Featured Article

Molecular Prediction of Response to 5-Fluorouracil and Interferon-α Combination Chemotherapy in Advanced Hepatocellular Carcinoma

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ABSTRACT

Purpose: **The prognosis of hepatocellular carcinoma (HCC) is very poor, particularly in patients with tumors that have invaded the major branches of the portal vein. Combination chemotherapy with intra-arterial 5-fluorouracil and subcutaneous interferon-α has shown promising results for such advanced HCC, but it is important to develop the ability to accurately predict chemotherapeutic responses.**

Experimental Design: **We analyzed the expression of 3,080 genes using a polymerase chain reaction-based array in 20 HCC patients who were treated with combination chemotherapy after reduction surgery. After unsupervised analyses, a supervised classification method for predicting chemotherapeutic responses was constructed. To minimize the number of predictive genes, we used a random permutation test to select only significant ($P < 0.01$) genes. A leave-one-out cross-validation confirmed the gene selection. We also prepared an additional 11 cases for validation of predictive performance.**

Results: **Hierarchical clustering analysis and principal component analysis with all 3,080 genes revealed distinct gene expression patterns in responders (those with complete response or partial response) and nonresponders (those with stable disease or progressive disease) to the combination chemotherapy. Using a weighted-voting classification method with either all genes or only significant genes as assessed by permutation testing, the objective responses to treatment were correctly predicted in 17 of 20 cases (accuracy, 85%; positive predictive value, 100%; negative predictive value, 80%). Moreover, patients in the validation dataset could be classified into two distinct prognostic groups using 63 predictive genes.**

Conclusions: **Molecular analysis of 63 genes can predict the response of patients with advanced HCC and major portal vein tumor thrombi to combination chemotherapy with 5-fluorouracil and interferon-α.**

INTRODUCTION

Hepatocellular carcinoma (HCC), the predominant histologic subtype of primary liver cancer, is one of the major causes of death from malignancy worldwide. Although recent progress in both diagnostic and surgical techniques has resulted in considerable improvement in the morbidity and mortality rates, the overall outcome remains far from satisfactory. The prognosis is miserable, particularly in patients with tumors that have invaded the major branch of the portal vein, and the median survival time is several months (1–3). Indeed, the 1-year survival rate was <50% even in resectable cases (3, 4). We and others have recently developed a new treatment regimen for such advanced HCC patients: combination chemotherapy of 5-fluorouracil (5-FU) and interferon (IFN)-α (5, 6). In follow-up studies, about 40% to 50% of patients showed promising responses to the therapy and lived >2 years. However, for patients who did not respond to this treatment, survival was too short to receive another type of treatment. Furthermore, this chemotherapy may have multiple adverse effects, including leukopenia, thrombocytopenia, and depression. Therefore, accurate prediction of chemosensitivity is desirable, not only so that nonresponding patients do not lose a limited chance to take advantage of other possible treatments, but also to eliminate suffering of these patients due to debilitating side effects. Unfortunately, there are currently no useful indicators to distinguish between patients who are likely to respond to this combination chemotherapy and patients who are not.

In this study, genes possessing the ability to predict patient responses to 5-FU and IFN-α combination chemotherapy were selected by gene expression profile analysis using adaptor-tagged competitive polymerase chain reaction (ATAC-PCR) technology, a polymerase chain reaction (PCR)-based array system (7). The

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performance of this prediction method was estimated with both a complete cross-validation and an external validation dataset.

MATERIALS AND METHODS

Tissues and Patients. Between August 1998 and January 2003, 20 HCC patients with multiple tumors spreading to bilateral lobes with tumor thrombi in the major branches of the portal vein underwent palliative surgery to reopen the portal flow and recover liver function. After obtaining informed consent, we collected HCC tissue from the main resected tumors and isolated the total RNA from these samples for PCR-based array experiments. All 20 patients had visible tumors in the remaining liver. The patients were then subjected to a treatment regimen of combination chemotherapy with 5-FU and IFN-α as described previously (5). Briefly, patients received continuous arterial infusion of 5-FU (450–500 mg/d) for the initial 2 weeks and subcutaneous IFN-α injection (5 million IU) 3 times per week for 4 weeks. The sizes of the remaining tumors in the liver, which are thought to be intrahepatic metastases from the resected primary tumor, were measured before and after treatment by either abdominal computed tomography scan or ultrasonography. The chemotherapeutic response was clinically evaluated according to the Eastern Cooperative Oncology Group (ECOG) criteria (8). In this study, responders were defined as patients with complete response (CR) or partial response (PR); nonresponders were defined as patients with stable disease or progressive disease (PD).

Furthermore, as an independent validation dataset, we collected liver tissue specimens and clinical data from 11 HCC patients who underwent hepatic resection of all visible tumors between October 1998 and October 2002. Although these patients had tumor(s) with major portal vein tumor thrombi, the area of spreading tumor(s) was limited to two segments of the liver. The treatment regimen of combination chemotherapy and the method of follow-up in the validation dataset were the same as the procedures used in the original dataset. All aspects of our study protocol were approved by the ethics committee of Osaka University Medical School.

