *HMBS* inactivation synergizes with Wnt/ $\beta$ -catenin activation and gives rise to a homogenous molecular entity, preferentially developed in the non-fibrotic liver of females without classical risk factors.

## **Discussion**

Our data identify *HMBS* as a tumor suppressor in HCC with 2 modes of inactivation: (i) germline *HMBS* mutation carriers, with or without diagnosed AIP, acquiring a second somatic mutation, or (ii) patients with bi-allelic somatic alterations. The fact that these tumors mostly develop in the absence of fibrosis and classical HCC risk factor suggests that *HMBS* inactivation may be the initiating event that triggers tumorigenesis. The enrichment

of truncating mutations (10/18 *HMBS* mutations), the frequency of bi-allelic inactivation (9/11 tumors) and the massive PBG accumulation that we detected indicate that the heme synthesis pathway is blocked in these tumors. Similarly, a bi-allelic inactivation of protoporphyrin oxidase (PPOX) was previously described in a case of HCC that developed in a patient with variegate porphyria.<sup>26</sup> However, complete loss of heme synthesis is not compatible with cell viability, as shown in animal models. We have 2 hypotheses to explain how tumor cells can survive with bi-allelic *HMBS* mutations. First, missense mutations may lead to enzymes retaining a minor enzymatic activity. For example, *in vitro* experiments demonstrated that *HMBS* enzymes with the R26H, L30F and R149L mutations identified in our HCC series retained respectively 3%, 3% and 5% of the wild-type enzyme activity.<sup>31</sup> Second, tumor cells may import heme produced by surrounding non-tumor hepatocytes. Although *HMBS*-mutated HCC displayed higher expression of the heme importer *FLVCR2*, they also displayed lower expression of the heme importer *UNC119* (encoding HRG4) and higher expression of the heme exporter *ABCG2*. These data do not support an adaptation of *HMBS*-mutated tumors to increased heme import. However, *HMBS*-mutated tumor cells do express several heme importers and are certainly able to import heme from their environment.

The tumorigenic effect of heme synthesis pathway blockage may result from the accumulation of porphyrin precursors. The leading hypothesis for acute attacks in AIP is that porphyrin precursors, notably ALA, are neurotoxic, although the precise mechanism of this toxicity remains elusive.<sup>32</sup> Hepatotoxicity has not been reported in AIP to our knowledge, but the higher level of PBG resulting from bi-allelic *HMBS* inactivation may become toxic to hepatocytes and promote tumorigenesis. Alternatively, the tumorigenic effect of heme pathway blockage may be due to the depletion of heme or late pathway intermediates. In particular, PPIX, the last intermediate before heme, is a dual inhibitor of p53 interactions with MDM2 and MDM4.<sup>33</sup> Treating human cells with PPIX leads to the overexpression of pro-apoptotic p53 target genes and induces cell death.<sup>34</sup> Thus, PPIX depletion in *HMBS*-inactivated cells may promote p53 inhibition by MDM2/4. Such mechanism would also explain why *HMBS* and *TP53* mutations are exclusive. We found only a non-significant trend towards decreased PPIX in *HMBS*-mutated tumors, but the baseline concentration in non-tumor liver is so low that it could be challenging to detect the decrease in tumor cells. This hypothesis should be explored further in mechanistic experiments. Finally, *HMBS* inactivation may have indirect consequences on other metabolic processes.<sup>35</sup> We observed an upregulation of several tricarboxylic acid cycle enzymes (*IDH3B*, *OGDH*, *SUCLA2*, *SUCLG2*, *SDHA/B/C/D*, *FH* and *CS*) in *HMBS*-mutated HCCs with respect to other HCCs, as well as a downregulation of early glycolysis enzymes (*HK1/2* and *GPI*) and an upregulation of late glycolysis enzymes (*PGM1* and *PKLR*) (Fig. S3 and Table S3). However, these changes were also observed in *CTNNB1*-mutated HCCs, so it is not clear if they were due to Wnt/ $\beta$ -catenin pathway activation and/or *HMBS* defects. *HMBS*-mutated tumors also displayed an upregulation of serine-glycine synthesis pathway enzymes (*PSAT1* and *SHMT2*) that may allow tumor cells to counterbalance the consumption of glycine used to produce of ALA and PBG.

