Argininosuccinate Synthase 1 (ASS1): A Marker of Unclassified Hepatocellular Adenoma and High Bleeding Risk

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Hepatocellular adenomas (HCAs) are rare benign tumors divided into three main subgroups defined by pathomolecular features, HNF1A (H-HCA), mutated β-catenin (b-HCA), and inflammatory (IHCA). In the case of unclassified HCAs (UHCAs), which are currently identified by default, a high risk of bleeding remains a clinical issue. The objective of this study was to explore UHCA proteome with the aim to identify specific biomarkers. Following dissection of the tumoral (T) and nontumoral (NT) tissue on formalin-fixed, paraffin-embedded HCA tissue sections using laser capture methodology, we performed mass spectrometry analysis to compare T and NT protein expression levels in H-HCA, IHCA, b-HCA, UHCA, and focal nodular hyperplasia. Using this methodology, we searched for proteins which are specifically deregulated in UHCA. We demonstrate that proteomic profiles allow for discriminating known HCA subtypes through identification of classical biomarkers in each HCA subgroup. We observed specific up-regulation of the arginine synthesis pathway associated with overexpression of argininosuccinate synthase (ASS1) and argininosuccinate lyase in UHCA. ASS1 immunohistochemistry identified all the UHCA, of which 64.7% presented clinical bleeding manifestations. Interestingly, we demonstrated that the significance of ASS1 was not restricted to UHCA, but also encompassed certain hemorrhagic cases in other HCA subtypes, particularly IHCA. Conclusion: ASS1 + HCA combined with a typical hematoxylin and eosin stain aspect defined a new HCA subgroup at a high risk of bleeding. (HEPATOLOGY 2017; 66:2016-2028).

Hepatocellular adenomas (HCAs) are rare benign tumors.1,2 The risk of complications, such as hemorrhage (26%)3 or malignant transformation to hepatocellular carcinoma (HCC) (7%),4 is higher when the tumors are larger than 5 cm leading to the recommendation to resect when the HCA reaches that size.5-7 Based on clinical, radiological, histological, immunohistochemical...
(IHC), and molecular features, HCA are classified into three major groups.\(^{(1,2,8,9)}\) (1) H-HCA, with inactivating mutations of \(HNF1A\) (hepatocyte nuclear factor 1A), account for 30%-35% of HCAs. \(HNF1A\) encodes a transcription factor involved in hepatocyte differentiation. Liver-type fatty acid binding protein (LFABP), whose expression is controlled by \(HNF1A\), is lost in H-HCA tumor cells and is used for the identification of H-HCA by IHC.\(^{(8,10)}\) (2) Inflammatory HCAs (IHCAs) account for 30%-35% of HCAs and present diverse mutations, which activate the Janus kinase/signal transducer and activator of transcription pathway. The neoplastic hepatocytes show strong and diffuse immunoreactivity of acute-phase inflammatory reactants serum amyloid A (SAA) and C-reactive protein (CRP).\(^{(11,12)}\) (3) \(\beta\)-catenin mutated HCAs (b-HCAs) with activating \(\beta\)-catenin mutations (exon 3 and exons 7-8) are found in 10%-15% of HCAs.\(^{(9,13,14)}\) The \(\beta\)-catenin target gene, \(GLUL\), encoding glutamine synthetase (GS) is diffusely or heterogeneously detected in exon 3 mutated b-HCA,\(^{(8,15)}\) which presents an increased risk of malignant transformation in HCC,\(^{(14)}\) particularly if associated with telomerase reverse transcriptase promoter mutations.\(^{(16)}\) In addition, half of the \(\beta\)-catenin activated HCAs also exhibited inflammatory features (representing roughly 10% of IHCAs) and, overall, \(\beta\)-catenin mutated and inflammatory HCAs (b-IHCAs) represent 15% of all HCA.\(^{(8,9,15)}\) Because of molecular classification of HCA and the identification of specific biomarkers for each subtype, IHC techniques have greatly improved the diagnosis of benign hepatocellular nodules.\(^{(8)}\) Indeed, it is possible to classify majority of HCA by IHC.

