A Unique Metastasis Gene Signature Enables Prediction of Tumor Relapse in Early-Stage Hepatocellular Carcinoma Patients

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Abstract

Metastasis-related recurrence often occurs in hepatocellular carcinoma (HCC) patients who receive curative therapies. At present, it is challenging to identify patients with high risk of recurrence, which would warrant additional therapies. In this study, we sought to analyze a recently developed metastasis-related gene signature for its utility in predicting HCC survival, using 2 independent cohorts consisting of a total of 386 patients who received radical resection. Cohort 1 contained 247 predominantly HBV-positive cases analyzed with an Affymetrix platform, whereas cohort 2 contained 139 cases with mixed etiology analyzed with the NCI Oligo Set microarray platform. We employed a survival risk prediction algorithm with training, test, and independent cross-validation strategies and found that the gene signature is predictive of overall and disease-free survival. Importantly, risk was significantly predicted independently of clinical characteristics and microarray platform. In addition, survival prediction was successful in patients with early disease, such as small (<5 cm in diameter) and solitary tumors, and the signature predicted particularly well for early recurrence risk (<2 years), especially when combined with serum alpha fetoprotein or tumor staging. In conclusion, we have shown in 2 independent cohorts with mixed etiologies and ethnicity that the metastasis gene signature is a useful tool to predict HCC outcome, suggesting the general utility of this classifier. We recommend the use of this classifier as a molecular diagnostic test to assess the risk that an HCC patient will develop tumor relapse within 2 years after surgical resection, particularly for those with early-stage tumors and solitary presentation. *Cancer Res; 70(24); 10202–12. ©2010 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the most frequent malignant tumor in the liver and the third leading cause of cancer-related deaths worldwide (1). It is most prevalent in developing countries, but its incidence is increasing in developed countries due to chronic infection with hepatitis C virus (HCV) and resulting liver cirrhosis (2). In the United States, liver cancer has the fastest growing cancer death rate, even

though the overall cancer mortality rate has declined during the past years (3). The poor outcome of HCC patients is mainly caused by the high frequency of late-stage disease, metastasis, and *de novo* tumor formation in the diseased liver, the so-called "field effect" (4, 5). Currently, surgery is the most effective treatment, but the recurrence rate is high, mainly due to the dissemination of malignant cells (6). Although early-stage tumors can be treated by resection, liver transplantation, or local ablation, few patients present with early-stage disease and many patients still suffer from recurrence after treatment of early-stage tumors (7).

Metastasis contributes to 90% of all cancer-related deaths, emphasizing the importance of metastasis risk prediction (8). Unlike other tumor types, HCC metastasis occurs mainly within the liver itself, with new tumor colonies frequently invading into the major branches of the portal vein or to other parts of the liver (9–11). It is believed that *de novo* development of primary HCC in the remnant liver occurs with a lower frequency (12, 13). Recurrence by metastasis seems to occur mainly in an early period, that is, within the first 2 years after resection, whereas recurrence due to new primary lesions often occurs after a longer period (5, 14–17). Consistently, Chen et al. found that tumors that recurred late often showed clonal origins different from the original tumors, suggesting a *de novo* second primary HCC (18). The comparison of early

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and late recurrences of HCC after hepatectomy revealed that early recurrence is associated with nonanatomic resection, microscopic vascular invasion, and high alpha fetoprotein (AFP) levels (14). In contrast, late recurrence is associated with the level of chronic hepatitis, multinodularity, and tumor classification (14).

In a recent pilot study, we identified a metastasis signature consisting of 153 genes that could distinguish HCC patients with portal venous metastases from those without (19). This metastasis signature was developed on the basis of cDNA microarray profiling of 20 well-defined HCC cases, of which 10 patients presented with tumor thrombi in the major branches of the portal vein at surgery whereas 10 patients with metastasis-free HCC at the time of surgery and at follow-up. In this study, we used 2 independent cohorts consisting of a total of 386 HCC patients to analyze the utility of this signature as a risk classifier for HCC recurrence and survival.

Materials and Methods

Study cohorts and patient characteristics

Cohort 1 hepatic tissues were obtained from the Liver Cancer Institute (LCI), with informed consent from patients who underwent radical resection between 2002 and 2003 at the Liver Cancer Institute and Zhongshan Hospital (Fudan University). The study was approved by the Institutional Review Board of the participating institutes. A total of 247 HCC patients were recruited. Cases were mainly patients with a history of hepatitis B virus (HBV) infection or HBV-related liver cirrhosis; all were diagnosed with HCC by 2 independent pathologists, with detailed information on clinical presentation and pathologic characteristics. For 242 patients, disease-free survival and overall survival as well as the cause of death were available.

The gene expression data of cohort 2 has been published earlier (20, 21). Briefly, gene expression profiling of cohort 2 was carried out by the Laboratory of Experimental Carcinogenesis (LEC) and analyzed using NCI's Human Array-Ready Oligo Set microarray platform (GPL1528). The microarray data are publicly available at the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) with accession numbers GSE1898 and GSE4024.

