Published in final edited form as:
Hepatology. 2012 May ; 55(5): 1443–1452. doi:10.1002/hep.24813.

65-gene–based risk score classifier predicts overall survival in hepatocellular carcinoma

Soo Mi Kim[1,13], **Sun-Hee Leem**[2], **In-Sun Chu**[3], **Yun-Yong Park**[1], **Sang-Cheol Kim**[3], **Sang-Bae Kim**[1], **Eun-Sung Park**[4], **Jae Yun Lim**[5], **Jeonghoon Heo**[6], **Yoon Jun Kim**[7], **Dae-Ghon Kim**[8], **Ahmed Kaseb**[9], **Young Nyun Park**[10], **Xin Wei Wang**[11], **Snorri S. Thorgeirsson**[12], and **Ju-Seog Lee**[1]

[1]Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas 77230

[2]Department of Biological Science, Dong-A University, Busan, Korea

[3]Korean Bioinformation Center, Korea Research Institute of Bioscience, Biotechnology, Daejeon, Korea

[4]Institute for Medical Convergence, Yonsei University College of Medicine, Seoul, Korea Biotechnology, Daejeon, Korea

[5]Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea Biotechnology, Daejeon, Korea

[6]Kosin University College of Medicine, Busan, Korea

[7]Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, Korea

[8]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chonbuk National University Medical School and Hospital, Jeonju, Korea

[9]Department of GI Medical Oncology, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas 77230

[10]Department of Pathology, Yonsei University College of Medicine, Seoul, Korea Biotechnology, Daejeon, Korea

[11]Lab of Human Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, MD

[12]Lab of Experimental Carcinogenesis, National Cancer Institute, National Institute of Health, Bethesda, MD

[13]Department of Physiology, Chonbuk National University Medical School and Hospital, Jeonju, Korea

Address request reprint to: Ju-Seog Lee, PhD, Department of Systems Biology, Division of Cancer Medicine, Unit 950, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77054, USA. jlee@mdanderson.org.
S. M. Kim. S-H. Leem., and I-S. Chu contributed equally to this study.

Conflict of Interest: none

Microarray data: GSE1898, GSE4024, GSE5975, GSE9843, GSE14520, and GSE16757.

Abstract

Clinical application of the prognostic gene expression signature has been delayed due to the large number of genes and complexity of prediction algorithms. In current study, we aim to develop an easy-to-use risk score with a limited number of genes that can robustly predict prognosis of patients with HCC. The risk score was developed by using Cox coefficient values of 65 genes in the training set (n=139) and its robustness was validated in test sets (n=292). The risk score was a highly significant predictor of overall survival (OS) in the first test cohort ($P = 5.6 \times 10^{-5}$, n = 100) and the second test cohort ($P = 5.0 \times 10^{-5}$, n = 192). In multivariate analysis, the risk score was significant risk factor among clinical variables examined together (hazard ratio [HR], 1.36; 95% confidential interval [CI], 1.13-1.64; $P = 0.001$ for OS).

Conclusion—The risk score classifier we have developed can identify two clinically distinct HCC subtypes at early and late stage of the disease in a simple and highly reproducible manner across multiple data sets.

Keywords

Hepatocellular Carcinoma; Gene expression signature; Microarrays; Prognostic biomarkers

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide and accounts for an estimated 600,000 deaths annually.[1] Although surgical resection for HCC provides best chance for cure, the prognosis after surgery differs considerably among patients. Because of this clinical heterogeneity, predicting the recurrence or survival of HCC patients after surgical resection remains challenging. An accurate stratification reflecting the prognosis of HCC patients would help select the therapy with the potential to confer the best survival, so considerable effort has been devoted to establishing such a stratification (or staging) model for HCC by using clinical information and pathological criteria.[2, 3] Currently several clinical classification systems, including Cancer of the Liver Italian Program, the Barcelona-Clinic Liver Cancer (BCLC), the Chinese University Prognostic Index, and the Japanese Integrated Staging schema have been developed and used in clinics.[4-7] Although these staging systems have proved to be useful,[8] their predictive accuracy remains limited and they failed to provide biological characteristics of HCC that might account for the clinical heterogeneity.

With the recent advances in gene expression profiling technology, improvement in prediction models for risk assessment in HCC has been reported.[9-18] However, although these gene expression signatures might better reflect the biological characteristics of HCC tumors, the complexity of prediction models based on such signatures has hampered their clinical usefulness. To overcome this limitation, we have developed a simple risk scoring system that can predict overall survival (OS) of patients after surgical resection for HCC.

MATERIALS AND METHODS

Patients and Gene Expression Data

Gene expression and clinical data from the National Cancer Institute (NCI), Mount Sinai Hospital (MSH), and Liver Cancer Institute (LCI) HCC cohorts, as reported in previous studies, were acquired from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession numbers GSE1898, GSE4024, GSE9843, and GSE14520).[11, 13, 15-17] Gene expression data from HCC patients at the French National Institute for Health and Medical Research (INSERM) were obtained from ArrayExpress, another public microarray database (accession number E-TABM-36).[9]

In addition to these gene expression data from previous studies, we included gene expression data from 100 patients with HCC (the Korean cohort) as an independent validation cohort for the risk score. Tumor specimens and clinical data were obtained from HCC patients undergoing hepatectomy as primary treatment for HCC at Seoul National University, Seoul, and Chonbuk National University, Jeonju, Korea. One hundred surgically removed frozen HCC specimens were used for microarray experiments. Samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction. The study protocols were approved by the Institutional Review Boards at both institutions, and all participants provided written, informed consent. Gene expression data from the Korean cohort were generated using the Illumina microarray platform (Illumina, San Diego, CA). Patients in the Korean cohort were followed up prospectively at least once every 3 months after surgery.

Most of the patients in the two validation cohorts were men (83% for Korean cohort and 87.5% for LCI cohort), Child-Pugh class A (92% for Korean cohort and 87% for LCI cohort), and had cirrhosis (64% for Korean cohort and 92.0% for LCI cohort). HBV infection was determined by serological positivity for HBV surface antigen (HBsAg) or anti-HBe antibodies. All patients were received surgical resection and majority of patients had a single tumor at the time of resection (96% for Korean cohort and 78% for LCI cohort). Patients were staged according to TNM sixth edition (2006) and Barcelona Clinic Liver Cancer staging system.[19] Tumor size was based on the largest dimension of the tumor specimen. Tumor grade was scored using the modified nuclear grading scheme outlined by Edmondson and Steiner.[20] Grades 1 and 2 were defined as well-differentiated, and grades 3 and 4 as moderately/poorly differentiated. Majority of patients in three cohorts were not received anti-Hepatitis B virus treatment after surgery. ECOG performance score of all patients was 0 or 1. Presence of cirrhosis was also confirmed on the surgical specimen. Overall survival (OS) was defined as the time from surgery to death, and censored when a patient was alive at last contact.