The remaining unclassified HCAs (UHCAs), which represent 10% of HCAs, are currently characterized by default, when all features known for the other HCA subtypes are negative, including IHC markers.\(^{(8)}\) In addition, there are still few cases with abnormal GS staining in which no \(\beta\)-catenin mutation is found. A new subgroup among UHCA has recently been described, named sonic hedgehog HCA (shHCA), and is characterized by the activation of the sonic hedgehog pathway.\(^{(9)}\) This subgroup accounts for 4% of HCAs and is associated with histological hemorrhage. However, the biomarker associated with this subgroup, prostaglandin-H2 D-isomerase, is not reliable for IHC in routine diagnosis in our experience.\(^{(9)}\) The absence of specific biomarkers in UHCA, including shHCA, prompted us to analyze their proteome by combining laser microdissection with mass spectrometry. We analyzed and compared the deregulation of protein expression in the tumoral (T) and nontumoral (NT) parts of each UHCA patient and then compared them with the other HCA subgroups.

**Materials and Methods**

In our center, all HCAs are routinely classified by IHC. UHCAs are defined by default (when all specific HCA markers are negative). From a total of 218 resected cases (189 female/29 male) included in the study, between 1984 and 2016, there were 70 \(HNF1A\) mutated HCAs (H-HCAs), 69 IHCAs, 22 b-HCAs (including 15 ex3), 32 b-IHCAs (including 20 ex3),

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17 UHCA cases (1 case was associated with two IHCAs and was counted in the IHCA list), 8 unclassifiable cases attributed to unusable material (enormous hemorrhage/necrosis), and 3 cases are still awaiting identification (with abnormal GS staining, usually focal, and with no β-catenin mutation by molecular analysis). In our series of 17 UHCA cases, there were 11 shHCA characterized by molecular tools (including the 4 cases of the proteome-analyzed group and 7 cases which were identified at a later stage), 1 UHCA non-shHCA (by molecular analysis), and, finally, 5 cases which were not available for molecular biology. We reviewed the chart of these 17 UHCA cases, including age, sex, body mass index (BMI), oral contraception (duration), reason for the discovery of the tumor, associated diseases (hypertension, diabetes, etc.), number of nodules and their maximum size, and gross pathology (in particular, necrosis and hemorrhage). The slides were reviewed (hematoxylin and eosin stain [H&E], CD34, reticulin, GS, and additional markers, if necessary), and all the standard pathological criteria were analyzed in the tumor, including areas of necrosis/hemorrhage, peliosis, steatosis, cholestasis, sinusoidal dilatation, vessels (veins and arteries), and NT tissue, in particular, steatosis.

Sample preparation, laser microdissection, and mass spectrometry analysis, bioinformatics analysis, RNA isolation, qRT-PCR, western blotting, IHC analysis, and statistical analyses were performed as described in Supplementary Materials and Methods.

Results

CLINICAL AND PATHOLOGICAL FEATURES IN PATIENTS

All 17 UHCA patients (from 218 cases of our cohort) were women; mean age was 40 years (range, 27–48). All patients were using oral contraceptives, with a mean duration of 21 years (range, 7-30), the latter known for 10 of 16 patients. BMI showed an increase in 13 cases with a mean of 30 (range, 19.7-45.7; Supporting Table S1). The mean size of the largest nodule was 7 cm (range, 1.3-23.0). The mode of discovery was either by death, hemorrhagic rupture of the liver, or severe hemorrhaging (intratumoral, intrahepatic, or peritoneal), or abdominal pain, and or by chance, for 1, 7, 4, and 5 of all cases, respectively. The number of nodules was 1, 2, and ≥5 for 10, 5, and 2 of all cases, respectively. Associated diseases were type 1 and 2 diabetes and arterial hypertension for 1, 4, and 5 of all cases (1 of renal origin), respectively. Hemorrhage with or without necrosis (Table 1 and Supporting Table S2) was detectable based on the investigation of clinical, radiological, and/or pathological information: (1) macroscopic (small or large, recent or old areas) visible in 15 cases and (2) microscopic in 1 additional case. In all 17 cases, tumors had the same aspect: there was nonencapsulated, well-differentiated hepatocellular proliferation composed of clear or often hypereosinophilic and packed cells (Supporting Fig. S1). Tumors were well vascularized by numerous arteries and veins, without noticeable inflammation, ductular reaction, or steatosis (except in 1 case with 30% steatosis). The criteria for malignant transformation were present in 2 cases: 1 was classified as borderline HCA (case 217) and another 1 had several HCC foci (case 162). NT liver was steatotic in 12 of 17 cases: ≥60%, 30%-60%, and 10%-30% for 7, 4, and 1 of all cases, respectively.