*HMBS* inactivation defines a homogeneous HCC subgroup with distinct clinical and molecular features. Notably, *HMBS*-mutated HCCs were strongly enriched in females (7/10

vs. 24% in HCC without *HMBS* mutation), and this association holds true in patients without AIP (5/7 females). Thus, women are more prone than men to develop HCC upon *HMBS* inactivation. Similarly, germline *HMBS* mutations induce more frequent and severe AIP attacks in women.<sup>36,37</sup> Indeed, female sex hormones increase the production of porphyrin precursors by inducing ALA-synthase (the first enzyme in the heme synthesis pathway), and GnRH agonists have been shown to prevent cyclical attacks.<sup>38,39</sup> Contrary to AIP, which mostly affects young and middle-aged women, *HMBS*-related HCCs were diagnosed in post-menopausal women. Thus, the role of sex hormones in interaction with heme pathway blockage in liver tumorigenesis remains to be explored. At the molecular level, we identified a striking association between *HMBS* and *CTNNB1* mutations, indicating a synergy between the pathways in liver tumorigenesis. It was previously shown in mice that *Cttnb1* knockout induces downregulation of the heme biosynthesis enzymes ALA-S and ALA-D and protects from porphyria-associated liver injury,<sup>40</sup> whereas  $\beta$ -catenin activation enhances the expression of heme synthesis-related genes.<sup>41</sup> Consistently, we observed a significant upregulation of 5 heme pathway enzymes (*ALAS1*, *ALAD*, *UROS*, *PPOX* and *FECH*) in sporadic HCC with *CTNNB1*-activating mutations (Fig. S4 and Table S3). *HMBS*-mutated tumors displayed an even stronger upregulation of *ALAS1* and *ALAD*, suggesting that tumor cells react to pathway blockage by increasing the expression of these early enzymes, reinforcing the accumulation of toxic PBG. Both female sex and *CTNNB1* activation may thus promote liver tumorigenesis by intensifying the consequences of *HMBS* inactivation. Consistently, a recent large-scale study of 1,244 individuals with acute hepatic porphyrias identified a strong association between clinical AIP activity, elevated urinary PBG, and primary liver cancer risk.<sup>24</sup>

Metabolic reprogramming is considered a hallmark of cancer.<sup>42</sup> Frequent metabolic alterations include the shift from oxidative phosphorylation to aerobic glycolysis (Warburg effect), deregulated glutamine uptake or hyperactivation of the serine/glycine biosynthetic pathway.<sup>43</sup> Yet, only few metabolic genes have been identified as direct cancer drivers. These include *IDH1/2* in glioma and AML,<sup>44,45</sup> genes encoding SDH subunits in paraganglioma,<sup>46</sup> *FH* in renal cell cancer<sup>47</sup> or *FUT9* in colorectal cancer.<sup>48</sup> Herein, we identify *HMBS* as an HCC driver gene and highlight a tumorigenic role of heme biosynthesis pathway blockage in the liver. *HMBS* mutations should be investigated in patients with HCC without typical risk factors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Data availability statement

Whole-genome sequencing and RNA-seq data generated for this study have been deposited to the European Genome Archive (EGA) under study accession number [EGAS00001005986].

### Abbreviations

<b>AIP</b>	acute intermittent porphyria
<b>ALA</b>	aminolevulinic acid
<b>HCC</b>	hepatocellular carcinoma
<b>HMBS</b>	hydroxymethylbilane synthase
<b>PBG</b>	porphobilinogen
<b>PPIX</b>	protoporphyrin IX
<b>PPOX</b>	protoporphyrin oxidase
<b>SBS</b>	single base substitution
<b>RNA-seq</b>	RNA-sequencing
<b>TCGA</b>	The Cancer Genome Atlas
<b>UPLC-QTOFMS</b>	ultra-high performance liquid chromatography with quadrupole time-of-flight mass; spectrometry
<b>WES</b>	whole-exome sequencing
<b>WGS</b>	whole-genome sequencing

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Author names in bold designate shared co-first authorship