Table 1 shows the pathologic and clinical characteristics of the patients in all five cohorts. All patients had undergone surgical resection as their primary treatment. Patient data were retrospectively collected from medical records. BCLC staging is based on pre-operation data, and vasculature invasion is pathologically defined as presence of endolymphatic or lymphovascular tumor emboli within tumor. Survival data are not publically available for the MSH and INSERM cohorts; thus, these patients were not used for survival analyses.

RNA isolation, microarray experiments, and gene expression data

For generation of gene expression data from the Korean cohort, total RNA was isolated from tissue samples using a mirVana RNA Isolation labeling kit (Ambion, Austin, TX). Five-hundred nanograms of total RNA were used for labeling and hybridization, in accordance with the manufacturer's protocols (Illumina, San Diego, CA). After the bead chips were scanned with an Illumina BeadArray Reader (Illumina), the microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data package in the R language environment (http://www.r-project.org).[21] The expression level of each gene was transformed into a log-2 base for further analysis. Primary microarray data are available from the NCBI GEO public database (accession number GSE16757).

Statistical analysis

BRB-ArrayTools were primarily used for statistical analysis of gene expression data,[22] and all other statistical analyses were performed in the R language environment. We estimated patient prognoses using Kaplan-Meier plots and the log-rank test. Stratification of patients in NCI cohort according to Seoul National University (SNU) recurrence signature was done as described in previous study.[18]

Receiver-operating characteristic (ROC) curve analyses were carried out to estimate discriminatory power of the prognostic gene expression signatures and clinical variables. We calculated the area under the curve (AUC), which ranges from 0.5 (for a noninformative predictive marker) to 1 (for a perfect predictive marker) and a bootstrap method (1000 re-sampling) was used to calculate the 95% confident internal (CI) for AUC.

We used multivariate Cox proportional hazards regression analysis to evaluate independent prognostic factors associated with OS, and as covariates we used a 65- gene risk score, tumor stages, and pathologic characteristics.[23] A P-value <0.05 indicated statistical significance, and all statistical tests were two-tailed. Heat map of gene expression was generated by using Cluster and TreeView software.[24]

GoMiner was used to group genes based gene ontology (GO) characteristics of them.[25]

Development and validation of 65-gene risk scoring system

To generate a risk score, we adopted a previously developed strategy using the Cox regression coefficient of each gene among a 65-gene set from the NCI cohort.[26] The risk score for each patient was derived by multiplying the expression level of a gene by its corresponding coefficient (Risk score = sum of Cox coefficient of Gene Gi X expression value of Gene Gi). The patients were thus dichotomized into groups at high or low risk using the 50^{th} percentile (median) cutoff of the risk score as the threshold value. The median risk score in the NCI cohort was 8.36. The coefficient and the threshold value (8.36) derived from the NCI cohort were directly applied to gene expression data from the Korean, LCI, MSH, and INSERM cohorts to divide the rest of the patients into high-risk and low-risk groups. Gene expression data and master prediction model is available as Supporting Data 1.

RESULTS

Sixty-five gene expression signature in HCC and development of the 65-gene risk score

To identify a limited number of genes whose expression pattern is significantly associated with the prognosis of HCC, we used two previously identified gene expression signatures. The NCI proliferation signature (1016 gene features) was identified when two major clusters of HCC patients were uncovered by hierarchical clustering method and the signature was found to be significantly associated with OS and recurrence-free survival (RFS).[13, 15, 16] The Seoul National University (SNU) recurrence signature (628 gene features) was developed to predict the likelihood of recurrence after surgical treatment of HCC.[18] We hypothesized that the genes present in both signatures would be better predictors than genes only present in one signature. Therefore, expression patterns of these genes would be sufficient to predict the prognosis of HCC patients. When the two gene lists were compared with each other, only 65 genes overlapped (Fig. 1A).

In order to develop new risk assessment model for prognosis with 65 genes, we adopted a previously developed strategy that generate the risk score using the Cox regression coefficient of each gene in the prognostic signature.[26] The risk score for each patient was calculated by using the regression coefficient of each gene in the 65-gene signature (Table 2). HCC patients in the NCI cohort were then dichotomized into a highrisk and low-risk group for death using the 50^{th} percentile cutoff (8.36) of the risk score as the threshold value (Fig. 1B). The OS rates were significantly lower in the patient group with the high risk score ($P = 1.0 \times 10^{-4}$ by the log-rank test; Fig. 1C). When predicted outcomes of new and reduced model were compared with those from original prognostic signatures, the statistical significance of the 65-gene risk score in discriminating between HCC patients with different prognoses is similar to the discriminatory power of the two original gene expression signatures (Fig. 1D and E). We also assessed predictive performance of three-year OS of three prognosis models by calculating areas under curve (AUC) from receiver operating characteristic (ROC) analysis. Not surprisingly, AUC of 65-gene risk score (0.68; 95% CI, 0.604 – 0.761) is highly similar to those from original prognosis models (Supporting Fig. 1). This result strongly suggests that the expression patterns of the 65 genes are sufficient to predict the prognosis of HCC patients, although this data set represents only 5.8% of genes in the NCI proliferation signature and 10.3% of genes in the SNU recurrence signature.

To test whether genes not shared by two prognostic signatures have similar discriminatory power, two additional risk scores were generated from 65 genes that were randomly selected from non-overlapped gene lists in each prognostic signature, and applied to NCI and SNU cohorts. As expectedly, NCI proliferation signature risk score showed significant predictive performance on patients in NCI cohorts (Supporting Fig. 2B). However, it failed to show significant predictive performance on patients in SNU cohorts (Supporting Fig. 2C). SNU recurrence signature risk score also showed opposite predictive performance on patients from two different cohorts (Supporting Fig. 2B & C). However, common gene risk score showed consistent predictive performance on patients from both cohorts. These data suggest that genes shared in two independent prognostic signatures might be more robust than those only present in one signature.

Validation of the 65-gene risk score

We next sought to validate the risk score using expression data of the 65 genes from independent HCC cohort. Gene expression data for 100 tumors from Korean patients with HCC were collected and used as an independent test set. The coefficient and threshold value (8.36) derived from the NCI cohort were directly applied. When patients in the Korean cohort were stratified according to their risk score, the patient group with a low risk score had a significantly better prognosis ($P = 5.6 \times 10^{-5}$ for OS, log-rank test) (Fig. 2A) than patients with a high risk score. The risk score was further validated in another independent cohort (LCI cohort, $P = 5.0 \times 10^{-4}$ for OS, log-rank test) (Fig. 2B). Taken together, these results demonstrate that it is possible to determine a risk score on the basis of the expression of a small number of genes.