Tumor samples and microarray processing

Total RNA was extracted from frozen tissues using TRIzol (Invitrogen) according to the manufacturer's protocol. Only RNA samples with good RNA quality as confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies) and agarose gel electrophoresis were included in the study. For microarray profiling, tumors, and paired nontumor tissues were profiled separately using a single-channel array platform. Gene expression profiling of 22 tumor samples was carried out on Affymetrix GeneChip HG-U133A 2.0 arrays according to the manufacturer's protocol. The fluorescent intensities were determined with an Affymetrix GeneChip Scanner 3000, controlled by GCOS Affymetrix software. The remaining 225 tumor samples were processed on the 96 HT HG-U133A 2.0 microarray platform. The fluorescent intensities were determined

with an Affymetrix GeneChip HT Array Plate Scanner, controlled by GCOS Affymetrix software. Quality controls included image inspection as well as Relative Log Expression (RLE) and Normalized Unscaled Standard Error (NUSE) implemented in the affyPLM package available at the Bioconductor (www.bioconductor.org). In accordance with Minimum Information About a Microarray Experiment (MIAME) guidelines, we deposited the microarray data and additional patient information into the GEO repository with accession number GSE14520.
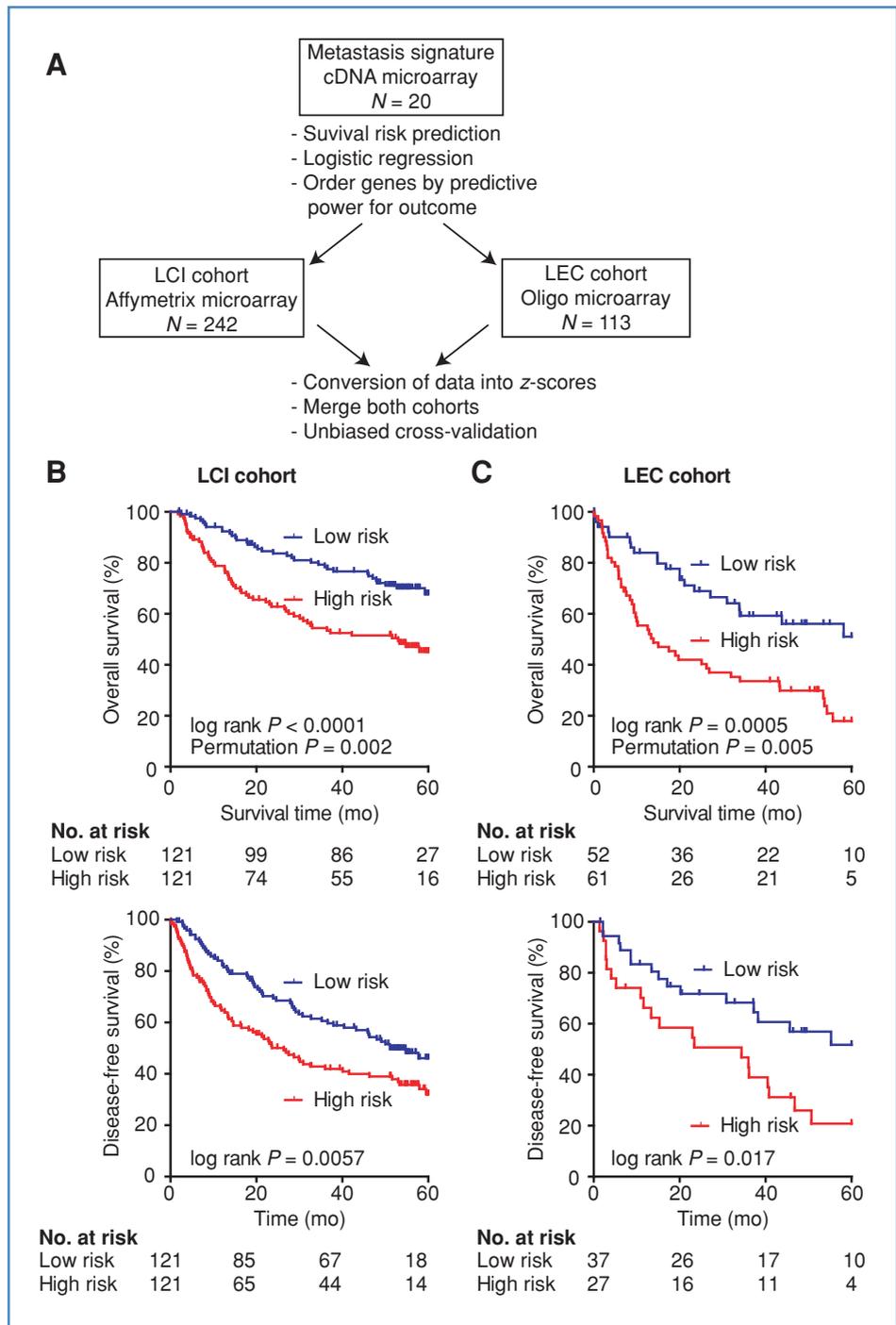
Affymetrix gene expression arrays obtained from different platforms were combined with the match probes package in R (http://www.R-project.org; ref. 22). Raw gene expression data were normalized using the Robust Multi-array Average (RMA) method and global median centering (23). For genes with more than 1 probe set, the mean gene expression was calculated.

Statistical analysis

Class comparison and survival risk prediction of the gene expression data was done with the BRB-Array Tools software (http://linus.nci.nih.gov/BRB-ArrayTools.html; Version 3.7.0). For survival risk prediction, we identified genes whose expression was significantly related to survival by applying univariate Cox proportional hazards regression followed by principal component analysis, which is a computational procedure that transforms a number of possible correlated variables into a significantly smaller number of uncorrelated variables called principal components. This resulted in a regression coefficient (weight) related to survival time based on 2 principal components. Next, to compute a prognostic index, the weighted average of the principal component values was calculated using the regression coefficients derived from the Cox regression, described above. Finally, this prognostic index was used to split samples into 2 groups of equal size by the median of the prognostic index. Thereby, a high value of the prognostic index corresponded to a high value of hazard of death (high risk) and consequently a relatively poor predicted survival.

Kaplan–Meier survival curves for the predicted cases to have above-average risk and the cases predicted to have below-average risk were plotted. To evaluate the predictive value of the method, 10-fold cross-validation with 1,000-fold random permutation of the Cox–Mantel log-rank test was conducted.

