

A three-gene predictor for early intrahepatic recurrence of hepatocellular carcinoma after curative hepatectomy

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Received July 30, 2007; Accepted September 24, 2007

Abstract. We previously developed a DNA microarray-based system that out-performs traditionally used clinical parameters for prediction of early intrahepatic recurrence (IHR) of hepatocellular carcinoma (HCC). Because DNA microarray is too expensive for daily clinical use, we used a quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) to develop a lower-cost predictor for early IHR. From the 12 early IHR-related genes integrated in the previous predictor, we selected 6 genes whose levels showed the strongest association between data from the 2 distinct DNA microarray platforms with the same sample set. Expression of these 6 genes relative to that of *GAPDH* was measured by QRT-PCR in 82 HCCs. Of the 82 HCCs, 39 and 43 were assigned to training and independent test sets, respectively. By searching all combinations ($n=2-6$) of the 6 genes, we found an optimal combination of 3 genes (*HLADRA*, *DDX17* and *LAPTM5*) that minimized the leave-one-out error for prediction of early IHR in the training set. The 3-gene predictor constructed with the Fisher linear classifier correctly predicted early IHR or non-recurrence in 35 (81.4%) of 43 HCCs in the independent test set and had a high positive predictive value of 72.7% and a high negative predictive value of 84.4%. Multivariate analysis with the stepwise logistic regression showed that the 3-gene predictor [$F(x)<0$] was an independent risk

factor for early IHR (risk ratio, 13.6; $p=0.006$), indicating its potential as an easy-to-use predictor for accurate prediction of early IHR of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, with an estimated 564,000 new cases worldwide in 2000 (1), and represents a major international health problem because the incidence is increasing in many countries (2,3). Major obstacles in the surgical treatment of HCC are the low resectability rate at time of diagnosis and the high rate of intrahepatic recurrence (IHR) after treatment. Indeed, IHR is observed in 30-50% of HCC patients even after curative surgery (4-6). There are two representative modes of IHR after surgery, early IHR and late IHR (3-6). Most cases of early IHR can be attributed to intrahepatic metastasis of cancer cells and are detected within 1 to 2 years of surgery. However, early IHR limits the potential for surgical cure of HCC (6,7). To avoid unnecessary overtreatment of patients who have been cured by surgery alone and to provide more personalized therapeutic options, we must accurately predict early IHR or non-recurrence. Accurate prediction of early IHR may lead to an improvement of the prognoses of HCC.

In the post-genomic era, DNA microarray technology has created a new avenue in the research field of biomedical science (8). One can now use DNA microarray technology, which provides a snapshot of comprehensive genetic alterations, not only to gain insights into the molecular basis of cancer biology, but also to stratify cancer patients according to their clinical course. Several researchers, including ourselves, have attempted to predict postoperative recurrence of HCC by means of genome-wide information from DNA microarray analysis (6,7,9). Prior to clinical use of microarrays as multiple-gene predictors, the predictive performance must be validated in a larger cohort (10); however, none of the previous studies (6,7,9) addressed this issue. One possible reason is that array-based strategies are too expensive for confirmation studies or routine clinical use, and therefore such an expensive strategy

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Key words: hepatocellular carcinoma, microarray, RT-PCR, intrahepatic recurrence, surgery