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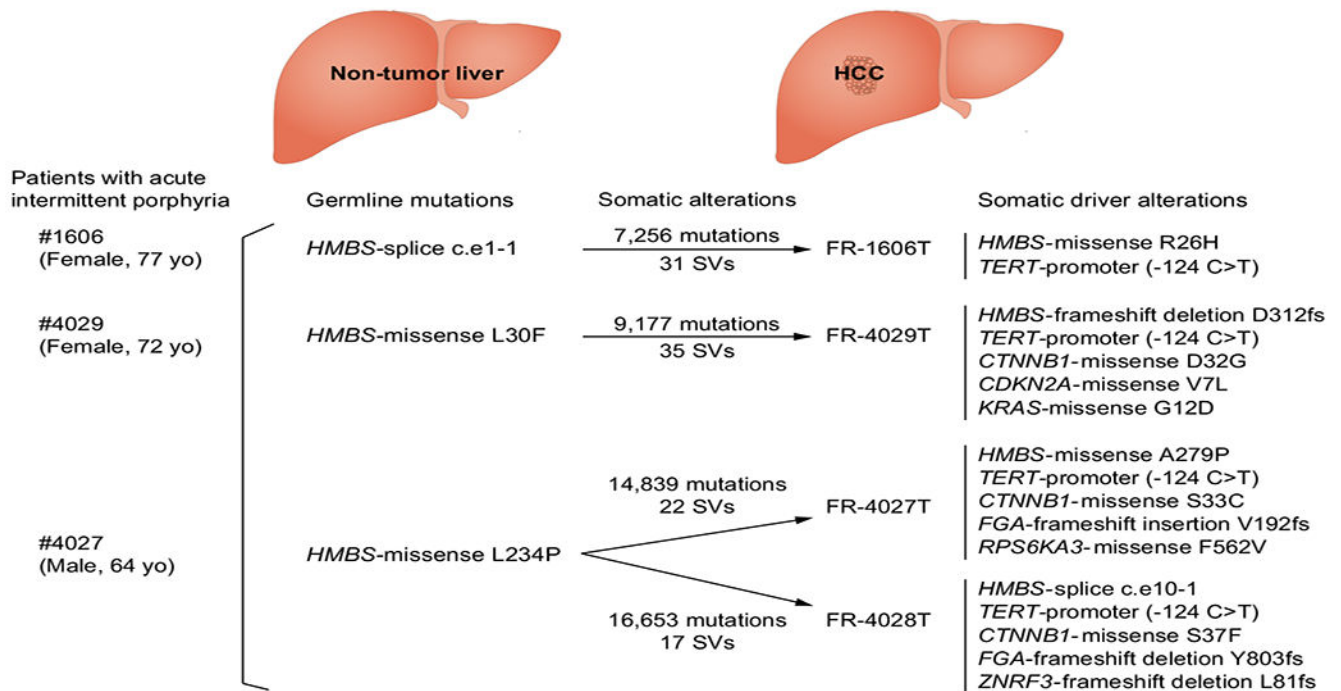
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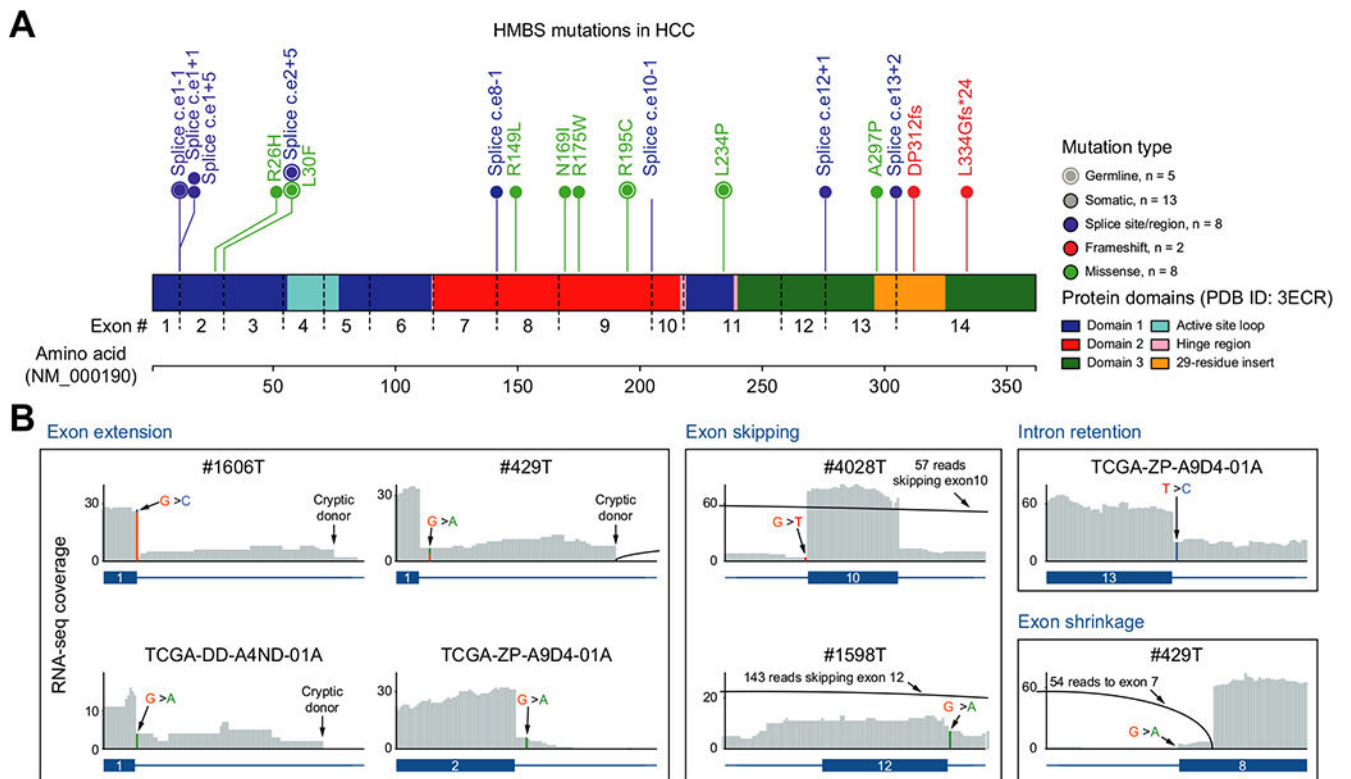
### Highlights

