

Differentially Expressed Genes between Early and Advanced Hepatocellular Carcinoma (HCC) as a Potential Tool for Selecting Liver Transplant Recipients

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Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. Liver transplantation (LT) represents a curative treatment for "small" HCC. Preoperative staging is critical in selecting optimal candidates for LT to optimize the use of this scarce resource. From December 1997 to February 2004, 148 patients diagnosed with cirrhosis and HCC were evaluated at our center. After staging, the patients were listed for LT according to United Network for Organ Sharing (UNOS) criteria. When pretransplant liver MRIs were compared with the findings of the explanted livers, 8 of 35 patients (22.8%) were understaged. Three of the 8 patients (37.5%) had recurrence post-LT. A retrospective gene expression profiling study was done using microarray technology for tumor samples in the pretransplant hepatitis C virus (HCV)-HCC understaged patients and in a contemporaneous group of HCV-HCC patients that were accurately staged. Two sample *t* tests comparing the early versus advanced HCV-HCCs with respect to gene expression showed an important set of genes differentially expressed among the samples. Hierarchical clustering analysis of the gene expression profiling classified 93.8% of the total tumor samples and 85.7% of the understaged samples in concordance with the explanted pathological staging. We found a distinctive pattern of gene expression between early and advanced HCV-HCCs. These results suggest that gene expression profiling could improve the pre-LT HCC staging to more closely mimic the explant pathology. Whether gene expression profiling of HCC will be refined to the point of predicting potential metastatic biologic behavior to predict post-LT recurrence will require longitudinal prospective study of this gene array technology.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary solid tumor of the liver (1). Cirrhosis of the liver, regardless the etiology, is considered to be the main risk factor for the onset of HCC. In the United States, hepatitis C virus (HCV) is a major cause of cirrhosis of the liver in patients with HCC (2). In addition, there is a well-defined risk group (cirrhosis of the liver) and curative therapeutic techniques are available that can increase patient survival (resection, local ablation therapy, and liver transplantation).

To date, both ultrasonography (US) and the serum α -fetoprotein (AFP) assay are the principal methods of screening for HCC. The diagnostic sensitivity of AFP for HCC is reported to range from 39% to 64% and specificity from 76% to 91% (3-7). The sensitivity of US for the detection of HCC is directly related to tumor size. Its diagnostic sensitivity for tumors smaller than 1 cm is about 42% (7,8) and reaches 95% for tumors of larger size (9). To confirm the suspicion of HCC by US in a patient with cirrhosis of the liver, the use of computed tomography (CT) is suggested (9). Its diagnos-

tic efficacy depends on technical factors (injection of contrast) and on factors inherent to the tumor (tumor size and vascularity) (10,11). Magnetic resonance imaging (MRI) has been used to obtain a better characterization of hepatic lesions suggestive of HCC and also for their differentiation from benign lesions (12).

After the diagnosis of HCC is confirmed, preoperative accurate staging of HCC is a critical step. The main objective of tumor staging is to determine the prognosis of the disease and to establish the best therapeutic options for the patient.

The selection of HCC therapy depends on the functional reserve of the liver, usually assessed by Child-Turcotte-Pugh classification (13), and the extent of tumor growth, for which the 2 most popular staging systems are the Okuda system (14) and a modification of the

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tumor-node-metastasis (TNM) classification (15) of the International Union Against Cancer.

Surgical resection (SR) and liver transplantation (LT) represent potentially curative treatments for early stages of HCC (16-20). LT is the treatment of choice in patients with HCC limited to the liver that cannot be submitted to surgical resection owing to poor hepatic reserve or to technical impossibility. When strict selection criteria (single, small tumor [< 5 cm] without satellite nodules, no vascular invasion, no metastases) are used, excellent transplant survival can be achieved (16, 18,19). Pre-LT adjuvant treatment to prevent tumor progression until the time of the surgical procedure has been adopted at most transplant centers where the time on the waiting list can be more than 6 months. In our experience, patient survival and cancer-free survival in patients undergoing orthotopic LT after adjuvant multimodality ablation treatment are 92.9% and 95.24%, respectively, at 32 months of followup (17).