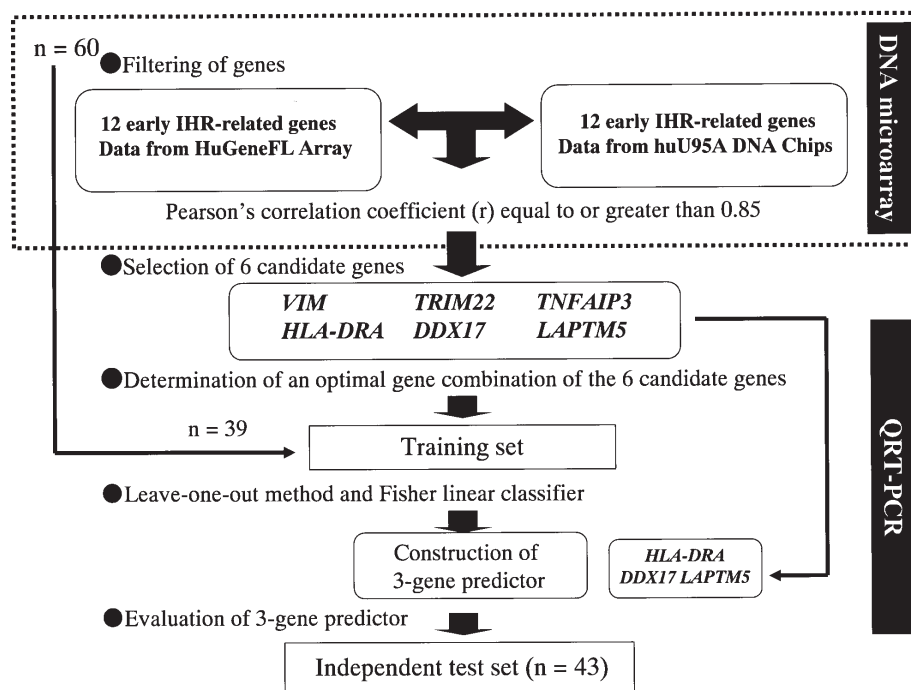


Figure 1. Schematic diagram of the design our study. For HuGeneFL Array, the clinicopathologic features and gene expression data of the 60 HCCs are available on our website (<http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/hcc-recurrence/index.html>). QRT-PCR, quantitative real-time RT-PCR.

must be replaced with a lower-cost method of analysis such as reverse transcription-polymerase chain reaction (RT-PCR).

To address this issue, it is crucial to decrease the number of genes for prediction. In our previous study, we successfully reduced the dimensionality by 12 without loss of predictive power (6). In the present study, we attempted to decrease the number of genes to a minimum without loss of predictive power. We developed a 3-gene quantitative real-time RT-PCR (QRT-PCR) assay that allows simple, inexpensive and accurate prediction of early IHR of HCC.

Materials and methods

Selection of 6 candidate genes related to early IHR from pooled DNA microarray data. We previously analyzed the expression of 7070 genes in 60 HCC samples using high-density oligonucleotide arrays (HuGeneFL Array, Affymetrix, Santa Clara, CA) and pooled the data (6,11-13). The clinicopathologic features and gene expression data of the 60 HCCs are available on our website (<http://surgery2.med.yamaguchi-u.ac.jp/research/DNAchip/hcc-recurrence/index.html>). Our array analysis yielded a robust predictive panel of 12 genes for early IHR (6). More recently, we used a new DNA microarray platform, huU95A DNA Chips® (Affymetrix) to analyze the expression of 12600 genes in RNAs isolated from the same 60 HCCs (14).

As shown in the schematic diagram of our current study (Fig. 1), we evaluated the reproducibility of the 12 early-IHR-related genes between the 2 microarray platforms described above. Of the 12 genes, we selected 6 candidate genes (*TNFAIP3*, *HLADRA*, *TRIM22*, *DDX17*, *VIM* and *LAPTM5*) whose levels showed strongest association ($r \geq 0.85$) between the 2 DNA microarray platforms (Fig. 2). Levels of

all 6 genes were significantly lower in HCC with early IHR than in HCC without early IHR. We focused our investigation on the 6 genes and measured their levels by means of quantitative real-time RT-PCR (QRT-PCR).

Subjects. Eighty-two HCC samples from 82 patients were selected from our collection and subjected to QRT-PCR. HCC was histopathologically diagnosed in all 82 patients who underwent curative hepatectomy. Written informed consent was obtained from all patients before surgery. The study protocol was approved by the Institutional Review Board for Human Use at Yamaguchi University School of Medicine (Japan).

Of the 82 HCCs, 39, which were included in our previous DNA microarray study (6), were defined as training samples, and the remaining 43 were defined as test samples. Clinicopathologic features of the training and test sets based on the UICC TNM classification are shown in Table I. Patient follow-up was performed as reported previously (6).