The main clinical data for the other HCA subtypes and focal nodular hyperplasia (FNH) analyzed by proteomics and/or IHC are presented in Supporting Table S3. The data are those usually observed in benign hepatocellular nodules.

COMBINATION OF LASER CAPTURE AND MASS SPECTROMETRY ANALYSIS FOR PROTEOMIC ANALYSIS OF HCA

We performed proteomic analysis for 9 of the 17 UHCA cases as well as for 3 H-HCA, 3 IHCA, 3 b-HCA (exon 3), and 3 FNH cases. In this approach, the H-HCA, IHCA, and b-HCA cases did not present hemorrhagic features. We implemented a method combining laser microdissection and mass spectrometry analysis, tailored to compare protein expression levels in T and NT tissue derived from the formalin-fixed and paraffin-embedded (FFPE) tissue sections from the same patient. Following histopathological selection, NT and T areas of the same size were microdissected from tissue sections. Three replicates were made from three serial sections of the same block to ensure technical robustness. After protein extraction and fixation reversion, proteins were digested by trypsin and the peptides were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). The amounts of protein from NT and T tissue were compared by label free quantification (17) (Fig. 1A). Using this method, we compared the T and NT proteomes of HCA. We retained only
proteins identified with at least two peptides and with the highest identification scores; we then considered their expression as variant if the ratio of T versus NT protein counts was \( \leq 0.5 \) or \( \geq 2 \). For all tumor types, we identified a number of proteins averaging 1,383 \( \pm 243 \). In total, 315 proteins were (commonly) quantified in all the pairs of T/NT samples. Among the identified proteins, 5% were up-regulated in tumors and 6% down-regulated in UHCA with a very similar general quantification profile for FNH (4% up-regulated, 3% down-regulated). A large number of proteins was deregulated in H-HCA (9% up-regulated, 9% down-regulated), IHCA (15% up-regulated, 12% down-regulated) and b-HCA (9% up-regulated, 7% down-regulated; Fig. 1b).

The UHCA group appeared to be both homogeneous within the group (Supporting Fig. S2) and separated from the rest of the tumor groups (Fig. 1C) based on the hierarchical clustering analysis of differential protein expression profiles. Moreover, mean intracluster distance of the UHCA group (9.42) showed the compactness of the corresponding cluster.

To validate our approach, we first analyzed the proteomic data (Supporting Table S4) for known biomarkers used for HCA characterization by IHC. As expected, we identified CRP and SAA in IHCA only. We confirmed the up-regulation of GS in b-HCA (44.4 \( \pm 29.8 \)) and FNH (12.5 \( \pm 10.6 \)) and absence of LFABP expression in H-HCA (0.07 \( \pm 0.02 \)). Surprisingly, LFABP was detected, but systematically
down-regulated, in UHCA (ratios T/NT, 0.4 ± 0.1) in comparison with IHCA, b-HCA, and FNH, whereas a total extinction of the expression was observed for H-HCA (Fig. 2A). All these data validate our methodology and show that proteomic expression data can be used to distinguish HCA sub-groups.

The T/NT protein expression ratios were then compared for UHCA and other HCAs as well as for FNH.
Supporting Table S4). Gene enrichment analysis of the 70 significantly differentially expressed proteins showed that the UHCA group was characterized by the enrichment of the arginine biosynthesis pathway (Fig. 2B, which shows the results for 30 of the 70 proteins with the adjusted $P$ value <0.0007).
Furthermore, among the 30 top-ranking differentially expressed proteins (adjusted \( P \) value, \(<0.0007\)) that differentiated the UHCA from the other subgroups, we found three key members of the urea cycle, argininosuccinate synthase (ASS1) being the top-ranking protein (adjusted \( P \) value \( = 1.14E-07 \)) as well as argininosuccinate lyase (ASL) and carbamoyl phosphate synthetase I (CPS1; Fig. 2C).