Polymerase Chain Reaction-Based Array System. To select genes expressed in liver tissues, we constructed three cDNA libraries: one from a mixture of HCC and nontumorous livers, one from normal livers, and one from metastatic liver cancers, as described previously (9). We designed PCR primers for ATAC-PCR reactions for a total of 2,666 genes from these expressed sequence tag collections. In total, we prepared 3,080 primers for ATAC-PCR; this total includes an additional 414 genes established in previous literature. The specificity of this gene selection provides an advantage over more universal gene sets, such as those selected from the UniGene database, which include genes not expressed in liver tissues. The ATAC-PCR experimental procedure was performed as described previously (10). The complete list of genes and detailed protocols for the ATAC-PCR experimental procedure are available on our web site.[4]

Analysis of Polymerase Chain Reaction-Based Array Data. The relative expression level of each gene was calculated by calibration using a standard mixture of normal liver tissues, as described previously (11–15). After conversion to a logarithmic scale (base 2), the data matrix was normalized to a median of 0 by standardizing each sample.

As a supervised classification method, we adopted a weighted-voting (WV) algorithm generally used in gene expression profiling (16–19). Briefly, we calculated the signal to noise (S2N) ratio, $S_i = (\mu_R - \mu_N)/(\sigma_R + \sigma_N)$, where μ and σ represent the mean and SD of expression for responders and nonresponders, respectively. The magnitude of the gene vote (v_i) reflects the deviation of the test sample X_i value from the average of the two groups: $v_i = S_i \times (X_i - (\mu_R + \mu_N)/2)$. We summed the v_i values to obtain the total votes for a "good signature" group (V_G) and a "poor signature" group (V_P). The prediction strength is $(V_G - |V_P|)/(V_G + |V_P|)$, and we adopted a threshold of 0. If the strength is a positive number, the test sample belongs to the good signature group. If the strength is a negative number, the test sample belongs to the poor signature group. This model was evaluated by leave-one-out cross-validation (16–21), wherein one sample was randomly withheld, and the model was regenerated with the remaining samples and then used to predict the group of the withheld sample. This process was repeated until every sample was tested, and the cumulative accuracy was recorded. When an external validation dataset was used to evaluate this WV model, we used the S2N ratio (S_i) obtained from the original dataset. In calculating the weighted vote (v_i), the mean expression level of the entire validation dataset was substituted for the average of the two groups ($(\mu_R + \mu_N)/2$) in the original dataset.

Permutation testing, which involves randomly permuting group labels to determine gene-group correlations, was used to determine statistical significance (16–19). The original score, $So = |\mu_R - \mu_N|$, of each gene was calculated without permuting the labels (responder or nonresponder). The labels of all of the samples were then randomly permuted, and the scores were recalculated between two groups consisting of the new members. Repetition of this permutation 50,000 times provides a score number (No) larger than the original score (So). For each gene, the permutation P value was determined by $P = No/50{,}000$. To estimate the false discovery rate, which is the percentage of genes identified by chance (22), we calculated the Q value of every gene using downloaded software.[5] The Q value provides a measure of each feature's significance, automatically taking into account the problem of multiple testing (23).

Hierarchical cluster analysis using an unweighted pair group method using arithmetic mean method with Pearson's correlation and principal component analysis (PCA) was performed using GeneMaths 2.0 software. Other statistical analyses were performed using StatView 5.0J software. Correlations between the responses to our combination chemotherapy and clinicopathological parameters were evaluated by the χ^2 test with the Yates correction. Overall and disease-free survival rates were calculated using the Kaplan-Meier method. Differences in survival curves were estimated by the log-rank test.

[4] http://love2.aist-nara.ac.jp/laboratory/index_frame.html.

[5] http://faculty.washington.edu/~jstorey/qvalue.

Table 1 Characteristics of 20 patients

Case no.	Age (y)/sex	HBs Ag	HCV Ab	Child grade	Cirrhosis	AFP*	Edmondson classification	Main tumor size (cm)	Cycles of chemotherapy	Response†
1	74/M	−	+	A	Present	Low	3	3.0	3	CR
2	67/F	−	+	A	Present	High	3	9.2	3	CR
3	49/F	+	−	A	Absent	High	4	6.8	3	PD
4	47/M	+	−	A	Present	High	3	12.0	3	PD
5	51/F	−	−	B	Absent	High	3	15.0	3	PR
6	56/M	−	−	B	Present	Low	2	0.8	3	PD
7	69/M	−	+	A	Absent	High	3	8.0	3	CR
8	66/M	+	+	A	Present	High	3	4.0	3	CR
9	65/M	−	+	B	Present	High	3	1.3	2	PD
10	53/M	+	−	A	Present	High	3	17.0	3	PD
11	52/M	+	−	B	Absent	High	3	18.0	3	CR
12	66/M	−	−	B	Present	High	3	3.5	1	PD
13	39/M	+	−	B	Present	High	3	4.5	1	PD
14	56/M	−	+	A	Present	High	3	5.5	3	SD
15	67/M	−	+	A	Present	High	3	3.2	2	PD
16	47/M	+	−	A	Absent	High	3	13.0	3	PD
17	70/M	−	+	A	Absent	Low	3	9.4	3	CR
18	70/M	−	+	A	Absent	High	3	9.8	3	CR
19	71/M	−	+	A	Absent	High	3	9.0	3	PD
20	29/M	+	−	A	Absent	High	3	18.0	2	PD

Abbreviations: HBs Ag, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; SD, stable disease.
* Divided into either low (≤50 ng/mL) or high (>50 ng/mL).
† Assessment based on ECOG criteria.