- We identify recurrent *HMBS*-inactivating mutations in hepatocellular carcinoma.
- Bi-allelic *HMBS* inactivation occurs both in patients with acute intermittent porphyria and sporadic HCC.
- *HMBS* inactivation induces a massive accumulation of its toxic substrate porphobilinogen.
- *HMBS*-mutated HCC mostly develop in females, in the absence of fibrosis and classical HCC risk factors.
- *HMBS*-mutated HCC display activating *CTNNB1* mutations and Wnt/ $\beta$ -catenin pathway activation.



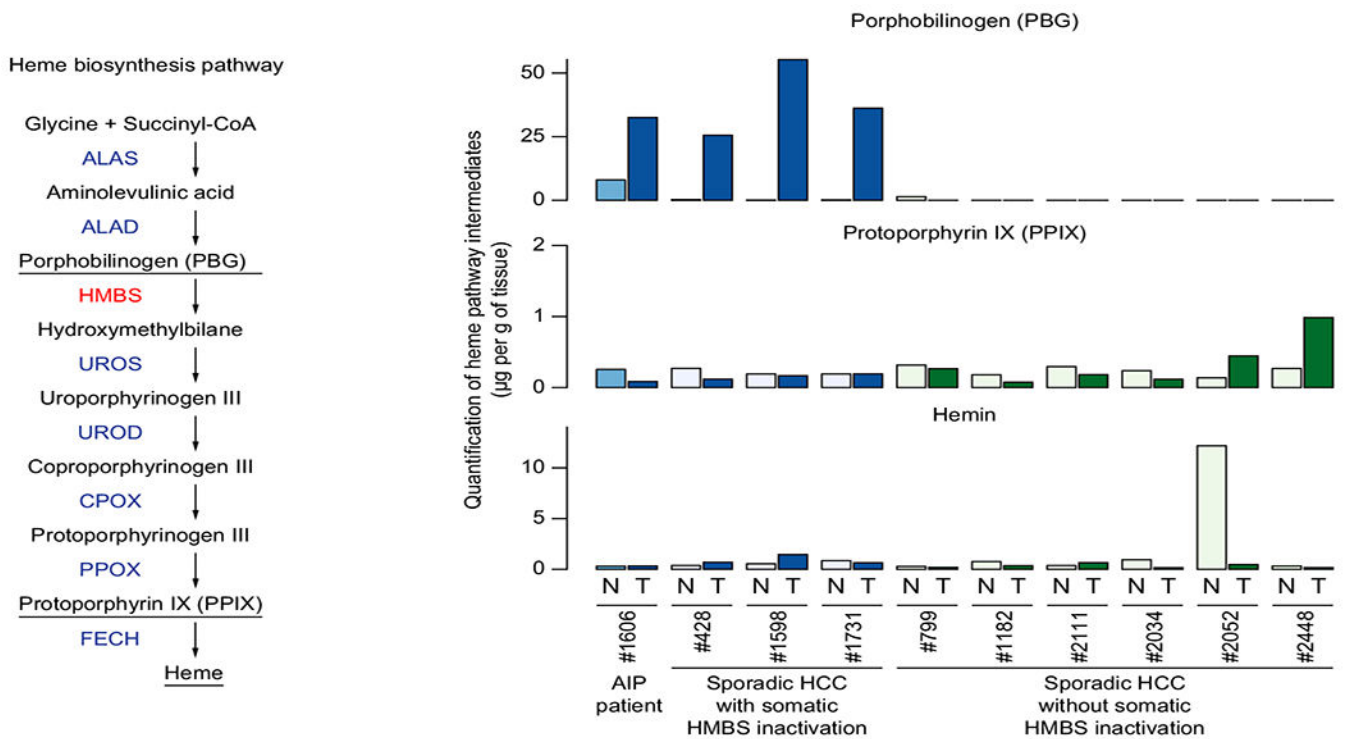
**Fig. 1. Molecular history of HCC developed in patients with AIP.**