Worldwide attempts to improve the classification and broaden transplant application and prognostic prediction for HCC are still evolving (21), and there is no agreement on the best staging system to be recommended.

Recently, we showed multiple molecular alterations during HCV-HCC hepatocarcinogenesis using gene expression analysis (22). Moreover, we observed differences in the gene expression profiles between early and advanced stages of HCV-HCC.

In the present study, we demonstrate the potential clinical utility of molecular studies as gene expression profiling using microarrays in the staging of the tumor at the pretransplantation time and its potential effectiveness for safely broadening the selection of HCC patients for liver transplantation.

MATERIALS AND METHODS

Patients

From December 1997 to February 2004, 148 patients diagnosed with cirrhosis

and HCC were evaluated at our center. After staging and tumor ablation, based on serial liver MRI, chest CT, bone scan, AFP, and a physical examination every 3 months, the patients were listed for LT according to United Network for Organ Sharing (UNOS) criteria. Thirty-five patients received ablation therapy (2.4 ± 1.4 procedures per patient) followed by LT. The HCC etiology included 29 HCV (82.8%), 1 HBV (2.8%), 2 ETOH (5.7%), and 3 with other etiologies (8.6%). AFP < 20 ng/dL was found in 37.1% of the patients. When pretransplant liver MRIs were compared with the findings of the explanted livers, 22.8% of the patients ($n = 8$, 7 HCV-HCC and 1 HBV-HCC) were understaged. Three of the 8 patients (37.5%) (all HCV) had recurrence or metastasis after LT.

This study was restricted to the HCV-HCC patients to avoid confounding factors involved in HCC etiology. The characteristics of the patients and tumors are described in Table 1. Twenty-four tumor tissues samples (22 from single and 2 from multifocal tumors) were collected from the diagnostic biopsies (2 samples) and explanted livers (22 samples) from 23 HCV-HCC patients (including all 7 HCV-HCC understaged cases). For patient 19, needle biopsies were performed from the recurrent tumor. Samples were freshly snap-frozen and the tumor samples (with more than 85% of tumor cell content) were used for the microarray studies. Expression profiling was performed on 1) 2 pools comprised of RNA from early HCV-HCC (tumor samples from patients 1 to 5) and advanced HCV-HCC (tumor samples from patients 6 to 9) and 2) individual RNA samples from the understaged group and a control group of patients (tumor samples from patients 10 to 23). As it can be noted (Table 1), tumor samples used for the pools were from different patients that those tumor samples used for the individual microarray evaluations. Histopathological classification was performed according to the Edmondson grading system; clinical stages were de-

termined according to the American Tumor Study Group modified TNM classification mandated by UNOS (23).

Written informed consent was obtained from all patients before surgery. The Institutional Review Board at Virginia Commonwealth University approved the study protocol.

Ablation Techniques

Three ablation techniques were utilized as primary treatment for ablating HCC, including trans catheter hepatic arterial chemoembolization (TACE), trans catheter hepatic arterial chemoinfusion (TACI), and radiofrequency microwave ablation (RFA). Individual decisions on the safest and most effective ablation techniques to use were based on the evaluation of HCC size, location, and multiplicity; limits of imaging localization for accurate, total ablation; and estimation of the zone of thermal and chemical injury in relation to the estimated hepatocellular reserve and risk of systemic morbidity of each ablation technique.

RNA Isolation, cDNA Synthesis and In Vitro Transcription (IVT) for Labeled cRNA Probe

The sample preparation protocol follows the Affymetrix GeneChip Expression Analysis Manual (Santa Clara, CA, USA). Total RNA was extracted using TRIzol (Life Technologies, Rockville, MD, USA) and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Using 5 μ g total RNA; we created double-stranded cDNA by using the SuperScript Choice system (Life Technologies). Two pools were made from equal amounts of total RNA from liver tissues including early (pool 1 [T1-T2 tumors] = 5 tissues) and advanced HCV-HCC tumors (pool 2 [T3-T4 tumors] = 4 tissues) (Table 1). First-strand cDNA synthesis was primed with a T7-(dT24) oligonucleotide. From 2 μ g cDNA, cRNA was synthesized using T7 MegaScript In Vitro Transcription (IVT) kit (Ambion, Austin, TX, USA). To label the cRNA with biotin, we added nu-

Table 1. Characteristics of the patients and tumors included in the microarray analysis.

