Prediction of Response to Sorafenib in Hepatocellular Carcinoma: A Putative Marker Panel by Multiple Reaction Monitoring-Mass Spectrometry (MRM-MS)*

Hyunsoo Kim‡§‡‡, Su Jong Yu¶‡‡, Injun Yeo¶, Young Youn Cho¶, Dong Hyeon Lee¶, Yuri Cho¶, Eun Ju Cho¶, Jeong-Hoon Lee¶, Yoon Jun Kim¶, Sungyoung Lee¶, Jongsoo Jun**, Taesung Park***, Jung-Hwan Yoon¶¶¶, and e Youngsoo Kim‡§§

Sorafenib is the only standard treatment for unresectable hepatocellular carcinoma (HCC), but it provides modest survival benefits over placebo, necessitating predictive biomarkers of the response to sorafenib. Serum samples were obtained from 115 consecutive patients with HCC before sorafenib treatment and analyzed by multiple reaction monitoring-mass spectrometry (MRM-MS) and ELISA to quantify candidate biomarkers. We verified a triple-marker panel to be predictive of the response to sorafenib by MRM-MS, comprising CD5 antigen-like (CD5L), immunoglobulin J (IGHJ), and galectin-3-binding protein (LGALS3BP), in HCC patients. This panel was a significant predictor (AUROC > 0.950) of the response to sorafenib treatment, having the best cut-off value (0.4) by multivariate analysis. In the training set, patients who exceeded this cut-off value had significantly better overall survival (median, 21.4 months) than those with lower values (median, 8.6 months; p = 0.001). Further, a value that was lower than this cutoff was an independent predictor of poor overall survival [hazard ratio (HR), 2.728; 95% confidence interval (CI), 1.312–5.672; p = 0.007] and remained an independent predictive factor of rapid progression (HR, 2.631; 95% CI, 1.448–4.780; p = 0.002). When applied to the independent validation set, levels of the cut-off value for triple-marker panel maintained their prognostic value for poor clinical outcomes. On the contrast, the triple-marker panel was not a prognostic factor for patients who were treated with transarterial chemoembolization (TACE). The discriminatory signature of a triple-marker panel provides new insights into targeted proteomic biomarkers for individualized sorafenib therapy. Molecular & Cellular Proteomics 16: 10.1074/mcp.M116.066704, 1312–1323, 2017.

Hepatocellular carcinoma (HCC) 1 is the third leading cause of cancer-related death worldwide. Even when diagnosed at the earlier stages, most patients with HCC eventually progresses to the advanced stage (1, 2). The therapeutic options for patients with advanced HCC are limited and generally considered to be chemoresistant, and the results of systemic chemotherapy have been unsatisfactory (3, 4).

Sorafenib is a multikinase inhibitor that blocks angiogenesis, tumor growth, and cell proliferation and, in the Sorafenib

---

1 The abbreviations used are: HCC, hepatocellular carcinoma; SHARP, sorafenib hepatocellular carcinoma assessment randomized protocol; AFP, alpha-fetoprotein; ELISA, enzyme-linked immunosorbent assay; MRM-MS, multiple reaction monitoring-mass spectrometry; TACE, transarterial chemoembolization; AASLD, American Association for the Study of Liver Diseases; EASL, European Association for the Study of the Liver; RECIPE, reporting recommendations for tumor marker prognostic studies; NCI-CTCAE, national cancer institute-common terminology criteria of adverse events; OS, overall survival; TTP, time to progression; mRECIST, modified response evaluation criteria in solid tumors; CE-CT, contrast-enhanced computed tomography; MRI, magnetic resonance imaging; BOR, best overall response; QqQ, triple quadrupole; LC, liquid chromatography; SIS, stable isotope-labeled synthetic; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CI, confidence interval; PFS, progression-free survival; OS, overall survival; TTP, time to progression; NIST, National Institute of Standards and Technology; CV, coefficients of variation; AUROC, area under the receiver operating characteristic; RF, random forest; SVM, support vector machine; LR, logistic regression; CD5L, CD5 antigen-like; IGHG1, Ig gamma-1 chain C region; IGHG3, Ig gamma-3 chain C region; IGHJ, immunoglobulin J chain; LGALS3BP, galectin-3-binding protein; NRP1, neuropilin-1; BCLC, Barcelona clinic liver cancer; PPV, positive predictive value; NPV, negative predictive value; HR, hazard ratio; ECOG, Eastern cooperative oncology group.
Prediction of Response to Sorafenib on Advanced HCC

Molecular & Cellular Proteomics 16.7

Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) and Asia-Pacific trials, was the first systemic chemotherapeutic agent that was found to lengthen the survival of patients with unresectable (intermediate to advanced) HCC (5, 6). However, these trials demonstrated a modest survival benefit of ~3 months over placebo, and several patients with advanced HCC remained refractory to sorafenib (5–7). Moreover, the clinical parameters that determine which patients benefit from sorafenib therapy remain unknown (8, 9).

Several studies have examined the prognostic factors of sorafenib treatment (8, 10–12). In the sorafenib cohort of the SHARP trial, sorafenib trended toward increasing survival in patients with high s-c-KIT or low hepatocyte growth factor concentrations at baseline, but none of the biomarkers significantly predicted the response to sorafenib (8). In other studies, the response to alpha-fetoprotein (AFP) and a decrease in vascular endothelial growth factor were independent predictors of responsiveness to sorafenib, alone or in combination (12, 13). However, these on-treatment biomarkers fail to identify who will benefit from sorafenib before treatment is commenced. Thus, pretreatment biomarkers, including serum cholinesterase, FGFR3/FGF4 gene amplification, and galectin-1, have been evaluated but have not been fully validated (14–16). Moreover, these studies included a narrow range of candidate proteins in the biomarker discovery stage.

Traditionally, the most widely used technique for protein quantification has been enzyme-linked immunosorbent assay (ELISA). The advantages of this method are its speed, sensitivity, specificity, and compatibility with standard clinical laboratory equipment, allowing it to be applied routinely in clinical practice (17). However, the ELISA has significant constraints, including its cost and time-consuming development of specific antibodies and its technical limitations regarding multiplex quantitation. Multiplex immunoassays have been developed to obtain quantitative data by parallel analyses for multiple antigens, but they have increased cross-reactivity because of the presence of several antibodies (18, 19).

In contrast, high-throughput “omics” technologies are now available, allowing one to measure the relative abundance of thousands of molecular targets in their assessment as biomarkers (20). In addition to antibody-based technologies, there are alternative methods for the quantitative analysis and validation of potential biomarkers, such as multiple reaction monitoring-mass spectrometry (MRM-MS), which is a highly selective and sensitive approach to quantitating targeted proteins or peptides in samples—a potential substitute method for screening diseases. MRM-MS is a targeted proteomics technology that does not require antibody and simultaneously measures at least 100 protein targets per sample (21, 22). Further, MRM-MS generates consistent, precise, and reproducible datasets between laboratories in highly complex samples (23).

The MRM-MS assay has not been applied to biomarker discovery regarding the response to sorafenib. This study aimed to develop pretreatment serum protein biomarkers to predict sorafenib response by MRM-MS in patients with unresectable HCC. To this end, we established a triple-marker panel to predict the response to sorafenib, based on a quantitative proteomics approach, and verified its prognostic value in patients with advanced HCC.