**UP-REGULATION OF THE ENDOGENOUS SYNTHESIS OF ARGinine IN ALL UHCA TUMORS**

We then focused on the urea cycle located primarily in the periportal hepatocytes of the liver.\(^{(18,19)}\) The urea cycle produces urea from ammonia and consists of five reactions: two mitochondrial and three cytosolic.
CPS1, also known as Hep Par 1, is a mitochondrial ligase that converts glutamine or glutamate into carbamate which, when phosphorylated with adenosine triphosphate, results in carbamyl phosphate (CP). The second mitochondrial enzyme, ornithine transcarbamylase (OTC), catalyzes the reaction between CP and ornithine to form citrulline. Subsequently, in the cytosol, the urea cycle crosses the arginine biosynthesis pathway, which uses glutamate or proline as a substrate and citrulline as an intermediate. The conversion of citrulline into arginine involves two enzymes: ASS1 and ASL. Arginase, whose isoform 1 is essentially expressed in hepatocytes, catalyzes the fifth and final cytosolic step in the urea cycle, converting arginine to ornithine and urea.

Compared to NT liver, CPS1 was significantly up-regulated in UHCA (2.0 \pm 0.8; adjusted p value UHCA vs. other HCA = 3.1E-03), down-regulated in the other HCA subgroups (0.6 \pm 0.4), and unregulated in FNH (1.0 \pm 0.3; Fig. 3A,B). ASS1 and ASL were significantly up-regulated (5.5 \pm 2.3; adjusted P value UHCA vs. other HCA = 7.4E-06 and adjusted P value UHCA vs. FNH = 8.4E-03 and 2.4 \pm 1.0; adjusted P value UHCA vs. other HCA = 1.9E-04 and adjusted P value UHCA vs. FNH = 1.4E-03, respectively) in all UHCA cases, whereas they were down-regulated in the other HCA subgroups (0.5 \pm 0.4 for ASS1 and 0.7 \pm 0.4 for ASL) and FNH (0.4 \pm 0.2 for ASS1 and 0.7 \pm 0.1 for ASL; Fig. 3A,B). Arginase 1 was not deregulated in UHCA tumors whereas it was down-regulated in the other HCA subgroups (0.5 \pm 0.1) as well as in FNH (0.5 \pm 0.2). OTC remained unchanged in all subgroups (Fig. 3A,B). Altogether, the proteomic data suggest specific hyperactivation of arginine production in all the UHCA tumors without activation of its degradation in urea and ornithine cycles.

Interestingly, L-arginine is the nitric oxide synthase (NOS) substrate, which generates nitric oxide (NO) with L-citrulline as a by-product. Arginine is the only source for NO production; several studies have reported that supplemental L-arginine is sufficient for increasing NO production. NO synthesis is believed to have a role in the regulation of vascular permeability, and overabundant production of NO has been correlated to bleeding events. NO plays important roles in hepatic and pathophysiology, and NO system disturbance seems to play a key role in the pathogenesis of chronic liver disease. Furthermore, NO is proposed to be one of the major endogenous vasodilators in portal hypertension. NOS2, expressed in the liver, was not identified in our proteomic analysis.

We analyzed the protein and mRNA expression of ASS1 and ASL1 using protein extracts and corresponding mRNA, respectively, from frozen blocks.
FIG. 5. ASS1 immunostaining in hemorrhagic HCA subtypes. (A) Two cases of hemorrhagic H-HCA: H&E (left panel), ASS1 (right panel); case 122 (up) does not express ASS1; case 50 (down) expresses ASS1. (B) Two cases of hemorrhagic (H) IHCA: case 57 (up) and 115 (down) expressing CRP (left panel) with a heterogeneous overexpression of ASS1, in comparison with NT liver (right panel). The dotted yellow line highlights the periphery of the tumor 57.
from 11 UHCA patients. We confirmed both mRNA and protein up-regulation of ASS1 and ASL in all UHCA tumors (Fig. 3C,D).

VALIDATION OF ASS1 AS A BIOMARKER FOR ALL UHCA

We performed IHC using an anti-ASS1 antibody in all 17 UHCA cases, in the other HCA subtypes and in FNH analyzed previously using proteomics. ASS1 was detected in the normal liver, highlighting the lobular architecture with a periportal and septal staining of variable intensity (Fig. 4A). In some cases, and because of steatosis, or tissue compression (tumor or hematoma), this pattern was not homogeneous and was better observed at a certain distance from the tumor. In UHCA, ASS1 immunoreactivity was stronger than in the corresponding NT liver (Fig. 4B). Staining was either homogeneous or heterogeneous, but it was positive for all the UHCAs tested. Staining difference between T and NT was also observed in three biopsies of the UHCA performed before surgery (Fig. 4C). In the other HCA subtypes and FNH tested previously, there was none or less ASS1 staining in the T part compared to the NT liver (Fig. 4D-G). These data demonstrated that ASS1 could be considered as a biomarker for all UHCA.

