Gd-EOB-DTPA-Enhanced Magnetic Resonance Imaging and Alpha-Fetoprotein Predict Prognosis of Early-Stage Hepatocellular Carcinoma

Taro Yamashita,1,2 Azusa Kitao,3 Osamu Matsui,4 Takehiro Hayashi,2 Kouki Nio,2 Mitsumasa Kondo,2 Naoki Ohno,4 Toshiaki Miyati,4 Hickari Okada,2 Tatsuya Yamashita,2 Eishiro Mizukoshi,2 Masao Honda,2 Yasunori Nakanuma,5 Hiroyuki Takamura,6 Tetsuo Ohta,6 Yasunari Nakamoto,7 Masakazu Yamamoto,8 Tadatoshi Takayama,9 Shigeki Arii,10 XinWei Wang,11 and Shuichi Kaneko2

The survival of patients with hepatocellular carcinoma (HCC) is often individually different even after surgery for early-stage tumors. Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI) has been introduced recently to evaluate hepatic lesions with regard to vascularity and the activity of the organic anion transporter OATP1B3. Here we report that Gd-EOB-DTPA-enhanced MRI (EOB-MRI) in combination with serum alpha-fetoprotein (AFP) status reflects the stem/maturational status of HCC with distinct biology and prognostic information. Gd-EOB-DTPA uptake in the hepatobiliary phase was observed in ~15% of HCCs. This uptake correlated with low serum AFP levels, maintenance of hepatocyte function with the up-regulation of OATP1B3 and HNF4A expression, and good prognosis. By contrast, HCC showing reduced Gd-EOB-DTPA uptake with high serum AFP levels was associated with poor prognosis and the activation of the oncogene FOXM1. Knockdown of HNF4A in HCC cells showing Gd-EOB-DTPA uptake resulted in the increased expression of AFP and FOXM1 and the loss of OATP1B3 expression accompanied by morphological changes, enhanced tumorigenesis, and loss of Gd-EOB-DTPA uptake in vivo. HCC classification based on EOB-MRI and serum AFP levels predicted overall survival in a single-institution cohort (n = 70), and its prognostic utility was validated independently in a multi-institution cohort of early-stage HCCs (n = 109). Conclusion: This noninvasive classification system is molecularly based on the stem/maturation status of HCCs and can be incorporated into current staging practices to improve management algorithms, especially in the early stage of disease. (HEPATOLOGY 2014;60:1674-1685)

Liver cancer is the fifth most commonly diagnosed cancer and the second most frequent cause of cancer death in men worldwide.1 Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, accounting for 70-86% of cases of primary liver cancer.1 Several staging systems are currently available for HCC classification and include Tumor Node
Metastasis (TNM) and Barcelona Clinic Liver Cancer (BCLC) staging, which are based on tumor number and size, vascular invasion, metastatic status, hepatic reserve, and performance status. These systems can provide an approximate estimate of patients’ survival, but patients diagnosed at the same disease stage sometimes show a different prognosis. This is most likely because these systems do not include an assessment of the malignant phenotype of the tumor, which would be especially important in those patients diagnosed at the early stage of disease. To overcome these limitations, gene expression profiling technologies have been applied to classify HCC. In particular, the stemness of HCC is currently of great interest because its gene expression profile reflects the malignant nature of the tumor. However, the application of these new technologies still needs to be validated externally prior to their implementation in clinical practice.

The hallmark of HCC diagnosis has been image analysis based on vascularity. Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) is a liver-specific magnetic resonance imaging (MRI) contrast agent introduced specifically to improve the detection of liver lesions. Gd-EOB-DTPA-enhanced MRI (EOB-MRI) has been used to evaluate liver tumors in Europe since 2004, in the USA and Japan since 2008, and in China since 2010. Gd-EOB-DTPA is characterized by its rapid and specific uptake by hepatocytes by way of organic anion transporting polypeptides (OATPs) expressed in the sinusoidal membrane. Therefore, Gd-EOB-DTPA uptake in the liver is considered to reflect hepatocyte function. Among OATP1A2, 1B1, 1B3, and 2B1, only OATP1B3 expression was found to correlate with the enhancement ratio on EOB-MRI, indicating that it transports Gd-EOB-DTPA into HCC cells. It is generally accepted that ~85% of HCCs show hypointensity in the hepatobiliary phase of EOB-MRI compared to the noncancerous background liver, with a reduction of OATP1B3 protein or OATP1B3 gene expression in the tumor. However, atypical Gd-EOB-DTPA uptake in the hepatobiliary phase is observed in the remaining 15% of HCCs, and the molecular phenotype and clinical features of these HCCs remain to be elucidated.

We hypothesized that EOB-MRI findings may vary in different tumor subtypes with distinct biology. Therefore, in this study we evaluated the molecular profiles of HCCs in a single-institute cohort determined from the EOB-MRI findings using quantitative reverse-transcription polymerase chain reaction (qRT-PCR), microarray, and immunohistochemistry (IHC) analyses. To clarify the clinical utility of the EOB-MRI findings, we also evaluated the prognosis of a multicenter cohort of patients with early-stage HCC who underwent radical resection.