QRT-PCR analysis. RNA extraction and RT reaction were carried out as reported previously (15,16). Following the RT step, real-time PCR (LightCycler System Version 3.39; Roche Diagnostics, Mannheim, Germany) was performed with 10 pmol of each primer set (described below), cDNA equivalent to 10 ng initial RNA and 2 ml LightCycler Fast Start DNA Master SYBR-Green I (Roche Diagnostics).

The following real-time PCR protocol was employed: denaturation for 10 min at 95°C and 40 cycles of 10-sec denaturation at 95°C, 10-sec annealing at 60°C and 20-sec elongation at 72°C. We quantified mRNA levels for the 6 experimental genes and *GAPDH* with the crossing point (CP), which was calculated with the second derivative maximum

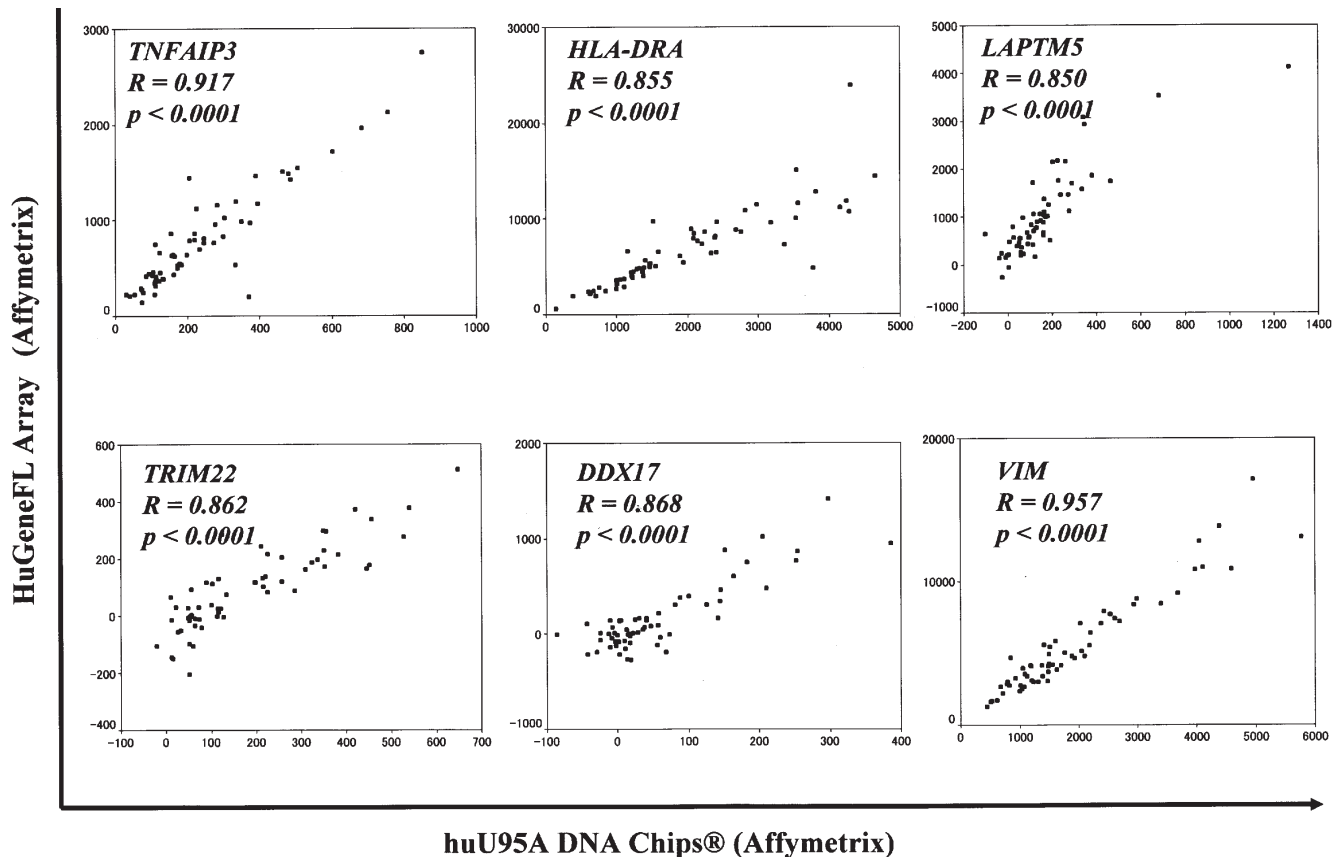


Figure 2. Relation of levels of 6 early IHR-related genes between 2 distinct microarray platforms (HuGeneFL Array and huU95A DNA Chips). Gene symbols were obtained from Entrez Gene (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>).

Table I. Clinicopathologic features of HCC.

Early intrahepatic recurrence	Training set (n=39)			Test set (n=43)		
	Positive (n=17)	Negative (n=22)	P-value	Positive (n=13)	Negative (n=30)	P-value
Sex (male/female)	12/5	18/4	N.S.	11/2	23/7	N.S.
Age in years (mean \pm SD)	62.4 \pm 9.7	63.7 \pm 7.8	N.S.	65.9 \pm 8.2	64.3 \pm 9.4	N.S.
Viral infection (HBV/HCV/non-B non-C)	4/11/2	5/13/4	N.S.	4/9/0	10/19/3	N.S.
Tumor size in cm (mean \pm SD)	5.0 \pm 2.9	3.2 \pm 1.5	0.017	4.7 \pm 2.7	3.4 \pm 1.6	N.S.