**EXPERIMENTAL PROCEDURES**

**Study Population**—This study was based on 115 sorafenib-treated patients who were enrolled into a prospective cohort at Seoul National University Hospital (Seoul, Republic of Korea) as part of an ongoing study that identified biomarkers that were associated with treatment response and prognosis in HCC (Table I). Serum samples were collected from a single center according to standard operating procedures (24). All serum samples were collected immediately prior to the commencement of sorafenib treatment and were obtained by incubating the vials for 30 min (clotting time, at room temperature), centrifuging them at 1200 g for 20 min at room temperature, and separating the serum supernatant into aliquots (100 μl) for storage at −80 °C.

The inclusion criteria were patients with unresectable (intermediate to advanced) HCC who have undergone sorafenib treatment (see details in Supplemental Data). Patients without a radiological response to sorafenib were excluded. The training set consisted of 65 patients who were enrolled between May 2013 and December 2014, and the validation set comprised 50 patients who were recruited between January 2014 and August 2014. For further validation in patients who were treated with another modality, 100 patients who underwent transarterial chemoembolization (TACE) were enrolled, as detailed in (supplemental Table S1). All serum samples were collected just prior to TACE; there were no differences between the sorafenib and TACE sample sets. HCC was diagnosed by histological or radiological evaluation per American Association for the Study of Liver Diseases (AASLD) (25) or European Association for the Study of the Liver (EASL) (26) guidelines. The institutional review board of Seoul National University Hospital approved the study protocol (IRB No. 0506–150-005), and written informed consent was obtained from each patient or a legally authorized representative. The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria were followed throughout the study (27).

**Sorafenib Administration**—In principle, sorafenib was initiated at 400 mg twice daily (800 mg/day). Patients were administered the initial dose during the first 2 weeks, and thereafter, sorafenib was continued every 4 weeks if there were no side effects. However, treatment interruptions and dose reductions were permitted for drug-related adverse events, which were graded per the National Cancer Institute Common Terminology Criteria of Adverse Events (NCI-CTCAE) ver.4.03 (http://ctep.cancer.gov).

**Outcomes and Assessment of Response to Sorafenib**—The primary end-point was overall survival (OS), defined as the time from the commencement of sorafenib treatment to death from any cause. The secondary end-point was time to progression (TTP), measured from the commencement of sorafenib treatment until the first documented evidence of tumor progression in imaging studies per the modified Response Evaluation Criteria in Solid Tumors (mRECIST) (28) by contrast-enhanced computed tomography (CE-CT) or magnetic resonance imaging (MRI) by 2 independent radiologists who were blinded to the clinical information.

Treatment was continued until symptomatic progression, unacceptable adverse events, or death. Clinical and laboratory assessments were performed monthly, and a radiological tumor evaluation was conducted initially at Week 4 and every 8 weeks afterward. In this study, we prospectively determined the best response during

**Notes**

For a detailed description of the study population, see Supplemental Data. The study population was based on 115 sorafenib-treated patients who were enrolled into a prospective cohort at Seoul National University Hospital (Seoul, Republic of Korea) as part of an ongoing study that identified biomarkers that were associated with treatment response and prognosis in HCC (Table I). Serum samples were collected from a single center according to standard operating procedures (24). All serum samples were collected immediately prior to the commencement of sorafenib treatment and were obtained by incubating the vials for 30 min (clotting time, at room temperature), centrifuging them at 1200 g for 20 min at room temperature, and separating the serum supernatant into aliquots (100 μl) for storage at −80 °C.

The inclusion criteria were patients with unresectable (intermediate to advanced) HCC who have undergone sorafenib treatment (see details in Supplemental Data). Patients without a radiological response to sorafenib were excluded. The training set consisted of 65 patients who were enrolled between May 2013 and December 2014, and the validation set comprised 50 patients who were recruited between January 2014 and August 2014. For further validation in patients who were treated with another modality, 100 patients who underwent transarterial chemoembolization (TACE) were enrolled, as detailed in (supplemental Table S1). All serum samples were collected just prior to TACE; there were no differences between the sorafenib and TACE sample sets. HCC was diagnosed by histological or radiological evaluation per American Association for the Study of Liver Diseases (AASLD) (25) or European Association for the Study of the Liver (EASL) (26) guidelines. The institutional review board of Seoul National University Hospital approved the study protocol (IRB No. 0506–150-005), and written informed consent was obtained from each patient or a legally authorized representative. The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria were followed throughout the study (27).

**Sorafenib Administration**—In principle, sorafenib was initiated at 400 mg twice daily (800 mg/day). Patients were administered the initial dose during the first 2 weeks, and thereafter, sorafenib was continued every 4 weeks if there were no side effects. However, treatment interruptions and dose reductions were permitted for drug-related adverse events, which were graded per the National Cancer Institute Common Terminology Criteria of Adverse Events (NCI-CTCAE) ver.4.03 (http://ctep.cancer.gov).

**Outcomes and Assessment of Response to Sorafenib**—The primary end-point was overall survival (OS), defined as the time from the commencement of sorafenib treatment to death from any cause. The secondary end-point was time to progression (TTP), measured from the commencement of sorafenib treatment until the first documented evidence of tumor progression in imaging studies per the modified Response Evaluation Criteria in Solid Tumors (mRECIST) (28) by contrast-enhanced computed tomography (CE-CT) or magnetic resonance imaging (MRI) by 2 independent radiologists who were blinded to the clinical information.

Treatment was continued until symptomatic progression, unacceptable adverse events, or death. Clinical and laboratory assessments were performed monthly, and a radiological tumor evaluation was conducted initially at Week 4 and every 8 weeks afterward. In this study, we prospectively determined the best response during
Prediction of Response to Sorafenib on Advanced HCC

sorafenib treatment using the mRECIST criteria and adopted it as the best overall response (BOR).

**Serum Sample Preparation and MRM-MS Analysis**—Serum samples were randomized statistically and subjected to immunodepletion, denaturation, trypsin digestion, and desalting, followed by reversed-phase liquid chromatography. All MRM-MS analyses were performed on an Agilent 6490 triple quadrupole (QqQ) mass spectrometer with a Jetstream electrospray source, coupled to a 1260 Capillary LC system (Agilent Technologies, Santa Clara, CA). Peptides were separated on a reversed-phase analytical column (150 mm × 0.5 mm i.d., 3.5 μm particle size, Agilent Zorbax SB-C18) with mobile phases A [water 0.1% (v/v) formic acid] and B [acetonitrile 0.1% (v/v) formic acid]. Five microliters of tryptic digest was injected into a column and separated using a binary gradient. Skyline (McCoss Lab, University of Washington, WA) was used to import and align all MRM-MS raw data files and quantitate features (see details in supplemental Data).

**Enzyme-Linked Immunosorbent Assay**—Serum levels of 6 proteins were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit. All ELISAs for CD5 antigen-like (SunRed, Shanghai, China), Ig gamma-1 chain C region (SunRed), Ig gamma-3 chain C region (SunRed), immunoglobulin J chain (SunRed), galectin-3-binding protein (CUSABIO, Hubei, China), and neutrophil-1 (USCN Life Science, Houston, USA) were performed per the manufacturers’ instructions.