For cross-validation of the LCI and LEC cohorts, we converted the gene expression data into z-scores and then conducted class prediction in BRB-Array Tools. First, we used the LEC cohort for training/testing and predicted the outcome of the LCI cohort and then used the LCI cohort for training/testing and predicted the outcome of the LEC cohort. Six class prediction algorithms, Support Vector Machines (SVM), Nearest Centroid (NC), 3-Nearest Neighbor (3-NN), 1-Nearest Neighbor (1-NN), Linear Discriminant Analysis (LDA), and Compound Covariate Predictor (CCP), were used to determine whether mRNA expression patterns could accurately discriminate good and poor survival HCC groups in an independent data set. The accuracy of the prediction was calculated after 1,000 repetitions of this random partitioning process to control the number and proportion of false discoveries.

A

Metastasis signature
cDNA microarray
N = 20

- Suvival risk prediction
- Logistic regression
- Order genes by predictive
  power for outcome

LCI cohort
Affymetrix microarray
N = 242

LEC cohort
Oligo microarray
N = 113

- Conversion of data into *z*-scores
- Merge both cohorts
- Unbiased cross-validation

Figure 1. Survival risk prediction analysis and application of the metastasis gene signature. A, schematic overview of the study design. B, Kaplan–Meier survival curves showing the overall survival (top; *N* = 242) and the disease-free survival (bottom; *N* = 242) of the predicted high- and low-risk groups in the LCI cohort. C, Kaplan–Meier survival curves showing the overall survival (top; *N* = 113) and the disease-free survival (bottom; *N* = 64) of the predicted high- and low-risk groups in the LEC cohort. Displayed are the Cox–Mantel log-rank, the permutation *P*-values and the number of patients at risk for each Kaplan–Meier survival curve.

Kaplan–Meier survival analysis was done using GraphPad Prism software 5.0 (GraphPad Software) and the statistical *P* values were generated by the Cox–Mantel log-rank test. Cox proportional hazards regression was used to analyze the effect of clinical variables on patient survival, using STATA 9.2. Clinical variables included age, gender, HBV active status, preresection AFP, cirrhosis, alanine aminotransferase (ALT), tumor size or size of the largest tumor when multiple tumors are present, nodular type, and the HCC prognosis staging systems Barcelona Clinic Liver Cancer (BCLC), Cancer Liver Italian Program (CLIP), or Tumor Node Metastasis (TNM) classification (24–26). An AFP cutoff of 300 ng/mL, ALT of 50 U/L, and tumor size of 5 cm were used in Cox regression analysis and are clinically relevant values used to distinguish patient survival. A univariate test was used to examine the influence of the "metastasis" gene predictor or each clinical

Table 1. Clinical characteristics of patients in the LCI and LEC cohorts at the time of surgery

Clinical variable	LCI	LEC	P value[a]
	(*N* = 247)	(*N* = 139)	
Etiology (HBV/HCV/HBV + HCV/nonviral/NA)	(236/0/5/2/4)	(58/17/4/32/28)	<0.0001
AVR-CC (yes/no/NA)	62/179/6	NA	NA
Gender (male/female/NA)	214/31/2	102/37/0	**0.0008**
Age (≥50 y/<50 y/NA)	136/109/2	92/47/0	0.0515
AFP (>300 ng/mL/≤300 ng/mL/NA)	111/130/6	55/73/11	0.5844
ALT (>50 U/L/≤50 U/L/NA)	101/144/2	NA	NA
Cirrhosis (yes/no/NA)	224/21/2	69/70/0	<0.0001
Tumor size (>5 cm/≤5 cm /NA)	89/155/3	72/67/0	**0.0038**
Multinodular (yes/no /NA)	52/193/2	NA	NA
Encapsulation (no/yes/NA)	114/129/4	NA	NA
Microscopic vascular invasion (yes/no/NA)	107/90/50	NA	NA
BCLC staging (B–C/A–0/NA)	53/174/20	NA	NA
CLIP staging (1–5/0/NA)	128/99/20	NA	NA
TNM staging (II–III/I/NA)	130/97/20	NA	NA
Survival at 60 mo (events/censored/NA)	95/147/5	67/46/26	**<0.0001**[b]

Abbreviations: NA, not available; AVR-CC, active viral replication chronic carrier.
[a]Fsher's exact test.
[b]Cox-Mantel log-rank test.

variable on patient survival. A multivariate analysis was done to estimate the hazards ratio of the predictor while controlling for clinical variables that were significantly associated with survival in the univariate analysis. Because tumor size and nodular type were collinear with tumor staging, these variables were not included in the multivariate analysis. It was determined that the final model met the proportional hazards assumption. Receiver operating characteristic (ROC) curves were computed by using the tumor expression level for compound covariate prediction and the ROCR package (27). The statistical significance was defined as $P < 0.05$.

Endpoints

We analyzed the *overall survival*, which was defined as time from surgery to death from any disease, as well as the *disease-free survival*, which was defined as the time from surgery to any recurrence, distant metastasis, or death from any cause. The Kaplan–Meier estimator was used to display time-to-event curves for these 2 endpoints.

Results

Redefining the metastasis gene signature

We reanalyzed the data from our pilot study on 20 well-defined HCC cases used to identify our recently published 153 gene HCC metastasis signature with the updated gene annotation, sequence data, and software (19). Class comparison identified 181 differentially expressed cDNA probes ($P < 0.001$, FDR < 0.05). Thirty-six of the 181 probes did not have any gene annotation available in the original study (19). Alignment of the probe sequences to the human genome (NCBI BLAST)

resulted in the annotation information of 8 additional genes. Therefore, 161 of 181 probes matched to annotated genes (including all original 153 genes; Supplementary Table 1). This new 161 gene signature is referred to as a metastasis risk classifier and was used for subsequent analysis.