RESULTS

Patient Characteristics. The characteristics of the 20 HCC patients in the original dataset are shown in Table 1. All patients had multiple tumors spreading to bilateral lobes with tumor thrombi in the major branches of the portal vein and were treated with 5-FU and IFN-α combination chemotherapy after reduction surgery. Fifteen of the 20 patients were treated with three cycles of the above-mentioned regimen. The remaining five patients received only one or two cycles of 5-FU due to extensive progression of the tumors. According to the ECOG criteria of objective response, 8 patients (40%) were classified as responders, demonstrating either CR or PR for at least 4 weeks. The remaining 12 patients (60%) were classified as nonresponders, exhibiting either stable disease or PD. The clinicopathological characteristics of responders (CR and PR) and nonresponders (stable disease and PD) were compared by the χ^2 test (Table 2). There were no significant differences ($P < 0.05$) in any factor between the two response groups.

Unsupervised Analyses. We performed a hierarchical cluster analysis of the samples using all 3,080 genes. When the clinical samples were sorted on the basis of similarity in gene expression, the samples could be separated according to the response to 5-FU and IFN-α combination chemotherapy, *i.e.*, responders and nonresponders, with only a few exceptions (Fig. 1). Next, we applied PCA, a statistical method for reducing the number of data dimensions, to more simply present the relationships between the samples. On displaying the expression patterns of all 3,080 genes in three-dimensional space, we observed that most responders and nonresponders were located separately, indicating distinct gene expression patterns (Fig. 2).

Supervised Analyses. To construct a molecular prediction system, we used a supervised approach, a WV algorithm using S2N correlation ratios. The ability of this method to

Table 2 Clinicopathological characteristics between responders and nonresponders

Characteristics	Responders ($n = 8$)	Nonresponders ($n = 12$)	P
Age (y)			>0.05
<65	2	8	
≥65	6	4	
Sex			>0.05
Male	6	11	
Female	2	1	
HBs Ag			>0.05
Negative	6	6	
Positive	2	6	
HCV Ab			>0.05
Negative	2	8	
Positive	6	4	
Child grade			>0.05
A	6	8	
B	2	4	
Liver cirrhosis			>0.05
Absent	5	4	
Present	3	8	
AFP (ng/mL)			>0.05
≤ 50	2	1	
>50	6	11	
Histological grade			>0.05
Moderate	0	1	
Poor	8	10	
Undiff	0	1	
Tumor size (cm)			>0.05
≤5	2	5	
>5	6	7	

Abbreviations: Moderate, moderately differentiated hepatocellular carcinoma; Poor, poorly differentiated hepatocellular carcinoma; Undiff, undifferentiated carcinoma.