Whole-genome sequencing of HCC and matched non-tumor liver samples from 3 patients with AIP revealed germline *HMBS* mutations and somatic alterations acquired by cancer cells. Alterations in known liver cancer driver genes are annotated. Patient #4027 developed 2 independent synchronous HCCs. AIP, acute intermittent porphyria; HCC, hepatocellular carcinoma; SVs, structural variants.



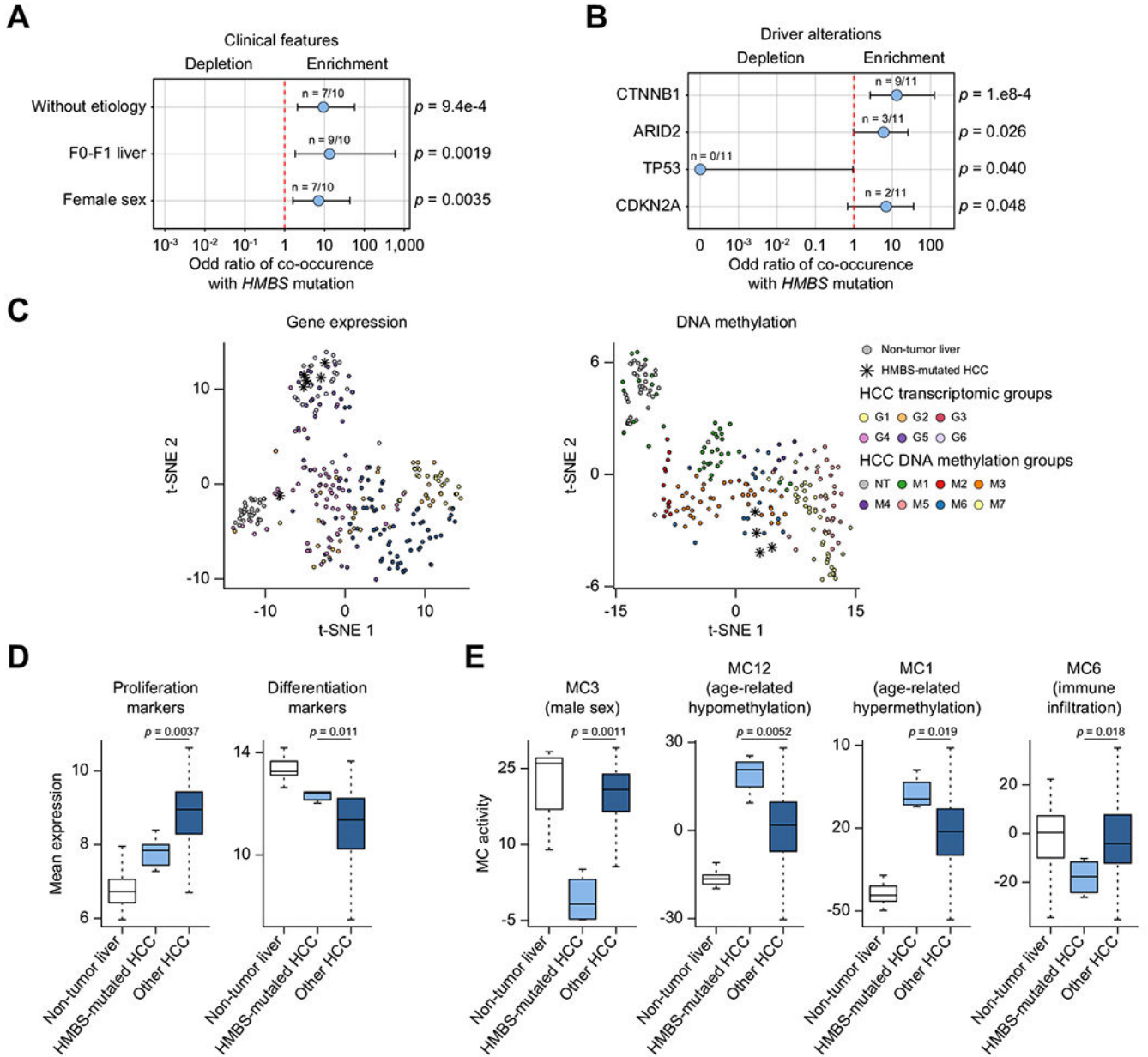
**Fig. 2. Recurrent inactivating HMBS mutations in HCC.**