Predicting HCC survival using 2 independent validation cohorts

Next, we developed a strategy for testing the metastasis risk classifier by incorporating 2 independent patient cohorts, that is, LCI and LEC cohorts (Fig. 1A). We aimed to determine whether this classifier can predict survival, as HCC metastasis is the main causative factor for poor outcome. The recruitment criteria of the LCI cohort were based on the characteristics of the 40 original patients previously described (19). In addition to the 2 different microarray platforms used, the LCI and LEC cohorts differed in their patient characteristics (Table 1). The LCI cohort mainly consists of HBV-positive Chinese patients (95.6%), whereas the LEC cohort is heterogeneous, containing a mixture of Chinese, European, and American patients with 41.7% HBV positive, 12.2% HCV positive, and 23.0% nonviral HCC. The 2 cohorts also differed in gender distribution, the number of patients with underlying cirrhosis, tumor size, and survival time (Table 1). The survival time of patients in the LEC cohort was significantly shorter than in the LCI cohort, which was consistent with the larger tumor size of the LEC cohort.

We tested the genes of the metastasis risk classifier for their survival association. The survival risk prediction based on 10-fold cross-validation classified patients into low- and high-risk groups, with a significant difference in survival as analyzed by

Table 2. Univariate and multivariate Cox regression analysis of clinical factors associated with overall survival of the LCI cohort (N = 242)[a]

Clinical variable Univariate analysis[b]	Hazard ratio (95% CI)	P value
Predictor (high vs. low risk)	2.25 (1.48–4.5)	**<0.001**
Gender (male vs. female)	1.86 (0.90–3.83)	0.094
Age (≥50 y vs. <50 y)	0.80 (0.53–1.19)	0.209
AFP (>300 ng/mL vs. ≤300 ng/mL)	1.64 (1.10–2.45)	**0.016**
ALT (>50 U/L vs. ≤50 U/L)	1.15 (0.77–1.73)	0.483
Cirrhosis (yes vs. no)	5.09 (1.25–20.7)	**0.023**
Tumor size (>5 cm vs. ≤5 cm)	2.01 (1.35–3.01)	**0.001**
Multinodular (yes vs. no)	1.65 (1.06–2.57)	**0.025**
Encapsulation (no vs. yes)	0.76 (0.50–1.14)	0.181
Microscopic vascular invasion (yes vs. no)	1.97 (1.26–3.09)	**0.003**
HBV (AVR-CC vs. CC)	1.36 (0.85–2.16)	0.196
BCLC staging (B–C vs. A–0)	3.69 (2.38–5.73)	**<0.001**
CLIP staging (1–5 vs. 0)	2.19 (1.38–3.48)	**0.001**
TNM staging (II–III vs. I)	3.08 (1.88–5.05)	**<0.001**
Multivariate analysis[c]		
Predictor (high vs. low risk)	1.64 (1.03–2.60)	**0.038**
AFP (>300 ng/mL vs. ≤300 ng/mL)	1.31 (0.84–2.03)	0.237
Cirrhosis (yes vs. no)	3.91 (0.96–16.0)	0.058
TNM staging (II–III vs. I)	2.72 (1.64–4.51)	**<0.001**
Multivariate analysis[d]		
Predictor (high vs. low risk)	1.91 (1.22–2.99)	**0.005**
AFP (>300 ng/mL vs. ≤300 ng/mL)	0.74 (0.42–1.29)	0.289
CLIP staging (1–5 vs. 0)	2.38 (1.29–4.40)	**0.006**
Multivariate analysis[e]		
Predictor (high vs. low risk)	1.79 (1.14–2.82)	**0.012**
AFP (>300 ng/mL vs. ≤300 ng/mL)	1.16 (0.75–1.80)	0.542
BCLC staging (B–C vs. A–0)	3.43 (2.19–5.36)	**<0.001**

NOTE: Bold indicates significant P values.

Abbreviations: AVR-CC, active viral replication chronic carrier; CC, chronic carrier.

[a]Analysis was done on the entire gene expression cohort.

[b]Univariate analysis, Cox proportional hazards regression.

[c]Multivariate analysis, Cox proportional hazards regression adjusting for AFP status, cirrhosis, and TNM staging.

[d]Multivariate analysis, Cox proportional hazards regression adjusting for AFP status and CLIP staging.

[e]Multivariate analysis, Cox proportional hazards regression adjusting for AFP status and BCLC staging.

Kaplan–Meier plot, with log-rank P values of P < 0.0001 and P = 0.0005 in LCI and LEC cohorts, respectively (Fig. 1B and C, top). The cross-validated misclassification rates were significantly lower than expected by chance (permutation P < 0.01; Fig. 1). Similar results were observed when disease-free survival was used as an endpoint (Fig. 1B and C, bottom). Thus, this signature was independently validated as a classifier to predict survival in addition to metastasis.

We conducted Cox proportional hazards regression analysis to determine whether the metastasis gene signature was confounded by the underlying clinical parameters. In univariate Cox analysis, the unadjusted hazard ratio for the overall survival in the high-risk versus the low-risk patient groups in the LCI cohort was 2.25 (95% CI = 1.48–4.5). The Cox analysis was stratified by several clinical factors and revealed that the AFP serum levels, underlying liver cirrhosis, tumor size, microscopic vascular invasion, and tumor staging such as BCLC, CLIP, or TNM were associated with overall survival (Table 2; ref. 24–26). Multivariate Cox regression analysis accounting for the prognostic clinical factors that were significant in the univariate analysis revealed that the gene signature is an independent predictor of survival (Table 2). Only limited clinical information was available for the Cox regression analysis in the LEC cohort (Table 1). Analysis of the LEC cohort showed that the gene signature was a strong prognostic factor for patient survival with a hazard ratio of 2.59 (95% CI = 1.61–4.17). The univariate Cox regression analysis of the available clinicopathologic data of the LEC cohort did not result in any significant clinical factor and thus no further multivariate analysis was done (data not shown).