65-gene risk score is an independent risk factor for OS

We next combined clinical data from two test cohorts and assessed the prognostic association between our newly developed 65-gene risk score and other known clinical risk factors using univariate Cox regression analyses. In addition to alpha-fetoprotein level, tumor size, grade, and vasculature invasion, which are already well-known risk factors, the risk score was a significant indicator for OS (Table 3). We then included all relevant clinical variables in a multivariate Cox regression analysis. Importantly, the risk score remained the significant prognostic risk factor (HR 1.36, 95% CI 1.13 – 1.64, $P = 0.001$ for OS) (Table 3).

We next carried out ROC analysis to assess predictive performance of three-year OS of 65-gene risk scores in pooled test cohort and compared it with other clinical variables that showed significance in univariate analysis (tumor size, vasculature invasion, grade, and AFP). AUC of risk score (0.699; 95% CI, 0.636 – 0.764) is highly close to that of tumor size (0.691; 95% CI, 0.628 – 0.755), most significant clinical variable in univariate analysis (Fig. 3). Taken together, these findings suggest that the risk score retains its prognostic relevance even after the classical clinicopathological prognostic features have been taken into account.

We further tested the independence of the risk score over current staging systems. When the risk score was applied to patients with early stage (BCLC stage A) and intermediate and advanced stage (BCLC stage B and C) HCC, it successfully identified high-risk patients in different BCLC stages (Fig. 4). The risk score was also independent of American Joint Committee on Cancer (AJCC) stages (Supporting Fig. 3). We next tested whether new risk score can improve the discrimination of prognosis over BCLC stages. Performance of combined model (BCLC and risk score) is substantially improved over the baseline models with only BCLC and risk score as evidenced by increase of AUC from ROC analysis (Supporting Fig. 4A). Moreover, subset ROC analysis within each BCLC stage clearly demonstrated incremental value of risk score over current staging system (Supporting Fig. 4B).

Because vasculature invasion is the clinical variable best known to be significantly associated with OS of HCC after surgical resection,[27-31] we next tested how independent the new risk score is of vasculature invasion. As expected, the prognosis of patients without

vasculature invasion was significantly better than that of patients with invasion (Supporting Fig. 5A). When the risk score–based stratification was applied separately to invasion-positive and -negative patients, it successfully identified high-risk patients in both subgroups (Supporting Fig. 5B and C). Importantly, when all stratifications were combined together, the risk score even identified patients without vasculature invasion whose risk was worse than or similar to that of patients with invasion (Supporting Fig. 5D).

We next examined potential association of risk score with underlying liver disease by including Child-Pugh class and cirrhosis information into analysis. As expectedly, Edmondson grade reflecting pathological characteristics of tumors showed incremental association with risk score. The number of patients with high risk score is slightly increased in higher grade. However, indices for underlying liver disease lack any association with risk score (Supporting Table 1), indicating that risk score does not reflect biological characteristics associated with underlying liver disease.

Molecular characteristics of HCC associated with 65-gene risk score

We grouped 65 genes in risk scores in the context of the Gene Ontology (GO) to summarize biological characteristics of risk score. Not surprisingly, genes involved in signaling transduction are enriched in those whose expression is positively associated with poor prognosis (high risk genes, Supporting Table 2), while genes associated with normal metabolic functions of liver are enriched in low risk genes (Supporting Table 3).

In addition, we used gene expression data from the MSH cohort, for whom many biological characteristics are available.[11] Ninety-one patients from the MSH cohort were stratified according to risk score by applying the coefficient and threshold values (8.36) derived from the NCI cohort. All three signaling events (phosphorylation) examined in the previous study with the MSH cohort were significantly associated with the risk score (Supporting Table 4). We found that a high risk score was significantly associated with enriched phosphorylation of *AKT* ($P = 0.003$, χ^2-test), *IGFR1* ($P = 2.2 \times 10^{-4}$, χ^2-test), and *RPS6* ($P = 3.6 \times 10^{-5}$, χ^2-test). Mutation of *TP53* is not associated with the risk score ($P = 0.93$), whereas a high frequency of mutations of *CTNNB1* (beta-catenin) was significantly associated with a low risk score (23/ 27 mutations, $P = 0.05$, χ^2-test). To validate the association between risk score and *CTNNB1* mutations in HCC, patients in the INSERM cohort (n = 57) were stratified by risk score using same 8.36 cutoff threshold.[9] Of 17 HCC tumors with *CTNNB1* mutations, 16 were in the low-risk group, and this association was statistically significant (Supporting Table 5; $P = 0.015$, χ^2-test).

DISCUSSION

By applying multi-step exploration and validation strategy (Supporting Fig. 6), we identified and validated a risk score based on expression patterns of 65 genes that can easily quantify the likelihood of OS in HCC patients who have undergone surgical resection as primary treatment.

Several lines of evidence strongly support that the risk score is an independent and significant predictor of prognosis. First, the risk score was the significant predictive factor

for OS in the combined validation cohort in multivariate analysis (Table 3). Second, the risk score can identify high risk patients in both patients with early stage HCC (BCLC stage A) and those with intermediate or advanced stage (BCLC stage B and C) (Fig. 4). The strength and independence of the risk score over the current staging systems remained significant even when the AJCC staging system was applied (Supporting Fig. 3). Third, the risk score identified poor prognosis patients without vasculature invasion, who are typically considered as good prognosis patients. (Supporting Fig. 5). Fourth, the risk score was the most significant predictor of 3-year survival of patients in ROC analysis (Fig. 3). Taken together, these results strongly support the notion that the risk score identifies clinical characteristics significantly associated with the prognosis of HCC that are not recognized by current staging criteria.

Although it is interesting to see that risk score reflects biological characteristics (Supporting Table 4), its associations need to be validated in future study. For example, activation of *AKT* is most commonly altered signaling event in many cancer and many genetic alterations would lead to activation of AKT.[32] Thus, it is currently uncertain whether *AKT* is the driver of tumor developments in patients with high risk score and would be potential therapeutic targets for these patients. However, the significant association of risk score with *CTNNB1* mutations is in good agreement with the results of previous studies demonstrating a significant correlation between *CTNNB1* mutations and a favorable prognosis among patients with HCC.[33, 34] Moreover, *TBX3*, one of the canonical downstream target genes of *CTNNB1*,[35] was included in our 65-gene signature, and its expression was associated with a better prognosis, which strongly supports the activation of *CTNNB1* in the low-risk group in all HCC patients examined. It is also noteworthy to point out that the risk score does not reflect the status of underlying liver disease, indicating that there might be room for improvement. Previous study identified prognostic gene expression signature from surrounding non-tumor tissues of patients with HCC that better reflects biological characteristics of underlying liver disease than tumors.[12] The risk score might be improved by incorporating genomic data from surrounding tissues that does not overlap with but complementary to those from tumor tissues.