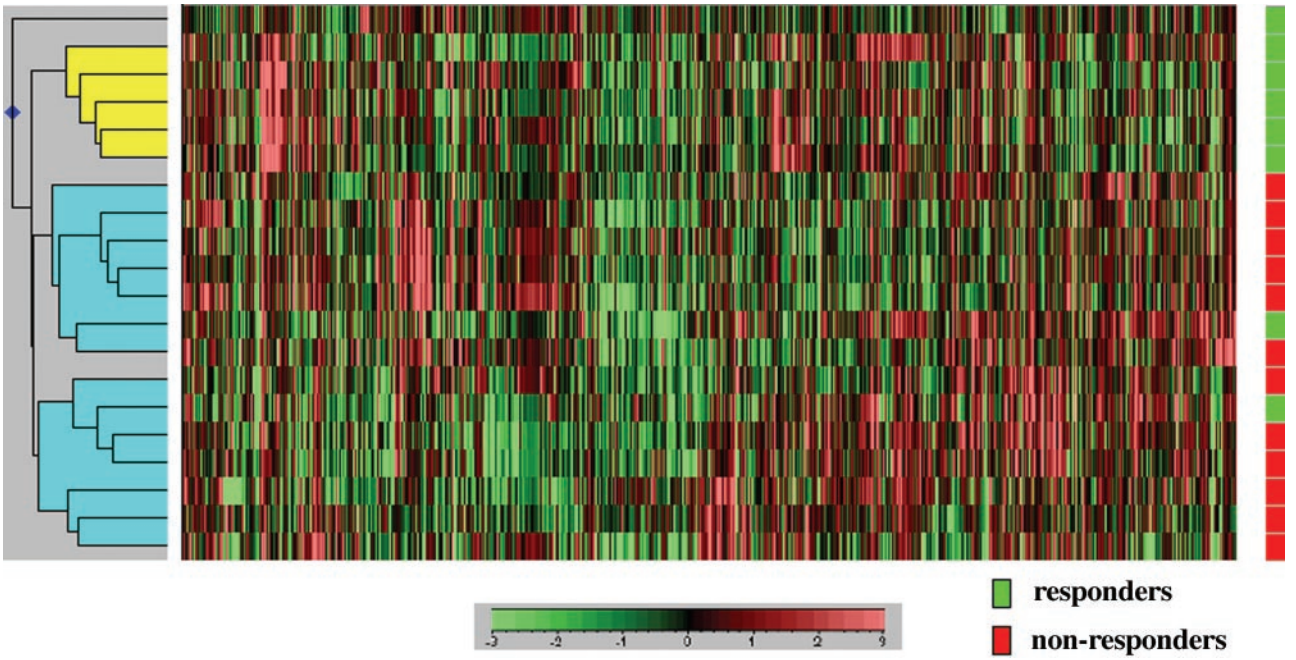

Fig. 1 Hierarchical clustering of 3,080 genes in 20 HCC cases. Each *column* represents a single gene, and each *row* represents a patient sample. The tissue type of each sample is designated by a *bar* at the *right*, and *green* and *red bars* indicate responders and nonresponders, respectively. The *color scale* at the *bottom* indicates the relative expression levels in terms of SDs from the mean.

predict patient responses to 5-FU and IFN-α combination chemotherapy was evaluated by leave-one-out cross-validation. In this first cross-validation analysis, we used all 3,080 genes to avoid any selection bias (Fig. 3*A*). The prediction accuracy of this WV method was 85.0%, and its 95% confidence interval ranged from 66.0% to 95.9%. The positive and negative predictive values were 100.0% and 80.0%, respectively.

These results demonstrate that this method provides a

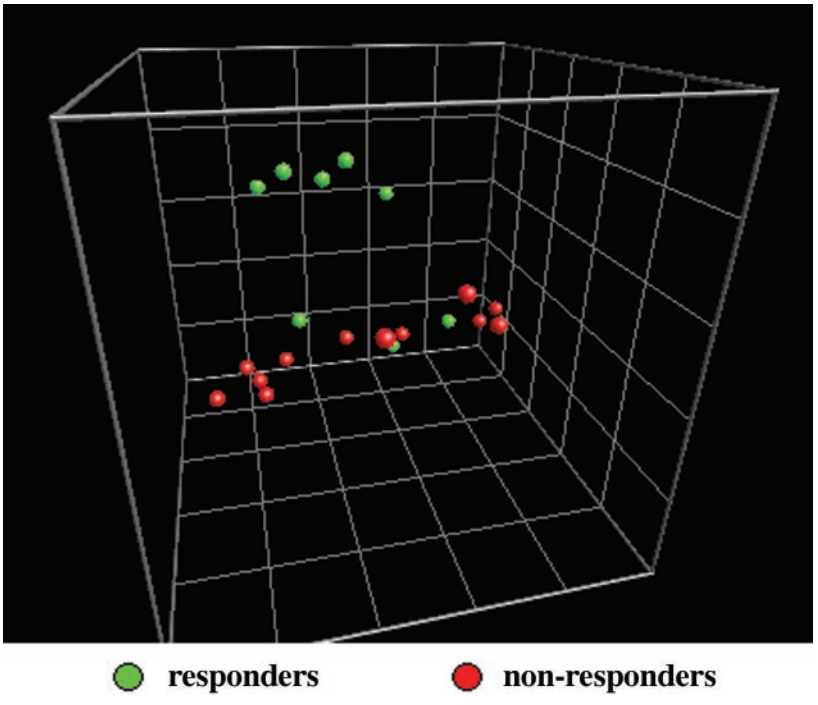

Fig. 2 PCA in 20 HCC cases. The variation is reduced to three-dimensional space; three components represent 37.0% of the total variance. Each *sphere* represents a single sample: *green* for a responder and *red* for a nonresponder.

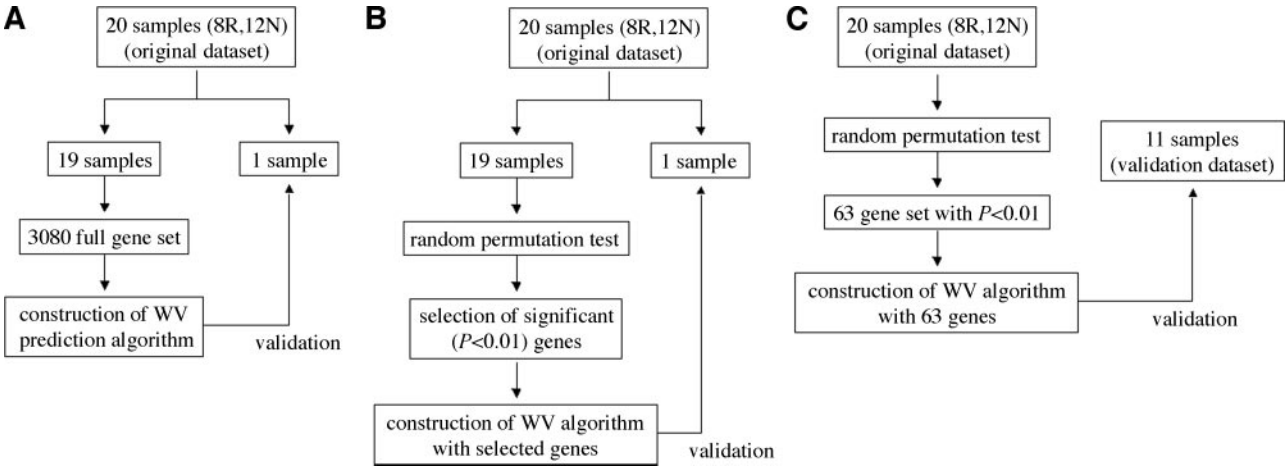

Fig. 3 Flow chart for selecting samples and gene set for analysis. *A*, the first approach, which used the original dataset and all genes. *B*, the second approach, which used the original dataset and only significant genes ($P < 0.01$). *C*, the third approach, which used the validation dataset and 63 significant genes selected with the original dataset.

