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BASIC AND TRANSLATIONAL—LIVER

Molecular Classification of Hepatocellular Adenoma Associates With Risk Factors, Bleeding, and Malignant Transformation

Jean-Charles Nault,[1,2,3] Gabrielle Couchy,[1] Charles Balabaud,[4] Guillaume Morcrette,[1] Stefano Caruso,[1] Jean-Frederic Blanc,[4,5] Yannick Bacq,[6] Julien Calderaro,[1,7] Valérie Paradis,[8] Jeanne Ramos,[9] Jean-Yves Scoazec,[10] Viviane Gnemmi,[11] Nathalie Sturm,[12] Catherine Guettier,[13] Monique Fabre,[14] Eric Savier,[15] Laurence Chiche,[16] Philippe Labrune,[17] Janick Selves,[18] Dominique Wendum,[19] Camilla Pilati,[1] Alexis Laurent,[20] Anne De Muret,[21] Brigitte Le Bail,[4,22] Sandra Rebouissou,[1] Sandrine Imbeaud[1]; GENTHEP Investigators, Paulette Bioulac-Sage,[4,22] Eric Letouzé,[1] and Jessica Zucman-Rossi[1,23]

[1]Unité Mixte de Recherche 1162, Génomique Fonctionnelle des Tumeurs Solides, Institut National de la Santé et de la Recherche Médicale, Université Paris Descartes, Université Paris Diderot, Paris, France; [2]Liver Unit, Hôpital Jean Verdier, Hôpitaux Universitaires Paris-Seine-Saint-Denis, Assistance Publique Hôpitaux de Paris, Bondy, France; [3]Unité de Formation et de Recherche Santé Médecine et Biologie Humaine, Université Paris 13, Communauté d'Universités et Etablissements Sorbonne Paris Cité, Paris, France; [4]Université Bordeaux, Bordeaux Research in Translational Oncology, Bordeaux, France; [5]Service Hépato-Gastroentérologie et Oncologie Digestive, Centre Medico-Chirurgical Magellan, Hôpital Haut-Lévêque, Centre Hospitalier Universitaire de Bordeaux, Bordeaux, France; [6]Service d'Hépatogastroentérologie, Centre Hospitalier Régional Universitaire de Tours, Tours, France; [7]Service d'Anatomopathologie, Hôpital Henri Mondor, Université Paris Est Créteil, Institut National de la Santé et de la Recherche Médicale U955, Institut Mondor de Recherche Biomédicale, Créteil, France; [8]Service d'Anatomopathologie, Hôpital Beaujon, Clichy, France; [9]Service d'Anatomopathologie, Gui de Chauliac, Montpellier, France; [10]Service d'Anatomopathologie, Institut Gustave Roussy, Villejuif, France; [11]Institut de Pathologie, Centre Hospitalier Régional Universitaire de Lille, UMR-S 1124, Jean-Pierre Aubert Research Center, Lille, France; [12]Service d'Anatomopathologie, Centre Hospitalier Universitaire de Grenoble, Grenoble, France; [13]Service d'Anatomopathologie, Hôpitaux Paul Brousse et Bicêtre, Le Kremlin Bicêtre, Institut National de la Santé et de la Recherche Médicale U1193 Université Paris-Sud, Orsay, France; [14]Service d'Anatomopathologie, Hôptal Necker-Enfants Malades, Paris, France; [15]Service de Chirurgie Hépato-Bilio-Pancréatique, Centre Hospitalier Universitaire, Pitié Salpétrière, Université Pierre et Marie Curie, Paris, France; [16]Service de Chirurgie Digestive, Centre Medico-Chirurgical Magellan, Hôpital Haut-Lévêque, Centre Hospitalier Universitaire Bordeaux, Bordeaux, France; [17]Assistance Hôpitaux Publique de Paris, Hôpitaux Universitaires Paris-Sud, Hôpital Antoine Béclère, Centre de Référence des Maladies Héréditaires du Métabolisme Hépatique, Clamart, and Université Paris-Sud, and Institut National de la Santé et de la Recherche Médicale U 1169, Orsay, France; [18]Département d'Anatomopathologie, Institut Universitaire du Cancer-Oncopole, Toulouse, France; [19]Service d'Anatomie Pathologique, Assistance Hôpitaux Publique de Paris Hôpital St Antoine, Sorbonne Universités, Université Pierre et Marie Curie 06, Paris, France; [20]Service de Chirurgie Digestive, Hôpital Henri Mondor, Créteil, Institut National de la Santé et de la Recherche Médicale U955, Créteil, France; [21]Service d'anatomopathologie, Centre Hospitalier Régional Universitaire de Tours, Tours, France; [22]Service de Pathologie, Hôpital Pellegrin, Centre Hospitalier Universitaire de Bordeaux, Bordeaux, France; and [23]Assistance Publique Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Département d'Oncologie, Paris, France