**Statistical Analysis**—The raw data were processed in Skyline to visually inspect the traces of the MRM-MS data and calculate the peak areas of the transitions. The normalized peak areas of endogenous peptides [endogenous peptide/corresponding stable isotope-labeled synthetic (SIS) peptide] were used to compare the relative abundance of each peptide between samples. The Savitzky-Golay method was applied to smooth the data points. All statistical analyses were performed using SPSS ver. 19.0 (IBM Corp., New York, NY), Graph Pad Prism ver. 6.0 (Graph PAD, San Diego, CA), and R ver. 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria) (see details in Supplemental Data).

**RESULTS**

**Baseline Characteristics**—The baseline characteristics of the sorafenib-treated study population are summarized in Table I. A total of 115 subjects were drawn from our prospective cohort, matched for gender, age, and other clinical factors between groups to avoid bias. Of the 115 subjects, 65 constituted the training set, and 50 patients formed the validation set. The 2 sets had similar baseline characteristics—58 males and 7 females with a median age of 60 years at diagnosis (range 34–84) in the training set versus 44 males and 6 females with a median age of 59 years at diagnosis (range 38–77) in the validation set.

The predominant etiology of liver disease was hepatitis B in the training (n = 51, 78.5%) and validation sets (n = 40, 80.0%). One hundred patients were Child-Pugh class A in the training (n = 61, 93.8%) and validation sets (n = 39, 78.0%). One hundred one patients had a history of previous therapy for HCC before sorafenib treatment in the training (n = 57, 87.7%) and validation sets (n = 44, 88.0%) (supplemental Table S2).

**Sorafenib Administration and Treatment Interruption**—The sorafenib regimen for the enrolled patients is shown in supplemental Table S3. Fifty-five patients (47.8%) were treated with sorafenib for less than 12 weeks, whereas 23 patients (20%) were given sorafenib for over 24 weeks. Sorafenib was administered at an initial daily dose of 800 mg. The median daily dose of sorafenib in the first 6 weeks was 800 mg (Fig. 1).

**Treatment Outcomes of Sorafenib**—Radiological tumor response to sorafenib was assessed initially at Week 4 and every 8 weeks afterward. Three patients (2 in the training set and 1 in the validation set) achieved a complete response (CR), 6 patients (3 and 3, respectively) obtained a partial response (PR), 31 patients (16 and 15, respectively) achieved a stable disease (S.D.), and 75 patients (44 and 31, respectively) attained a progressive disease (PD). The disease control (+) rate with sorafenib treatment was 34.8% (40 patients) (supplemental Table S4).

The median follow-up time was 9.5 months (range, 1.6–30.0 months) in the training set and 11.9 months (range, 1.3–22.6 months) in the validation set; the median treatment duration was 3.2 months (range, 1.6–21.1 months) and 3.1 months (range, 1.3–14.3 months), respectively. In the general population, the median progression-free survival (PFS) was 2.6 months (95% confidence interval (CI), 1.79–3.35) (progression: 107 patients, 93.0%), whereas the median overall survival (OS) was 9.5 months (95% CI, 7.26–14.60) (death: 82 patients, 71.3%). In the training set, 21 patients (32.3%) were non-PD, and the median time to progression (TTP) was 4.0 months (95% CI, 3.34–8.57). In the validation set, 19 patients (38.0%) were non-PD, and the median TTP was 6.0 months (95% CI, 4.95–9.26).

**Selection of Candidate Protein Biomarkers**—Candidate protein biomarkers were chosen, based on the LiverAtlas database (http://liveratlas.hupo.org.cn), which is the most comprehensive resource of biomedical knowledge on liver and various hepatic diseases (29). The following criteria were used to filter candidate biomarkers: (1) From the LiverAtlas database, 50,265 proteins were downloaded. (2) We focused on proteins on which high-throughput experiments and highly focused biochemical studies have been performed in the 50,208 proteins (Reliability score ≥ 3). (3) Then, we selected 1683 proteins that were secreted or might be secreted, per UniProt Knowledgebase (UniProtKB, http://www.uniprot.org/) database. (4) Next, 930 proteins were filtered with MS/MS spectra from the National Institute of Standards and Technology (NIST) MS/MS library for empirical evidence of MS spectrum detectability. (5) For the 930 proteins, we selected 20 proteolytic peptides per protein, and 754 peptides were selected to represent the 347 proteins that were detectable in pooled serum samples. (6) Subsequently, 210 proteins (339 peptides) were filtered, based on reproducibility (coefficient of variation (CV) ≤ 20%) (7) Ultimately, 124 protein targets (231 peptide targets) (supplemental Table S5) were examined in 115 serum samples using the MRM-MS assay, the transitions of which did not interfere with each other; MRM-MS quantitation was performed in a blinded manner (30). The procedure
of screening candidate markers for triple-marker panel is summarized in Fig. 2 (see details in supplemental Data).

**Peptide Marker Discovery Using MRM-MS Dataset**—A total of 65 subjects (21 non-PD, 44 PD) constituted the training set. For the validation set, the 231 peptides (124 proteins) were rescreened in the dataset, consisting of 50 subjects (19 non-PD, 31 PD).

In the single-marker analysis for the training set, the best marker was LVGGDNLCSGR (CD5L); its area under the receiver operating characteristic (AUROC) curve value was 0.942, 0.950, and 0.958 by random forest (RF), support vector machine (SVM), and logistic regression (LR), respectively. For the validation set, the best marker was IIVPLNNR (Immunoglobulin J chain (IGJ)), with an AUROC value of 0.990, 0.991, and 0.976, respectively, whereas LVGGDNLCSGR (CD5 antigen-like (CD5L)) ranked fourth (top 1.7%), with AUROC values of 0.980, 0.987, and 0.966 (supplemental Table S6).

### Table I

Demographic and clinical characteristics of the studied population (n = 115)