Figure 2. Analysis of the performance of the survival risk prediction dependent on HCC tumor recurrence over time after surgery. A, cumulative HCC recurrence rate over time. B, smoothed recurrence rate per month over time. C, Forest plots showing hazard ratios for high-risk patients in the indicated clinical groups of patients. Hazard ratios are shown for the overall survival at 5 years, (D) the overall survival at 2 years, (E) the disease-free survival at 5 years, and (F) the disease-free survival at 2 years of follow-up of the high-risk subgroup as compared with the low-risk group. Hazard ratios above 1.0 indicate significantly worse outcome. ND, not determined.
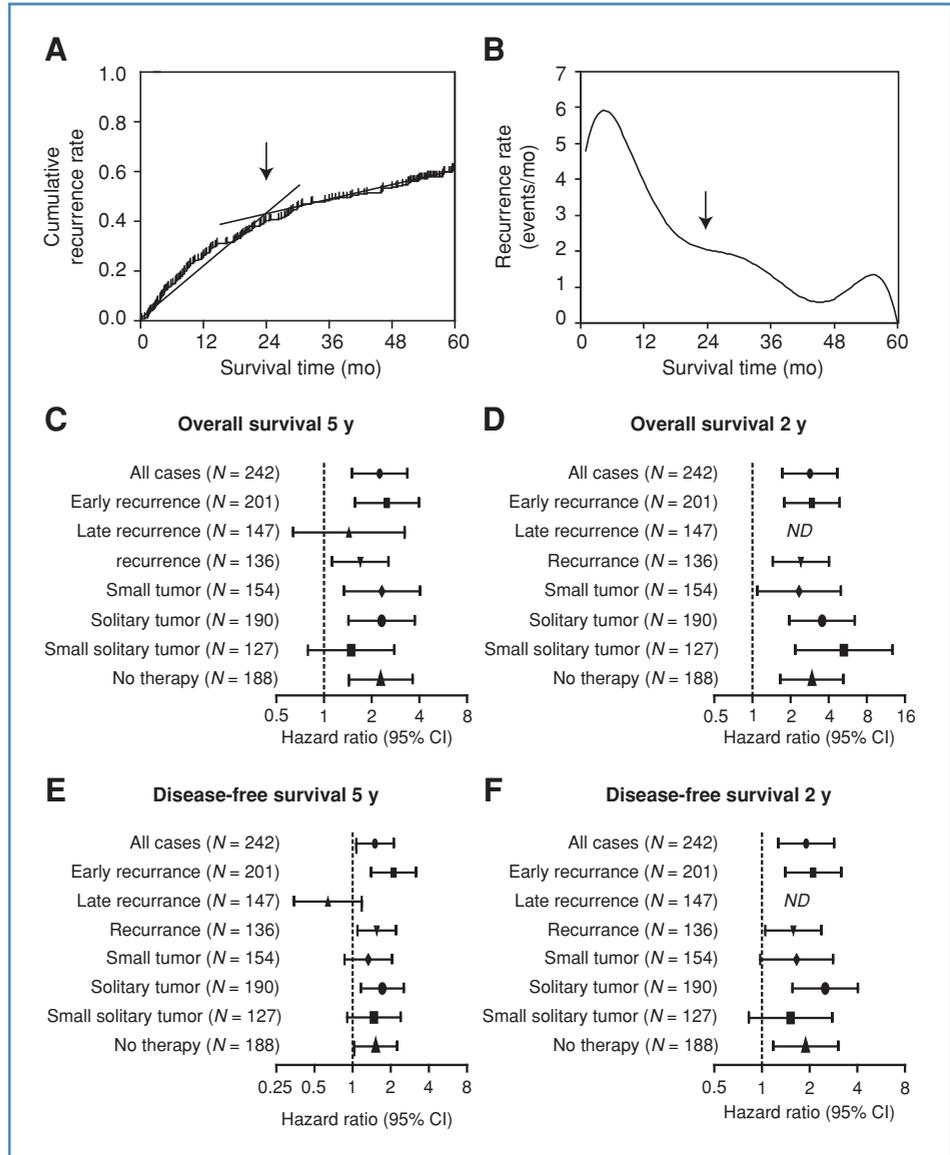
Performance of the metastasis risk classifier

It has been suggested that there are 2 biologically different forms of HCC recurrence, that is, early and late recurrences (5, 14–17). Early recurrence is believed to occur within the first 2 years after HCC treatment, mainly contributed by dissemination of metastatic HCC cells. In contrast, late recurrence is thought to originate *de novo* in the at-risk liver and early recurrence is generally more common than late recurrence (18, 28). Consistently, when we analyzed the cumulative recurrence in the LCI cohort, we found that the HCC recurrence rate is biphasic (Fig. 2A and B). The cumulative recurrence rate was 20.35% per year during the first 2 years after diagnosis, whereas the rate beyond 2 years after diagnosis decreased to 6.77% per year (Fig. 2A). In agreement with these data, the recurrence rate peaked during the first year and

persisted through the following years (Fig. 2B). We did not analyze the LEC cohort due to the lack of sufficient recurrence data in this cohort.