ASS1 STAINING IN HEMORRHAGIC HCA

Because arginine metabolism is related to NO production and consequently correlated to vascular permeability, we investigated to see whether ASS1 and bleeding in HCAs were correlated. To do this, we increased the cohort size tested for ASS1 staining by adding 31 cases (H-HCA, IHCA, b-HCA, and b-IHCA), including hemorrhagic cases, to the 12 cases, which had already been analyzed and validated by ASS1 IHC (Supporting Table S3). ASS1 staining was negative in all FNH, b-HCA, and b-IHCA tested and in 10 of 11 H-HCA cases, including 1 hemorrhagic case (15-cm nodule; case 122). In case 50, there were two nodules: one (1 cm, nonhemorrhagic) was ASS1 negative and one (6 cm, hemorrhagic) was ASS1 positive (Fig. 5A). ASS1 staining was positive in 45% of IHCA nodules (Fig. 5B). In relation to hemorrhage or massive congestion (liver pathology), ASS1 was negative in 3 cases (five nodules, three <5 cm); ASS1 was positive in 5 cases (eight nodules, three <5 cm). In cases without hemorrhagic manifestation, ASS1 was negative in 7 cases (seven nodules, two <5 cm); ASS1 was positive in 1 case (case 68) with two nodules (one <5 cm). In all the ASS1-positive cases, the staining was weaker than in UHCA. These data show that ASS1 is not restricted to UHCA, but encompasses certain hemorrhagic cases in other HCA subtypes. In all tested nodules (n = 79), ASS1 up-regulation is significantly associated to hemorrhage (P value = 0.006054), mainly for UHCA (P value = 1.019e-13) and also for IHCA tumors <5 cm (P value = 0.006434; Supporting Table S5). We then analyzed ASS1 and ASL1 protein expression in IHCA s. We used protein extracts from frozen blocks of 11 IHCA ASS1+/−, with or without bleeding. We observed that ASS1 and ASL1 were co-regulated (Supporting Fig. S3A,B). Moreover, there is a significant correlation between expression level of ASS1/ASL and bleeding (Supporting Fig. S3C). However, as previously observed by IHC, ASS1 tumoral expression level in IHCA was lower than in UHCA ASS1− tumors (Fig. 3D and Supporting Fig. S3A).

Discussion

In this study, we used a combination of laser microdissection and mass spectrometry to compare T and NT tissues from HCA to identify biomarkers that are specifically deregulated in UHCA. Owing to the experimental design used and to laser capture, we analyzed T and NT tissues excised from the same patient with great accuracy. In addition, this technique enabled us to rule out interindividual variability and thus allowed for strong computational evidence to be generated despite the small size of the cohort. First, regarding protein expression, UHCA appeared as a homogeneous subgroup, which was different from the others. Second, we observed that the arginine biosynthesis pathway, including ASS1 and ASL, is significantly up-regulated in UHCA when compared to NT tissue. These data were confirmed by IHC, qPCR, and western blotting analyses in a larger cohort. Consequently, we demonstrated the power of mass spectrometry coupled with laser microdissection to identify tumoral signatures and robust biomarkers.

Regarding HCA classification, genomic and transcriptomic analyses defined different HCA subtypes: H-HCA, IHCA, b-HCA, b-IHCA, and UHCA.9 Using IHC, in UHCA subtype, CRP and GS expression was not observed; LFABP was expressed but often decreased (10 of 17 cases), a finding that was also
observed in proteomic analysis. The new subgroup—shHCA—is characterized by the activation of sonic hedgehog signaling, defined by focal deletions that fuse the promoter of \textit{INHBE} gene with \textit{GLI1} gene.\(^9\) We observed that all 17 UHCA cases in our cohort expressed ASS1. Of the 17 cases, 11 correspond to shHCA (characterized by molecular tools), 1 UHCA does not fall in the shHCA group, and 5 were not available for molecular analysis. Interestingly enough, we found no difference regarding protein expression, based on clinical or pathological criteria, in shHCA and non-shHCA cases within our cohort of UHCA patients. In the absence of molecular argument to prove a link between sonic hedgehog activation and ASS1 overexpression, it is reasonable to label the new subgroup ASS1-HCA. We observed approximately the same percentage of different subtypes using IHC in comparison to the molecular analysis performed in a multicenter French study\(^9\) (Table 3).