| Variable                  | Training set (n = 65) | Validation set (n = 50) | Total (n = 115) |
|---------------------------|-----------------------|-------------------------|-----------------|
| Age (years)               |                       |                         |                 |
| < 60                      | 29 (44.6%)            | 27 (54.0%)              | 56 (48.7%)      |
| ≥ 60                      | 36 (55.4%)            | 23 (46.0%)              | 59 (51.3%)      |
| Gender                    |                       |                         |                 |
| Male                      | 58 (89.2%)            | 44 (88.0%)              | 102 (88.7%)     |
| Female                    | 7 (10.8%)             | 6 (12.0%)               | 13 (11.3%)      |
| Etiology                  |                       |                         |                 |
| HBsAg-positive            | 51 (78.5%)            | 40 (80.0%)              | 91 (79.1%)      |
| Anti-HCV-positive         | 3 (4.6%)              | 4 (8.0%)                | 7 (6.1%)        |
| Alcohol                   | 4 (6.1%)              | 2 (4.0%)                | 6 (5.2%)        |
| Unknown                   | 7 (10.8%)             | 4 (8.0%)                | 11 (9.6%)       |
| ECOG                      |                       |                         |                 |
| 0                         | 63 (96.9%)            | 48 (96.0%)              | 111 (96.5%)     |
| 1                         | 2 (3.1%)              | 2 (4.0%)                | 4 (3.5%)        |
| Body mass index (kg/m²)   | 20.5 ± 7.56           | 22.0 ± 5.68             | 21.1 ± 6.82     |
| CTP classification        |                       |                         |                 |
| A                         | 61 (93.8%)            | 39 (78.0%)              | 100 (87.0%)     |
| B                         | 4 (6.2%)              | 11 (22.0%)              | 15 (13.0%)      |
| MELD score                | 8.82 ± 1.65           | 9.81 ± 3.14             | 9.25 ± 2.45     |
| WBC (x 10³/µl)            | 5.96 ± 2.31           | 4.74 ± 1.59             | 5.43 ± 2.11     |
| Neutrophil (%)            | 62.6 ± 11.5           | 61.9 ± 11.0             | 62.3 ± 11.2     |
| Platelet (x 10³/µl)       | 175.4 ± 106.3         | 143.0 ± 86.1            | 161.3 ± 98.9    |
| AFP (ng/ml)               |                       |                         |                 |
| < 200                     | 44 (67.7%)            | 29 (58.0%)              | 73 (63.5%)      |
| ≥ 200                     | 21 (32.3%)            | 21 (42.0%)              | 42 (36.5%)      |
| DCP (mAU/ml)              | 8,301 ± 18,651        | 3,705 ± 11,022          | 6,825 ± 15,870  |
| Tumor size (cm)           |                       |                         |                 |
| < 5                       | 53 (81.5%)            | 40 (80.0%)              | 93 (80.9%)      |
| ≥ 5                       | 12 (18.5%)            | 10 (20.0%)              | 22 (19.1%)      |
| Tumor number              |                       |                         |                 |
| ≤ 3                       | 32 (49.2%)            | 31 (62.0%)              | 63 (54.8%)      |
| 4–9                       | 13 (20.0%)            | 11 (22.0%)              | 24 (20.9%)      |
| ≥ 10                      | 20 (30.8%)            | 8 (16.0%)               | 28 (24.3%)      |
| Vascular invasion         |                       |                         |                 |
| No                        | 47 (72.3%)            | 32 (64.0%)              | 79 (68.7%)      |
| Yes                       | 18 (27.7%)            | 18 (36.0%)              | 36 (31.3%)      |
| Lymph node                |                       |                         |                 |
| No                        | 53 (81.5%)            | 41 (82.0%)              | 94 (81.7%)      |
| Yes                       | 12 (18.5%)            | 9 (18.0%)               | 21 (18.3%)      |
| Metastasis                |                       |                         |                 |
| No                        | 31 (47.7%)            | 29 (58.0%)              | 60 (52.2%)      |
| Yes                       | 34 (52.3%)            | 21 (42.0%)              | 55 (47.8%)      |
| BCLC stage                |                       |                         |                 |
| B (intermediate)          | 15 (23.1%)            | 15 (30.0%)              | 30 (26.1%)      |
| C (advanced)              | 50 (76.9%)            | 35 (70.0%)              | 85 (73.9%)      |

HBsAg, hepatitis B surface antigen; Anti-HCV, antibody against hepatitis C virus; ECOG, eastern cooperative oncology group; CTP, child-turcotte-pugh; MELD, model for end-stage liver disease; WBC, white blood cell; AFP, alpha-fetoprotein; DCP, des-gamma-carboxyprothrombin; BCLC, barcelona clinic liver cancer.
FIG. 1. Timeline of sorafenib treatment scheduled for prospective cohort patients enrolled in the study. Patients were administrated the initial dose (800 mg/day) of sorafenib during the first 2 weeks, and thereafter, sorafenib treatment was continued every 4 weeks until symptomatic progression, side effects, or death. A radiological CT (or MRI) was taken before initiation of sorafenib treatment in patients with unresectable HCC. Radiological tumor evaluation was conducted initially at Week 4 and every 8 weeks afterward, and 115 serum samples were collected before sorafenib treatment.

FIG. 2. Multistage, targeted MRM-MS pipeline for triage and validation of biomarker candidates. Overview of the workflow used to triage and validate candidate biomarkers, showing the flux of candidates at each stage of the pipeline. Number of targets pared from 50,265 candidates by MRM-MS and ELISA—930 candidate biomarkers were subjected to semi-quantitative MRM-MS, of which 124 candidates were detected with interference free-transitions and applied to quantitative MRM-MS. Next, AUROC values were used to prioritize candidates for marker selection. Based on these data, 11 markers were selected (AUROC > 0.800), and 6 markers were ultimately selected for immunoassay (ELISA). Finally, we established a multiple marker panel that comprised of 3 proteins.
Validation of Protein Marker by ELISA—Candidate proteins were selected for further validation, based on AUROC values of more than 0.800 by quantitative MRM-MS analysis and the availability of an antibody for the ELISA kit. Per these criteria, 6 proteins—CD5 antigen-like (CD5L), Ig gamma-1 chain C region (IGHG1), Ig gamma-3 chain C region (IGHG3), immunoglobulin J chain (IGJ), galectin-3-binding protein (LGALS3BP), and neutrophilin-1 (NRP1)—were chosen for validation by ELISA.

From the training and validation sets, 80 subjects (40 non-PD and 40 PD) were randomly selected for the ELISA analysis. To maintain the quality of the ELISA dataset, subjects who lay outside of the dynamic range were discarded. Based on the best cutoff values, the sensitivity and specificity of the 6 proteins were calculated (supplemental Fig. S3).

Diagnostic Accuracy of Multiple-Marker Panel—We performed a multiple-marker analysis for the MRM-MS and ELISA datasets. For the MRM-MS dataset, we applied a step-wise method to reduce the computational burden of testing all possible multiple-marker sets. The diagnostic sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) of the single marker and multiple-marker panel are shown in supplemental Table S7.

Of the 4083 MRM-MS multiple-marker panels (66 panels in with 2 markers; 220 panels with 3 markers; and 3797 panels with 4 or more markers), the best panel for the training set comprised NTCNHDTEDWCEDEPDFLR, IVVPLNRR, and SDLAVPSEALLK (CD5L, IGJ, and LGALS3BP, respectively); the AUROC values were 0.954, 0.970, and 0.949 by RF, SVM, and LR, respectively. For the validation set, the best multiple-marker panel was LVGGDNLCSGR, IVLVDNK, and LR, respectively. For the validation set, the best multiple-marker panel was LGALS3BP and Neutrophilin-1 (NRP1). We also determined the interpeptide correlation for the data on all individual samples that were analyzed by MRM-MS assay. The results confirmed the robust correlation (R² ≥ 0.8) between peptides, derived primarily from the same protein (supplemental Fig. S4).

For the ELISA dataset, the best multiple-marker panel was IGHG3, IGJ, and LGALS3BP, with AUROC values of 0.796, 0.807, and 0.843 by RF, SVM, and LR, respectively. The AUROC values of CD5L, IGJ, and LGALS3BP, corresponding to the best multiple-marker panel of the MRM-MS data set, were 0.793, 0.811, and 0.841 by RF, SVM, and LR, respectively.

The Delong test (based on LR analysis) was used to compare the ELISA results of MRM-MS dataset derived optimal multiple-marker panel (CD5L + IGJ + LGALS3BP) to that of ELISA dataset derived optimal multiple-marker panel (IGHG3 + IGJ + LGALS3BP). As a result, we confirmed that the difference in AUROC values was not statistically significant (p = 0.522; 95% CI, 0.021–0.041).

The MRM-MS assay has greater diagnostic power (difference in AUROC of 0.2) than ELISA assay for the multiple-marker panel (consisting of the same combination of proteins) and can analyze several proteins simultaneously, whereas ELISA assay examines each of the 3 proteins separately. Thus, only the optimal multiple-marker panel (CD5L + IGJ + LGALS3BP) from the MRM-MS data set was analyzed further.