Patient	Sex	Age, y	Race	Cirrhosis	Tumor size, cm	Vascular invasion	Tumor number	Lobar distribution	TNM score
1	M	60	W	Yes	< 5 ^a	Microvascular	1	Right lobe	T1N0M0
2	M	56	B	Yes	< 5	Microvascular	1	Right lobe	T1N0M0
3	F	69	H	Yes	< 5	Microvascular	2	Right lobe	T2N0M0
4	M	58	W	Yes	< 5	Microvascular	2	Right lobe	T2N0M0
5	M	56	W	Yes	< 5	Microvascular	2	Right lobe	T2N0M0
6	F	62	W	Yes	> 5 ^b	Microvascular	1	Left lobe	T3N0M0
7	M	61	W	Yes	> 5	Microvascular	2	Right lobe	T3N0M0
8	F	60	W	Yes	> 5	Microvascular	3	Bilobar	T3N0M0
9	M	54	H	Yes	> 5	Macrovascular	> 3	Bilobar	T4N0M0

Patients 1-5: Pool 1 Early HCV-HCC

Patients 6-9: Pool 2 Advanced HCV-HCC

HCV-HCC individual samples^c

Patient	Sex	Age, y	Race	Tumor size, cm	Vascular invasion ^d	Tumor number	Lobar distribution	Enlisted stage	Explanted stage
10	M	47	B	< 5	Microvascular	1	Right lobe	T2N0M0	T2N0M0
11	M	44	W	< 5	Microvascular	1	Right lobe	T1N0M0	T1N0M0
12	M	44	B	< 5	Microvascular	1	Right lobe	T2N0M0	T2N0M0
13	M	49	H	< 5	Microvascular	1	Right lobe	T2N0M0	T2N0M0
14	F	54	W	< 5	Microvascular	2	Right lobe	T2N0M0	T2N0M0
15 ^e	M	52	W	> 5	Microvascular	2	Bilobar	T2N0M0	T3N0M0
16 ^e	M	56	W	> 5	Macrovascular	2	Bilobar	T2N0M0	T4N0M0
17 ^e	M	66	W	> 5	Microvascular	1	Right lobe	T2N0M0	T3N0M0
18 ^e	M	68	W	> 5	Microvascular	1	Right lobe	T2N0M0	T3N0M0
19 ^e	M	58	H	> 5	Microvascular	2	Bilobar	T3N0M0	T4N0M0
20 ^e	M	56	W	> 5	Microvascular	2	Bilobar	T2N0M0	T4N0M0
21 ^e	M	58	W	> 5	Microvascular	3	Right lobe	T2N0M0	T4N0M0
22	M	66	W	< 5	Microvascular	1	Right lobe	T2N0M0	T2N0M0
23	M	41	W	> 5	Microvascular	2	Right lobe	T3N0M0	T3N0M0

^aSingle tumor < 5 cm or up to 3 tumors all < 3 cm.

^bSingle tumor > 5 cm or multiple tumors (4 or more).

^cAll the patients had cirrhosis.

^dVascular invasion according to explant findings.

^eUnderstaged patients at pretransplantation.

cleotides Bio-11-CTP and Bio-16-UTP (Enzo Diagnostics, Farmingdale, NY, USA) to the reaction. The cRNA was fragmented and hybridized on the HG-U133A and HG-U133Av2 arrays for 18 to 20 h at 60 rpm in a 45°C hybridization oven. The chips were washed and stained with streptavidin phycoerythrin (SAPE) (Molecular Probes, Eugene, OR, USA) in Affymetrix fluidics stations. Hybridization to the probe arrays was detected by fluorometric scanning (Hewlett Packard Gene Array Scanner; Hewlett Packard, Palo Alto, CA, USA).

Quality Control

RNA integrity was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies). To be considered for microarray analysis, the RNA samples needed to pass quality control criteria as 28S/18S ratios greater than 1.5 and A260nm/A280nm greater than 2.0. We established a cutoff value of 30% rRNA contribution to the total area under the electropherogram for RNA samples to be considered as intact or undegraded (24).