Primary lesion (single/multiple)	4/13	12/10	0.051	5/8	22/8	0.043
Histological grading (G1/G2/G3) ^a	0/11/6	2/18/2	N.S.	0/12/1	8/19/3	N.S.
Venous invasion (-/+)	5/12	18/4	0.001	7/6	25/5	0.061
Stage (I/II/III) ^a	2/10/5	11/9/2	0.034	1/7/5	18/8/4	0.004

HBV, hepatitis B virus; HCV, hepatitis C virus; non-B non-C, patient negative for both hepatitis B antigen and HCV antibody. G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated. Minor, subsegmentectomy and partial hepatectomy. P-value was evaluated by Fisher's exact test, chi-square test or Student's t-test. ^aAssessment based on pTNM classification of UICC. N.S., not significant.

method included in the LightCycler Software 3.39 (Roche Diagnostics), as described previously (15,16). The CP is the number of PCR cycles when maximal acceleration of the increase in fluorescence is reached. We also calculated the PCR efficiency (E) for each gene in titration experiments with various amounts of cDNA according to the method of

Neuvians *et al* (17). The investigated transcripts showed PCR efficiencies (E) of 1.937 for *GAPDH*, 2.020 for *TNFAIP3*, 1.937 for *HLADRA*, 2.030 for *TRIM22*, 1.910 for *DDX17*, 2.010 for *VIM* and 1.927 for *LAPTM5*. Target gene expression relative to *GAPDH* (N_{target}), was determined with the following formula: $N_{\text{target}} = E_{\text{target}}^{[40(\text{maximal cycle number}) - \text{CPT}_{\text{target}}]} / E_{\text{GAPDH}}^{[40 - \text{CP}_{\text{GAPDH}}]}$.