To study the prognostic capacity of the metastasis risk classifier with respect to the time of recurrence, we compared the hazards ratios of patient groups with early and late recurrences. The metastasis risk classifier significantly predicted overall survival and disease-free survival only in patients with early but not with late recurrence (Fig. 2C–F). In addition, the classifier was not affected by postoperative adjuvant therapy and was able to predict overall survival within the first 2 years in patients with small solitary tumors (tumor size ≤5 cm; Fig. 2D). These results are consistent with the hypothesis that early recurrence and late recurrence differ in their gene expression profiles and indicate that the metastasis
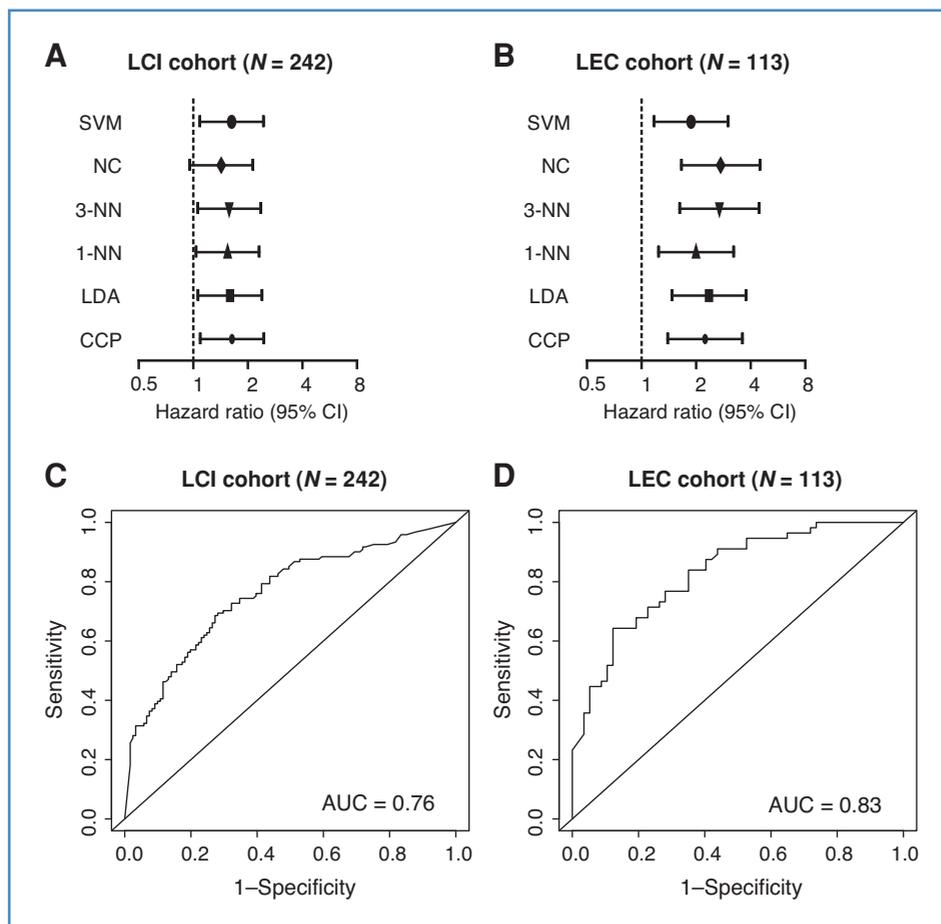
Figure 3. Unbiased cross-validation of the survival risk prediction and analysis of the sensitivity and specificity by ROC curves. A, 6 class prediction algorithms, that is, SVM, NC, 3-NN, 1-NN, LDA, or CCP, were used to predict good and poor survival HCC groups in the independent validation data set. Forest plots show hazard ratios for high-risk patients in clinical groups of patients. Hazard ratios are shown for the overall survival for the LCI cohort at 5 years using the LEC cohort as a training/test set and predicting outcome in the LCI cohort. B, hazard ratios of the LEC cohort are shown using LCI as the training/test set and prediction of the LEC cohort are depicted. C, ROC curve of the LCI cohort and (D) ROC curve of the LEC cohort applying the compound covariate predictor. AUC, area under the curve.

risk classifier is applicable only to metastasis-related relapse and can be used to classify early HCC recurrence.

Independent cross-validation and analysis of sensitivity and specificity

To determine whether the signature has any practical measure, we carried out a new sample assignment/prediction simulation strategy by independently cross-validating the 2 cohorts. We converted the gene expression data of both cohorts into z-scores. The resulting survival risk prediction was then used for unbiased cross-validation of both cohorts. We used 6 class prediction algorithms, SVM, NC, 3-NN, 1-NN, LDA, or CCP, to predict good and poor survival HCC subgroups. To assess outcome prediction, we used one of the cohorts as a template and the second cohort as an independent validation set, and vice versa. After using the LEC cohort as the template cohort and the LCI cohort as the validation cohort, Cox proportional hazards regression analysis showed that 5 of the 6 prediction algorithms were able to significantly predict outcome (Fig. 3A). Next, we used the LCI cohort as template and the LEC cohort as validation cohort and found that all 6 prediction algorithms significantly predicted the patient outcome (Fig. 3B). Therefore, even though the 2

cohorts differed in their patient characteristics and were analyzed on 2 different microarray platforms, the metastasis risk classifier was consistently able to predict survival. Of note, both cohorts could prospectively serve as templates for patient classification in the future. ROC curves showed that the predictive accuracy of the CCP had high sensitivity (i.e., a low probability of falsely classifying a patient as at low risk; the sensitivity is 0.760 and 0.839 for the LCI and LEC cohorts, respectively) and good specificity (i.e., a low probability of falsely classifying a patient as at high risk; the specificity is 0.603 and 0.649 for the LCI and LEC cohorts, respectively) in both cohorts (Fig. 3C and D).

Improving prediction by combining clinical prognostic factors and the metastasis risk classifier

Currently, the only clinically available marker for HCC is AFP, whose serum levels have been linked to HCC prognosis (refs. 20, 29; Table 2 and Supplementary Fig. S1). We sought to determine whether prognostic prediction of the LCI cohort (Fig. 4A) and the LEC cohort (Fig. 4B) could be improved by combining AFP and the metastasis risk classifier. We divided patients into subgroups based on an AFP level cutoff of 300 ng/mL and the survival risk determined by the metastasis risk