Multiple-Marker Panel as an Independent Prognostic Factor after Sorafenib Therapy—The prognostic value of the multiple-marker panel (CD5L + IGJ + LGALS3BP) was initially analyzed as a continuous variable. By univariate analysis, poor Child-Pugh score, duration of sorafenib treatment, multiple tumors, and the triple-marker panel were significantly associated with OS in the training set. However, Barcelona Clinic Liver Cancer (BCLC) stage was not a risk factor for poor OS in the training set. In the multivariate Cox analysis, poor Child-Pugh score, short duration of sorafenib treatment, a large number of tumors, and low level of the triple-marker panel were independent risk factors for poor OS in the training set (Table II).

Because the triple-marker panel was identified as an independent predictor of the response to sorafenib treatment, we stratified the patients into 2 groups using the best cutoff point of 0.4, as calculated solely by LR method, which provided the maximum sum of specificity and sensitivity (sensitivity, 95.3%; specificity, 91.3%; AUROC, 0.949). Consequently, of the training set patients, 44 were included in the low-triple-marker panel group, compared with 21 in the high-triple-marker panel group. At a cutoff of 0.4, 36 patients (78.3%) in the low-triple-marker panel group and 10 (21.7%) in the high-triple-marker panel group died during the follow-up. Patients with high-triple-marker panel levels (≥ 0.4) had significantly better OS (median, 21.4 months; 95% CI, 7.470–28.530) than those with low levels (< 0.4; median OS, 8.6 months; 95% CI, 5.303–10.697; p = 0.001; Fig. 3A). The prognostic factors for OS using multivariate analysis were poor Child-Pugh score (hazard ratio [HR], 1.857; 95% CI, 1.156–2.982; p = 0.010), short duration of sorafenib treatment (HR, 4.063; 95% CI, 1.581–10.441; p = 0.004 for < 12 versus > 24; HR, 1.904; 95% CI, 0.656–5.258; p = 0.237 for 12–18 versus > 24; HR, 1.774; 95% CI, 0.488–6.447; p = 0.384 for 18–24 versus > 24), a large number of tumors (HR, 1.566; 95% CI, 0.685–3.582; p = 0.288 for 4–9 versus ≤ 3; HR, 3.129; 95% CI, 1.509–6.488; p = 0.002 for ≥ 10 versus ≤ 3), and low level of the triple-marker panel (HR, 2.510; 95% CI, 1.144–5.070; p = 0.022) (Table II).

The levels of the triple-marker panel were also significantly associated with TTP, Kaplan-Meier estimates are shown in Fig. 3B. By univariate analysis, duration of sorafenib treatment and low-triple-marker panel level correlated significantly with TTP in the training set (Table III). In the multivariate analysis, gender was also included (p < 0.10), but short sorafenib treatment (HR, 3.645; 95% CI, 1.537–8.645; p = 0.003 for < 12 versus > 24; HR, 2.147; 95% CI, 0.901–5.119; p = 0.085
### Table II
Factors identified by univariate and multivariate analyses that affect overall survival (OS) in HCC patients treated with sorafenib

| Variable                      | Training set (n = 65) | Validation set (n = 50) |
|-------------------------------|-----------------------|-------------------------|
|                               | Univariate Analysis   | Multivariate Analysis   | Univariate Analysis   | Multivariate Analysis   |
|                               | HR (95% CI)           | P                       | HR (95% CI)           | P                       |
| Age (≤ 60 years)              | 1.211 (0.675–2.174)   | 0.521                   | 1.184 (0.614–2.282)   | 0.614                   |
| Male                          | 0.982 (0.387–2.490)   | 0.969                   | 1.183 (0.418–3.348)   | 0.752                   |
| Etiology                      |                       |                         | 1.183 (0.418–3.348)   | 0.752                   |
| HBV vs. NBNC                  | 1.033 (0.479–2.229)   | 0.933                   | 0.458 (0.109–1.921)   | 0.286                   |
| HCV vs. NBNC                  | 0.332 (0.041–2.670)   | 0.300                   | 0.458 (0.109–1.921)   | 0.286                   |
| Child-Pugh score              | 2.21 (1.390–3.514)    | 0.001                   | 1.857 (1.156–2.982)   | 0.010                   |
|AFP (ng/ml) ≥ 200              | 1.516 (0.837–2.744)   | 0.170                   | 0.866 (0.443–1.696)   | 0.675                   |
| Log(DCP) (mAU/ml)             | 1.178 (0.903–1.536)   | 0.228                   | 1.542 (1.108–2.147)   | 0.010                   |
| Sorafenib administration      |                       |                         | 1.542 (1.108–2.147)   | 0.010                   |
| Dose modification during treatment | 1.041 (0.528–2.051) | 0.908                   | 1.653 (0.637–4.290)   | 0.301                   |
| Duration of sorafenib treatment (weeks) | 0.005 | 0.013                   | 0.477 |                         |
| < 12 vs. > 24                 | 4.109 (1.665–10.138)  | 0.002                   | 4.063 (1.581–10.441)  | 0.004                   |
| 12–18 vs. > 24                | 1.897 (0.688–5.232)   | 0.216                   | 1.904 (0.656–5.528)   | 0.237                   |
| 18–24 vs. > 24                | 1.411 (0.397–5.05)    | 0.595                   | 1.774 (0.488–6.447)   | 0.384                   |
| Tumor size (cm) ≥ 5           | 1.18 (0.548–2.539)    | 0.672                   | 1.408 (0.640–3.099)   | 0.395                   |
| Tumor number                   | < 4 vs. ≥ 3           | 1.27 (0.570–2.829)      | 0.588                   | 1.566 (0.685–3.582)     | 0.288                   |
| Vascular invasion              | Yes                   | 3.852 (1.937–7.688)     | < 0.001                | 3.129 (1.509–6.488)     | 0.002                   |
| Extrahepatic spread            | Yes                   | 1.305 (0.674–2.528)     | 0.430                   | 2.017 (1.030–3.948)     | 0.041                   |
| BCLC stage                     | C                     | 1.176 (0.650–2.128)     | 0.591                   | 1.092 (0.566–2.108)     | 0.792                   |
| Triple-marker panel level      | Low (< 0.4)           | 0.933 (0.473–1.841)     | 0.842                   | 0.571 (0.273–1.196)     | 0.138                   |
|                               |                       | 3.089 (1.506–6.339)     | 0.002                   | 2.510 (1.144–5.507)     | 0.022                   |

HR, hazard ratio; CI, confidence interval; HBV, hepatitis B virus; NBNC, HBsAg-negative and anti-hepatitis C virus-negative; HCV, hepatitis C virus; AFP, alpha-fetoprotein; DCP, Des-gamma carboxyprothrombin; BCLC, barcelona clinic liver cancer.
for 12–18 versus > 24; HR, 1.805; 95% CI, 0.666–4.893; \( p = 0.246 \) for 18–24 versus > 24) and low-triple-marker panel level (HR, 2.631; 95% CI, 1.448–4.780; \( p = 0.002 \)) were independent risk factors for shorter TTP in the training set.