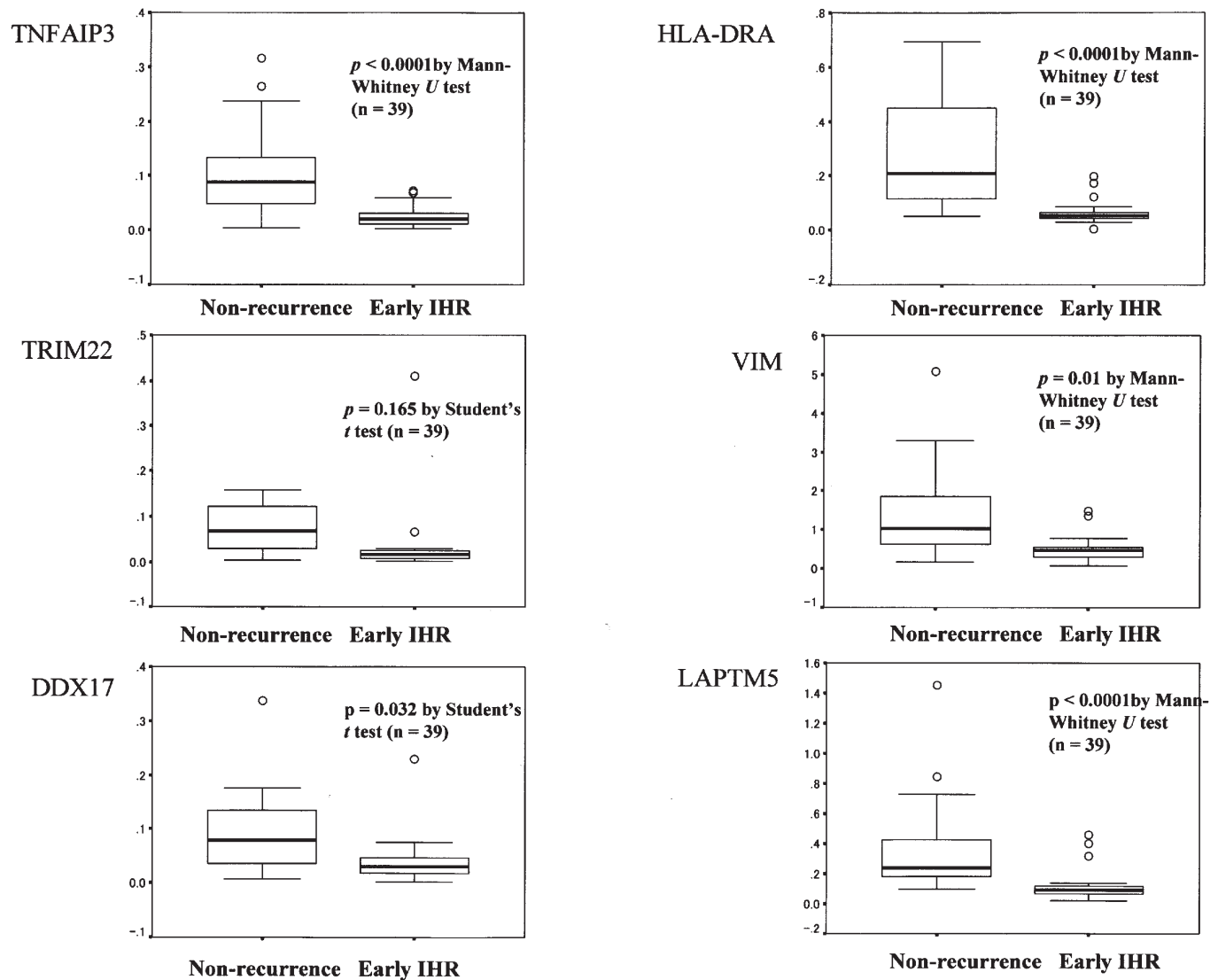


Figure 3. Box and whisker plot of expression patterns of the 6 genes in HCCs with early IHR and those without recurrence. Gene symbols were obtained from Entrez Gene (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>).