Products of cDNA synthesis and IVT were tested using the Agilent 2100

Bioanalyzer (cDNA synthesis 1.5 kb < cDNA < 5.0 kb; IVT 1.0 kb < cRNA < 4.5 kb) (24). Finally, the values for a ratio of 3'/5' for 2 housekeeping genes were checked: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin; ratios no higher than 3.00 were considered acceptable.

Data Analysis and Statistical Methods

Probe set expression summaries were calculated for all 18 GeneChips using 2 different previously described algorithms, Microarray Suite v5.0 (MAS 5.0

software) (25,26) and robust multi-array average method (RMA) (27). To avoid bias that may arise from chip type, probes were matched for the 2 different GeneChips (HG-U133A and HG-U133Av2) before calculating RMA expression summaries. To identify genes differentially expressed by tumor stage, the 2-pooled RNA GeneChips (pool 1 and pool 2) were compared using the *S*-score algorithm (28). The *S*-score comparative call algorithm was developed to leverage the probe pair intensity differences *I* within a probe set as replicate measurements for transcript intensity which can be used to compare 2 GeneChips. The *S* score is calculated as

$$S = \sum_{j=1}^J \frac{I_{j1} - I_{j2}}{\alpha \epsilon_j \sqrt{J}}$$

where I_{jk} represents the *j*th probe pair intensity difference ($PM_j - MM_j$) in samples $k = 1, 2$; ϵ_j is the estimated error, *J* is the number of probe pairs within the probe set, and α is a normalization factor that corrects for the effect of covariation among probe pair signals. The value of α is chosen so that the variance of *S* score values on an array is 1 when outliers are excluded. Probe sets with an absolute *S* score value ≥ 3.291 , corresponding to a $P < 0.001$, were further examined.

In addition, the dataset was restricted to the individual samples, and the 2-sample *t* test was used to compare the early versus advanced HCV-HCCs ($\alpha = 0.001$). Based on the recommendation from the Tumor Best Practices Working Group (29), the 2-sample *t* test was performed using both the MAS and RMA expression summaries.

Significant probe sets identified by the *S* score algorithm when comparing the 2-pooled samples were filtered, and cluster analyses were subsequently performed on the set of GeneChips to which individual samples were hybridized. Similarities were calculated using both MAS and RMA probe set expression summary methods. Average linkage hier-

archical clustering was applied after converting the similarities to a distance. Distance was calculated as $1 - |\rho|$, which treats both highly negatively correlated and highly positively correlated samples as similar, and $(1 - \rho)/2$, which treats highly positively correlated samples as similar. In addition, the random forest algorithm (RF) (30) was used to predict explant stage using 5000 trees and 20 randomly selected probe sets at each node from the 424 retained probe set predictor variables. The proximities from the RF algorithm were converted to a distance measure for clustering. Average linkage hierarchical clustering was applied after converting the similarities to a distance.

REAL TIME PCR ANALYSIS

For validating our microarray results, we carried out a quantitative reverse transcriptase (RT) real-time PCR for a selected group of genes including *CD24*, *AFP*, and *GPC3* from the RNA samples that were subjected to microarray study. These genes were previously found to be overexpressed in HCV-HCC samples (22). Real-time RT-PCR reactions were performed using QuantiTect SYBR Green RT-PCR (Qiagen), forward and reverse primers for each system, and RNA from the different samples. Data was analyzed according to the comparative cycle threshold method and normalized by β -actin expression in each sample.

Statistical Analysis

Pearson's correlation coefficient was calculated to examine the relation between microarray and real-time PCR results. $P < 0.05$ were considered significant.

RESULTS

Quality Control

All the RNA samples met the quality control criteria for sample preparation. cDNA synthesis and IVT showed satisfactory results in concordance with pre-established quality control criteria (24) (Figure 1).