Materials and Methods

Patients. A total of 417 patients who received surgical resection for HCC were enrolled in this study. Seventy patients underwent EOB-MRI for the diagnosis of HCC and received surgical resection at Kanazawa University Hospital from 2008 to 2011. Survival analysis was performed in this single-institute cohort (Cohort 1) and prognosis was evaluated every 6 months. The final evaluation of survival was performed in October 2011. From these 70 patients, 62 tumor and nontumor samples were snap-frozen in liquid nitrogen and used for qRT-PCR.

For microarray analysis, we assessed 238 patients who received surgical resection of HCC at the Liver Cancer Institute of Fudan University. EOB-MRI was not performed in these patients because Gd-EOB-DTPA had not yet been introduced in China. Their clinicopathologic characteristics and prognostic data have been described previously.

To evaluate the survival of early-stage HCCs, we enrolled 109 patients who received EOB-MRI and surgical resection at Tokyo Medical and Dental University Hospital, Tokyo Women’s Medical University Hospital, Nihon University School of Medicine Itabashi Hospital, Niigata University Medical & Dental Hospital, Hyogo College of Medicine Hospital, or Kurume...
University Hospital from 2008 to 2009 (Cohort 2). The prognosis of these patients was evaluated every year, and the final evaluation of survival was performed in February 2012.

This study was approved by the Institutional Review Board at each study center and all patients provided written informed consent.

**EOB-MRI.** EOB-MRI was performed before surgical resection using a 1.5 or 3.0 Tesla MRI system with a fat-suppressed 2D or 3D gradient echo T1-weighted sequence (relaxation time / echo time [TR/TE] = 3.2-3.6/1.6-2.3 ms, flip angle 10-15°, field of view 33-42 cm, matrix 128-192 × 256-512, slice thickness 4.0-8.0 mm). A dose of 0.025 mmol/kg Gd-EOB-DTPA (Primovist; Bayer Schering Pharma, Berlin, Germany) was injected intravenously and the hepatobiliary phase was obtained at 15-20 minutes after the injection. All abdominal MRI data of the HCC patients were generated at Kanazawa University Hospital and image analysis was performed retrospectively by two radiologists (A.K. and O.M.) without knowledge of the clinical and pathological results. The signal intensity (SI) of the tumor was measured within the region of interest, which was determined as the maximum oval area at the largest section of the tumor. The SI of the adjacent background liver was also measured within a region of interest of the same size, while avoiding large vessels. The nodules were classified into the two following types: hypointense HCC, which was defined as showing a lower SI than that of the surrounding liver (tumor SI/ background SI <1.0) in the hepatobiliary phase, and hyperintense HCC, which was defined as showing an equal or higher SI (tumor SI/ background SI ≥1.0).

For the mouse study, EOB-MRI was performed using a 0.4 T MRI system with a fat-suppressed 3D gradient echo T1-weighted sequence (TR/TE = 66.5/4.0 ms, flip angle 40°, field of view 10 cm, matrix 224 × 192, slice thickness 1.0 mm). A dose of 0.025 mmol/kg Gd-EOB-DTPA (Bayer Schering Pharma) was injected through the tail vein, and the hepatobiliary phase was obtained at 12-20 minutes after the injection.

**Xenotransplantation of Primary HCC in Immunodeficient Mice and HNF4A Knockdown.** Primary HCC tissue was dissected and digested in 1 mg/mL type 4 collagenase solution (Sigma-Aldrich Japan, Tokyo, Japan) at 37°C for 15-30 minutes. Contaminated red blood cells were lysed with an ammonium chloride solution (STEMCELL Technologies, Vancouver, BC, Canada) on ice for 5 minutes. CD45+ leukocytes and annexin V+ apoptotic cells were removed by an autoMACS-pro cell separator and magnetic beads (Miltenyi Biotec, Tokyo, Japan). The cells were suspended 1:1 in 200 μL Dulbecco’s modified Eagle’s medium (DMEM) and Matrigel (BD Biosciences) and injected subcutaneously into 6-week-old NOD/SCID mice (NOD/NCr.Crl-Pykdle<sup>sid</sup>) purchased from Charles River Laboratories (Wilmington, MA). EOB-MRI was performed to evaluate Gd-EOB-DTPA uptake in the subcutaneous tumor at the hepatobiliary phase, and the subcutaneous tumor was dissected and digested as described above, and subsequently cultured in DMEM. *HNF4A* knockdown was performed using pGFP-V-RS vectors (OriGene Technologies, Rockville, MD), allowing stable delivery of the short hairpin RNA (shRNA) expression cassette against *HNF4A* or scramble sequence into host cells by way of a replication-deficient retrovirus. Infected HCC cells were grown in DMEM containing 1 μg/mL puromycin (Sigma-Aldrich Japan) for 7 days to establish stable shRNA-expressing HCC cells. Western blotting and immunofluorescence analyses were performed using an antihuman HNF4α C11F12 antibody (Cell Signaling Technology, Danvers, MA) and a mouse monoclonal antihuman OATP1B3 MDQ/5F260 antibody (Novus Biologicals, Littleton, CO), essentially as described previously. Control or Sh-HNF4A-transfected HCC cells were injected subcutaneously into NOD/SCID mice, and tumor volume and survival were evaluated every 2-3 days. The protocol was approved by the Kanazawa University Animal Care and Use Committee and the Kanazawa University Genetic Modification Experiment Committee.