Figure 4. Combination of survival risk prediction applying the compound covariate predictor (CCP) and AFP (300 ng/mL cutoff) to stratify patient subgroups. A, Kaplan–Meier curves show overall survival of the LCI cohort (N = 238) and (B) LEC cohort (N = 104) subgrouped by survival risk prediction and AFP. Disconc., cases with discordant risk assessments, that is, high risk according to the metastasis risk classification and low-risk prediction by AFP, that is AFP less than 300 ng/mL.
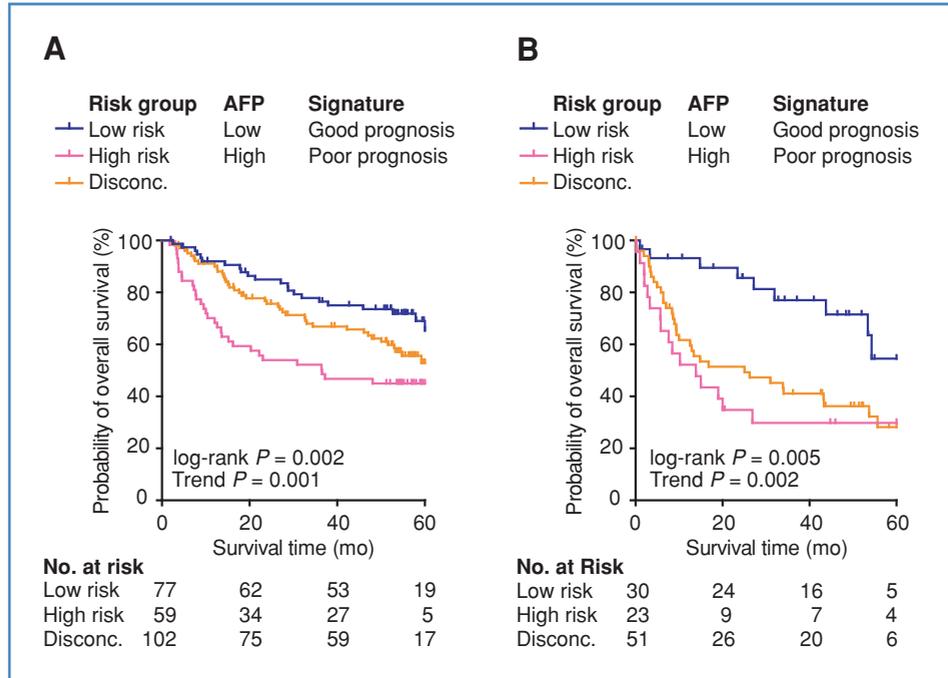
classifier (Fig. 4). This resulted in 3 outcome groups (low risk, high risk, and discordant). Although the low- and high-risk patients were both classified into the same outcome groups by AFP and the gene classifier, it seemed that there was a subset of patients misclassified by both methods (discordant cases, i.e., high risk according to the metastasis risk classification and low-risk prediction by AFP, or vice versa). Kaplan–Meier survival analysis showed that patients with discordant risk prediction have poorer outcome than low-risk patients and therefore might benefit from more rigid therapies. Stratification of discordant cases revealed that neither the gene signature nor AFP is a stronger predictor but that the combination of the gene signature classifier with AFP might improve prediction outcome (Supplementary Fig. S2).

We also sought to determine whether the gene classifier could improve BCLC staging as both were independent predictors of HCC survival. BCLC staging, which includes tumor size and liver function, is frequently used in the clinic to determine treatment options. BCLC stage A includes early-stage HCC patients with single tumors or 3 tumors smaller than 3 cm and Child-Pugh class A–B. Patients with BCLC stage A are suitable for radical therapies such as resection, transplantation, or percutaneous treatments. We carried out analyses only on the LCI cohort, as the LEC cohort lacked BCLC staging data. Similar to the results obtained with AFP, we found that the metastasis risk classifier improved survival prediction when combined with BCLC (Fig. 5A). Importantly, the gene signature was capable of significantly stratifying patients into low- and high-risk groups, especially among those with early-stage HCC as defined by BCLC stage A (Fig. 5B and Supplementary Fig. S3). Therefore, these results confirmed that the gene signature can significantly improve

BCLC recurrence risk assessment. Taken together, combination of the recurrence risk classifier with clinical staging as a molecular diagnostic test might be clinically useful to improve recurrence risk prediction and to determine treatment modality, particularly for those with early-stage tumors and solitary presentation.

Discussion

Recurrence is a common postsurgical event contributing to the poor prognosis of HCC patients. Currently, there are few effective therapeutic options to reduce metastasis-related recurrence. This is due, in part, to our inability to identify in advance the subgroup of HCC patients that are at high risk of developing metastatic disease. Risk stratification is particularly important for those patients with early stage of HCC who do not have vascular invasion and regional tumor cell dissemination at the time of diagnosis. This problem has hindered our ability to identify a specific therapeutic regimen that could improve the outcome of HCC, as no "one-size-fits-all" therapeutic strategy has been shown to be effective. Recent findings from 2 phase III randomized control trials on the use of Sorafenib as a therapeutic agent for advanced HCC are encouraging, but the survival benefit seems modest and its value in the prevention and treatment of postoperative metastatic recurrence is still under investigation (30, 31). There is an urgent need to develop genetic profiling tools to stratify patients with respect to prognosis and response to therapy, an essential step toward personalized medicine-based cancer management. For this purpose, we recently identified miR-26 as a biomarker to predict HCC survival and response to adjuvant IFN therapy (32).
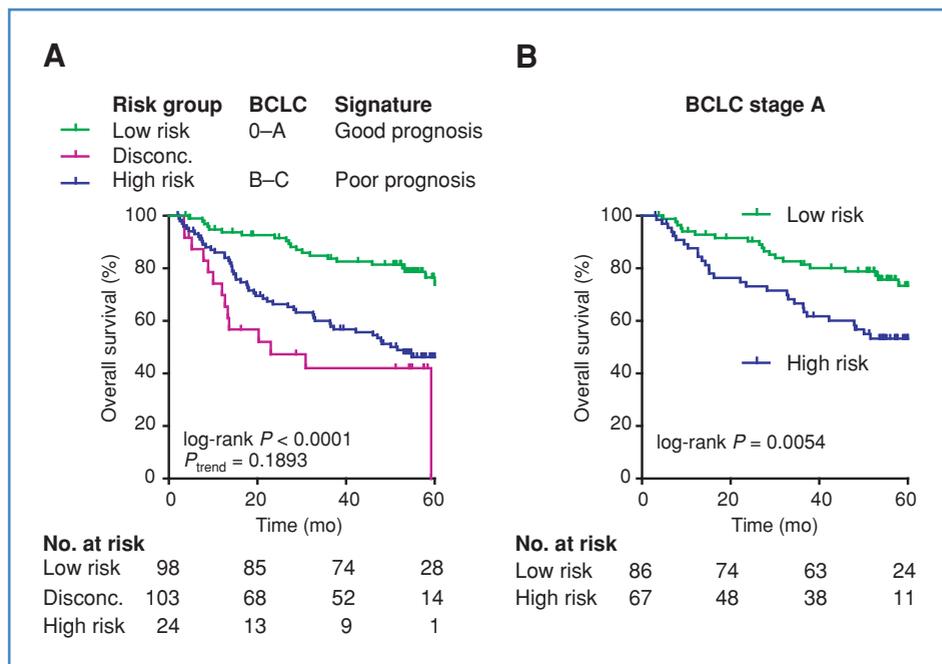
Figure 5. Patient stratification using survival risk prediction and BCLC staging. A, Kaplan–Meier curves are showing overall survival of the LCI cohort (N = 225) by subgrouping according to CCP class prediction of good or poor prognosis and BCLC stage 0–A or B–C. B, Kaplan–Meier curves of patients with BCLC staging A (N = 153) stratified by CCP survival risk prediction. Disconc., cases with discordant risk assessments, that is, high risk according to the metastasis risk classification and early-stage prediction by BCLC.

