Clinical Cancer Research

ESM1 as a Marker of Macrotrabecular-Massive Hepatocellular Carcinoma

Julien Calderaro[1,2,3], Léa Meunier[4], Cong Trung Nguyen[2,3], Marouane Boubaya[5], Stefano Caruso[4], Alain Luciani[2,3,6], Giuliana Amaddeo[2,3,7], Hélène Regnault[7], Jean-Charles Nault[4,8,9], Justine Cohen[1,2], Frédéric Oberti[10], Sophie Michalak[11], Mohamed Bouattour[12], Valérie Vilgrain[13], Georges Philippe Pageaux[14], Jeanne Ramos[15], Nathalie Barget[16], Boris Guiu[17], Valérie Paradis[18], Christophe Aubé[19], Alexis Laurent[20], Jean-Michel Pawlotsky[2,3,21,22], Nathalie Ganne-Carrié[4,8,9], Jessica Zucman-Rossi[4,22,23], Olivier Seror[24], and Marianne Ziol[4,9,25]

Abstract

Purpose: Macrotrabecular-massive hepatocellular carcinoma (MTM-HCC) is a novel morphological subtype of HCC associated with early relapse after resection or percutaneous ablation, independently of classical clinical and radiological prognostic factors. The aim of the present study was to identify immunohistochemical markers of MTM-HCC, to ease its diagnosis and implementation into clinical practice.

Experimental Design: To identify potential biomarkers of MTM-HCC, we first analyzed gene expression profiling data from The Cancer Genome Atlas study and further selected two candidate biomarkers. Performance of both biomarkers for diagnosis of MTM-HCC was further tested by immunohistochemistry in two independent series of 67 and 132 HCC biopsy samples.

Results: Analysis of RNA sequencing data showed that MTM-HCC was characterized by a high expression of neoangiogenesis-related genes. Two candidate biomarkers, Endothelial-Specific Molecule 1 (ESM1) and Carbonic Anhydrase IX (CAIX), were selected. In the discovery series, sensitivity and specificity of ESM1 expression by stromal endothelial cells for the detection of MTM-HCC were 97% (28/29), and 92% (35/38), respectively. Sensitivity and specificity of CAIX were 48% (14/29) and 89% (34/38). In the validation set, sensitivity and specificity of ESM1 for the identification of MTM-HCC were 93% (14/15) and 91% (107/117), respectively. Interobserver agreement for ESM1 assessment was good in both series (Cohen Kappa 0.77 and 0.76).

Conclusions: Using a molecular-driven selection of biomarkers, we identified ESM1 as a reliable microenvironment immunohistochemical marker of MTM-HCC. The results represent a step toward the implementation of HCC morpho-molecular subtyping into clinical practice.

