observed in hyper-intense HCCs. Since serum AFP levels are reportedly related to the stem/maturation subtypes of HCCs with different gene expression profiles (12), 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, Figure 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: hyper-intense HCC, class B: hypo-intense and AFP-low [<100 ng/mL] HCC, and class C: hypo-intense 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 (Figure 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 ($P < 0.0001$) (Figure 2D). The differences in FOXM1 and HNF4α expression among class A, B, and C HCCs were statistically significant (Figure 2E).

We further investigated the expression of 5 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 ($P = 0.06$) (Figure 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).

Regulation of Gd-EOB-DTPA uptake and tumorigenic capacity by HNF4α in hyper-intense HCC

Microarray and IHC analyses suggested the activation of transcription factor HNF4α in hyper-intense HCC, but its role in the maintenance of hepatocyte function and Gd-EOB-DTPA uptake has not yet been clarified. To explore directly the role of HNF4α in Gd-EOB-DTPA uptake and tumorigenic capacities, we transplanted tumor cells from hyper- and hypo-intense primary HCC

specimens into NOD/SCID mice (Figure 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 (Figure 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 hyper-intense HCC. We confirmed the reduction of HNF4α protein expression in Sh-HNF4A-transfected cells compared with Sh-Scr-transfected cells by western blotting (Figure 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 (Figure 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 (Figure 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 observed in Sh-HNF4A-transfected cells, whereas Sh-Scr-transfected cells still showed Gd-EOB-DTPA uptake with less tumorigenic capacity (Figure 3E). Mice xenotransplanted with Sh-HNF4A-transfected cells had a worse prognosis compared with those xenotransplanted with Sh-Scr-transfected cells (Figure 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 hyper-intense 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 Supplementary 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 (Figure 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 (Figure S4A–D); nevertheless, the EOB-AFP classification system robustly stratified HCCs according to survival with statistically significant differences between the classes (Figure 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 (Figure 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 (2), the BCLC system is recommended because it is linked to treatment strategy (22). The assessment of the malignant nature of tumors coupled with current staging systems will supplement the management of early stage HCC (23) 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 (24). Molecular profiling approaches have tried to evaluate the malignant features of HCCs and the surrounding non-cancerous liver tissue (3–6, 12, 18), 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) (12) and others (hepatoblast-type and hepatocyte type) (4) (Figure 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, namely HNF4α and FOXM1 (Figure 4E).

HNF4α 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 (25). The liver-specific loss of *HNF4A* in adult mice results in hepatocyte

proliferation (26), whereas the introduction of *HNF4A* suppresses HCC growth (27, 28). Furthermore, a recent study suggested a role for *HNF4A* as a tumor suppressor in inflammation-related hepatocarcinogenesis through the regulation of microRNAs (29). The present study demonstrated a crucial role for HNF4α 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 (30). The pivotal role of FOXM1 in liver development and regeneration has been reported previously (17). FOXM1 was also required for HCC development in a mouse hepatocarcinogenesis model (31) and acted as an oncogene in a transgenic mouse model (32). It was recently shown that FOXM1 levels are elevated in various cancers including HCC (32, 33). A prognostic role for FOXM1 in HCC patients after liver transplantation was also reported (34); this may be associated with the metastatic capacity of tumors regulated by FOXM1 (35). 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 HNF4α 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 hyper-intense HCCs (36) 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 (37), integration of the EOB-AFP classification system into current staging practices may provide additional therapeutic options for early-stage HCC patients who will receive surgery.

A limitation of the present study is that we utilized 3 different cohorts to reveal the molecular portraits associated with clinical imaging and prognosis (i.e., the microarray cohort of 238 HCCs of various stages for the evaluation of molecular profiling; Cohort 1 for the validation 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 hyper-intense 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 non-invasive Gd-EOB-DTPA-enhanced MRI and serum AFP levels can be used preoperatively to classify resectable HCCs into 3 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.

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Figure Legends

Figure 1 Molecular profiles of HCCs corresponding to the EOB-MRI findings.

(A) Representative MRI scans of hypo- and hyper-intense HCCs in the precontrast, arterial, and hepatobiliary phases. The T/N signal intensity ratios of the images in the hepatobiliary phase were 0.47 (upper panel) and 1.07 (lower panel). (B) Upper panel: Representative photomicrographs of IHC staining with an anti-OATP1B3 antibody in hypo- and hyper-intense HCCs. Lower panel: *OATP1B3* expression in hypo- and hyper-intense HCCs. (C) The expression