Validation of Results—We then determine whether triple-marker panel levels maintained their predictive value in the internal validation set. Like the findings from the training set, patients with low-triple-marker panel levels had a significantly shorter OS (median, 7.3 months; 95% CI, 2.0–12.7; Fig. 3C) and TTP (median, 1.9 months; 95% CI, 1.7–2.1; Fig. 3D) than those with high levels (median OS, 20.9 months; 95% CI, 10.0–31.8; \( p = 0.006 \); median TTP, 9.0 months; 95% CI, 4.1–13.9; \( p < 0.001 \)). However, BCLC stage was not a risk factor for poor OS or short TTP in the validation set, either. Although the duration of sorafenib treatment was a risk factor for rapid progression in the univariate analysis (\( p = 0.035 \)), it was not an independent risk factor for it by multivariable analysis (\( p = 0.179 \)). The multivariable analysis indicated that poor Child-Pugh score (HR, 1.373; 95% CI, 1.016–1.855; \( p = 0.039 \)), presence of vascular invasion (HR, 2.525; 95% CI, 1.198–5.324; \( p = 0.015 \)), and a low level of triple-marker panel (HR, 2.639; 95% CI, 1.216–5.730; \( p = 0.014 \)) were an independent risk factors for poor survival in the validation set (Table II). In addition, the multivariable analysis indicated that a low level of triple-marker panel (HR, 18.399; 95% CI, 4.007–84.483; \( p < 0.001 \)) was an independent risk factor for rapid progression in the validation set (Table III).

Subgroups Analysis and Additional Validation Study—Next, we performed a subgroup analysis according to BCLC stage for the entire study population. Most subjects had stage BCLC-C disease (\( n = 85; 73.9\% \)), and the number of patients in BCLC-B was too low (\( n = 30; 26.1\% \)). Thus, we performed a subgroup analysis of our entire cohort. In patients with BCLC-B disease, the triple-marker panel was not a significant risk factor for poor OS (HR, 1.524; 95% CI, 1.009–2.781; \( p = 0.046 \)) and rapid progression (HR, 2.615; 95% CI, 1.581–4.325; \( p < 0.001 \)).

When we performed an additional validation study in patients who were treated with TACE, the high- and low-triple-
| Variable | Training set (n = 65) | Validation set (n = 50) |
|----------|-----------------------|------------------------|
|          | Univariate Analysis   | Multivariate Analysis  | Univariate Analysis   | Multivariate Analysis  |
|          | HR (95% CI) P         | HR (95% CI) P          | HR (95% CI) P         | HR (95% CI) P          |
| Age (≥ 60 years) | 1.238 (0.743–2.064) 0.412 | 0.709 (0.381–1.322) 0.280 |
| Male     | 0.460 (0.201–1.057) 0.067 | 0.909 (0.355–2.326) 0.843 |
| Etiology | 0.498                | 0.694                  |
| HBV vs. NBNC | 0.421 (0.098–1.802) 0.244 | 1.536 (0.540–4.370) 0.422 |
| HCV vs. NBNC | 1.045 (0.537–2.032) 0.898 | 0.902 (0.317–2.564) 0.847 |
| Child-Pugh score | 1.108 (0.701–1.750) 0.660 | 0.963 (0.760–1.221) 0.756 |
| AFP (ng/ml) | ≥ 200 | 0.932 (0.536–1.620) 0.802 | 0.822 (0.444–1.521) 0.532 |
| Log(DCP) (mAU/ml) | 0.944 (0.761–1.172) 0.604 | 1.222 (0.899–1.661) 0.201 |
| Sorafenib administration | | | | |
| Dose modification during treatment | 1.168 (0.650–2.098) 0.603 | 0.901 (0.353–2.302) 0.828 | |
| Duration of sorafenib treatment (weeks) | 0.001 | 0.030 |
| < 12 vs. > 24 | 4.993 (2.222–11.219) <0.001 | 3.645 (1.537–8.645) 0.003 | 3.962 (1.554–10.105) 0.004 | 2.062 (0.735–5.788) 0.169 |
| 12–18 vs. > 24 | 2.555 (1.093–5.973) 0.030 | 2.147 (0.901–5.119) 0.085 | 2.693 (0.923–7.856) 0.070 | 1.446 (0.449–4.662) 0.537 |
| 18–24 vs. > 24 | 1.872 (0.691–5.071) 0.217 | 1.805 (0.666–4.893) 0.246 | 3.764 (1.024–13.841) 0.046 | 4.109 (1.057–15.972) 0.041 |
| Tumor size (cm) | 0.597 (0.300–1.190) 0.143 | 0.885 (0.409–1.919) 0.758 |
| ≥ 5 | 0.303 | 0.666 |
| 4–9 vs. ≤ 3 | 1.296 (0.674–2.491) 0.437 | 1.293 (0.618–2.704) 0.495 |
| ≥ 10 vs. ≤ 3 | 1.582 (0.877–2.854) 0.128 | 0.823 (0.337–2.011) 0.669 |
| Vascular invasion | 0.921 (0.511–1.660) 0.784 | 1.022 (0.534–1.954) 0.948 |
| Extrahepatic spread | 1.301 (0.778–2.176) 0.315 | 1.228 (0.672–2.244) 0.503 |
| BCLC stage | 1.001 (0.557–1.797) 0.999 | 0.869 (0.450–1.680) 0.677 |
| Low (< 0.4) | 2.749 (1.525–4.955) 0.001 | 2.631 (1.448–4.780) 0.002 | 19.322 (4.444–84.006) <0.001 | 18.399 (4.007–84.483) <0.001 |

HR, hazard ratio; CI, confidence interval; HBV, hepatitis B virus; NBNC, HBsAg-negative and anti-hepatitis C virus-negative; HCV, hepatitis C virus; AFP, alpha-fetoprotein; DCP, Des-gamma carboxyprothrombin; BCLC, barcelona clinic liver cancer.
marker panel groups had similar clinical outcomes regarding OS ($p = 0.140$, supplemental Fig. S5A) and progression of HCC ($p = 0.869$, supplemental Fig. S5B), unlike in patients who were given sorafenib. Thus, we concluded that the triple-marker panel was a predictor of the response to sorafenib and a prognostic factor in patients with advanced HCC—not a general prognostic factor of HCC.

**DISCUSSION**

The principal findings of this study relate to the prediction of a good response to sorafenib, based on a quantitative proteomics approach. Specifically, our triple-marker panel (CD5L + IGJ + LGALS3BP) was an independent risk factor of poor survival and rapid progression after sorafenib treatment. This association was independent of other well-known prognostic factors, such as Child-Pugh score, and was maintained in the subgroup analysis of Child-Pugh class A patients. In addition, the prognostic value of the triple-marker panel regarding OS and TTP was sustained in the subsequent validation set. This work is the first study to report the prognostic value of quantitative proteomics in HCC patients who have been treated with sorafenib.

Sorafenib is the first targeted agent to improve overall survival in unresectable HCC, rendering it the new standard for first-line treatments (31). However, several problems with sorafenib therapy have been reported. Despite the significant improvement in survival, the efficacy of sorafenib against HCC is modest, with objective tumor response rates as low as 2% to 3% (16). Further, like other targeted therapies, sorafenib is associated with a higher incidence of severe adverse events, such as liver failure, bleeding, and tumor lysis syndrome. Sorafenib therapy is also costly, but the median overall survival benefit is merely ~3 months. For these reasons, the development of a method for evaluating the therapeutic response early during the course of treatment is essential to maximize the clinical benefits of sorafenib (32). Early functional evaluation of the therapeutic response is paramount, because it identifies patients who should be treated, sparing unnecessary toxicity and costs. If molecular biomarkers can be used to predict the efficacy of sorafenib during the early stages of therapy, unnecessary treatment can be avoided, which would undoubtedly have a significant effect in reducing the physiological distress and financial burden of patients.