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[1]Assistance Publique-Hôpitaux de Paris, Département Pathologie, CHU Henri Mondor, F-94000 Créteil, France. [2]Université Paris-Est Créteil, Faculté de Médecine, Créteil, France. [3]Inserm, U955, Team 18, Créteil, France. [4]INSERM UMR-1162, génomique fonctionnelle des tumeurs solides, Paris, France. [5]Unité de Recherche Clinique, AP-HP, Hôpital Universitaire Avicenne, Bobigny, France. [6]Assistance Publique-Hôpitaux de Paris, Service de Radiologie, CHU Henri Mondor, F-94000 Créteil, France. [7]Assistance Publique-Hôpitaux de Paris, Service d'Hépatologie, CHU Henri Mondor, F-94000 Créteil, France. [8]Service d'Hépatologie, Groupe hospitalier Paris-Seine-Saint Denis, Hôpital Jean Verdier, AP-HP, Bondy, France. [9]Université Paris 13, Sorbonne Paris-Cité, Bobigny, France. [10]Hépato-gastroentérologie et oncologie digestive, Centre Hospitalier Universitaire d'Angers, France. [11]Service d'Anatomie et de Cytologie Pathologiques, Centre Hospitalier Universitaire d'Angers, France. [12]Assistance Publique-Hôpitaux de Paris, Service d'Oncologie Digestive, Hôpital Universitaire Beaujon, France. [13]Assistance Publique-Hôpitaux de Paris, Service d'Anatomie et de Cytologie Pathologiques, Hôpital Universitaire Beaujon, France. [14]Hépato-gastroentérologie et oncologie digestive, Centre Hospitalier Universitaire de Montpellier, France. [15]Service d'Anatomie et de Cytologie Pathologiques, Centre Hospitalier Universitaire de Montpellier, France. [16]Assistance Publique-Hôpitaux de Paris, Centre de ressources biologiques BB-0033-00027 du Groupe hospitalier Paris-Seine-Saint Denis, Hôpital Jean Verdier, Bondy, France. [17]Service de Radiologie, Centre Hospitalier Universitaire de Montpellier, France. [18]Assistance Publique-Hôpitaux de Paris, Service de Radiologie, Hôpital Universitaire Beaujon, France. [19]Service de Radiologie, Centre Hospitalier Universitaire d'Angers, France. [20]Assistance Publique-Hôpitaux de Paris, Département de Chirurgie Digestive et Hépato-Biliaire, CHU Henri Mondor, F-94000 Créteil, France. [21]Service de Virologie, Bactériologie-Hygiène, Mycologie-Parasitologie et Unité Transversale de Traitement des Infections, Assistance-Publique Hôpitaux de Paris, Groupe Hospitalier Henri Mondor, Créteil, France. [22]Université Paris Descartes, Université Paris Diderot, Université Paris 13, F-75010, France [23]Assistance Publique–Hôpitaux de Paris, Service d'Oncologie Médicale, Hôpital Européen Georges Pompidou, Paris, France. [24]Service de Radiologie, Groupe hospitalier Paris-Seine-Saint Denis, Hôpital Jean Verdier, AP-HP, Bondy, France. [25]Assistance Publique-Hôpitaux de Paris, Service d'Anatomie et de Cytologie Pathologiques, Groupe hospitalier Paris-Seine-Saint Denis, Hôpital Jean Verdier, Bondy, France.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Julien Calderaro, Hôpital Henri Mondor, 51 avenue du Maréchal de Lattre de Tassigny, 94010 Créteil, France. Phone: 334-981-2732; Fax: 334-981-2733; E-mail: julien.calderaro@aphp.fr

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Translational Relevance

Using a molecular-driven selection of biomarkers, we identified ESM1 as a reliable microenvironment immunohistochemical marker of macrotrabecular-massive HCC. The results represent a step toward the implementation of HCC morpho-molecular subtyping into clinical practice.

Introduction

Hepatocellular carcinoma is a leading cause of cancer-related death worldwide, with an increasing incidence in most Western countries (1). It most often arises in the context of chronic liver disease due to Hepatitis C or B virus infection, alcohol intake, or metabolic syndrome (1). Its prognosis remains poor, as approximately two thirds of patients are diagnosed with advanced disease not amenable to curative treatment (1). Our understanding of HCC biology has significantly improved over the last decade (1–5). Several transcriptomic subclasses linked to different clinical and biological pathways have been identified, and high-throughput sequencing technologies have allowed the identification of the mutational landscape of HCC (5, 6).

At the pathological level, most tumors are characterized by a trabecular pattern of growth with cords of neoplastic cells that mimic normal liver trabeculae (7, 8). HCC morphology is, however, highly heterogeneous, and the World Health Organization recognizes numerous architectural patterns and cytological variants (8). Several subtypes, defined by robust and homogenous histological features, have also been reported (2, 8). They have however aroused no or very little interest for physicians due to the lack of proved impact on therapeutic strategies in a context of non-invasive diagnosis.

We have recently identified a novel morphological subtype of HCC, designated as "macrotrabecular-massive" (MTM-HCC), characterized by very high rates of early relapse after resection or percutaneous ablation (9). This phenotype is also associated with a particular biology, with strong angiogenesis activation (2). Identification of such morphological variants holds promise for personalized treatment (2, 10). However, biomarkers are needed to develop a reproducible HCC subtyping. Immunohistochemical markers are indeed routinely performed to achieve morpho-molecular classification in various human malignancies, but remain a critical unmet need in HCC.