Classification of human cancers into more homogenous clinical groups such as stages and grades significantly improved the treatment of patients by standardizing patient care. Molecular classification of cancers further improved patient care by enabling the development of treatments tailored to the abnormalities present in each patient's cancer cells. Currently, decision-making for HCC treatment in the clinical setting is mainly based on clinical data, which is best reflected in BCLC staging and its associated treatment algorithm.[2] However, this staging method offers little or almost no information about biological characteristics of HCC that would be very critical for tailored treatment in future. Importantly, risk score may provide clues on biological characteristics of tumors (i.e., activation of *CTNNB1*) as well as prognostic characteristics. Thus, it would provide opportunity for developing rationalized clinical trials based on molecular characteristics of tumor that are supplemental to current staging systems. Since our data showed that small number of genes (65 genes) is sufficient to identify patient with poor prognosis (Supporting Fig. 1), it will open up possibility that simpler and easily accessible technology in clinics

like quantitative reverse transcription polymerase chain reaction can replace complicated microarray technologies to develop easy-to-use prognostic model with small samples from biopsies.

Our current stratification strategy is limited by its assumption that there are two major prognostic HCC subgroups. Although this assumption is largely supported by the results of previous studies,[10, 12, 13, 15, 16, 18] we cannot rule out the possibility that there are more than two prognostic groups of HCC patients, given the genetic heterogeneity of the disease. However, because our method generates continuous risk scores, it is easy to adjust cutoff criteria to restratify HCC patients according to the degree of genetic heterogeneity. Future studies should clarify this result.

In conclusion, the use of a risk score as defined by an expression pattern of 65 genes can identify a poorer prognosis in HCC patients in a reliable and reproducible manner across independent patient cohorts. However, due to the heterogeneity in both ethnic backgrounds and potential differences in patient care in different hospitals, conclusions of current study should be validated in larger independent cohort. Moreover, at present it is unclear whether the risk score offers information about the potential benefits of adjuvant therapies after surgical resection. Thus, prospective validation using tissues from patients having received adjuvant therapies is necessary in future studies with proper incorporation of analyses to correlate it with underlying liver diseases, identify patterns of recurrence, and determine impact of subsequent therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding/Support: The study was funded by the intramural faculty fund of The University of Texas MD Anderson Cancer Center to J-S Lee, MD Anderson Cancer Center is partially funded by NCI CCSG Core Grant CA106672.

Reference List

1. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer. 2001 Oct 15; 94(2):153–156. [PubMed: 11668491]
2. Bruix J, Llovet JM. HCC surveillance: who is the target population? Hepatology. 2003 Mar; 37(3): 507–509. [PubMed: 12601346]
3. Okuda K, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, et al. Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. Cancer. 1985 Aug 15; 56(4):918–928. [PubMed: 2990661]
4. Calvet X, Bruix J, Gines P, Bru C, Sole M, Vilana R, et al. Prognostic factors of hepatocellular carcinoma in the west: a multivariate analysis in 206 patients. Hepatology. 1990 Oct; 12(4 Pt 1): 753–760. [PubMed: 2170267]
5. CLIP investigators. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. Hepatology. 1998 Sep; 28(3):751–755. [PubMed: 9731568]
6. Kudo M, Chung H, Osaki Y. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). J Gastroenterol. 2003; 38(3):207–215. [PubMed: 12673442]

7. Leung TW, Tang AM, Zee B, Lau WY, Lai PB, Leung KL, et al. Construction of the Chinese University Prognostic Index for hepatocellular carcinoma and comparison with the TNM staging system, the Okuda staging system, and the Cancer of the Liver Italian Program staging system: a study based on 926 patients. Cancer. 2002 Mar 15; 94(6):1760–1769. [PubMed: 11920539]
8. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology. 2005 Nov; 42(5): 1208–1236. [PubMed: 16250051]
9. Boyault S, Rickman DS, de R A, Balabaud C, Rebouissou S, Jeannot E, et al. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology. 2007 Jan; 45(1):42–52. [PubMed: 17187432]
10. Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, et al. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer Cell. 2006 Aug; 10(2):99–111. [PubMed: 16904609]
11. Chiang DY, Villanueva A, Hoshida Y, Peix J, Newell P, Minguez B, et al. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma. Cancer Res. 2008 Aug 15; 68(16):6779–6788. [PubMed: 18701503]
12. Hoshida 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 Nov 6; 359(19):1995–2004. [PubMed: 18923165]
13. Lee JS, Chu IS, Heo J, Calvisi DF, Sun Z, Roskams T, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. Hepatology. 2004 Sep; 40(3): 667–676. [PubMed: 15349906]
14. Lee JS, Thorgeirsson SS. Genome-scale profiling of gene expression in hepatocellular carcinoma: classification, survival prediction, and identification of therapeutic targets. Gastroenterology. 2004 Nov; 127(5 Suppl 1):S51–S55. [PubMed: 15508103]
15. Lee JS, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, et al. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. Nat Genet. 2004 Dec; 36(12):1306–1311. [PubMed: 15565109]
16. Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med. 2006 Apr; 12(4):410–416. [PubMed: 16532004]
17. Roessler S, Jia HL, Budhu A, Forgues M, Ye QH, Lee JS, et al. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. Cancer Res. 2010 Dec 15; 70(24):10202–10212. [PubMed: 21159642]
18. Woo HG, Park ES, Cheon JH, Kim JH, Lee JS, Park BJ, et al. Gene expression-based recurrence prediction of hepatitis B virus-related human hepatocellular carcinoma. Clin Cancer Res. 2008 Apr 1; 14(7):2056–2064. [PubMed: 18381945]
19. Llovet JM, Bru C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis. 1999; 19(3):329–338. [PubMed: 10518312]
20. EDMONDSON HA, STEINER PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. Cancer. 1954 May; 7(3):462–503. [PubMed: 13160935]
21. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003 Jan 22; 19(2): 185–193. [PubMed: 12538238]
22. Simon R, Lam A, Li M-C, Ngan M, Menenzes S, Zhao Y. Analysis of gene expression data using BRB-Array Tools. Cancer Inform. 2007; 3:11–17. [PubMed: 19455231]
23. Cox DR. Regression models with life tables. J Royal Statis Soc. 1972; 34:187–220.
24. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A. 1998 Dec 8; 95(25):14863–14868. [PubMed: 9843981]
25. Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, Sunshine M, et al. GoMiner: a resource for biological interpretation of genomic and proteomic data. Genome Biol. 2003; 4(4):R28. [PubMed: 12702209]

26. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med. 2004 Dec 30; 351(27):2817–2826. [PubMed: 15591335]
27. Okada S, Shimada K, Yamamoto J, Takayama T, Kosuge T, Yamasaki S, et al. Predictive factors for postoperative recurrence of hepatocellular carcinoma. Gastroenterology. 1994 Jun; 106(6): 1618–1624. [PubMed: 8194710]
28. Adachi E, Maeda T, Matsumata T, Shirabe K, Kinukawa N, Sugimachi K, et al. Risk factors for intrahepatic recurrence in human small hepatocellular carcinoma. Gastroenterology. 1995 Mar; 108(3):768–775. [PubMed: 7875479]
29. Kumada T, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, et al. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. Hepatology. 1997 Jan; 25(1):87–92. [PubMed: 8985270]
30. Vauthey JN, Lauwers GY, Esnaola NF, Do KA, Belghiti J, Mirza N, et al. Simplified staging for hepatocellular carcinoma. J Clin Oncol. 2002 Mar 15; 20(6):1527–1536. [PubMed: 11896101]
31. Poon RT, Fan ST. Evaluation of the new AJCC/UICC staging system for hepatocellular carcinoma after hepatic resection in Chinese patients. Surg Oncol Clin N Am. 2003 Jan; 12(1):35–50. viii. [PubMed: 12735128]
32. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer. 2009 Aug; 9(8):550–562. [PubMed: 19629070]
33. Mao TL, Chu JS, Jeng YM, Lai PL, Hsu HC. Expression of mutant nuclear beta-catenin correlates with non-invasive hepatocellular carcinoma, absence of portal vein spread, and good prognosis. J Pathol. 2001 Jan; 193(1):95–101. [PubMed: 11169521]
34. Hsu HC, Jeng YM, Mao TL, Chu JS, Lai PL, Peng SY. Beta-catenin mutations are associated with a subset of low-stage hepatocellular carcinoma negative for hepatitis B virus and with favorable prognosis. Am J Pathol. 2000 Sep; 157(3):763–770. [PubMed: 10980116]
35. Renard CA, Labalette C, Armengol C, Cougot D, Wei Y, Cairo S, et al. Tbx3 is a downstream target of the Wnt/beta-catenin pathway and a critical mediator of beta-catenin survival functions in liver cancer. Cancer Res. 2007 Feb 1; 67(3):901–910. [PubMed: 17283120]

Abbreviation

HCC	hepatocellular carcinoma
OS	overall survival
AUC	area under the curve
LOOCV	leave-one-out-cross-validation

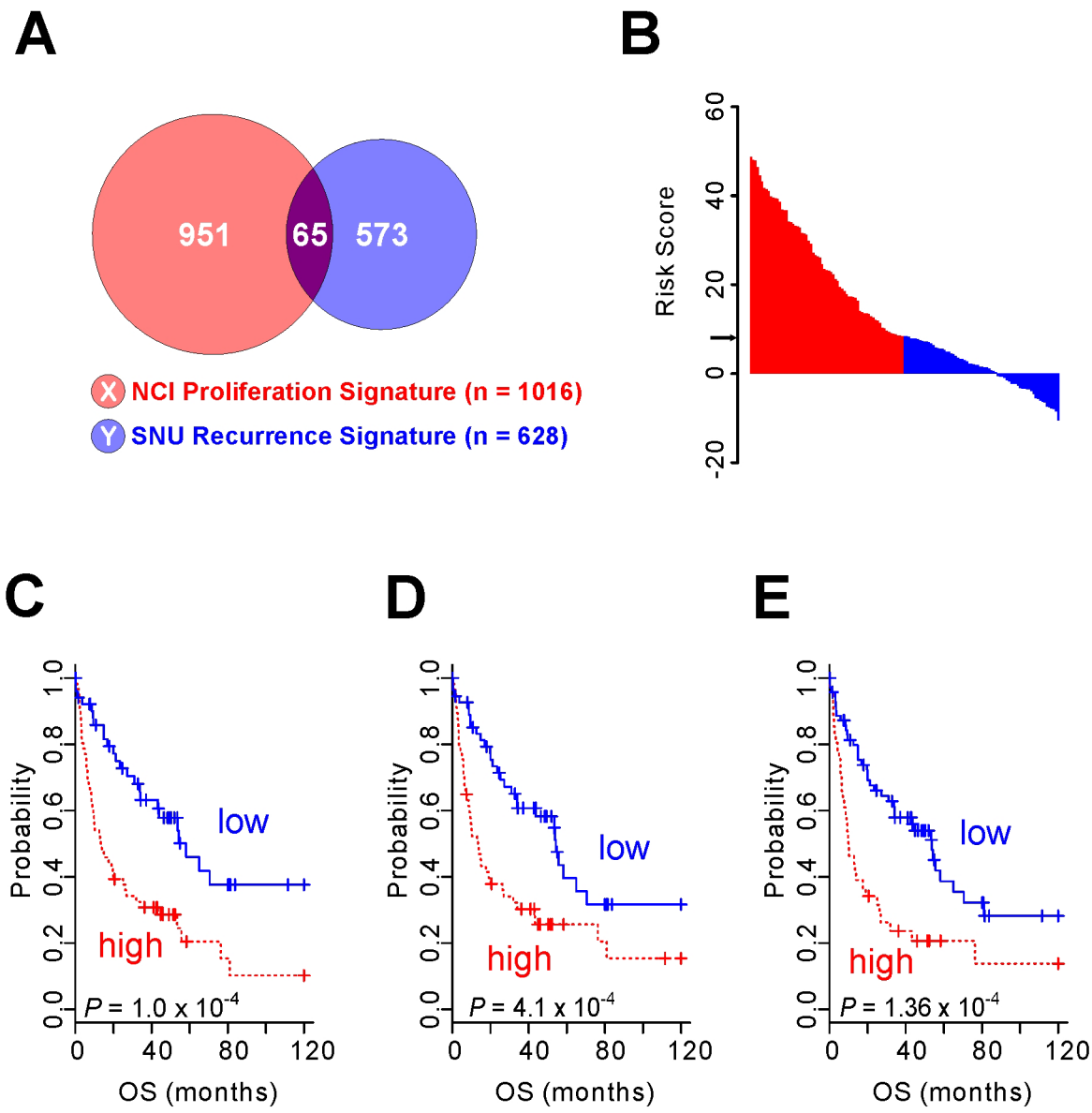

Figure 1. Stratification of HCC patients in NCI cohort with 65-gene risk score

(A) Venn diagram of gene lists from two independently generated prognostic expression signatures. **(B)** Risk scores in the NCI cohort. Each bar represents the risk score for an individual patient. (**C**) Kaplan-Meier plots of the two subgroups in the NCI cohort stratified by risk score. (**D**) Kaplan-Meier plots of the two subgroups in the NCI cohort stratified by NCI proliferation signature. (**E**) Kaplan-Meier plots of the two subgroups in the NCI cohort stratified by SNU recurrence signature. Percentages in parenthesis indicate recurrence rate in each subgroup. OS, overall survival.