**Microarray Analysis.** The 238 HCC cases from the Liver Cancer Institute of Fudan University with available microarray data and clinicopathologic and prognostic data have been described previously. BRB-ArrayTools software (v. 3.8.1) was used for class comparison analysis. Hierarchical clustering analysis was performed with Genesis software (v. 1.6.0 beta). Canonical pathway and transcription factor analyses were performed using MetaCore software (http://www.genego.com). Interaction network analysis was performed using Ingenuity Pathway Analysis software (http://www.ingenuity.com).

**qRT-PCR Analysis.** Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The expression of selected genes was determined in triplicate using the Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and the ΔΔCT method. The following probes were used: *AFP*, Hs00173490_m1; *FOXM1*, Hs01073586_m1; *OATP1B3*, Hs00251986_m1; *CYP3A4*, Hs00430021_m1; and *18S*, Hs99999901_s1 (Applied Biosystems).
**IHC Analysis.** IHC was performed using Envision+ kits (Dako Japan, Tokyo, Japan) as described previously. Mouse monoclonal antihuman Ki-67 antigen MIB-1 (Dako Japan), mouse monoclonal antihuman OATP1B3 MDQ/5F260 (Novus Biologicals), rabbit monoclonal antihuman HNF4α C11F12 (Cell Signaling Technology), mouse monoclonal antihuman FOXM1 0.T.181 (Abcam, Cambridge, MA), mouse monoclonal antihuman glypican-3 1G12 (BioMosaics, Burlington, VT), and mouse monoclonal antiglutamine synthetase clone GS-6 (Millipore, Billerica, MA) antibodies were used. The staining area and intensities were evaluated in each sample and graded from 0-3 (0, 0-5%; 1, 5-25%; 2, 25-50%; 3, >50%) and 0-2 (0, negative; 1, weak; 2, strong), respectively. The sum of the area and intensity scores of each marker (IHC score) were calculated. Samples were defined as marker-high (IHC score ≥3) or -low (IHC score ≤2). The Ki-67 labeling index was calculated as described previously.

**Statistical Analysis.** Mann-Whitney, χ², Fisher’s exact, and Kruskal-Wallis tests were used to compare the clinicopathologic characteristics and gene expression data. The correlation of the gene expression data was evaluated by Spearman’s rank correlation coefficient. Kaplan-Meier survival analysis with the log-rank test was performed to compare patient survival. All analyses were performed using GraphPad Prism software v. 5.0.1 (GraphPad Software, San Diego, CA).

**Results**

**EOB-MRI Findings and Molecular Characteristics of HCC.** Nine of the 70 HCC cases (12.9%) in Cohort 1 were diagnosed with hyperintense HCC on EOB-MRI (Fig. 1A). Analysis of the clinicopathologic characteristics of hyper- or hypointense HCCs revealed that hyperintense HCCs were significantly associated with low serum alpha-fetoprotein (AFP) levels (Table 1). There was no significant difference between hyper- and hypointense HCCs in terms of other factors, including tumor size, number, TNM and BCLC stages, surgical procedures, and elapsed time between MRI and surgery. We confirmed the overexpression of OATP1B3, a transporter responsible for the uptake of Gd-EOB-DTPA in hepatocytes, in hyperintense HCCs by qRT-PCR and IHC (Fig. 1B).

To understand the transcriptomic characteristics of HCCs overexpressing OATP1B3, we analyzed the microarray data of an additional 238 HCC cases. OATP1B3-high and -low HCCs were defined as HCCs with a T/N ratio ≥1.0 and <1.0, respectively, as used for the evaluation of hyperintense HCCs (tumor SI / background SI ≥1.0). The frequency of OATP1B3-high HCCs was 15.1% (36 of the 238 HCC cases), almost comparable to the frequency of hyperintense HCCs reported thus far. Class-comparison analysis yielded a total of 974 genes that were differentially expressed between OATP1B3-high and -low HCCs (P < 0.001). Hierarchical cluster analysis of this 974 gene set (OATP1B3 gene signature) separated HCCs into two branches (B1 and B2) (Fig. 1C). Thirty-four of the 36 OATP1B3-high HCCs (blue box) were classified in the left branch (B1), while OATP1B3-low HCCs were clustered in both branches. The prognosis of HCC patients clustered in B1 was significantly better than those clustered in B2 (P = 0.02) (Supporting Fig. S1). Genes associated with mature hepatocyte function such as ALB and CYP3AA4 were significantly up-regulated in the HCCs clustered in B1, and the known hepatic stem/progenitor markers KRT19 and EPcam, as well as the G1/S cell cycle marker MKI67, were significantly up-regulated in the HCCs clustered in B2 (Fig. 1D).