The aim of the present study was to identify immunohistochemical markers of MTM-HCC, to ease its diagnosis and implementation into clinical practice. Therefore, we first selected candidate markers using RNA sequencing data from The Cancer Genome Atlas (TCGA) dataset and further performed immunostaining in two independent series of HCC biopsy samples.

Materials and Methods

Analysis of RNA sequencing data from The Cancer Genome Atlas

We included 331 tumors (MTM-HCC $n = 53$ and non MTM-HCC $n = 278$) with available histological slides and RNA sequencing data from the TCGA public database (Flow chart of the study available in Fig. 1; ref. 11). Cases with equivocal morphological features suggestive of either combined HCC-cholangiocarcinoma or cholangiocarcinoma were excluded from the analysis. Gene expression level 3 (number of reads overlapping a given gene) datasets, aligned to the reference human genome hg19/GRCh37 using BWA (Burrows-Wheeler Alignment Tool), were downloaded (12). The import of raw reads counts for each sample into R software was performed using Bioconductor DESeq2 package (13). FPKM (fragments per kilo base per million mapped reads) scores were calculated by normalizing the count matrix for the library size and the coding length of each gene. We used the Bioconductor limma package to test for differential expression of all genes expressed in at least 5 samples (FPKM > 0.1, $n = 28{,}640$) between MTM-HCC and non-MTM HCC (14). Differentially expressed genes were defined by a q value threshold of ≤ 0.05.

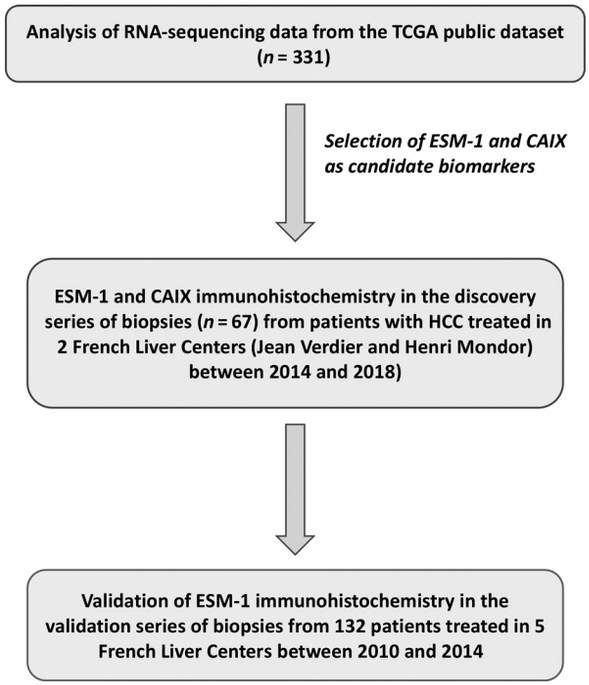

Figure 1.
Flow-chart of the study.

Prediction of HCC subclasses according to Boyault classification was performed using the open-source R package (https://github.com/cit-bioinfo/MS.liverK, www.biorxiv.org/content/ 10.1101/540005v1). Qualitative data were compared using the χ^2 test with non-binary categorical variables. For Gene Set Enrichment Analysis experiments (GSEA), we used a R script adapted from that of the study of Subramanian and collaborators (15). This computational method allows the identification of gene sets over-represented among up- and downregulated genes, based on the p-value and fold change of the previous analysis. We used the MSigDB v6 database to identify genes that share common biological function, chromosomal location, or regulation.

Patients from the discovery set

The discovery set retrospectively included 67 patients who underwent liver biopsy for HCC in 2 French tertiary liver centers

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(Jean Verdier and Henri Mondor University Hospitals), between 2014 and 2018 (Fig. 1). The series was enriched in MTM-HCC to first explore the sensitivity and specificity of the selected immunohistochemical markers. Inclusion criteria were as follows: (i) lack of antitumor treatment before the biopsy, (ii) available material for immunohistochemical analyses, and (iii) unequivocal morphological features of HCC. Cases with histological appearance suggestive of combined HCC-cholangiocarcinoma were excluded. The following clinical and biological features were recorded: age, gender, risk factors of liver disease, Barcelona Clinic of Liver Cancer (BCLC) disease stage and alpha feto-protein (AFP) serum levels. The study was approved by an institutional review board, and was performed in accordance with the Declaration of Helsinki. Informed consent of patients was obtained.