Differentially Expressed Genes between Early and Advanced HCV-HCCs Pooled Samples

From the analysis of the gene expression profiling between pools 1 and 2, we found 423 probe sets (corresponding to 394 genes: 272 up- and 122 downregulated) that were differentially expressed using *S* score algorithm. The Gene Ontology Consortium terms classified the overexpressed genes as fundamentally associated with functions related to cell proliferation and cell cycle (*DNAJB1*, DnaJ [Hsp40] homolog; *PLU-1*, putative DNA/chromatin binding motif; *ID2*, inhibitor of DNA binding 2, dominant negative helix-loop-helix protein; *POLB*, polymerase beta; among others) and transcription (*STAT1*, signal transducer and activator of transcription 1; *TCEA1*, transcription elongation factor A [SII], 1; *NR2F2*, nuclear receptor subfamily 2, group F, member 2; *RBPMS*, RNA binding protein with multiple splicing; among others). In addition, the genes that were upregulated in pool 2 included IFN-inducible genes (*IFI27*, interferon α -inducible protein 27; *IFI30*, interferon γ -inducible protein 30; *GIP2*, interferon α -inducible protein; *MX1*, myxovirus [influenza virus] resistance 1, interferon-inducible protein p78 (mouse); among others). The transcript *DLK1* (delta-like 1 homolog) was upregulated 86-fold in pool 2 versus pool 1. The expression of *AFP* in pool 2 was more than 50-fold higher than in pool 1. Moreover, glypican 3 (*GPC3*) was highly expressed in pool 2 (more than 20-fold overexpressed). The gene expression of *CK19* (keratin 19), *CK7* (keratin 7), *FOS* (v-fos FBJ murine osteosarcoma viral oncogene), and *S100A11* (S100 calcium binding protein A11 [calgizzarin]) were downregulated in the pool 2 versus pool 1. We also identified a set of genes differentially expressed between the pools that encode secreted (e.g., *GPC3* and *LCN2*) or membrane-bound (e.g., *GPC3*, *IGSF1*, and *EMP3*) proteins, which may be attractive candidates for the staging of HCC.

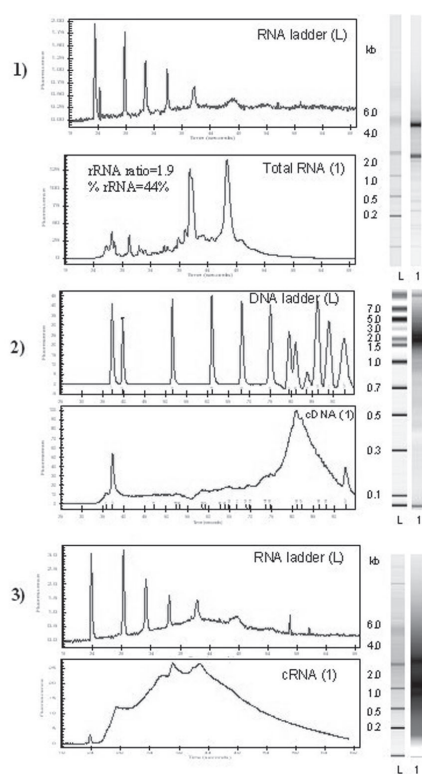


Figure 1. Quality control (QC) of the reaction products for microarray analysis. 1. Evaluation of RNA integrity for an individual RNA HCV-HCC sample (sample 5). 2. Capillary electrophoresis of cDNA synthesis product from sample 5. 3. Capillary electrophoresis of IVT product from the same sample.

Differentially Expressed Genes among Early and Advanced HCV-HCC Individual Samples

Two sample *t* tests comparing the early versus advanced HCV-HCCs with respect to gene expression was performed using both the MAS and RMA expression summaries. Probe set expression was considered significantly different at the $\alpha = 0.001$ level. From this analysis we found 31 genes among the samples for both RMA and MAS expression summary methods (Table 2). The genes with functions related to cell proliferation and cell cycle (*RFC5*, replication factor C; *RGS10*, regulator of G-protein signaling 10; *CDC34*, cell division cycle 34; among others) and inflammatory and immune response (*IGSF6*, immunoglobulin superfamily, member 6; *AIF*, allograft inflam-

matory factor 1; *MSR1*, macrophage scavenger receptor 1; among others) were found to be highly expressed in the advanced HCV-HCC samples. In addition we found some common differentially expressed genes between pooled and individual samples (*PPT1*, palmitoyl-protein thioesterase 1; *CTSC*, cathepsin C; *LIPA*, lipase A, lysosomal acid, cholesterol esterase; among others).