Pathway analysis indicated that OATP1B3-high HCCs showed maintenance of mature hepatocyte function and decreased cell proliferation and Wnt signaling (Fig. 1E), which are known to be activated during liver development and regeneration. Transcription factor analysis identified eight genes (HNF4A, NFIA, NR3C1, NRIH3, ESR1, NR1H3, MLXIPL, and NFE2L2) as candidate transcription factors that were significantly activated in OATP1B3-high HCCs (P < 0.005) (Fig. 1F). These transcription factors are known to play a pivotal role in liver development and in the regulation of hepatocyte functions including lipid, bile, carbohydrate, and xenobiotic metabolism. By contrast, only one gene (FOXM1) was identified as a candidate transcription factor activated in OATP1B3-low HCCs. The forkhead box M1 (FOXM1) transcription factor is known to be activated during liver regeneration and regulation of the cell cycle. We investigated the expression of the two transcription factors most strongly activated (HNF4A encoding hepatocyte nuclear factor 4 alpha [HNF4α]) or inactivated (FOXM1) in hyperintense HCCs (Fig. S2) and validated the results using microarray analyses (Fig. 2A,B).

Although the microarray data revealed distinct molecular portraits associated with liver development and the maturation programs present in hyper- and hypointense HCCs, hierarchical cluster analysis further indicated that a subset of hypointense HCCs (corresponding to the OATP1B3-low HCCs clustered in B1)
might show similar gene expression profiles to those observed in hyperintense HCCs. Since serum AFP levels are reportedly related to the stem/maturity subtypes of HCCs with different gene expression profiles, we analyzed the characteristics of OATP1B3-low HCCs in 238 cases according to serum AFP levels. Interestingly, OATP1B3-low HCCs assigned to the left branch (B1) had low serum AFP
levels (<100 ng/mL: orange box, Fig. 1C), while the majority of AFP-high (≥100 ng/mL) HCCs (red box, Fig. 1C) were clustered in the right branch (B2). Consistently, the OATP1B3 gene signature significantly predicted the serum AFP status of 238 HCCs (P < 0.05) (Tables S1-3).

### OATP1B3 and AFP Expression in HCC Subtypes Related to Stem/Maturational Status.

Molecular profiling of tissue samples may be useful for predicting the survival of HCC patients, as reported previously.18,19 However, such an approach should be established before being applied routinely in a clinical setting. The above data prompted us to hypothesize that EOB-MRI findings and serum AFP levels, in place of molecular profiling techniques, have the potential to categorize HCCs (EOB-AFP classification), thus serving as predictors of survival. We categorized HCCs into three groups (class A: hyperintense HCC, class B: hypointense and AFP-low [<100 ng/mL] HCC, and class C: hypointense and AFP-high [≥100 ng/mL] HCC). The clinicopathologic characteristics of patients with class A, B, and C HCCs in Cohort 1 are shown in Table S4.

We investigated the expression of HNF4α and FOXM1 as well as the G1/S marker Ki-67 by IHC according to the EOB-AFP classification system in Cohort 1 (Fig. 2C). HNF4α was most abundantly expressed in class A HCCs, but its expression was decreased in class B and C HCCs. By contrast, the expression of FOXM1 and Ki-67 was highest in class C HCCs, significantly decreased in class B HCCs, and not detected in class A HCCs. The mean Ki-67 labeling indices in class A, B, and C HCCs were 2.8%, 9.4%, and 18.2%, respectively (P < 0.0001) (Fig. 2D).

The differences in FOXM1 and HNF4α expression among class A, B, and C HCCs were statistically significant (Fig. 2E).

We further investigated the expression of five markers (glypican 3, GPC-3; lymphatic vessel endothelial hyaluronan receptor 1, LYVE-1; survivin; heat shock 70 kDa protein, HSP70; and glutamine synthetase, GS), known to be differentially expressed between dysplastic nodule and well-differentiated HCC,20,21 to clarify if the molecular alterations in early-stage hepatocarcinogenesis can be detected differentially in EOB-AFP class A, B, and C HCCs. IHC analysis suggested no differential expression of LYVE-1, survivin, and HSP70 among the EOB-AFP classes (data not shown). Interestingly, GS was most abundantly expressed in class A HCCs, and its expression was relatively decreased in class B and C HCCs with borderline significance (P = 0.06) (Fig. S3A,B). In contrast, GPC-3 expression was highest in class C HCCs and relatively decreased in class A and B HCCs with statistical significance (P = 0.03). We investigated the microarray data of 238 independent HCC cases and validated the positive correlation between OATP1B3 and GLUL (encoding GS) and the weak negative correlation between OATP1B3 and GPC3 (encoding GPC-3).