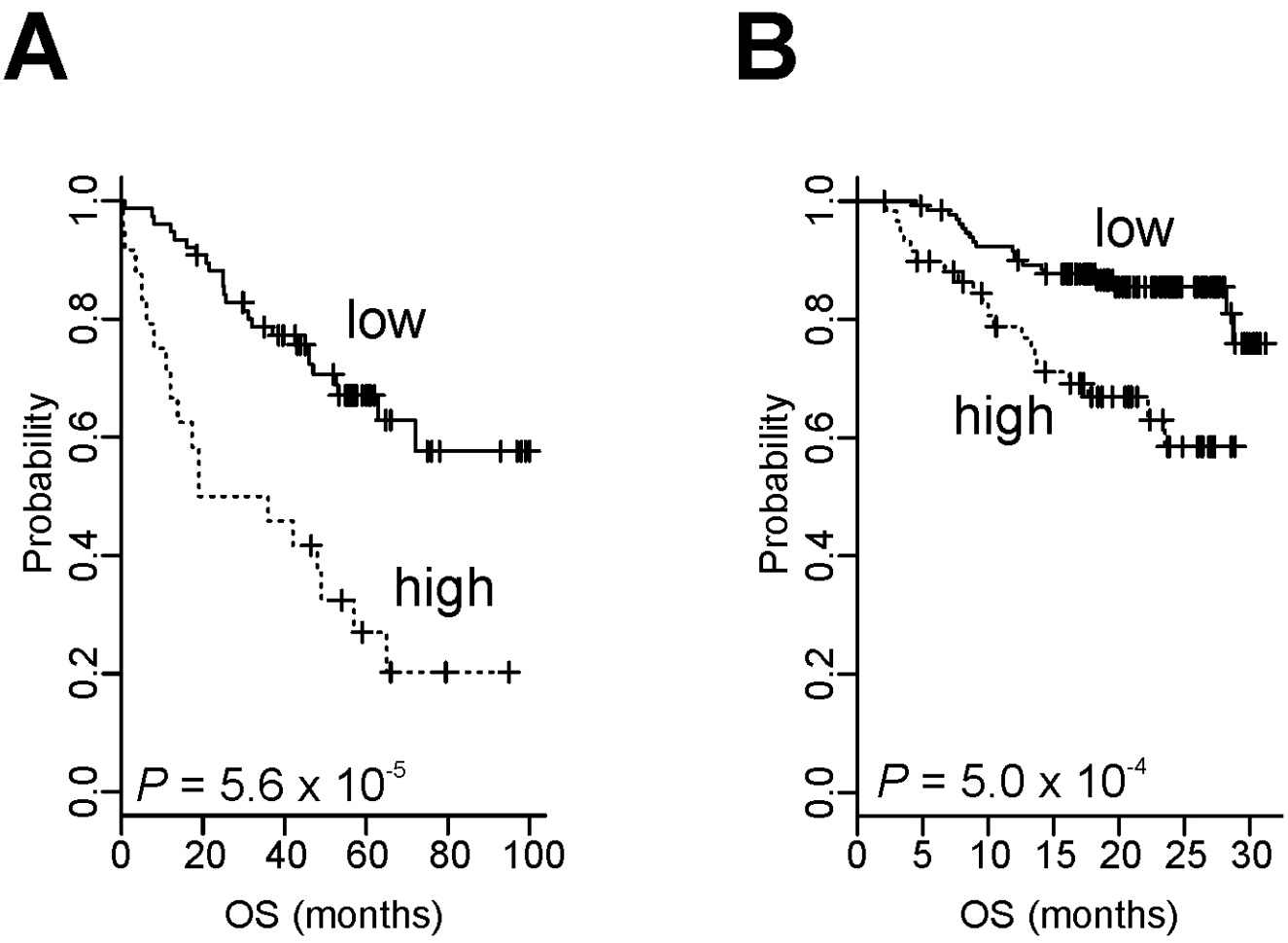

Figure 2. Overall survival of HCC patients stratified by risk score in the Korean and LCI HCC cohorts

HCC patients in the Korean cohort (**A**) and LCI cohort (**B**) were stratified by 65-gene risk score. OS, overall survival.

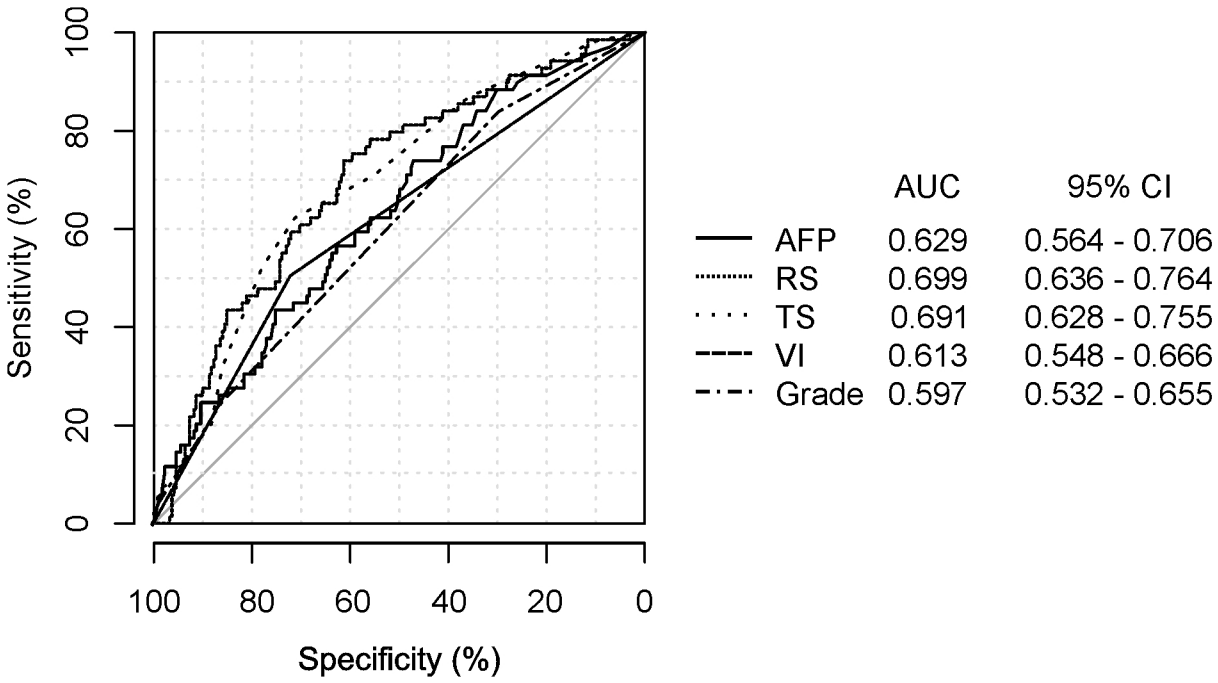

Figure 3. Comparison of ROC curves of clinical variables and risk score in validation cohorts