Patients from the validation set

For the validation set, the R package "MKmisc" was used for sample size and power calculation. It consisted of 132 consecutive patients who underwent liver biopsy before radiofrequency ablation procedure in 5 French liver centers between 2010 and 2014 (Jean Verdier Hospital, Bondy; Henri Mondor Hospital, Créteil; Beaujon Hospital, Clichy, Angers Hospital, Angers; CHU Montpellier Hospital, Montpellier). Inclusion criteria were (i) compensated cirrhosis (Child-Pugh A or B7), (ii) first treatment of a single HCC $\leq$ 5 cm without detectable portal extension or distant metastasis (BCLC 0/A), and (iii) available tumor biopsy sample (Fig. 1). Clinical and biological features were collected.

Histology and immunohistochemistry

For each case, hematein–eosin and saffron-stained slides were reviewed by two pathologists specialized in liver disease (J. Calderaro and M. Ziol) using a multipipe microscope. Tumors were classified as MTM-HCC if at least one foci of neoplastic cells arranged in a macrotrabecular architectural pattern (trabeculae > 6 cells thick, lined by sinusoidal cells) could be identified, according to formerly published criteria (9). These features are illustrated in Fig. 2.

For immunohistochemical experiments, 3-μm-thick sections were cut from each paraffin embedded biopsy samples. All stainings were performed on whole sections. Slides were processed on automated immunostainers (Leica Bond-Max; Leica Biosystems). After dewaxing and rehydratation, antigen retrieval was performed with Bond Epitope Retrieval Solution 1 for 20 minutes at pH8. Slides were further incubated with primary antibodies against Carbonic Anhydrase IX (CAIX; Novocastra NCL-L-CAIX, clone TH22, dilution 1/100) and Endothelial-Specific Molecule 1 (ESM1; anti-human Endocan monoclonal antibody MEP08, Lunginnov, dilution 1/500 Lille, France). Polymer reagents were further applied to sections for immunodetection (Bond Polymer Refine detection, Leica Biosystems), with di-amino benzidine as the chromogen.

As shown in Fig. 2, cases were classified as $ESM1^+$ when at least 5 stained endothelial cells could be identified in a high-power field (×400). A weak cytoplasmic background staining was occasionally observed in neoplastic cells. Tumors were considered $CAIX^+$ if positive neoplastic cells could be observed, regardless of their percentage/number (Fig. 3). This study complies with the REMARK guidelines for biomarker discovery (16).

Results

RNA sequencing analysis of TCGA

To identify potential biomarkers of MTM-HCC, we first analyzed gene expression profiling data from the TCGA study. RNA-sequencing data of 331 tumors (MTM-HCC $n = 53$ and non MTM-HCC $n = 278$) were available and downloaded. Consistent with former reports showing that angiogenesis activation is a hallmark biological feature of MTM-HCC, we identified several markers of hypoxia/neoangiogenesis among the most upregulated genes compared with non MTM-HCC, including *CAIX*, *ESM1*, and *EPO* (adjusted *P* value of <0.01 for all 3 genes; Supplemental Table S1).

We further selected two of these candidate biomarkers, CAIX and ESM1, as (i) they have a molecular/biological relevance, (ii) validated antibodies were available, and (iii) their expression has been investigated before in HCC and has been linked to an adverse outcome, as reported for MTM-HCC (17–19). Other biomarkers, such as VEGFA or EPO, may have been considered. However, previously published studies show that most HCC stain positive for these proteins, and their assessment would have required to take into account staining intensity, which is highly dependent on technical protocols (https://www.proteinatlas.org/; refs. 20–22). It may also lead to poor standardization and inter-observer agreement.

Carbonic anhydrases are a family of enzymes that catalyze the conversion of cell-generated carbon dioxide into protons and bicarbonate to maintain the acid-base balance in blood and other tissues (23). CAIX, a transmembrane isoform with an extracellular-facing catalytic site, is mainly expressed in neoplastic tissues and is considered one of the major hypoxia-inducible genes.