HCC Staging Using Gene Expression Profiles

The data set was restricted to the 16 individual samples and the 424 probe sets identified as significant when comparing the early versus advanced HCV-HCC pools. Cluster analyses were performed on this set of GeneChips (individual samples). The cluster analyses were applied to both MAS and RMA expression summaries using 2 different distances (RF proximities and correlation). Figure 1 shows the cluster results for RMA expression summaries using RF proximities using the previously described analysis. From this analysis we observed that 15 of the 16 tumor samples (93.8%) clustered together in concordance with the explant histopathological classification. The samples from 6 of 7 patients (85.7%) that were understaged prior to liver transplantation (Table 1) clustered in higher association with the corresponding tumor classification (early or advanced HCV-HCC) according to the histopathological explanted analysis.

One of the understaged samples (from patient 20), clustered in closer association to the early HCV-HCC samples. From the analysis of the levels of gene expression, we found that for this sample the levels of *KT 19* and *KR 7* were importantly decreased, but increased in the entire remnant individual and pooled advanced HCV-HCC samples. For patient 19, tumor tissue from 2 independent pretransplant liver tumors (TS1 and TS2) and tumor tissue from the recurrent posttransplant tumor (TS3) were studied using microarray analysis. From the analysis of the dendrogram

shown in Figure 2, we observed that the 3 samples from patient 19 clustered together, showing a similar gene expression profile.

Gene Expression Quantitation Using Real-Time PCR

Expression levels of the *CD24*, *AFP*, and *GPC3* mRNAs were further confirmed using a quantitative RT-real time PCR. The results from the microarray were reproduced by RT-real time PCR ($r = 0.74$, $P < 0.001$; $r = 0.88$, $P < 0.001$; and $r = 0.94$, $P = 0.012$ respectively).

Validation of Sample Pooling

To validate the results derived from the analysis of pooled samples, we performed real-time PCR analysis using *GPC3* to assess the expression of individual gene expression in the individual RNA samples that formed the pools. We found correlation between individual sample and pool mRNA quantitation ($r = 0.892$, $P < 0.001$). This suggested that pooling samples could, in a single assay, provide results that summarize the expression of individual samples.

DISCUSSION

LT has been considered the best treatment option for patients with early-stage HCC. Its use, however, is limited by the shortage of grafts (31-33). Until organ availability improves, transplantation for HCC can only be offered to patients whose survival is predicted to be similar to that in non-HCC transplant patients. Moreover, there is no agreement on the selection criteria that could simultaneously maximize the number of viable HCC candidates for transplantation and reject the smallest number of those who could have benefited (34-36). Molecular genetic analyses have shown that genomic changes accumulate during the development and progression of HCC (37-40). Establishment of a molecular-based method for the classification of HCV-HCC pretransplantation will permit the detection of distinct subgroups of HCC patients with different prognoses, allowing greater accuracy in selection of

Table 2. The 31 Genes for Which Levels Differed between Early and Advanced HCCs.