The traditional tumor evolution model suggests that a primary tumor is initially benign and over time acquires mutations that give a few tumor cells the ability to metastasize (8, 33). If a tumor is detected and treated before it spreads, the chances of long-term survival should be increased (34). Therefore, early detection is crucial to improve patient outcome. However, recent publications show that even if tumors are detected early, they might have already completed most of the steps on their way to metastasis (35). For example, genome analyses of primary colon tumors and paired metastases suggest that the genetic machinery that causes metastases may be hardwired into the tumor from the beginning (36). Similarly, copy number analysis of prostate cancers and their metastasis revealed that lethal metastatic prostate cancer is of monoclonal origin and that most metastatic cancers arise from a single cell (37). Consistently, our recent studies revealed that global gene expression patterns are very similar between primary HCCs and their paired metastases (19). These results provide a rationale for profiling primary tumors to predict patient prognosis.

In this study, we have validated our recently identified metastasis risk classifier by profiling primary HCC tissues in 2 independent cohorts with mixed etiologies as a tool to predict recurrence and survival attributed to metastatic HCC. Multivariate analyses including various clinical risk factors and clinical staging indicate that the molecular classifier is an independent prognostic predictor, especially applicable to early recurrence, and a poor prognostic factor mainly associated with metastatic dissemination of HCC cells but not late recurrence, an outcome contributed mainly by high carcinogenic activities of diseased livers. These results indicate that early and late recurrences differ in their molecular profile. Importantly, the gene classifier could predict poor outcome in patients with small solitary tumors, which has been traditionally viewed as having low risk for tumor recurrence. Therefore, the metastasis risk classifier adds independent prognostic value to the recurrence risk assessment, especially in early-stage HCC patients in whom current clinical staging fails to provide an accurate assessment. The ability to identify patients at high risk for recurrence in advance would reduce unnecessary economic burden and side effects for those low-risk patients who may not benefit from these treatments.

As our molecular signature is independent of other prognostic clinical factors, we also tested whether an improved prediction can be achieved by combining the signature with clinically relevant serum AFP or tumor staging (38, 39). Our data confirmed this hypothesis in 2 independent cohorts when the gene signature was combined with AFP. Encouraging results were also obtained in the LCI cohort in which the gene classifier improves HCC survival prediction when combined with BCLC staging, especially for those with early-stage HCC. These data require further validation in additional cohorts, as tumor staging data were not available in the LEI cohort. As the combination of the metastasis risk classifier and either AFP or BCLC staging leads to the identification of discordant cases that have poorer outcome than low-risk cases, we suggest that patients with discordant risk prediction should receive more rigid therapies.

It should be noted that osteopontin (OPN) was the top-ranked gene in our classifier (i.e., the most highly overexpressed in metastatic HCC; ref. 19). Further studies indicated that OPN may be a potential therapeutic target for metastatic HCC, as inhibition of OPN by neutralizing antibody, small peptides, or lentivirus-mediated RNA interference can block HCC cell invasion *in vitro* and inhibit pulmonary metastasis in mice (19, 40, 41). Further studies are warranted to determine

whether the application of the metastasis risk classifier in combination with novel agents such as inhibitors of OPN can improve HCC outcome.

For other cancer types, there are already gene classifiers used in the clinic. For breast cancer, there are 2 commercial reference laboratory tests based on gene-expression profiling (MammaPrint and Oncotype DX) that are either agency-approved or widely accepted by the oncology community (42–44). The Oncotype DX assay measures the expression of 16 genes by qRT-PCR and requires a routinely processed formalin-fixed, paraffin-embedded tumor tissue block. The MammaPrint assay measures the expression of 70 genes with a microarray and requires snap-frozen tumor tissue or fresh tumor tissue procured in a special buffer. Therefore, we suggest that the metastasis gene signature, similarly to the MammaPrint assay, can be used as an assay in the clinic, as we showed that the metastasis gene signature can be applied to different microarray platforms.

In conclusion, we have validated the metastasis risk classifier as a tool to predict HCC outcome in 2 independent cohorts with mixed etiologies and ethnicity, suggesting the general utility of this classifier. In addition, the gene classifier was able to predict disease-free survival and early recurrence. In combination with serum AFP levels or BCLC staging, the gene classifier may improve survival risk prediction. Thus, we recommend the use of this classifier as a molecular diagnostic test to assess the recurrence risk of HCC patients, particularly those with early stage after curative resection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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