valuable prediction of the chemotherapeutic response. To determine whether all 3,080 genes are necessary for prediction, we compared the prediction accuracy of a small number of genes to the prediction accuracy of all 3,080 genes. When the predictive genes were defined as genes with significant *P* values ($P < 0.01$) by the random permutation tests (Fig. 3*B*), the complete cross-validation without information leakage showed an identical performance with the initial cross-validation using all 3,080 genes. We therefore selected only 63 genes with significant *P* values between the 8 responder patients and the 12 nonresponder patients (Table 3). The median of their Q values, which is a measure in terms of the false discovery rate, was <25%. The expression patterns of these 63 genes exhibited distinct profiles between the two groups (Fig. 4).

To further evaluate our prediction system, we prepared an independent dataset, which consisted of 11 HCC patients with major portal vein tumor thrombi. Although these patients had no visible tumors after resection before combination chemotherapy, the risk of recurrence within the early postoperative period due to intrahepatic micrometastasis was very high (3, 4, 24). Moreover, in these advanced HCC cases with major portal vein tumor thrombi, the survival time was strongly correlated to the chemotherapeutic response. In fact, the median survival times of responders and nonresponders in the original dataset were 28 and 7 months, respectively, and the difference was statistically significant ($P = 0.002$). We therefore performed overall survival analysis and disease-free survival analysis instead of estimation to determine the prediction accuracy of objective response. Our prediction method using 63 genes classified patients into either a good signature group (those who were predicted to have good responses to this chemotherapy) or a poor signature group (those who were predicted to have poor responses; Fig. 3*C*). The overall survival rates were significantly different between these two predicted groups ($P = 0.001$; Fig. 5*A*). In addition, the good signature group had a distinctly better prognosis for disease-free survival than the poor signature group ($P = 0.002$; Fig. 5*B*).

DISCUSSION

HCC remains a major problem worldwide and appears to be increasing in developed Western countries (25). HCC is notorious for its poor prognosis because the overall 5-year survival rate after resection has remained as poor as 35% to 50%, even if curative surgery is performed (26–28). Moreover, once the tumor invades the major branch of the portal vein, the prognosis is extremely poor (1–4). Various complications including gastrointestinal bleeding, ascites, and hemorrhage from esophageal varices are provoked by stenosis of the portal vein. Until now, there has been no standard treatment for advanced HCC with portal tumor thrombi. However, we and others have recently developed a new combination chemotherapy regimen using intra-arterial 5-FU and subcutaneous IFN-α (5, 6), which has proven to be effective in about half of such advanced HCC patients. In this study, we have demonstrated that a molecular prediction method using expression data of 63 genes was able to distinguish patients who responded to this therapy from those who did not respond.

DNA microarray technology allows parallel expression analysis of thousands of genes to address complex questions in tumor biology. Many trials predicting the prognosis of various human malignancies have been reported using DNA microarrays (29–31). Moreover, a novel prediction method using gene expression profiling has recently been reported for the treatment of breast cancer patients with the taxanes (docetaxel and paclitaxel; ref. 21). Although DNA microarrays have contributed to such expression profiling studies to some degree, DNA microarrays can detect only a fraction of the changes in gene expression detectable by reverse transcription-PCR (32). We therefore performed a high-throughput quantitative PCR based on ATAC-PCR (7) to analyze the genetic differences in HCC. This assay requires smaller amounts of RNA than DNA microarray analysis. PCR-based analysis of selected genes costs much less than DNA microarrays, which are likely to require at least several hundred spotted genes for diagnosis. The aforementioned ben-