### Table 1. Characteristics of HCCs Classified by EOB-MRI in Cohorts 1 and 2

| Characteristics            | Cohort 1 |          | Cohort 2 |          |
|-----------------------------|----------|----------|----------|----------|
|                            | Hyperintense | Hypointense | P*       | Hyperintense | Hypointense | P*       |
| Age (years, mean ± SE)      | 66.2 ± 3.6 | 64.6 ± 1.2 | 0.21     | 67.2 ± 2.0  | 66.2 ± 1.0  | 1.0      |
| Sex (male/female)           | 7/2       | 44/17    | 0.72     | 9/0        | 79/21       | 0.13     |
| Etiology (HBV/HCV/other)    | 2/3/4     | 14/23/24 | 0.95     | 1/6/0/2    | 22/56/2/20  | 0.52     |
| Liver cirrhosis (yes/no)    | 5/4       | 33/28    | 0.94     | 2/7        | 42/58       | 0.25     |
| AFP (ng/mL, mean ± SE)      | 12.4 ± 1.9 | 2,157 ± 866 | 0.03    | 7.0 ± 2.2  | 188.4 ± 74  | 0.03     |
| Histologic grade†           | I-II      | 1        | 12       | 2        | 16        |          |
|                            | II-III    | 8        | 38       | 7        | 74        |          |
|                            | III-IV    | 0        | 11       | 0        | 10        | 0.57     |
| Tumor size (cm, mean ± SE)  | 4.0 ± 0.9  | 4.4 ± 0.4 | 0.79     | 3.3 ± 0.4  | 2.6 ± 0.1  | 0.09     |
| Tumor number (single/multiple) | 7/2   | 48/13    | 0.95     | 8/1       | 86/14      | 0.81     |
| Microscopic portal vein invasion (yes/no) | 1/8 | 5/56 | 0.58 | 0/9 | 0/100 |          |
| Microscopic portal vein invasion (yes/no) | 2/7 | 27/34 | 0.21 | 0/9 | 11/89 | 0.59     |
| Tumor-node-metastasis classification ([I/II/III)] | 6/2/1 | 29/28/4 | 0.40 | 7/2/0 | 75/25/0 | 0.85     |
| BCLC stage ([0/A/B/C])      | 0/1/1/1   | 4/30/22/5 | 0.34 | 0/9/0/0 | 27/73/0/0 | 0.07     |
| Elapsed time between MRI and surgery (days, mean ± SE) | 47.0 ± 8.4 | 51.5 ± 3.2 | 0.73 | 17.3 ± 5.0 | 20.6 ± 3.0 | 0.50     |
| Surgical procedure (partial resection or segmentectomy/lobectomy or extended lobectomy) | 6/3 | 35/26 | 0.60 | 8/1 | 86/14 | 1.0      |

* Mann–Whitney test, Fisher’s exact test, or χ² test.
† Edmondson–Steiner.
Regulation of Gd-EOB-DTPA Uptake and Tumorigenic Capacity by HNF4α in Hyperintense HCC. Microarray and IHC analyses suggested the activation of transcription factor HNF4α in hyperintense HCC, but its role in the maintenance of hepatocyte function and Gd-EOB-DTPA uptake has not yet been clarified. To directly explore the role of HNF4α in Gd-EOB-DTPA uptake and tumorigenic capacities, we transplanted tumor cells from hyper- and hypointense primary HCC specimens into NOD/SCID mice (Fig. 3A). We confirmed on EOB-MRI that Gd-EOB-DTPA uptake capacity was relatively maintained in the secondary xenotransplanted tumors that developed in the subcutaneous lesions of the mice (Fig. 3B).

Using a retrovirus system in vitro, we then introduced shRNA targeting HNF4A (Sh-HNF4A) or scramble (Sh-Scr) into tumor cells obtained from a hyperintense HCC. We confirmed the reduction of HNF4α protein expression in Sh-HNF4A-transfected cells compared with Sh-Scr-transfected cells by western blotting (Fig. 3C, left panel). Interestingly, HNF4A knockdown resulted in a modest increase in AFP and FOXM1 expression and a dramatic decrease in CYP3A4 and OATP1B3 expression (Fig. 3C, right panel). It also resulted in the loss of OATP1B3 protein expression, and striking morphological changes were confirmed by immunofluorescence and phase-contrast microscopy (Fig. 3D). Sh-HNF4A-transfected cells displayed long, thin cell shapes with neurite-like extensions, whereas Sh-Scr-transfected cells were relatively smooth and round. Sh-Scr- or Sh-HNF4A-transfected cells were further injected subcutaneously into NOD/SCID mice, and aggressive tumor growth accompanied with the loss of Gd-EOB-DTPA uptake capacity was
Fig. 3. HNF4α regulates a mature hepatocyte-like, less aggressive HCC phenotype coupled with Gd-EOB-DTPA uptake in hyperintense HCC.

(A) MRI scans of hyperintense (a) and hypointense (b) HCCs in the hepatobiliary phase before surgery. The T/N signal intensity ratios of the images in the hepatobiliary phase were 1.02 (left panel) and 0.49 (right panel). Surgically resected specimens were subsequently used for mouse xenotransplantation. (B) MRI scans of NOD/SCID mouse xenotransplanted with hyperintense (a) and hypointense (b) HCCs in the hepatobiliary phase. The T/N signal intensity ratios of the images were 0.82 (upper panel) and 0.45 (lower panel). (C) Left panel: Expression of HNF4α protein by western blotting. Hyperintense HCC cells were harvested in dishes and treated with retroviruses encoding an expression cassette against HNF4A (Sh-HNF4A) or scramble sequence (Sh-Scr). Right panel: qRT-PCR of AFP, FOXM1, CYP3A4, and OATP1B3 in hyperintense HCC cells transfected with Sh-Scr or Sh-HNF4A. (D) Left panel: Immunofluorescence analysis of HNF4α (red) and OATP1B3 (green) in hyperintense HCC cells transfected with Sh-Scr or Sh-HNF4A (scale bar = 100 μm). Right panel: Representative photomicrographs of hyperintense HCC cells transfected with Sh-Scr or Sh-HNF4A (scale bar = 100 μm). (E) MRI scans of NOD/SCID mouse xenotransplanted with hyperintense HCC cells transfected with Sh-Scr (day 49 after transplantation) or Sh-HNF4A (day 43 after transplantation). The T/N signal intensity ratios of the images in the hepatobiliary phase were 0.65 (left panel) and 0.34 (right panel). (F) Survival of NOD/SCID mice xenotransplanted with hyperintense HCC cells transfected with Sh-Scr (n = 5) or Sh-HNF4A (n = 5).
observed in Sh-HNF4A-transfected cells, whereas Sh-Scr-transfected cells still showed Gd-EOB-DTPA uptake with less tumorigenic capacity (Fig. 3E). Mice xenotransplanted with Sh-HNF4A-transfected cells had a worse prognosis compared with those xenotransplanted with Sh-Scr-transfected cells (Fig. 3F), indicating a crucial role for HNF4α in the maintenance of a mature hepatocyte-like, less aggressive HCC phenotype coupled with Gd-EOB-DTPA uptake capacity.