ESM1, which belongs to a family of proteoglycans, is expressed in a subset of endothelial cells and is commonly used as a biomarker of their activation. In this line, several studies showed that ESM1 promotes endothelial cell migration and thus neoangiogenesis (24).

Further analyses of RNA sequencing data were able to confirm the association between MTM-HCC subtype and the G3 transcriptomic subclass (Supplementary Fig. S1; ref. 2). GSEA experiments also revealed activation of various oncogenic pathways involved in cell proliferation and survival (Supplemental Table S2).

Discovery series

As described in Table 1, patients of the discovery series were mainly male (85%, 57/67). The main risk factors of liver disease were non-alcoholic steatohepatitis (35%, 19/54) and alcohol intake (32%, 17/54). BCLC disease stage was intermediate or advanced in 36% of the patients (24/67). Elevated AFP levels were detected in 44% of the cases (20/45). Twenty-nine tumors were classified as MTM-HCC (43%). Immunohistochemical staining against ESM1 and CAIX was performed in the whole series of 67 HCC biopsies.

Tumors were classified as ESM1 and CAIX positive in 46% (31/67) and 27% (18/67) of the cases, respectively (Figs. 2 and 3). ESM1 expression was observed in sinusoïdal stromal cells lining HCC and formed nearly a continuous positive surrounding of macrotrabeculae (Fig. 2). Inter-observer agreement was 0.77 for ESM1 and 0.71 for CAIX (Cohen kappa). Its expression was significantly associated with an older age at diagnosis ($P = 0.02$,

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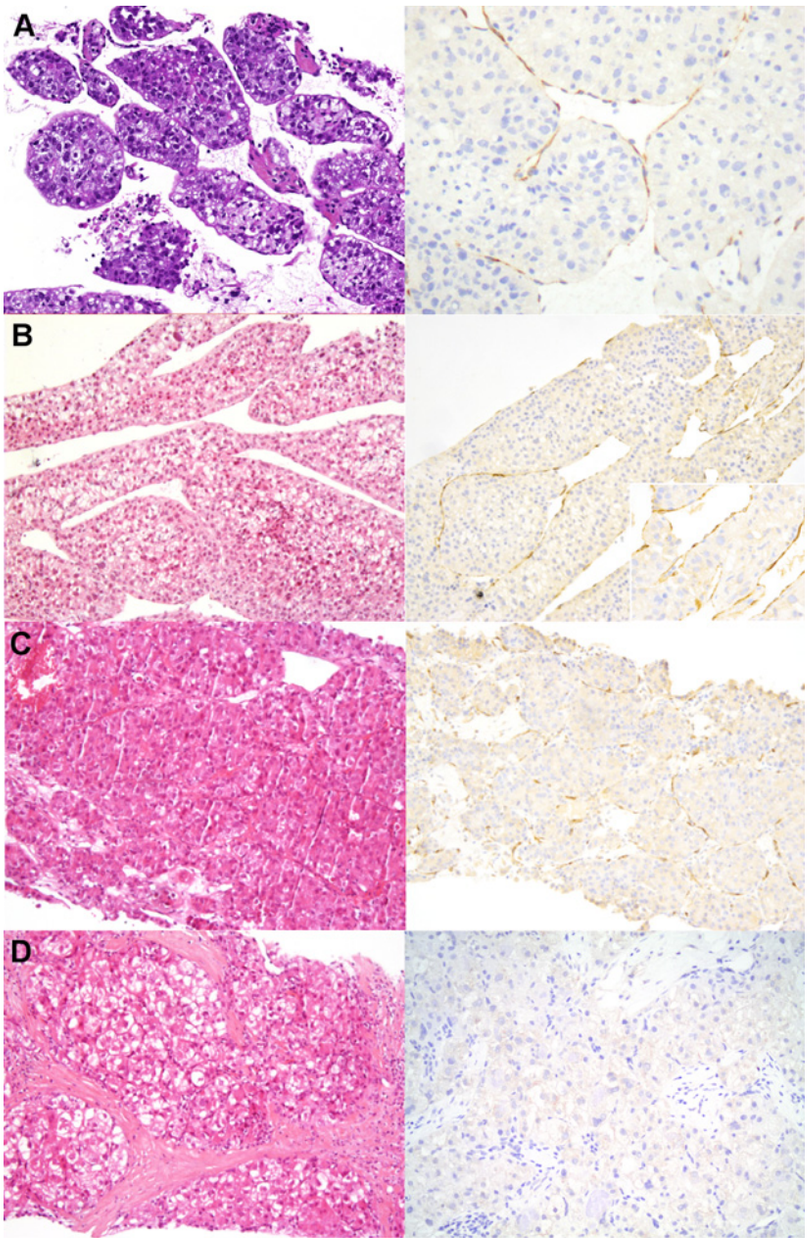