We performed MRM-MS quantitative proteomics, allowing us to determine broad, systematic changes in the proteome that were associated with the acquisition of sorafenib resistance. We established a multiple marker panel that comprised 3 proteins (CD5L + IGJ + LGALS3BP) with fair robustness (AUROC > 0.950) in differentiating PD from non-PD—high levels of the triple-marker panel were significantly associated with good treatment efficacy in advanced HCC patients who were treated with sorafenib. Moreover, this panel correlated independently with poorer survival and more rapid progression in sorafenib-treated HCC patients. Our report is the first study to identify a triplet of serum proteins that predicts the response to sorafenib and clinical outcomes.

Although sorafenib inhibits several tyrosine kinases, recent studies have suggested that sorafenib has immune-modulatory functions that elicit antitumor responses (33). In this context, it might be possible to predict sorafenib responsiveness, based on the 3 markers that we identified (CD5L, IGJ, and LGALS3BP), which regulate immune responses (34–36). CD5L promotes antitumor immune activity by lowering the regulatory T cell population and increasing natural killer cells in melanoma tumor-bearing mice (34). Transcription of IGJ in the lungs decreases substantially during tumorigenesis, which can be explained by the immunosuppressive effects of the tumor cells (35). In the immune system, LGALS3BP stimulates natural killer cells and lymphokine-activated killer cells, mediated in part by the induction of IL-2 (36). Thus, we postulate that our immune-related triple-marker set has prognostic value in sorafenib-treated patients. Consequently, up-regulation of immune-modulating proteins might augment the anti-tumor immune responses of sorafenib.

Our study has several limitations. First, it was conducted at a single institution, and an external validation could not be performed. Second, the study included primarily HBV-related HCC and thus might lack a representative sample of all HCCs in chronic liver diseases (often related to alcohol consumption or metabolic syndrome). Additional large-scale validation studies with external cohorts that include nonviral etiologies of chronic liver diseases are warranted to confirm our results. Third, the number of patients was small. Although the characteristics of the patients in the training and validation sets were similar, the clinical factors that affected OS differed significantly by univariate analysis. Several studies have reported that some of our markers are poor prognostic factors in cancers other than HCC (37–39), necessitating additional functional studies to determine the mechanisms of these immune-related markers in sorafenib-treated patients with HCC.

However, these results demonstrate a novel function of our triple-marker panel in sorafenib resistance that might have therapeutic value in detecting sorafenib-resistant HCCs. We believe that our findings will provide additional insight into the mechanisms of the sensitivity and resistance to sorafenib in HCC patients, which, in turn, can help discover therapeutic targets and biomarkers that facilitate patient stratification for optimal therapy. The introduction of new and efficient biomarkers can enable the early assessment of patient responses to sorafenib, reducing unnecessary costs and adverse events and improving outcomes (40).

* This work was supported by the Multi-omics Research Program, a National Research Foundation grant (No. 2011-0030740), the Industrial Strategic Technology Development Program (#10045352), and the Ministry of Science and ICT of the Republic of Korea (National Research Foundation, grant No. 2019R1A2C1005634).
Prediction of Response to Sorafenib on Advanced HCC

the Korea Health Technology R&D Project (No. H14C2640), and grants from the SNUH Research Fund (No. 04-2013-0830) and the Liver Research Foundation of Korea.

This article contains supplemental material.

**To whom correspondence should be addressed: Dr. Youngsoo Kim; Tel.: 82-2-740-8073; Fax: 82-2-741-0253; E-mail: biolab@snu.ac.kr or Dr. Jung-Hwan Yoon; Tel.: 82-2-2072-2228; Fax: 82-2-743-6701; E-mail: yoonjh@snu.ac.kr.**

**REFERENCES**

1. Dhankasakaran, R., Limaye, A., and Cabrera, R. (2012) Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis, and therapies. Hepat. Med. 4, 19–37

2. Waly Raphael, S., Yangde, Z., and Yuxiang, C. (2012) Hepatocellular carcinoma: focus on different aspects of management. ISRN Oncol. 2012, Article ID 421673, DOI 10.5402/2012/421673

3. He, A. R., and Goldenberg, A. S. (2013) Treating hepatocellular carcinoma using cytokines for prediction of outcomes in patients with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol. 10, 25–34

4. Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Bianchini, F., de Oliveira, A. C., Santoro, A., Balsano, L., Forner, A., Schwartz, M., Porta, C., Zeuzem, S., Bolondi, L., Xu, J., Sun, Y., Liang, H., Liu, J., Wang, J., Tak, W. Y., Pan, H., Burock, K., Zou, J., Vlckovit, D., and Guan, Z. (2009) Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol. 10, 25–34

5. Llovet, J. M., Pena, C. E., Lathia, C. D., Shan, M., Meinhardt, G., Bruix, J., and SHARP Investigators Study Group. (2008) Sorafenib in advanced hepatocellular carcinoma. N. Engl. J. Med. 359, 379–390

6. Abou-Alfa, G., K., Schwartz, L., Ricci, S., Amadori, D., Santoro, A., Figuer, A., De Greve, J., Douillard, J. Y., Lathia, C., Schwartz, B., Taylor, I., Moschov, M., and Saitz, L. B. (2006) Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. J. Clin. Oncol. 24, 4293–4300

7. Llovet, J. M., Pena, C. E., Lathia, C. D., Shan, M., Meinhardt, G., Bruix, J., and SHARP Investigators Study Group. (2012) Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. Clin. Cancer Res. 18, 2390–2300

8. Villanueva, A., Hernandez-Gea, V., and Llovet, J. M. (2013) Medical therapies for hepatocellular carcinoma: a critical view of the evidence. Nat. Rev. Gastroenterol. Hepatol. 10, 34–42

9. Faloppi, L., Scartozzi, M., Bianconi, M., Svegliati Baroni, G., Toniutto, P., Tsuchiya, K., Asahina, Y., Matsuda, S., Muraoka, M., Nakata, T., Suzuki, S., and Takeshima, M. (2013) Tumor microarray-to-microarray transfer of reagents by swapping of two chips for cross-reactivity-free multiplex immunoassays. Anal. Chem. 84, 4776–4783

10. Pla-Roca, M., Leulmi, R. F., Tourekhanova, S., Bergeron, S., Laforte, V., Moreau, E., Gosline, S. J., Bertos, N., Hallett, M., Park, M., and Juncker, D. (2012) Antibody colocalization microarray: a scalable technology for multiplex protein analysis in complex samples. Mol. Cell. Proteomics 11, 11110111460

11. Wheelock, C. E., Goss, V. M., Balgoma, D., Brandsma, J., Skipp, P. J., Snowden, S., Burg, D., D’Amico, A., Horvat, I., Chaibonchoe, A., Ahmed, H., Ballereau, S., Rossios, C., Chung, K. F., Montuschki, P., Fowler, S. J., Adcock, I. M., Postle, A. D., Dahlen, S. E., Rowe, A., Sterk, P. J., Auffray, C., Djukovics, R., and U-BIOPRED Study Group. (2013) Application of ‘omics technologies to biomarker discovery in inflammatory lung diseases. Eur. Respir. J. 42, 802–825