Clinical variables and 65-gene risk score were applied to patients in pooled validation cohorts and their prognostic significance was estimated by AUC from ROC analysis for 3-year OS. AUC: area under curve, CI: 95% confident internal of AUC. TS: tumor size, VI: vasculature invasion. RS: risk score, AFP:alpha-feto protein

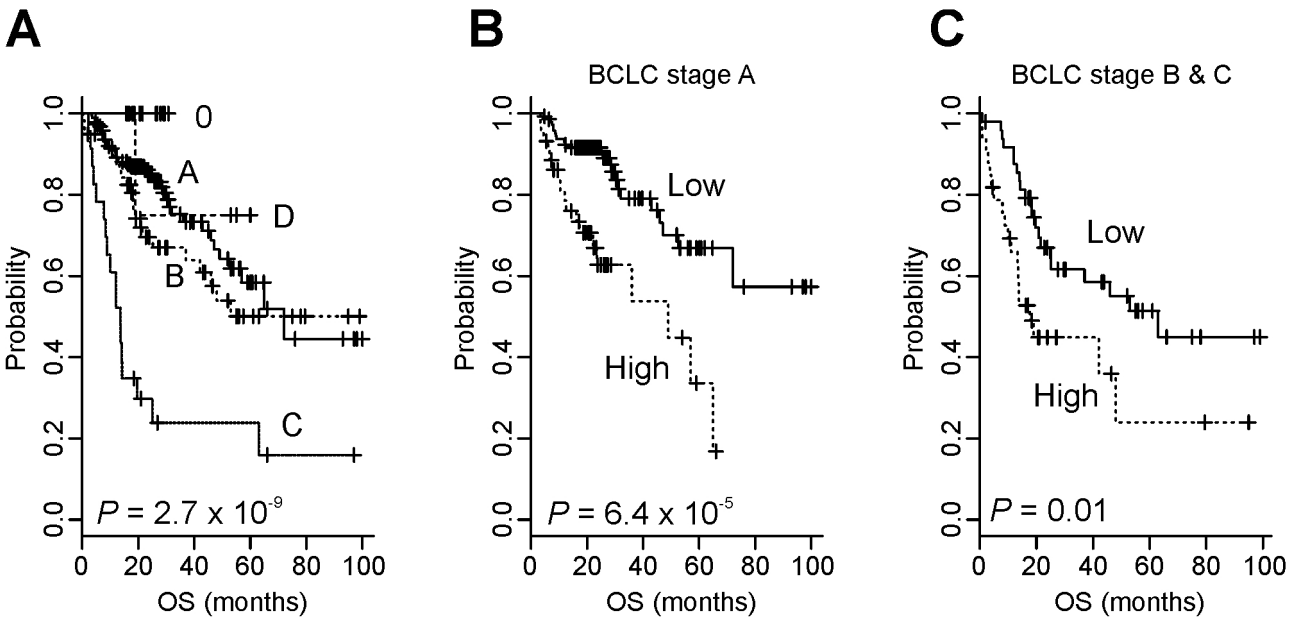

Figure 4. Kaplan–Meier plots of OS of HCC patients stratified by BCLC stages and risk score

Patients were stratified by BCLC stage (**A**) or risk score (**B** and **C**). P-values were obtained from the log-rank test.

Hepatology. Author manuscript; available in PMC 2014 June 17.

Table 1

Clinical and pathological features of HCC patients

Variable		NCI cohort	Korean cohort	LCI cohort	MSH cohort	INSERM cohort
Number of Patients		139	100	192	91	57
	male	102 (73%)	83 (83%)	168 (87.5%)	27 (30%)	46 (81%)
	female	37 (27%)	17 (17%)	24 (12.5%)	54 (59%)	11 (19%)
	NA				10 (11%)[+]	
Age	Median	57	55	50	68	66
	range	19-85	25-70	21-77	42-80	18-79
AFP[*] (>300 ng/ml)	+	55 (40%)	32 (32%)	88 (46%)	15 (17%)	
	-	73 (52%)	68 (68%)	104 (54%)	54 (59%)	
	NA	11 (8%)	0		22 (24%)	57
HBV[#]	+ (%)	63 (45%)	58 (58%)	187 (97.4%)		16 (28%)
	-(%)	76 (55 %)	17 (17 %)	5 (2.6%)		41 (72%)
	NA		25 (25%)		91 (100%)	
Edmonson Grade	1	2 (1.4%)	10 (10%)	65 (34%)		
	2	57 (41%)	51 (51%)	116 (60%)		
	3	74 (53.3%)	33 (33%)	9 (5%)		
	4	6 (4.3%)	6 (6%)	0 (0%)		
	NA			2 (1%)	91	57
Cirrhosis	Yes	69 (49.6%)	64 (64%)	176 (91.6%)		
	No	70 (50.4%)	34 (34%)	16 (8.4%)		
	NA	0	2 (2%)	0	91	57
Child-Pugh Class	A		92 (92%)	167 (87%)		
	B		4 (4%)	25 (13%)		
	C		4 (4%)			
	NA	139			91	57
Vasculature Invasion	Yes		48 (48%)	42 (21%)		
	No		52 (52%)	126 (66%)		
	NA	139		24 (13%)	91	57
AJCC Stage	I		35 (35%)	85 (44%)		

Variable		NCI cohort	Korean cohort	LCI cohort	MSH cohort	INSERM cohort
	II		17 (17%)	76 (40%)		
	III		48 (48%)	31 (16%)		
	IV		0 (0%)	0		
	NA	139			91	57
BCLC Stage	0		0 (0%)	17 (9%)		
	A		53 (53%)	136 (71%)		
	B		37 (37%)	21 (11%)		
	C		6 (6%)	17 (9%)		
	D		4 (4%)	0 (0%)		
	NA	139			91	57
Death		74	43	40	NA	NA

*Alpha-fetoprotein.

#Hepatitis B virus

+gender information is not available for patient who received liver transplantation

Table 2

Regression coefficients of 65 genes from univariate Cox regression analysis.