Figure 2.
Morphological features and ESM1 immunostaining of HCC in liver biopsy samples**. A,** This is a characteristic morphological aspect of MTM-HCC, with large trabeculae (>6 cells thick) separated by large empty spaces (left, HES, ×200). ESM1 shows a nearly continuous staining of stromal endothelial cells (right, ×400). **B,** Microscopic examination of this MTM-HCC shows foci of clear cells (left, HES, ×100). Stromal cells display ESM1 expression (right, ×100, higher magnification at bottom right). **C,** The macrotrabecular architecture of this sample is more difficult to diagnose on this HES section, because macrotrabeculae are closely packed together (left, HES, ×200). ESM1 staining however helps to identify the macrotrabeculae by lining their shapes (right, ×200). **D,** This is an example of a non–MTM-HCC, classified as steatohepatitic HCC (left, HES, ×200). No ESM1-positive endothelial cells are detected in this case (right, ×200). HES: Hematein-Eosin-Saffron.

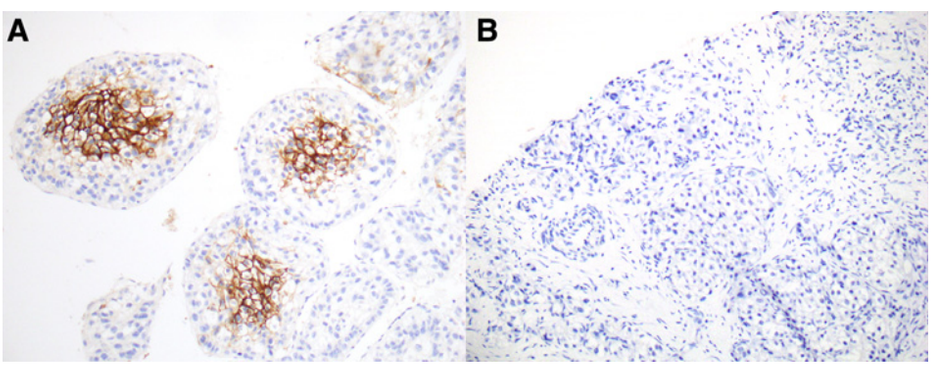

Figure 3.
Carbonic Anhydrase 9 expression in MTM and non MTM-HCC. **A,** In MTM-HCC, a particular expression pattern of CAIX was observed, with a predominant staining at the center of trabeculae, in cells located away from the blood stream. **B,** This conventional HCC does not display CAIX expression.

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Table 1. Clinical and pathological features of the discovery and validation sets (Fischer exact test)

	Discovery set				Validation set			
Variables	**Available data (*n*)**	**MTM-HCC**	**Non MTM-HCC**	***P***	**Available data (*n*)**	**MTM-HCC**	**Non MTM-HCC**	***P***
Age > 60 years	67	15/29	9/38	0.02	132	12/15	81/117	0.55
Gender: male	67	23/29	34/38	0.31	132	13/15	87/117	0.52
BCLC 0-A	67	17/29	26/38	0.45	132	15/15	117/117	1
Alcohol intake	54	6/20	11/34	1	132	5/15	54/117	0.35
Hepatitis C virus	54	5/20	6/34	0.73	132	7/15	50/117	0.77
Hepatitis B virus	54	7/20	5/34	0.10	132	2/15	8/117	0.32
NASH	54	5/20	14/34	0.26	132	5/15	22/117	0.19
Undetermined	54	1/20	2/34	1	132	0/15	0/117	1
Other	54	1/20	0/34	0.37	132	1/15	6/117	1
AFP serum levels >20 ng/mL	45	10/18	10/27	0.24	115	7/12	25/103	0.03

Supplemental Table S3). As depicted in Fig. 4, sensitivity and specificity of ESM1 for detection of MTM-HCC was 97% (28/29), and 92% (35/38), respectively. Specificity of CAIX was 89% (34/38); however, its sensitivity was rather low (48%, 14/29). These results prompted us to select ESM1 as the most promising marker, and to validate its value in the second series of samples.