12. Gillette, M. A., and Carr, S. A. (2013) Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. Nat. Methods 10, 28–34

13. Whiteaker, J. R., Lin, C., Kennedy, J., Hou, L., Trute, M., Sokal, I., Yan, P., Schoenher, R. M., Zhao, L., Voytovich, U. J., Kelly-Spratt, K. S., Krasnoselsky, A., Galfken, P. R., Hogan, J. M., Jones, L. A., Wang, P., Amon, L., Chodosh, L. A., Nelson, P. S., McIntosh, M. W., Kemp, C. J., and Paulovitch, A. G. (2011) A targeted proteomics-based pipeline for verification of biomarkers in plasma. Nat. Biotechnol. 29, 625–634

14. Kennedy, J. J., Abbatelli, S. E., Kim, K., Yan, P., Whiteaker, J. R., Lin, C., Kim, J., Zhang, Y., Wang, X., Ivey, R. G., Zhao, L., Min, H., Lee, Y., Yu, M. H., Yang, E. G., Lee, C., Wang, P., Rodriguez, H., Kim, Y., Carr, S. A., and Paulovitch, A. G. (2014) Demonstrating the feasibility of large-scale development of standardized assays to quantify human proteins. Nat. Methods 11, 149–155

15. Tuck, M. K., Chan, D. W., Chia, D., Godwin, A. K., Grizzle, W. E., Krueger, K. E., Rom, W., Sanda, M., Sorbara, L., Stass, S., Wang, W., and Brenner, D. E. (2009) Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. J. Proteome Res. 8, 113–119

16. Bruix, J., Sherman, M., and American Association for the Study of Liver Disease (2011) Management of hepatocellular carcinoma: an update. Hepatology 53, 1020–1022

17. Ronot, M., Bouattour, M., Wassermann, J., Bruno, O., Dreyer, C., Larroque, B., Castera, L., Vilgrain, V., Belghiti, J., Raymond, E., and Faire, S. (2014) Alternative Response Criteria (Choi, European association for the study of the liver, and modified Response Evaluation Criteria in Solid Tumors [RECIST]) Versus RECIST 1.1 in patients with advanced hepatocellular carcinoma treated with sorafenib. Oncologist 19, 394–402

18. McShane, L. M., Altman, D. G., Sauerbrei, W., Taube, S. E., Gion, M., Clark, G. M., and Statistics Subcommittee of the, Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics. (2005) Reporting recommendations for tumor marker prognostic studies (REMARK). J. Natl. Cancer Inst. 97, 1180–1184

**14. Takeda, H., Nishikawa, H., Iguchi, E., Ohara, Y., Sakamoto, A., Hatanaru, K., Henni, S., Saito, S., Nasu, A., Komakeido, H., Kita, R., Kimura, T., and Osaki, Y. (2013) Impact of pretreatment serum cholinesterase level in unresectable advanced hepatocellular carcinoma patients treated with sorafenib. Mol. Clin. Oncol. 1, 241–248**
28. Therasse, P., Arbuck, S. G., Eisenhauer, E. A., Wanders, J., Kaplan, R. S., Rubinstein, L., Verweij, J., Van Glabbeke, M., van Oosterom, A. T., Christian, M. C., and Gwyther, S. G. (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J. Natl. Cancer Inst. 92, 205–216

29. Zhang, Y., Yang, C., Wang, S., Chen, T., Li, M., Wang, X., Li, D., Wang, K., Ma, J., Wu, S., Zhang, X., Zhu, Y., Wu, J., and He, F. (2013) LiverAtlas: a unique integrated knowledge database for systems-level research of liver and hepatic disease. Liver Int. 33, 1239–1248

30. Pan, S., Chen, R., Brand, R. E., Hawley, S., Tamura, Y., Gafken, P. R., Milless, B. P., Goodlett, D. R., Ruah, J., and Brentnall, T. A. (2012) Multiplex targeted proteomic assay for biomarker detection in plasma: a pancreatic cancer biomarker case study. J. Proteome Res. 11, 1937–1948

31. Blivet-Van Eggelpoel, M. J., Chettouh, H., Fartoux, L., Aoudjehane, L., Barbu, V., Rey, C., Priam, S., Housset, C., Rosmorduc, O., and Desbois-Mouthon, C. (2012) Epidermal growth factor receptor and HER-3 restrict cell response to sorafenib in hepatocellular carcinoma cells. J. Hepatol. 57, 108–115

32. Sugimoto, K., Moriyasu, F., Saito, K., Rognin, N., Kamiyama, N., Furuki, Y., and Imai, Y. (2013) Hepatocellular carcinoma treated with sorafenib: early detection of treatment response and major adverse events by contrast-enhanced US. Liver Int. 33, 605–615

33. Cabrera, R., Ararat, M., Xu, Y., Brusko, T., Wasserfall, C., Atkinson, M. A., Chang, L. J., Liu, C., and Nelson, D. R. (2013) Immune modulation of effector CD4+ and regulatory T cell function by sorafenib in patients with hepatocellular carcinoma. Cancer Immunol. Immunother. 62, 737–748

34. Fenutria, R., Martinez, V. G., Simoes, I., Postigo, J., Gil, V., Martinez-Florensá, M., Sintes, J., Naves, R., Cashman, K. S., Alberola-Ila, J., Ramos-Casais, M., Soldevila, G., Raman, C., Merino, J., Merino, R., Engel, P., and Lozano, F. (2014) Transgenic expression of soluble human CD5 enhances experimentally-induced autoimmune and anti-tumoral immune responses. PLoS ONE 9, e84895

35. Slizhikova, D. K., Zinov’eva, M. V., Kuz’mín, D. V., Snezhkov, E. B., Shakhproronov, M. I., Dmitriev, R. I., Antipova, N. V., Zavalova, L. L., and Sverdlov, E. D. (2007) [Decrease in expression of human J-chain in lung squamous cell cancer and adenocarcinoma]. Mol. Biol. 41, 659–665

36. Chen, R., Tan, Y., Wang, M., Wang, F., Yao, Z., Dong, L., Ye, M., Wang, H., and Zou, H. (2011) Development of glycoprotein capture-based label-free method for the high-throughput screening of differential glycoproteins in hepatocellular carcinoma. Mol. Cell. Proteomics 10, M110.006445

37. Marchetti, A., Tinari, N., Buttitta, F., Chella, A., Angeletti, C. A., Sacco, R., Mucilli, F., Ullrich, A., and Iacobelli, S. (2002) Expression of 90K (Mac-2 BP) correlates with distant metastasis and predicts survival in stage I non-small cell lung cancer patients. Cancer Res. 62, 2535–2539

38. Iacobelli, S., Sismondi, P., Giai, M., D’Egidio, M., Tinari, N., Amatetti, C., Di Stefano, P., and Natali, C. (1994) Prognostic value of a novel circulating serum 90K antigen in breast cancer. Br. J. Cancer 69, 172–176

39. Zeimet, A. G., Natali, C., Herold, M., Fuchs, D., Windbichler, G., Daxenbichler, G., Iacobelli, S., Sismondi, P., Dapunt, O., and Marth, C. (1996) Circulating immunostimulatory protein 90K and soluble interleukin-2-receptor in human ovarian cancer. Int. J. Cancer 68, 34–38

40. Sacco, R., Mismas, V., Romano, A., Bertini, M., Bertoni, M., Federici, G., Metrangolo, S., Parisi, G., Tumino, E., Bresci, G., Giacomelli, L., Marceglia, S., and Bargellini, I. (2015) Assessment of clinical and radiological response to sorafenib in hepatocellular carcinoma patients. World J. Hepatol. 7, 33–39