The specificity of the products was documented with analysis of the melting temperature (data not shown). The primers used in this study were GAPDH-S, 5'-TGGTATCGTGGAA GGAACA-3' and GAPDH-AS, 5'-GGGGCCATCCACAGT CTT-3'; TNFAIP3-S, 5'-TGCACACTGTGTTTCATCGAG-3' and TNFAIP3-AS, 5'-ACGCTGTGGGACTGACTTTC-3'; HLA-DRA-S, 5'-CAAGGGATTGCGCAAAAG-3' and HLA-DRA-AS, 5'-GATCTTCTCTCTAAGAAACACCATCA-3'; TRIM22-S, 5'-CTAAAGAGTGTATTCCGAGTACCAG AT-3' and TRIM22-AS, 5'-GCATCACGTCCACCCAGTA-3'; DDX17-S, 5'-AAACTGATGCAGCTTGTGGA-3' and DDX17-AS, 5'-CGGTAACGAGAACGACCAC-3'; VIM-S, 5'-TACAGGAAGCTGCTGGAAGG-3' and VIM-AS, 5'-TC AGGGAGGAAAAGTTTGGA-3'; and LAPTM5-S, 5'-TCA TCTTTCCATCGCCTTC-3' and LAPTM5-AS, 5'-TTCTC CTCCACCGAGTTCAT-3'. The predicted product sizes were 70, 76, 87, 94, 71, 69, and 109 bp for *GAPDH*, *TNFAIP3*, *HLA-DRA*, *TRIM22*, *DDX17*, *VIM* and *LAPTM5*, respectively.

Construction of predictor with training set. In the leave-one-out method, we selected 38 HCC samples from the 39 training HCC samples and constructed the Fisher linear classifier (FLC)

(6) of N genes with the 38 samples. The performance of the constructed classifier on the remaining one HCC sample was tested, and this procedure was repeated 39 times. Each exhaustive search was performed for all combinations ($n=2, 3, 4, 5$ and 6) of the 6 genes. As a result, we determined that the gene combination of *HLA-DRA*, *DDX17* and *LAPTM5* minimized the leave-one-out error rate and maximized prediction (data not shown). The formula for the optimal predictor given by FLC was follows: $F(x) = 9.783538 \times HLA-DRA + 8.288929 \times DDX17 - 1.684217 \times LAPTM5 - 1.547365$. If $F(x) < 0$, our predictor predicted early IHR. If $F(x) > 0$, it predicted non-recurrence.

Evaluation of the performance of the optimal predictor on an independent test set. Finally, the performance of our final predictive system $F(x)$ was tested on 43 independent HCC samples (test set) (Fig. 1).

Statistical analysis. The reproducibility of the expression patterns of the 12 genes between the 2 distinct array data sets was confirmed with Pearson's correlation coefficient.

Variable	Regression coefficient	Standard error	Risk ratio (95% CI)	P-value
$F(x) < 0$	2.61	0.95	13.6 (2.1-88.1)	P=0.006
Stage IIIA	1.37	0.59	3.9 (1.2-12.5)	P=0.019

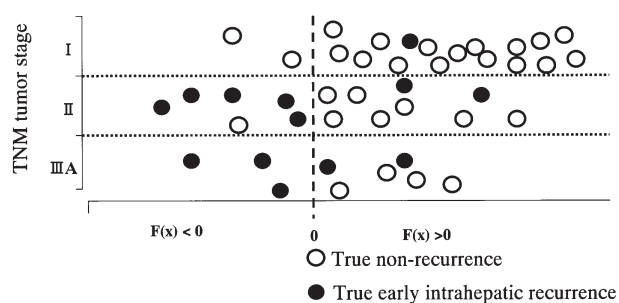


Figure 4. Performance of the 3-gene predictor on 43 independent test samples. $F(x)$, formula computed with the Fisher linear classifier. If $F(x) < 0$, our 3-gene predictor predicted early IHR. If $F(x) > 0$, it predicted non-recurrence. In total, our predictor correctly predicted early IHR or non-recurrence in 35 (81.4%) of 43 HCCs in the independent test set. The predictive accuracy was 84.2% (16/19) for stage-I HCCs, 80.0% (12/15) for stage-II HCCs, and 77.8% (7/9) for stage-III A HCCs.

QRT-PCR data for the 6 genes were analyzed by Student's t-test and Mann-Whitney U test. We carried out multivariate analysis to assess independent factors for early IHR in the 43 HCC samples using the logistic regression model (SPSS 11.0J software, SPSS, Inc., Chicago, IL). Five variables [$F(x)$, tumor size, venous invasion, number of primary lesions and TNM stage] were entered into a backward elimination model. Each model was tested for goodness-of-fit by -2 log likelihood and chi-square at each step. $P < 0.05$ was accepted as statistically significant.