**Prognosis of Early-Stage HCC by EOB-AFP Classification.** Finally, we evaluated the prognosis of patients with HCC diagnosed by EOB-MRI and serum AFP. To exclude the potential effect of lead-time bias on survival analysis for HCCs at different stages, we evaluated the power of the EOB-AFP classification system to predict the prognosis of patients with early-stage BCLC stage 0 or A HCCs diagnosed by EOB-MRI in an independent multicenter cohort (Cohort 2). Nine of the 109 HCC cases (8.3%) were diagnosed with hyperintense HCCs and were found to be significantly associated with low serum AFP levels (Table 1). The clinicopathologic characteristics of the patients defined by the EOB-AFP classification are shown in Supporting Table 5. The median follow-up times in Cohorts 1 and 2 were 569 and 932 days, respectively. The 3-year overall survival rates in Cohorts 1 and 2 were 77.7% and 90.9%, respectively (Fig. 4A,B). The prognosis of HCC patients was not separated by TNM or BCLC stages because most of these patients were diagnosed at early stages (Fig. S4A-D); nevertheless, the EOB-AFP classification system robustly stratified HCCs according to survival with statistically significant differences between the classes (Fig. 4C,D). EOB-AFP class A patients had 100% overall survival, whereas class C patients had 30% overall survival at 1,200 days after radical resection in Cohort 2.
The prognosis of HCC patients stratified by the EOB-AFP classification was most likely affected by the malignant nature of the tumor at surgical resection, because EOB-AFP class C patients showed a 40-60% recurrence-free survival rate, whereas class A patients had a 88-100% recurrence-free survival rate at 1 year after radical resection in both cohorts (Fig. S5).

Altogether, our data, for the first time, revealed that the prognosis of early-stage HCC patients is heterogeneous and related to the malignant phenotypes of the tumors, even after successful treatment by radical resection. The EOB-AFP classification system reflects the malignant nature of the tumor and predicts the survival of early-stage HCC patients prior to surgery.

Discussion

Among several HCC staging systems currently used, the BCLC system is recommended because it is linked to treatment strategy. The assessment of the malignant nature of tumors coupled with current staging systems will supplement the management of early-stage HCC because early recurrence after potentially curative treatment may be associated with the characteristics of the resected tumor rather than the development of a de novo HCC in the background liver. Molecular profiling approaches have tried to evaluate the malignant features of HCCs and the surrounding noncancerous liver tissue, although the evaluation of the potential clinical application of these approaches is ongoing. Our EOB-AFP classification system is molecularly related to the OATP1B3 gene signature, which can be used to classify HCCs according to their stem/maturational status. Interestingly, the differential expression of OATP1B3 was also noted in two HCC subtypes associated with the stem/maturational status, as reported recently by our group (hepatic stem cell-like and mature hepatocyte-like HCC) and others (hepatoblast-type and hepatocyte type) (Fig. S6). As expected, all class A HCCs were categorized as mature hepatocyte-like HCC in Cohort 1 (data not shown). The stem/maturational status defined by the EOB-AFP classification is most likely regulated by at least two transcription factors: HNF4a and FOXM1 (Fig. 4E).

HNF4a was first discovered as a liver-enriched nuclear orphan receptor activating the transcription of transthyretin genes, and it is known to regulate bile acid and cholesterol metabolism. The liver-specific loss of HNF4a in adult mice results in hepatocyte proliferation, whereas the introduction of HNF4a suppresses HCC growth. Furthermore, a recent study suggested a role for HNF4a as a tumor suppressor in inflammation-related hepatocarcinogenesis through the regulation of microRNAs. The present study demonstrated a crucial role for HNF4a in maintaining a hepatocyte-like, less aggressive phenotype coupled with Gd-EOB-DTPA uptake in a class A HCC by directly modifying HNF4a gene expression. Thus, HNF4a may work as a tumor suppressor gene and inhibit the progression of HCC, which may be related to the good prognosis of class A HCCs.

FOXM1 belongs to the forkhead superfamily of transcription factors and regulates a myriad of biologic processes including cell proliferation and differentiation. The pivotal role of FOXM1 in liver development and regeneration has been reported previously. FOXM1 was also required for HCC development in a mouse hepatocarcinogenesis model and acted as an oncogene in a transgenic mouse model. It was recently shown that FOXM1 levels are elevated in various cancers including HCC. A prognostic role for FOXM1 in HCC patients after liver transplantation was also reported, this may be associated with the metastatic capacity of tumors regulated by FOXM1.