The difference between the 4% of shHCA (and 7.5% of UHCA) in the multicenter French study\(^9\) and the 7.8% of ASS1-HCA (and 0% of UHCA ASS1 negative) in our present study (Supporting Table S6) could be tentatively explained by the (1) possibility that the % of shHCA is less than ASS1-positive UHCA and (2) difficulty to sample fresh tissue for molecular studies when the boundary between T and NT tissues is not obvious (which is frequent in both UHCA and in presence of hemorrhagic nodules). The second point highlights the advantage of working on fixed tissue, enabling us to choose the analyzed tissue area accurately.

According to the recent literature\(^7\), hemorrhage in HCA has been observed in 27.2% of all patients,\(^3\) and acute rupture and intraperitoneal bleeding were reported in 17.5% of patients.\(^3\) Hemorrhage generally arose in the larger lesions (>5 cm), although smaller lesions could also bleed with a frequency range of 8.3%-11.5%.\(^5,31-33\) The risk of bleeding was inconsistent across the HCA subtypes, although IHCA showed a higher propensity for macroscopic hemorrhage (30%) than H-HCA (8%).\(^31\)

As reported,\(^7\) the present study confirms that the risk of bleeding is exceedingly high. In our UHCA series, 41.1% (7 of 17) present an acute bleeding syndrome. Furthermore, there were 64.7% (11 of 17) of cases that exhibited well-characterized clinical symptoms (acute syndrome and abdominal pain related to bleeding). It is interesting to note that among the 15 cases (88%) with macroscopic hemorrhagic criteria, there were 4 cases discovered by chance (of 5; Table 1). More important, this risk is not strictly dependent on size (bleeding sometimes occurred in small nodules). We also underline that ASS1 staining intensity did not correlate with amount of bleeding.

For the UHCA cases, we discovered that the endogenous arginine synthesis pathway involving ASS1 and ASL was up-regulated. Arginine is a basic amino acid required for the synthesis of proteins, which acts as a precursor for NO.\(^34\) In UHCA, excess arginine synthesis, which is known to increase NO production, was observed, which, in turn, is known to be involved in vascular permeability and a higher risk of hemorrhage.\(^21-23,26\) If our results are confirmed in a larger series, the clinical strategy could be modified, which is currently based on size (>5 cm) only. For HCAs in the 2- to 4-cm range without imaging diagnosis subtypes, a biopsy could be part of patient management.\(^35\) If there are arguments to suggest that the nodule is LFABP\(^+\), CRP\(^-\), and GS\(^-\) but ASS1\(^+\), based on clinical, pathological, and IHC data, a resection could be proposed at an earlier stage, or at least the patient could be informed and therefore be aware of a bleeding risk (Table 2).

Surprisingly, ASS1 was overexpressed in other HCA subtypes, almost exclusively in IHCA. Interestingly, some small IHCAs express ASS1, which is consistent with the fact that NO production and signaling are inter-related in inflammation.\(^36\) ASS1 negative in b-HCA could be explained by the balance between the two pathways involved in ammonia detoxification. On
one hand, GS activity, which removes ammonium ions at low concentrations by the condensation of glutamate and ammonia, forms glutamine in the perivenous area. On the other hand, the ureogenesis with a high-capacity system is predominantly localized in the periportal area. Hence, GS overexpression in b-HCA or b-IHCA might be predominant in the urea cycle and could explain the absence of ASS1 up-regulation. In this case, the bleeding risk could probably be attributed to other factors such as size.\(^{(37)}\)

Consequently, we need to increase the number of H-HCA or IHCA cases and ASS1 positive, which may lead to the same management as described above. This would enable establishing the precise clinical role of ASS1 and understanding the impact of ASS1 positivity in HCA in terms of bleeding risk, especially in nodules smaller than 5 cm.

To conclude, based on proteomic analysis, ASS1+ HCA combined with a typical H&E aspect define a new HCA subgroup at high risk of bleeding. In this situation, no other markers are needed. However, in practice, to avoid any doubt, all immunomarkers are used. The major message of this study is to propose ASS1 immunomarker as the fifth mandatory marker (in addition to LFABP, CRP, and GS/b cat) and to suggest that it can be useful for patient management.

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Supporting Information

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