Table 3 The list of 63 predictive genes

P	Up/down	UniGene ID	Gene symbol	Gene definition
0.0001	Down	Hs.74088	*EGR3*	Early growth response 3
0.0002	Up	Hs.432818	*MFAP3*	Microfibrillar-associated protein 3
0.0002	Down	-	EST	DNC15 *Homo sapiens* cDNA
0.0003	Up	Hs.151738	*MMP9**	Matrix metalloproteinase 9
0.0005	Down	Hs.257697	*PDCD4*	Programmed cell death 4
0.0006	Up	Hs.337534	*EST*	*H. sapiens* clone 25061 mRNA sequence
0.0009	Down	Hs.293189	*FLJ37853*	*H. sapiens* cDNA FLJ37853 fis
0.0010	Down	Hs.6838	*ARHE*	Ras homolog gene family, member E
0.0010	Down	Hs.79078	*MAD2L1*	MAD2 mitotic arrest deficient-like 1
0.0011	Down	Hs.158244	*NMNAT2*	Nicotinamide nucleotide adenylyltransferase 2
0.0012	Down	Hs.175483	*FLJ22016*	*H. sapiens* cDNA: FLJ22016 fis
0.0012	Down	Hs.511400	*SND1*	Staphylococcal nuclease domain containing 1
0.0012	Up	Hs.301242	*MIDORI*	Myocytic induction/differentiation originator
0.0013	Down	Hs.471660	*FLJ36229*	*H. sapiens* cDNA FLJ36229 fis
0.0014	Down	Hs.81874	*MGST2*	Microsomal glutathione S-transferase 2
0.0015	Down	Hs.414156	*CTPS2*	CTP synthase II
0.0015	Up	Hs.118633	*OASL*†	2′-5′-oligoadenylate synthetase-like
0.0016	Down	Hs.406266	*HK2**	Hexokinase 2
0.0017	Down	Hs.75309	*EEF2*	Eukaryotic translation elongation factor 2
0.0019	Down	Hs.121150	*Taxilin*	Taxilin
0.0019	Down	Hs.67201	*NT5C*	5′, 3′-nucleotidase, cytosolic
0.0021	Down	Hs.417369	*CLNS1A*	Chloride channel, nucleotide-sensitive, 1A
0.0023	Down	Hs.13350	*UNC5B*	Unc-5 homolog B
0.0024	Down	Hs.345908	*TSC*	Hypothetical protein FLJ20607
0.0025	Down	Hs.2007	*TNFSF6**	Tumor necrosis factor (ligand) superfamily, member 6
0.0025	Up	Hs.440899	*KIAA1691*	KIAA1691 protein
0.0025	Down	Hs.143873	*S100A10*	S100 calcium binding protein A10
0.0026	Down	Hs.272630	*ATP6V1D*	ATPase, H+ transporting, lysosomal 34 kDa, V1 subunit D
0.0027	Down	Hs.413226	*ASB4*	Ankyrin repeat and SOCS box-containing 4
0.0027	Down	Hs.226391	*AGR2*	Anterior gradient 2 homolog
0.0027	Down	Hs.3610	*KIAA0205*	KIAA0205 gene product
0.0029	Down	Hs.335118	*JJAZ1*	Joined to JAZF1
0.0031	Down	Hs.12840	*LOC285859*	Hypothetical protein LOC285859
0.0031	Down	Hs.82324	*KIAA0157*	KIAA0157 protein
0.0031	Up	Hs.421825	*PPA2*	Inorganic pyrophosphatase 2
0.0036	Up	Hs.143773	*eIF3k*	Eukaryotic translation initiation factor 3 subunit k
0.0036	Up	Hs.380877	*SGKL*	Serum/glucocorticoid regulated kinase-like
0.0038	Down	Hs.387183	*BLMH*	Bleomycin hydrolase
0.0038	Up	Hs.114648	*ERG-1*	Estrogen-regulated gene 1
0.0042	Down	Hs.34012	*BRCA2*	Breast cancer 2, early onset
0.0048	Down	Hs.512628	*PPP2R2A*	Protein phosphatase 2, regulatory subunit B, α isoform
0.0051	Up	Hs.377975	*IGKC*	Immunoglobulin κ constant
0.0053	Down	Hs.435765	*ENPEP*†	Glutamyl aminopeptidase
0.0055	Down	Hs.356076	*BIRC4*†	Baculoviral IAP repeat-containing 4
0.0055	Down	Hs.220745	*FLJ10244*	Ral-A exchange factor RalGPS2
0.0060	Down	Hs.376531	*LOC283167*	*H. sapiens* LOC283167
0.0061	Down	Hs.128410	*GLS*	Glutaminase
0.0061	Down	Hs.356876	*GPR125*	G protein-coupled receptor 125
0.0065	Down	Hs.250696	*KDELR3*	KDEL endoplasmic reticulum protein retention receptor 3
0.0066	Down	Hs.10949	*ERO1L*	ERO1-like
0.0068	Down	Hs.380446	*LENG8*	Leukocyte receptor cluster member 8
0.0070	Up	Hs.22129	*DJ1042K10.2*	Hypothetical protein DJ1042K10.2
0.0070	Down	Hs.93177	*IFNB1*	Interferon β1, fibroblast
0.0072	Down	Hs.389461	*SMAP-1*	Smooth muscle cell-associated protein 1
0.0074	Up	Hs.512654	*SET07*	PR/SET domain containing protein 07
0.0080	Down	Hs.432574	*POLR2H*	Polymerase II polypeptide H
0.0080	Down	Hs.183986	*PVRL2*	Poliovirus receptor-related 2
0.0088	Down	Hs.406351	*EST*	*H. sapiens* clone IMAGE:120162 mRNA sequence
0.0089	Down	Hs.2533	*ALDH9A1*	Aldehyde dehydrogenase 9 family, member A1
0.0090	Down	Hs.282410	*CALM1*†	Calmodulin 1
0.0091	Down	Hs.37288	*NR1D2*	Nuclear receptor subfamily 1, group D, member 2
0.0096	Down	Hs.11355	*TMPO*	Thymopoietin
0.0098	Up	Hs.299208	*COQ3*	Coenzyme Q3 homolog, methyltransferase

NOTE. *P* values were calculated by random permutation test. Up-regulation and down-regulation were defined as expression in responders compared with nonresponders.