As FOXM1 and AFP are known to be activated during liver regeneration and hepatocarcinogenesis, serum AFP levels may be a surrogate marker for the expression status of FOXM1 and thus facilitate the prognostic stratification of HCCs by the EOB-AFP classification.

Among the molecular markers reported to be differentially expressed between dysplastic nodule and well-differentiated HCC, we found preferential overexpression of GS in EOB-AFP class A and GPC-3 in class C HCCs. Our data suggest that class A and class C HCCs may follow different processes of early hepatocarcinogenesis events that might be associated with the differential activation of HNF4a and FOXM1, and further studies are required to obtain molecular insights into these processes.

Our overall survival data in Cohort 2 indicated that EOB-AFP class A patients had 100% overall survival, whereas class C patients had 30% overall survival at 1,200 days after radical resection. This suggests that the micro-dissemination of tumor cells in EOB-AFP class C HCC patients has already occurred by the time they are diagnosed with early-stage disease. Indeed, 50% of all class C patients showed tumor recurrence, whereas 88-100% of class A patients showed no recurrence within 1 year of resection; this is consistent with a recent study evaluating the clinical features of hyperintense HCCs and may be due to
the overexpression of FOXM1, which results in the activation of metastatic programs. Therefore, these patients might have survival benefits if they receive adjuvant therapies. As several adjuvant therapies might be beneficial for HCC patients after surgical resection, the multiclassification of molecular profiling and EOB-MRI findings in various stages of HCC; and Cohort 2 for evaluating the utility of EOB-MRI and serum AFP in predicting the prognosis of early-stage HCCs, which made the molecular and prognostic analyses complex. Another limitation of this study was in the evaluation of prognostic utility because it uses small retrospective cohorts. Direct evaluation of the molecular profiles and prognostic values of hyperintense HCCs should be performed in a prospective study using a large-scale HCC cohort.

Taken together, the present study demonstrates for the first time that the combined approach of noninvasive Gd-EOB-DTPA-enhanced MRI and serum AFP levels can be used preoperatively to classify resectable HCCs into three subgroups with distinct prognoses. This classification is molecularly related to the stem/maturation status of HCCs regulated by HNF4α and FOXM1. The multicenter early-stage HCC cohort that received radical resection revealed that the EOB-AFP classification is clinically useful to determine the prognosis of early-stage HCC patients. On the basis of these observations, we propose that the EOB-AFP classification system be incorporated into current HCC staging practices, especially for the management of early-stage HCCs.

Acknowledgment: We thank Drs. Yutaka Aoyagi (Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan), Hiroko Iijima (Division of Hepatobiology and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan), and Michio Sata (Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Japan) for help with patient enrollment. We also thank Ms. Masayo Baba and Nami Nishiyama for excellent technical assistance.

References

1. Jamal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
2. Sala M, Forner A, Varela M, Bruix J. Prognostic prediction in patients with hepatocellular carcinoma. Semin Liver Dis 2005;25:171-180.
3. Cairo S, Wang Y, de Reynies A, Duroure K, Dahan J, Redon MJ, et al. Stem cell-like micro-RNA signature driven by Myc in aggressive liver cancer. Proc Natl Acad Sci U S A 2010;107:20471-20476.
4. Lee JS, Heo J, Libbrecht L, Chui IS, Kaposi-Novak P, Calvisi DE, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 2006;12:410-416.
5. Marquardt JU, Raggi C, Andersen JB, Seo D, Avital I, Geller D, et al. Human hepatic cancer stem cells are characterized by common stemness traits and diverse oncogenic pathways. Hepatology 2011;54:1031-1042.
6. Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. Gastroenterology 2009;136:2020-2024.
7. Yamashita T, Wang XW. Cancer stem cells in the development of liver cancer. J Clin Invest 2013;123:1911-1918.
8. Reimer P, Schneider G, Schima W. Hepatobiliary contrast agents for contrast-enhanced MRI of the liver: properties, clinical development and applications. Eur Radiol 2004;14:559-578.
9. Kanki A, Tamada T, Higali A, Noda Y, Tanimoto D, Sato T, et al. Hepatic parenchymal enhancement at Gd-EOB-DTPA-enhanced MR imaging: correlation with morphological grading of severity in cirrhosis and chronic hepatitis. Magn Reson Imaging 2012;30:356-360.
10. Kitao A, Matsui O, Yoneda N, Kozaka K, Shinmura R, Koda W, et al. The uptake transporter OATP1 expression decreases during multiplet hepatocarcinogenesis: correlation with gadoxetic acid-enhanced MR imaging, Eur Radiol 2011;21:2056-2066.
11. Kitao A, Zen Y, Matsui O, Gabata T, Kobayashi S, Koda W, et al. Hepatocellular carcinoma: signal intensity at gadoxetic acid-enhanced MR imaging—correlation with molecular transporters and histopathologic features. Radiology 2010;256:817-826.
12. Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res 2008;68:1451-1461.
13. Yamashita T, Honda M, Nakamoto Y, Baba M, Nio K, Hara Y, et al. Discrete nature of EpCAM(+) and CD90(+) cancer stem cells in human hepatocellular carcinoma. Hepatology 2013;57:1486-1497.
14. Yamashita T, Honda M, Nio K, Nakamoto Y, Takamura H, Tani T, et al. Oncostatin m renders epithelial cell adhesion molecule-positive liver cancer stem cells sensitive to 5-Fluorouracil by inducing hepatocytic differentiation. Cancer Res 2010;70:4687-4697.
15. Lade AG, Monga SP. Beta-catenin signaling in hepatic development and progenitors: which way does the WNT blow? Dev Dyn 2011;240:486-500.
16. Trauner M, Halilbasic E. Nuclear receptors as new perspective for the management of liver diseases. Gastroenterology 2011;140:1120-1125 e1121-1112.
17. Wang X, Kiyokawa H, Dennewitz MB, Costa RH. The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. Proc Natl Acad Sci U S A 2002;99:16881-16886.
18. Hoshiba Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med 2008;359:1995-2004.
19. Ji J, Shi J, Budhu A, Yu Z, Forgues M, Roessler S, et al. MicroRNA expression, survival, and response to interferon in liver cancer. N Engl J Med 2009;361:1437-1447.
20. Di Tommaso L, Destro A, Seok JY, Balladore E, Terracciano L, Sangiovanni A, et al. The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. J Hepatol 2009;50:746-754.
21. Llovet JM, Chen Y, Wurmbach E, Roayaie S, Fiel MI, Schwartz M, et al. A molecular signature to discriminate dysplastic nodules from early hepatocellular carcinoma in HCV cirrhosis. Gastroenterology 2006;131:1758-1767.