Locus link ID	Probe set ID	Gene symbol	Unigene ID	Gene name	Chromosome
8826	200791_s_at	IQGAP1	Hs.1742	IQ motif containing GTPase activating protein 1	15
5538	200975_at	PPT1	Hs.3873	palmitoyl-protein thioesterase 1 (ceroid-lipofuscinosis, neuronal 1, infantile)	1
1075	201487_at	CTSC	Hs.128065	cathepsin C	11
3988	201847_at	LIPA	Hs.85226	lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	10
6643	202114_at	SNX2	Hs.11183	sorting nexin 2	5
5985	203209_at	RFC5	Hs.443227	replication factor C (activator 1) 5, 36.5kDa	12
1536	203923_s_at	CYBB	Hs.88974	cytochrome b-245, β polypeptide (chronic granulomatous disease)	X
8884	204087_s_at	SLC5A6	Hs.435735	solute carrier family 5 (sodium-dependent vitamin transporter), member 6	2
7305	204122_at	TYROBP	Hs.9963	TYRO protein tyrosine kinase binding protein	19
6001	204319_s_at	RGS10	Hs.82280	regulator of G-protein signaling 10	10
23421	205176_s_at	ITGB3BP	Hs.405465	integrin β 3 binding protein (beta3-endonexin)	1
5918	206392_s_at	RARRES1	Hs.82547	retinoic acid receptor responder (tazarotene induced) 1	3
10261	206420_at	IGSF6	Hs.135194	immunoglobulin superfamily, member 6	16
11181	207378_at	TREH	Hs.129712	trehalase (brush-border membrane glycoprotein)	11
5313	207858_s_at	PKLR	Hs.95990	pyruvate kinase, liver and RBC	1
54504	208146_s_at	CPVL	Hs.95594	carboxypeptidase, vitellogenic-like	7
199	209901_x_at	AIF1	Hs.76364	allograft inflammatory factor 1	6
9747	210529_s_at	KIAA0738	Hs.406492	KIAA0738 gene product	7
3382	210547_x_at	ICA1	Hs.380460	islet cell autoantigen 1, 69kDa	7
3113	211991_s_at	HLA-DPA1	Hs.914	major histocompatibility complex, class II, DP α 1	6
997	212540_at	CDC34	Hs.423615	cell division cycle 34	19
3120	212998_x_at	HLA-DQB2	Hs.375115	major histocompatibility complex, class II, DQ β 2	6
811	214315_x_at	CALR	Hs.353170	calreticulin	19
4481	214770_at	MSR1	Hs.436887	macrophage scavenger receptor 1	8
6844	214792_x_at	VAMP2	Hs.25348	vesicle-associated membrane protein 2 (synaptobrevin 2)	17
10113	217861_s_at	PREB	Hs.279784	prolactin regulatory element binding	2
51026	218193_s_at	CGI-141	Hs.62275	CGI-141 protein	12
64077	218523_at	LHPP	Hs.20950	phospholysine phosphohistidine inorganic pyrophosphate phosphatase	10
64757	218865_at	FLJ22390	Hs.195345	hypothetical protein FLJ22390	1
64123	219134_at	ELTD1	Hs.429761	EGF, latrophilin and 7 transmembrane domain containing 1	1
63910	219559_at	C20orf59	Hs.353013	chromosome 20 open reading frame 59	20

patients for treatment cure with transplantation.

Appropriate liver allocation needs to be based on natural history data and sensitive and specific diagnostic tests. So far, size of tumor by CT scan or MRI has been used for preoperative staging because size has been reported to correlate with vascular invasion, progression of tumor, and recurrence rate after transplantation (6-12). TNM staging is limited by the need to obtain pathologic information, and image-based tumor assessment can be misleading given the difficulty in visualizing HCCs in the cirrhotic liver (6-12).

Tumor recurrence rate and survival of patients with HCC treated by LT depend on tumor stage at transplant time. Recent developments in biomedical

technology enable us to detect tumor cells at a molecular level (41,42). Molecular/biologic information is beginning to be incorporated into current staging systems to better predict HCC recurrence (37,43,44). An important question is whether molecular staging will be able to augment the accuracy by which the prognosis of individual patients can be assessed.

In a previous study, we demonstrated a clearly different pattern of gene expression profiling between early HCV-HCC and advanced HCV-HCC (22). In the present study, we explored retrospectively the utility of the gene expression profile analysis in the staging process of the HCC patients. Following this goal, we analyzed tumor samples from

the 7 cases of HCV-HCC understaged at the pretransplantation time (3 cases presented recurrence of the disease after transplantation) using individual gene expression profiling. According to the stage of HCC accepted by UNOS criteria using liver MRI and full metastatic workup, the patients were listed for LT. Study of the explanted livers showed more advanced stage of HCC, changing the initial classification (Table 1). In addition, a contemporaneous group of HCV-HCC patients with explanted liver-verified HCC stages were used as control for the analysis. For patient 19, gene expression analysis was performed in 2 independent pretransplant liver tumors and in a recurrent liver tumor posttransplantation.