* Genes previously reported to be associated with sensitivity to 5-FU.

† Genes previously reported to be associated with sensitivity to IFN.

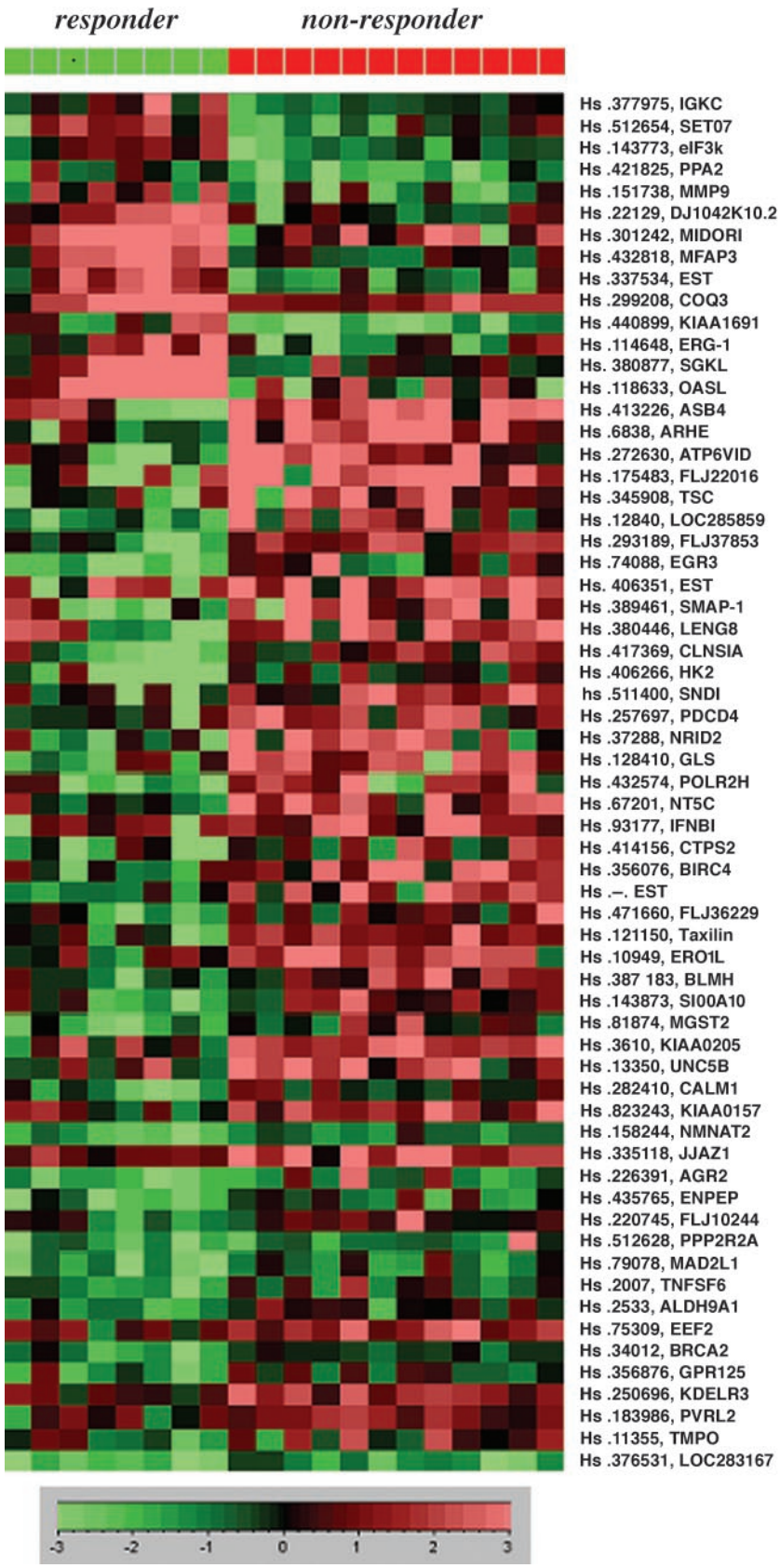

Fig. 4 The expression pattern of the 63 predictive genes in 20 HCC cases. Each *column* represents a patient sample, and each *row* represents a single gene.

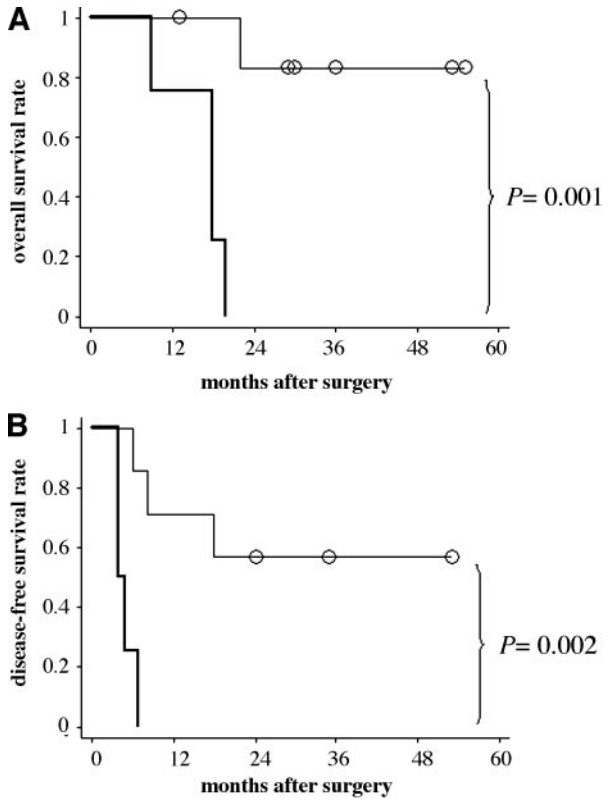

Fig. 5 Overall survival curves (*A*) and disease-free survival curves (*B*) calculated using the Kaplan-Meier method for the validation dataset. Differences in survival curves were estimated by the log-rank test.