Validation series

We estimated that the minimal number of patients required for the validation set was 119 (expected prevalence: 16%, assessed from the TCGA series; expected sensibility 0.97; power 0.80; delta 0.2) and included 132 patients. They were mainly male (76%, 100/132; Table 1). Elevated AFP serum levels were detected in 28% (32/115) of the cases. Compared with the discovery set, patients were older ($P > 0.01$), had lower AFP serum levels ($P = 0.01$) and a less advanced disease stage ($P < 0.01$; Supplemental Table S4). A higher rate of HCV infection ($P < 0.01$) and lower rates of HBV infection ($P = 0.01$) and NASH ($P = 0.04$) were also observed (Supplemental Table S4).

Fifteen tumors were classified as MTM-HCC during the pathological review (15/132, 11%). ESM1 immunohistochemistry was performed and 24 cases were classified as positive. Its expression was associated with elevated AFP serum levels (Supplemental Table S3). Interobserver agreement was good (Cohen kappa 0.76). Sensitivity and specificity of ESM1 for the identification of MTM-HCC were 93% (14/15) and 92% (107/117), respectively.

Discussion

We have previously identified a distinct HCC histological subtype, designated as macrotrabecular-massive and associated to particular molecular features. It is also noticeably characterized by an adverse clinical outcome, independently of classical HCC prognostic parameters (BCLC stage, AFP serum levels and pathological features; refs. 2, 9). Using a molecular-driven selection of biomarkers, we identify in the present study a microenvironment marker of this histological subtype, ESM1, strongly expressed by stromal endothelial cells lining macrotrabeculae. In a discovery and validation study of 67 and 132 HCC biopsy samples, ESM1 staining of stromal cells was associated with MTM-HCC subtype with 97 and 93% sensitivity, and 92 and 91% specificity, respectively. These results also provide a better insight into the biology of this clinically relevant HCC subtype.

Accurate classification and subtyping of tumors, based on integrated morphological and molecular studies, is a foundation of precision medicine and personalized therapies. The use of biomarkers is, however, advisable to reach a high interobserver agreement and a strong diagnostic confidence among different institutions and observers. Indeed, biopsy samples most often comprise a small amount of tumor, and assessment of tumor histological architecture may be difficult due to artifacts and/or tumor cell dissociation. As shown in Fig. 2, ESM1 staining by itself helped to identify the macrotrabecular architecture of HCC, by revealing the sinusoidal stromal cells surrounding the macrotrabeculae. ESM1 sensitivity was high with only 1 out of 25 MTM cases negative for ESM1 in the discovery study and 1 out of 15 MTM cases negative in the validation study. This high sensitivity observed in biopsy samples is likely to reinforce the reproducibility of diagnosis among laboratories. Specificity was also higher than 90% in both series. However, 10 biopsies in the validation study showed strong ESM1 staining without macrotrabecular-massive morphological features. Four showed compact architecture, and other showed pseudoglandular, steatohepatitic or microtrabecular patterns. We have previously showed that ESM1 staining was associated with an adverse outcome, and in the same line, Villa et al, by performing gene expression profiling of 132 pre-therapeutic HCC biopsies, also identified ESM1 as part of a five genes signature predictive for fast-growing HCC and poor overall survival (25). Large prospective studies will thus have to determine the most relevant prognostic indicator (MTM subtype or ESM1 staining). Our approach could be compared, in the field of breast cancer, with the wide use of the loss of E-cadherin expression to identify lobular carcinoma. The combined analysis of morphology and immunohistochemistry has indeed been shown to be very efficient to implement this tumor variant into clinical practice.