(A) Lollipop plot showing the type and distribution of HMBS mutations along the protein sequence. Amino acid and exon numbering is given with respect to the NCBI Refseq NM\_000190 isoform. (B) Impact of splice mutations on RNA structure. RNA-sequencing coverage is shown in grey. Arcs represent split reads indicating splicing events. Mutations are highlighted in each plot. HCC, hepatocellular carcinoma.



**Fig. 3. Massive porphobilinogen accumulation in HMBS-mutated HCC.**

Three heme biosynthesis pathway intermediates (porphobilinogen, protoporphyrin IX and hemin) were quantified by ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry. Porphobilinogen is the substrate of HMBS, protoporphyrin IX is the last intermediate before heme, and hemin results from the spontaneous oxidation of heme *in vitro*. These metabolites were quantified in 10 HCC/non-tumor liver pairs from 1 patient with AIP, 3 patients with HCC and somatic HMBS inactivation, and 6 patients with HCC without HMBS inactivation used as controls. AIP, acute intermittent porphyria; HCC, hepatocellular carcinoma.



**Fig. 4. Clinical and molecular features of HMBS-inactivated HCC.**

Forest plots showing odds ratios for enrichment of clinical features (A) and driver alterations (B) among HMBS-inactivated HCC. *P* values were obtained using Fisher’s exact test. (C) t-distributed stochastic neighbor embedding plots showing the unsupervised classification of gene expression (RNA-sequencing) and DNA methylation (Illumina Infinium HumanMethylation450 BeadChip) profiles in the LICA-FR series. Previously defined transcriptomic (G1-G6) and DNA methylation (M1-M7) subgroups are indicated with a color code, and HMBS-mutated HCCs are highlighted with stars. (D) Gene expression signatures showing differential activity in HCC with or without HMBS

mutations. (E) DNA methylation components showing differential activity in HCC with or without HMBS mutations. HCC, hepatocellular carcinoma.

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Table 1.

Ten patients harboring HMBS mutations.

Patient	AIP diagnosis	Sex	Age	HCC risk factors	Fibrosis score	HMBS alterations
#1606	Yes	F	77	Without common etiology	F0	Germline splice c.e1-1 <sup>#</sup> Somatic missense R26H <sup>#</sup>
#4027	Yes	M	64	Metabolic	F1	Germline missense L234P <sup>#</sup> Somatic missense A297P in tumor FR-4027T <sup>#</sup> Somatic splice c.e10-1 in tumor FR-4028T <sup>#</sup>
#4029	Yes	F	72	Without common etiology	F0	Germline missense L30F <sup>#</sup> Somatic frameshift DP312fs
TCGA-EP-A26S	No	M	70	Alcohol	F0	Germline missense R195C <sup>#</sup> Somatic frameshift L334Gfs <sup>#</sup>
TCGA-ZP-A9D4	No	F	64	Without common etiology	F0	Germline splice c.e2+5 Somatic splice c.e13+2 <sup>#</sup>
#1731	No	F	56	Without common etiology	F0	Somatic missense R149L <sup>#</sup> Somatic missense N169I
#428	No	F	65	Without common etiology	F0	Somatic splice c.e1+5 <sup>#</sup> Somatic splice c.e8-1 <sup>#</sup>
#1598	No	F	76	HBV	F0	Somatic splice c.e12+1 <sup>#</sup> Focal copy-neutral LOH
TCGA-DD-A4ND	No	F	56	Without common etiology	F0	Somatic splice c.e1+1 <sup>#</sup>
BCM683	No	M	75	Without common etiology	F4	Somatic missense R175W

Summary of germline and somatic mutations in each patient and their main clinical characteristics.

<sup>#</sup> Documented pathogenic variant causing AIP.