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BACKGROUND & AIMS: Hepatocellular adenomas (HCAs) are benign liver tumors that can be assigned to molecular subtypes based on inactivating mutations in hepatocyte nuclear factor 1A, activating mutations in β-catenin, or activation of inflammatory signaling pathways. We aimed to update the classification system for HCA and associate the subtypes with disease risk factors and complications. **METHODS:** We analyzed expression levels of 20 genes and sequenced exon regions of 8 genes (*HNF1A, IL6ST, CTNNB1, FRK, STAT3, GNAS, JAK1*, and *TERT*) in 607 samples of 533 HCAs from 411 patients, collected from 28 centers mainly in France from 2000 and 2014. We performed gene expression profile, RNA sequence, whole-exome and genome sequence, and immunohistochemical analyses of select samples. Molecular data were associated with risk factors, histopathology, bleeding, and malignant transformation. **RESULTS:** Symptomatic bleeding occurred in 14% of the patients (85% of cases were female, median age, 38 years); 7% of the nodules were borderline between HCA and hepatocellular carcinoma, and 3% of patients developed hepatocellular carcinoma from HCA. Based on molecular features, we classified HCA into 8 subgroups. One new subgroup, composed of previously unclassified HCA, represented 4% of HCAs overall and was associated with obesity and bleeding. These tumors were characterized by activation of sonic hedgehog signaling, due to focal deletions that fuse the promoter of *INHBE* with *GLI1*. Analysis of genetic heterogeneity among multiple HCAs, from different patients, revealed a molecular subtype field effect; multiple tumors had different mutations that deregulated similar pathways. Specific molecular subtypes of HCA associated with various HCA risk factors, including imbalances in estrogen or androgen hormones. Specific molecular subgroup of HCA with β-catenin and sonic hedgehog activation associated with malignant transformation and bleeding, respectively. **CONCLUSIONS:** Using sequencing and gene expression analyses, we identified a subgroup of HCA

characterized by fusion of the *INHBE* and *GLI1* genes and activation of sonic hedgehog pathway. Molecular subtypes of HCAs associated with different patients' risk factors for HCA, disease progression, and pathology features of tumors. This classification system might be used to select treatment strategies for patients with HCA.

Keywords: HCC; Tumor Progression; Benign; SHH.

Hepatocellular adenomas (HCA) are hormone-driven benign liver tumors developed mainly in young women, with an incidence around 3/100,000.[1,2] Exposure to estrogens and androgens has been associated with HCA occurrence.[3,4] Complications such as hemorrhage (15%–20%) or malignant transformation (5%)[5–7] increase with tumor size, leading to the recommendation to resect all HCAs >5 cm.[6,8]

We previously described a molecular classification of HCA dissecting the disease in 4 major subgroups strongly associated with risk factors, clinical features, and risk of complications, as well as histologic, immunohistochemical, and radiologic features.[2,9–11] Hepatocyte nuclear factor 1A (HNF1A) mutated hepatocellular adenomas (HHCAs) are defined by inactivating mutations of *HNF1A*[12] with rare *HNF1A* germline mutations that predispose to liver adenomatosis with >10 adenomas in the liver.[12–15] Inflammatory HCAs (IHCA) are defined by Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway activation driven by somatic mutations activating different actors of this signaling pathway,[16] such as gp130 (encoded by *IL6ST*, 60% of mutations), *STAT3* (5%), fyn-related kinase (*FRK*) (10%), JAK1 (3%), and guanine nucleotide binding protein α–timulating (GNAS) (5%).[16–20] Mutations of cadherin-associated protein β1 (*CTNNB1*) exon 3, activating β-catenin define the third group of tumors (b^{ex3}HCA).[21] These tumors have an increased risk of malignant transformation in hepatocellular carcinoma (HCC) linked to telomerase reverse transcriptase (*TERT*) promoter mutations.[2,20,22,23] Interestingly, a subgroup of HCAs shared both inflammatory phenotype and activating mutations of the exon 3 of *CTNNB1* (b^{ex3}IHCA). In contrast, mutations in *CTNNB1* exon 7 or 8 are associated with a mild activation of the Wnt/β-catenin pathway in β-catenin–mutated hepatocellular adenoma in exon 7 or 8 ($b^{ex7,8}$HCA) without an increased risk of malignant transformation.[20,24] Finally, around 10% of HCAs are currently unclassified (UHCA) according to the molecular analysis. Our study was constructed on a large series of 533 HCAs collected to refine the HCA molecular classification and to determine its potential uses in clinical practice.