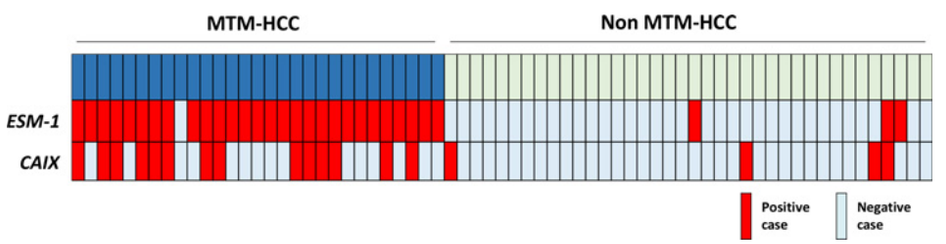

Figure 4.
Heatmap of ESM1 and CAIX immunohistochemical experiments in the discovery series.

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Interestingly, using immunohistochemistry, ESM1 expression has also been described in stromal endothelial cells of human tumors other than HCC, such as non–small cell lung carcinoma, glioblastoma, clear cell renal carcinoma or bladder carcinoma (24, 26–29). Several authors have hypothesized that ESM1 expression by endothelial cells reflects a switch from a dormant to an aggressive tumor phenotype with increased angiogenesis (25, 30). In animal models, ESM1 is one of the main genes involved in the angiogenic activation that occurs during hypoxia-induced retinal neovascularization (31). Altogether, these findings suggest that MTM-HCC is characterized by a highly activated angiogenic microenvironment highlighted by endothelial ESM1 expression.

These observations may also open research perspectives for the treatment of patients with this highly aggressive HCC subtype. Inhibition of ESM1 or other key regulators of angiogenesis also upregulated in MTM-HCC, such as ANG2 (angiopoietin 2) or VEGFA (Vascular Endothelial Growth Factor A), has been shown to have strong antitumor effects in experimental models. Roudnicky and collaborators showed *in vitro* that ESM1 knockdown was able to inhibit endothelial cell migration and tube formation, and, in a mice tumor xenograft model, administration of ANG2-blocking antibodies was able to reduce lymph node and lung metastasis, as well as tumor lymphangiogenesis (24, 32). Finally, inhibition of both VEGFA and ANG2 by a bispecific antibody was able not only to normalize intra-tumor blood vessels but also to enhance antitumor immunity by facilitating the extravasation of interferon-γ expressing cytotoxic T cells in mouse models of melanoma, breast and pancreatic neuroendocrine cancer (33). More basic and translational research is however required to assess the potential effects of such therapies on MTM-HCC.

In conclusion, using a molecular-driven selection of biomarkers, we have identified ESM1 expression by stromal sinusoidal cells as a robust marker of MTM-HCC in biopsy samples. Our results may help the implementation of morpho-molecular HCC subtyping into clinical staging systems.

Disclosure of Potential Conflicts of Interest

G.P. Pageaux reports receiving speakers bureau honoraria from Bayer. J.-M. Pawlotsky reports receiving speakers bureau honoraria from Abbvie and Gilead, and is a consultant/advisory board member for Merck and Siemens Healthcare. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J. Calderaro, M. Ziol
Development of methodology: J. Calderaro, M. Ziol
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Calderaro, A. Luciani, J.-C. Nault, J. Cohen, F. Oberti, S. Michalak, G.P. Pageaux, J. Ramos, N. Barget, V. Paradis, C. Aubé, A. Laurent, N. Ganne-Carrié, J. Zucman-Rossi, O. Seror, M. Ziol
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Calderaro, L. Meunier, C.T. Nguyen, M. Boubaya, S. Caruso, A. Luciani, V. Paradis, M. Ziol
Writing, review, and/or revision of the manuscript: J. Calderaro, C.T. Nguyen, A. Luciani, G. Amaddeo, H. Regnault, J.-C. Nault, M. Bouattour, V. Vilgrain, B. Guiu, V. Paradis, C. Aubé, A. Laurent, J.-M. Pawlotsky, N. Ganne-Carrié, O. Seror, M. Ziol
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):, N. Barget, O. Seror, M. Ziol
Study supervision: J. Calderaro, M. Ziol

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