Results

During the follow-up periods, 17 patients with HCC (43.6%) and 13 patients with HCC (30.2%) in the training and test sets, respectively, had early IHR within 1 year after curative hepatectomy (Table I). We found that tumor size, venous invasion and TNM stage were associated with early IHR and that the number of primary lesions tended to be associated with early IHR in the HCC training set (Table I). In the HCC test set, TNM stage and the number of primary lesions were associated with early IHR, but tumor size was not associated with early IHR (Table I). Thus, there was little difference in patient characteristics between the HCC training and test sets.

To validate the performance of our QRT-PCR analysis, we compared expression of the 6 genes between HCCs with early IHR and those without early IHR in the training set. The levels of *TNFAIP3*, *HLADRA*, *DDX17*, *VIM* and *LAPTM5* were significantly lower in HCC with early IHR than in HCC without (Fig. 3). Although there was no statistical difference

in levels of *TRIM22* between the 2 groups, the average levels were 1.85-fold lower in HCC with early IHR than in HCC without (Fig. 3). Thus, our QRT-PCR analysis reproduced the performance of the 6 genes from previous microarray analysis (6).

By screening for all combinations ($n=2-6$) of the 6 genes, we identified an optimal combination of 3 genes (*HLADRA*, *DDX17* and *LAPTM5*) that minimized the leave-one-out error for prediction of early IHR in the training set. The 3-gene predictor [$F(x)$] constructed with the Fisher linear classifier correctly predicted early IHR or non-recurrence in 35 (81.4%) of 43 HCCs in the independent test set (Fig. 4). It had a positive predictive value (PPV) of 72.7%, negative predictive value (NPV) of 84.4%, sensitivity of 61.5% and specificity of 90.0%. The predictive accuracy was 16 (84.2%) of 19 stage-I HCCs, 12 (80.0%) of 15 stage-II HCCs and 7 (77.8%) of 9 stage-III A HCCs, respectively (Fig. 4). Thus, our predictor worked well for early-stage HCC. There was no recurrence in 18 (94.7%) of 19 stage-I HCCs or in 12 (50.0%) of 24 HCCs of stages II-III A. The TNM staging system had a poor PPV of 49.9%, whereas it had a good NPV of 94.7%. When 5 variables [$F(x)$, tumor size, venous invasion, number of primary lesion and TNM stage] were entered into a backward elimination model of logistic regression, the 3-gene predictor was an independent risk factor for early IHR in the HCC test set [risk ratio, 13.6; 95% confidence interval (CI), 2.1-88.1; $p=0.006$] (Table II).

Discussion

DNA microarray technology has created a new means by which patients at distinct risk for recurrence of HCC can be identified (18). Although microarray analysis is a powerful technique, it is still relatively laborious and expensive and requires careful and complicated data analysis. In particular, most DNA microarray-based predictors have required large numbers of gene signatures. As a result, arrays remain a high-dimensional and expensive strategy. To confirm microarray results in larger cohorts (10), a cheaper, easier, and faster method, such as QRT-PCR, is needed. We previously developed an accurate predictor of IHR using only 12 genes (6). To extend this result, in our present study, we developed a 3-gene PCR-based predictor that will cost less than 10 US dollars per sample, and that accurately predicted early IHR in a set of independent HCC samples.