Material and Methods

Patients

Between 2000 and 2014, frozen tumor samples (n = 777) of benign liver tumors were collected in 28 centers mainly in France and analyzed in the laboratory (Table 1 and Supplementary Tables 1 and 2, Supplementary Figure 1). All patients gave informed consent according to French law and Paris Saint-Louis Institutional Review Board committee approved this study (Paris Saint-Louis, 2004; INSERM IRB 2010; the French Liver Biobanks Network, AFAQ NF S96-900; and Hepatobio Bank). All samples were frozen in nitrogen immediately after resection or biopsy and conserved at −80°C. After exclusion of focal nodular hyperplasia (n = 71), tumors without diagnosis (n = 19), or with poor RNA and DNA quality (n = 80), 607 samples of HCA were included in the study. Among them, 6 malignant transformations (13 samples) and 46 HCAs with multiple sampling (113 samples) were analyzed to assess intra-tumor heterogeneity. Finally, 533 different HCAs that had developed in 411 patients were analyzed in this study. Patients were treated by liver resection in 375 (92%) cases, by liver transplantations in 10 (2%) cases, or were only biopsied in 26 (6%) cases. Multiple HCAs analyzed in 73 patients (195 tumors) were used to study inter-tumor heterogeneity.

Gene Sequencing and Expression Analysis

HNF1A (exon 1 to 10), *IL6ST* (exon 6 and 10), *CTNNB1* (exon 2, 3, 4, 7, and 8), *FRK* (exon 6), *STAT3* (exon 2, 5, 16, and 20), *GNAS* (exon 7, 8, and 9), *JAK1* (exon 15 and 16), and *TERT* (promoter) were sequenced using Sanger sequencing (for detailed protocol see Pilati et al,[20] Rebouissou et al,[24] and Nault et al[25]). Somatic mutations were confirmed by sequencing a second amplification product of tumor and non-tumor samples. Microarray analysis was performed using Affymetrix U133.2 (accession number GSE88839). An robust multiarray averaging normalization was done and differential expression was investigated using a linear model, followed by moderated *t* test for the comparisons of interest, carried out with Limma package.[26] Correction for multiple testing used the Benjamini and Hochberg method.[27] Unsupervised cluster analysis was done over the top 1000 most variable genes and Spearman correlation with average linkage was measured using Gene Cluster 3.0 and Java TreeView.[28]

Gene set enrichment analysis was applied as described by Subramanian et al.[29] First, the genes were ranked according to their differential expression between the sonic hedgehog hepatocellular adenoma (shHCA) and normal liver classes. Then, hallmark gene sets were downloaded from the Molecular Signature Database and were screened against the gene set

Abbreviations used in this paper: **$b^{ex7,8}$HCA, β-catenin mutated hepatocellular adenoma in exon 7 or 8; $b^{ex7,8}$IHCA, β-catenin mutated inflammatory hepatocellular adenoma in exon 7 or 8; b^{ex3}IHCA, β-catenin mutated inflammatory hepatocellular adenoma in exon 3; b^{ex3}HCA, β-catenin mutated hepatocellular adenoma in exon 3; BMI, body mass index; CTNNB1, cadherin-associated protein β1; FRK, fyn-related kinase; GLI1, glioma-associated oncogene 1; GNAS, guanine nucleotide binding protein α stimulating; HCC, hepatocellular carcinoma; HHCA, HNF1A mutated hepatocellular adenoma; HNF1A, hepatocyte nuclear factor 1A; IL, interleukin; IHCA, inflammatory hepatocellular adenoma; INHBE, inhibin β E; JAK, Janus kinase; OC, oral contraception; OR, odds ratio; RT-PCR, reverse transcription polymerase chain reaction; SAA, serum amyloid A; shHCA, sonic hedgehog hepatocellular adenoma; STAT, signal transducer and activator of transcription; TERT, telomerase reverse transcriptase; UHCA, unclassified hepatocellular adenoma.**

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