GENE	Coefficient	SE	Z-score	p-value	HR	HR 95% CI
ACSL5	-0.349	0.12	-2.9	0.0037	0.706	0.558-0.893
ADH1B	-0.401	0.193	-2.08	0.038	0.67	0.459-0.978
ADH6	-0.258	0.169	-1.53	0.13	0.772	0.554-1.08
ALDOA	0.226	0.146	1.55	0.12	1.25	0.942-1.67
APOC3	-0.297	0.0885	-3.36	0.00079	0.743	0.625-0.884
AQP9	-0.269	0.066	-4.08	4.50E-05	0.764	0.671-0.87
ARPC2	0.242	0.207	1.17	0.24	1.27	0.849-1.91
BPHL	-0.645	0.215	-2.99	0.0028	0.525	0.344-0.8
C1orf115	-0.519	0.128	-4.06	4.90E-05	0.595	0.463-0.764
C4BPB	-0.353	0.0873	-4.04	5.30E-05	0.703	0.592-0.834
CDO1	-0.347	0.101	-3.43	6.00E-04	0.706	0.579-0.862
CHI3L1	-0.195	0.0652	-2.99	0.0028	0.823	0.724-0.935
COBLL1	-0.389	0.13	-3	0.0027	0.677	0.525-0.874
CRAT	-0.772	0.192	-4.02	5.80E-05	0.462	0.317-0.673
CRYL1	-0.439	0.112	-3.94	8.10E-05	0.644	0.518-0.802
CTSC	0.0338	0.139	0.244	0.81	1.03	0.788-1.36
CXCR4	0.296	0.175	1.69	0.091	1.34	0.954-1.89
CYB5A	-0.255	0.102	-2.5	0.012	0.775	0.634-0.946
CYP27A1	-0.38	0.114	-3.35	0.00081	0.684	0.547-0.854
CYP2J2	-0.613	0.165	-3.72	2.00E-04	0.541	0.392-0.748
CYP4F12	-0.198	0.125	-1.59	0.11	0.82	0.643-0.105
DDIT4	0.305	0.141	2.17	0.03	1.36	1.03-1.79
EPHX2	-0.382	0.154	-2.48	0.013	0.682	0.504-0.923
ETV5	0.228	0.181	1.26	0.21	1.26	0.881-1.79
F10	-0.325	0.0858	-3.79	0.00015	0.722	0.61-0.855
F3	0.788	0.174	4.54	5.70E-06	2.2	1.57-3.09
F5	-0.312	0.0946	-3.3	0.00096	0.732	0.608-0.88
GJB1	-0.421	0.096	-4.39	1.10E-05	0.656	0.544-0.792

GENE	Coefficient	SE	Z-score	p-value	HR	HR 95% CI
GPHN	-0.521	0.229	-2.27	0.023	0.594	0.379-0.93
HN1	0.414	0.147	2.82	0.0047	1.51	1.14-2.02
HNF4A	-0.567	0.213	-2.66	0.0078	0.567	0.373-0.862
IGFBP3	0.0252	0.0985	0.256	0.8	1.03	0.846-1.24
IQGAP1	0.34	0.186	1.83	0.068	1.4	0.976-2.02
IQGAP2	-0.539	0.134	-4.02	5.90E-05	0.583	0.449-0.759
ITPR2	-0.632	0.186	-3.4	0.00067	0.531	0.369-0.765
KHK	-0.514	0.14	-3.66	0.00025	0.598	0.455-0.787
LAMB1	0.53	0.147	3.6	0.00032	1.7	1.27-2.27
LECT2	-0.138	0.0702	-1.97	0.049	0.87	0.759-1.0
MST1	-0.312	0.0882	-3.54	0.00041	0.732	0.616-0.87
MTSS1	-0.435	0.118	-3.7	0.00022	0.647	0.514-0.815
PAH	-0.381	0.106	-3.58	0.00034	0.683	0.555-0.842
PFKFB3	0.521	0.168	3.11	0.0019	1.68	1.21-2.34
PKLR	-0.386	0.12	-3.22	0.0013	0.68	0.537-0.86
PKM2	0.358	0.126	2.85	0.0044	1.43	1.12-1.83
PLG	-0.256	0.0753	-3.4	0.00067	0.774	0.668-0.897
PLOD2	0.0948	0.134	0.709	0.48	1.1	0.846-1.43
PPT1	0.134	0.18	0.746	0.46	1.14	0.804-1.63
RALA	0.878	0.23	3.81	0.00014	2.41	1.53-3.78
RGN	-0.392	0.0965	-4.07	4.80E-05	0.675	0.559-0.816
RGS1	0.255	0.111	2.3	0.021	1.29	1.04-1.6
RGS2	0.268	0.0937	2.86	0.0043	1.31	1.09-1.57
RNASE4	-0.258	0.147	-1.76	0.079	0.772	0.579-1.03
SERPINA10	-0.391	0.143	-2.74	0.0061	0.676	0.511-0.894
SERPINC1	-0.228	0.0601	-3.79	0.00015	0.796	0.708-0.896
SERPINF2	-0.352	0.086	-4.1	4.20E-05	0.703	0.594-0.832
SFTPC	-0.269	0.183	-1.48	0.14	0.764	0.534-1.09
SLC22A7	-0.476	0.123	-3.87	0.00011	0.621	0.488-0.79
SLC2A2	-0.38	0.0736	-5.17	2.30E-07	0.684	0.592-0.79
SLC30A1	-0.337	0.144	-2.34	0.019	0.714	0.539-0.946

GENE	Coefficient	SE	Z-score	p-value	HR	HR 95% CI
SLC38A1	0.184	0.141	1.3	0.19	1.2	0.911-1.59
SPHK1	0.356	0.153	2.33	0.02	1.43	1.06-1.93
SULT2A1	-0.351	0.087	-4.04	5.40E-05	0.704	0.593-0.835
TBX3	-0.294	0.15	-1.95	0.051	0.745	0.555-1.0
TM4SF1	0.321	0.104	3.07	0.0021	1.38	1.12-1.69
TSPAN3	0.416	0.184	2.26	0.024	1.52	1.06-2.17

Table 3

Univariate and Multivariate Cox Proportional Hazard Regression Analyses of Clinical Variables Associated with Overall Survival of HCC Patients in Validation Cohort.

	Univariate		Multivariate	
	Hazard Ratio (95% CI)	**P-value**	**Hazard Ratio (95% CI)**	**P-value**
Gender (M or F)	1.22 (0.63 – 2.37)	0.54	0.87 (0.43 – 1.76)	0.71
Age (>60)	1.15 (0.7 -1.89)	0.57	1.27 (0.74 – 2.15)	0.38
AFP (>300 ng/ml)	1.9 (1.23 – 2.93)	0.003	1.63 (1.0 – 2.59)	0.04
Tumor size (quintiles)	1.57 (1.32 – 1.88)	3.1×10^{-7}	1.41 (1.16 – 1.71)	4.0×10^{-4}
Grade (1,2,3,4)	1.34 (0.99 – 1.82)	0.05	0.95 (0.69 -1.33)	0.79
Vasculature Invasion (Y, or N)	2.07 (1.33 – 3.22)	0.001	1.35 (0.85 – 2.16)	0.19
Risk Score (quintiles)	1.53 (1.28 – 1.82)	2.2×10^{-6}	1.36 (1.13 – 1.64)	0.001

AFP, alpha-fetoprotein