PCR technology has allowed for quantitative analysis of target genes in small amounts of DNA or RNA. Some investigators have used this technology to detect cancer-specific transcripts originating from HCC cells circulating in the bloodstream or HCC tissues (19-21). Cheung *et al* (22) used RT-PCR to select the Claudin-10 gene from a 23,000-

gene DNA microarray and showed that this gene has high predictive value for outcome of HCC patients after surgery. These studies (20-23) showed that the level of one specific gene correlated with the prognosis of HCC or detected transcripts such as α -fetoprotein and telomerase reverse transcriptase in blood from HCC patients; however, the predictive powers of these genes for recurrence was not satisfactory for clinical use. There has been only one study (7) of a PCR-based predictor that accurately classified early IHR or non-recurrence in independent HCC samples in a training-validation manner. The 20-gene PCR-based predictor of Kurokawa *et al* (7) correctly predicted early IHR or non-recurrence in 29 (72.5%) of 40 HCCs. In the present study, there were some differences in patient characteristics between the HCC training and test sets; however, the predictive accuracy of our 3-gene predictor was still 81.4% (35/43). This degree of accuracy was superior to that of the 20-gene predictor by Kurokawa *et al* (7) and, to our knowledge, it is the most robust PCR-based predictor for early IHR of HCC after hepatectomy. This superiority might be due to our unique gene selection procedure in which the combination of genes was considered and 2 distinct DNA microarray platforms were used to select the most reliable genes. Given that PCR technology can analyze target genes in small amounts of DNA or RNA, our methodology may enable us to evaluate the metastatic potential of HCC pre-operatively from small amounts of the biopsy specimens. The 3 genes used in our predictive system had no overlap with signature genes linked to patient backgrounds, such as hepatitis virus type, tumor differentiation grade or coexisting liver diseases (i.e., cirrhosis, hepatitis or normal liver) in our series (11,12,14). In addition, our 3-gene predictor was an independent risk factor for early IHR in the HCC test set. Thus, the lack of relation of the 3 genes to patient characteristics accounts for the high predictive value.

There are likely 2 opposite strategies for developing a multi-gene predictor for cancer outcome. One strategy decreases the dimensionality (i.e., the number of genes) in the multi-gene predictor, and the other strategy uses as many genes as possible. Our current study aimed to develop a predictor of early IHR of HCC with the decreased dimensionality strategy. It was reported that among the 36 feature genes obtained from data of several DNA microarray studies, measurement of the expression of only 6 genes by QRT-PCR was sufficient to predict overall survival for diffuse large-B-cell lymphoma (23). Likewise, Endoh *et al* (24) screened for genes, published in DNA microarray data and developed an 8-gene predictive model for outcome of pulmonary adenocarcinoma patients by QRT-PCR that worked well in an independent set of pulmonary adenocarcinomas. Ma *et al* (25) identified, from 22,000 genes, 2 genes related to outcome of breast cancer patients after tamoxifen treatment, and the 2-gene expression ratio yielded by QRT-PCR accurately predicted the treatment outcome of breast cancer patients. In conjunction with our present finding, these reports support the efficacy of a low-dimensional predictor for IHR of HCC in clinical practice.

As mentioned above, opposite conclusions were also drawn from several studies. For example, the 2-gene predictive model (25) for breast cancer patients could not be replicated in a new cohort of patients by other investigators (26). RT-PCR analysis by Shultz *et al* (27) also showed a lack of

reproducibility of a DNA microarray study for association with recurrence of urothelial cell carcinoma. It is apparent that patient heterogeneity may be one explanation for the lack of overlap between predictive signatures from different studies or institutes. To circumvent heterogeneity, Roepman *et al* (28) suggested that it is necessary to integrate information for many genes into the predictor. This concept might lead to development of a multi-gene predictor that can be used broadly; however, they did not evaluate the predictive performance in independent samples, and the predictive power may be decreased due to inclusion of many ineffective genes. Our current approach successfully decreased the number of genes while retaining predictive performance. As a result, our 3-gene predictor yielded a high accuracy on an independent cohort. Such a robust predictor with a small set of genes is advantageous for the clinical setting. Further large-scale studies are needed to elucidate the degree to which our 3-gene predictor works in predicting early IHR.

Acknowledgements

Grant sponsors were the Ministry of Education, Culture, Sports, Science and Technology (no. 18390366, no. 17591406 and Knowledge Cluster Initiative); the Venture Business Laboratory of Yamaguchi University; the New Energy and Industrial Technology Development Organization (no. 03A02018a).

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