22. Sherman M. Hepatocellular carcinoma: screening and staging. Clin Liver Dis 2011;15:323-334. vii-x.

23. Villanueva A, Hoshida Y, Toffanin S, Lachenmayer A, Albinet C, Savic R, et al. New strategies in hepatocellular carcinoma: genomic prognostic markers. Clin Cancer Res 2010;16:4688-4694.

24. de Lope CR, Tremosini S, Forner A, Reig M, Bruix J. Management of HCC. J Hepatol 2012;56 Suppl:S75-87.

25. Crestani M, De Fabiani E, Caruso D, Mitro N, Gilardi F, Vigil Chacon AB, et al. LXR (liver X receptor) and HNF-4 (hepatocyte nuclear factor-4): key regulators in reverse cholesterol transport. Biochem Soc Trans 2004;32:92-96.

26. Bonzo JA, Ferry CH, Matsubara T, Kim JH, Gonzalez FJ. Suppression of hepatocyte proliferation by hepatocyte nuclear factor 4alpha in adult mice. J Biol Chem 2012;287:7345-7356.

27. Ning BF, Ding J, Yin C, Zhong W, Wu K, Zeng X, et al. Hepatocyte nuclear factor 4 alpha suppresses the development of hepatocellular carcinoma. Cancer Res 2010;70:7640-7651.

28. Yin C, Lin Y, Zhang X, Chen YX, Zeng X, Yue HY, et al. Differentiation therapy of hepatocellular carcinoma in mice with recombinant adenovirus carrying hepatocyte nuclear factor-4alpha gene. HEPATOLOGY 2008;48:1528-1539.

29. Hatziapostolou M, Polytarchou C, Aggelidou E, Drakaki A, Poultsides GA, Jaeger SA, et al. An HNF4alpha-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. Cell 2011;147:1233-1247.

30. Koo CY, Muir KW, Lam EW. FOXM1: From cancer initiation to progression and treatment. Biochim Biophys Acta 2012;1819:28-37.

31. Kalinichenko VV, Major ML, Wang X, Petrovic V, Kuechle J, Yoder HM, et al. Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor. Genes Dev 2004;18:830-850.

32. Kalin TV, Ustijan V, Kalinichenko VV. Multiple faces of FoxM1 transcription factor; lessons from transgenic mouse models. Cell Cycle 2011;10:396-405.

33. Calvissi DF, Pinna F, Ladu S, Pellegrino R, Simile MM, Frau M, et al. Forkhead box M1B is a determinant of rat susceptibility to hepatocarcinogenesis and sustains ERK activity in human HCC. Gut 2009;58:679-687.

34. Sun H, Teng M, Liu J, Jin D, Wu J, Yan D, et al. FOXM1 expression predicts the prognosis in hepatocellular carcinoma patients after orthotopic liver transplantation combined with the Milan criteria. Cancer Lett 2011;306:214-222.

35. Raychaudhuri P, Park HJ. FoxM1: a master regulator of tumor metastasis. Cancer Res 2011;71:4329-4333.

36. Kitao A, Matsui O, Yoneda N, Kozaka K, Kobayashi S, Koda W, et al. Hypervascular hepatocellular carcinoma: correlation between biologic features and signal intensity on gadoxetic acid-enhanced MR images. Radiology 2012;265:780-789.

37. Zhong JF, Li H, Li QL, You XM, Zhang Y, Zhao YN, et al. Adjuvant therapy options following curative treatment of hepatocellular carcinoma: a systematic review of randomized trials. Eur J Surg Oncol 2012;38:286-295.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website.