efits and the strength of this system for cancer research, established in previous reports on HCC, breast, and colorectal cancers make this technique a powerful means to obtain a better understanding of the molecular characteristics of cancers (11–15).

In the unsupervised analyses with all examined genes, the difference of expression patterns between responders (CR and PR) and nonresponders (stable disease and PD) was clear. Of course, it was controversial how to divide two response groups. However, the response to this regimen with 5-FU and IFN-α was usually typical; that is, PR or stable disease cases were rare, as shown in Table 1. Clinically, radiologically, and biochemically, the PR case in this study revealed a near CR, and the stable disease case was similar to PD cases. We therefore classified the PR patient as a responder and the stable disease patient as a nonresponder due to the conventional grouping method (33, 34). In supervised analyses, we selected 63 significant genes by a random permutation test to minimize the number of predictive genes. These 63 predictive genes included some genes that have previously been reported to be associated with sensitivity to 5-FU or IFN. For example, a gene related to 5-FU sensitivity is tumor necrosis factor ligand superfamily member 6 (TNFSF6), also called FAS (CD95) ligand, which plays a major role in the induction of apoptosis in response to a variety of extracellular signals, including anticancer drugs. In hepatoma cells or human breast cancer, up-regulation of TNFSF6 was associated with poor sensitivity to 5-FU chemotherapy (35, 36). Matrix metalloproteinase 9 (MMP9), a member of the secreted zinc metalloprotease group, is also inhibited by 5-FU treatment (37). Hexokinase 2 (HK2) plays an important role in intracellular glucose metabolism and is strongly associated with the metabolism of 2′-fluoro-2′-deoxy-D-mannose, which has been correlated to 5-FU response (38). A gene involved in sensitivity to IFN-α is OASL, a member of the 2′-5′-oligoadenylate synthetase gene families. OASL plays an important role in the antiviral effects of IFN and is involved in apoptosis and control of cellular growth (39, 40). Baculoviral IAP repeat-containing 4 (BIRC4; XIAP), encodes a protein that significantly inhibits apoptosis. IFN inhibited BIRC4 function by inducing XIAP associated factor-1 (XAF-1; ref. 41). Glutamyl aminopeptidase is a differentiation-related kidney glycoprotein of 160 kDa (gp160), and the absence of glutamyl aminopeptidase expression was predictive of IFN-α sensitivity in renal cell carcinoma (42). Calmodulin-1 (CALM1) plays a role in growth and the cell cycle and in signal transduction. Calmodulin inhibitors affect not only the uptake and processing of IFN inducers but also the release of IFN from induced cells (43). Our selection approach to these predictive genes was confirmed with both a complete cross-validation and an external validation dataset.

In our univariate analysis with clinicopathological factors, there was no significant indicator that distinguished responders and nonresponders. Patt *et al.* (6) reported that low levels (≤50 ng/mL) of serum α-fetoprotein (AFP) were a predictor of response to 5-FU and IFN-α combination chemotherapy, but the serum AFP level was not a significant indicator of chemotherapeutic response in our study. Because we treated only extremely advanced HCC patients with major portal vein tumor thrombi with this combination therapy, only 3 of the 20 patients showed low levels of serum AFP. It may be too early to make conclusions regarding this factor, but we believe that another appropriate method will be required to more accurately predict the response to this treatment.

Indeed, this combination chemotherapy of 5-FU and IFN-α is promising for advanced HCC, but we need to consider the side effects. IFN-α and/or 5-FU sometimes induce severe adverse effects, including myelosuppression, fever, and depression (44). In particular, adverse myelosuppression is an important factor in HCC cases not only because thrombocytopenia and/or leukopenia are frequently present before chemotherapy, but also because treatment often has to be discontinued due to these side effects. Moreover, the survival of patients with such extremely advanced HCC was too short to allow a secondary treatment (1–4). Therefore, accurate prediction of sensitivity to the first-line chemotherapy is necessary for advanced HCC patients so that they do not lose their limited chance to receive treatment and the patients who will not respond to the therapy can be protected from debilitating side effects.

These results illustrate the potential of biological technology to advance diagnostic methods, allowing physicians to plan beyond empirical results toward a more molecularly well-defined, personalized therapy. Particularly for patients with poor prognosis, such as advanced HCC patients, accurate prediction of chemotherapeutic responses before treatment is necessary. Because our molecular prediction system involves only a small

number of predictive genes and a simplified algorithm that does not require either statistical software or specialists, this system should easily lead to future clinical application.

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