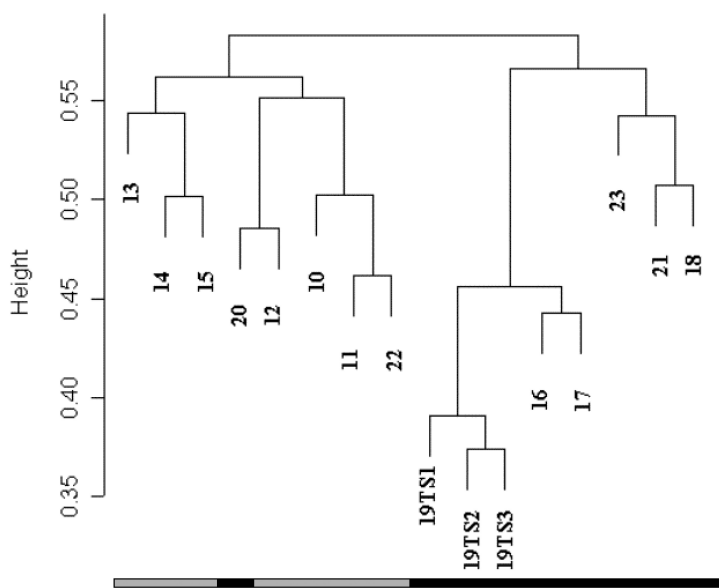


Figure 2. Cluster analysis. Cluster results for RMA expression summaries using RF proximities when the data set included the 16 individual samples and the 424 probe sets significant comparing the early versus advanced HCV-HCC pools. Black box indicates the advanced HCV-HCC samples (T3N0M0 and T4N0M0) and the gray box the early HCV-HCC samples (T1N0M0 and T2N0M0). *19TS1: pretransplant liver tumor sample 1 from patient 19; 19TS2: pretransplant liver tumor sample 2 from patient 19; 19TS3: recurrent tumor sample from patient 19 (posttransplantation).

Recent molecular studies confirm that HCC tissues from different individuals have many phenotypic differences. However, there are clearly features that unify HCC occurring in a background of viral hepatitis C (45,46). For this reason, this study was restricted to the HCV-HCC patients for the microarray analysis. From the analysis of the expression summaries between early and advanced HCV-HCC, we found an important set of differentially expressed genes. This finding supports our previous report (22) using pooled samples, indicating that early and advanced HCV-HCC samples could be differentiated using microarray analysis. Interestingly, a hierarchical clustering analysis classified 93.8% of the total tumor samples and 85.7% of the understaged patient samples at pretransplant time according to stage after the pathological evaluation of the explanted liver. These results suggest that the primary tumors (5 of 6 classified as early HCC at the diagnostic time) could have been classified as advanced HCC using

the gene expression profiling. In our cohort followup, even when 8 patients were understaged at pretransplant time, 5 of 8 patients (62.5% of the understaged HCC cases) did not present recurrence during the follow-up. These patients might have been excluded as liver transplant recipients if an appropriate HCC staging had been performed at pretransplantation time.

The real question is: what gene signature will select the 3 of 8 patients whose tumors cluster in the high-risk category but cause patient death due to metastases posttransplant? This question is raised assuming due diligence of pretransplant metastatic evaluation has eliminated “occult radiologic” metastatic disease. To invoke a strategy to biopsy every HCC, which we currently do not do, what gene signature teased from the advanced HCV-HCC gene expression profiling will classify these “advanced HCCs” as low risk for metastases? Or conversely, what percentage of this patient group should we not transplant? With this extensive

preliminary conceptual profiling and practical gene array experience, we have proposed a prospective, longitudinal, multicenter study to answer these questions. We are also aware of the limitations that can arise from the rigorous criteria for total RNA quality, cDNA and cRNA synthesis, and hybridization to ensure reproducible and accurate microarray data (24) and the need for multicenter patient contribution to eliminate sample size misleading associations as occurred for patient 20 in this study.

Expression profiling had been used previously for staging tumors where the prognostic factor for the cancer is tumor stage, or extent of disease at diagnosis (47-50). However, its utility for staging HCC tumors before therapy selection has not been explored in extension and awaits prospective, multicenter proof of concept as we propose.

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