Discovery of Differentially Expressed Genes Related to Histological Subtype of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common human malignancies in the world. To identify the histological subtype-specific genes of HCC, we analyzed the gene expression profile of 10 HCC patients by means of cDNA microarray. We proposed a systematic approach for determining the discriminatory genes and revealing the biological phenomena of HCC with cDNA microarray data. First, normalization of cDNA microarray data was performed to reduce or minimize systematic variations. On the basis of the suitably normalized data, we identified specific genes involved in histological subtype of HCC. Two classification methods, Fisher's discriminant analysis (FDA) and support vector machine (SVM), were used to evaluate the reliability of the selected genes and discriminate the histological subtypes of HCC. This study may provide a clue for the needs of different chemotherapy and the reason for heterogeneity of the clinical responses according to histological subtypes.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human malignancies in the world, and it usually develops in association with hepatitis B or C viral infection and cirrhosis (*1*, *2*). Development of HCC is associated with mutations in tumor suppressor genes, AXIN1 and β-catenin genes, as well as over- or under-expression of certain oncogenes (*3*, *4*). Although molecular analysis of individual candidate genes has revealed the biological nature of HCC and explained some causes of poor clinical response, the general characteristics and molecular mechanisms of HCC remain unknown (*4*). The recent introduction of microarray technology has allowed researchers to compare tens of thousands of genes from tumor tissues with corresponding genes from non-tumor tissues. This technique has been used in other cancers to elucidate the clinical heterogeneity of the disease, understand the basic biochemical processes, and identify new cancer-related genes (*5*−*11*). In particular, researchers are now focusing on class discovery and predicting the class of human malignancies, which is directly related to the efficacy of tumor therapy (*12*).

Recently, microarray technology has been used to examine the nature of HCC. Waring et al. (*13*) identified the relationship between clinical chemistry and histopathology based on cluster analysis and gene expression data. By comparing gene expression of noncancerous liver and HCC, Xu et al. (*14*) reported the characteristics of hepatitis B virus positive HCC and the cause and effect of the gene expression level involved in cell cycle regulation. Okabe et al. (*4*) reported the genetic mechanism in HCC with gene expression profiles. Also, they selected genes related to Edmonson grade using the Mann−Whitney test and then performed cluster analysis to evaluate them. These studies have shown the usefulness of the microarray technology in understanding the biological nature of HCC.

Here, we report on a new method for using microarray technology to determine discriminatory genes and HCC histological serotypes. Using the DNA microarray gene expression profiles of 10 HCC patients, we first normalized the cDNA microarray data to reduce systematic variations and then identified a number of genes involved in two histological subtypes of HCC. Two classification methods, Fisher's discriminant analysis (FDA) and support vector machine (SVM), were used to evaluate the selected genes and determine the histological subtypes. This new method provides yet another way in which DNA microarray technology is invaluable for cancer research.

Materials and Methods

Patients and Tissue Samples. Primary HCC and corresponding nontumorous liver specimens were obtained from 10 patients. According to pathological data, six patients suffered from poorly differentiated solid (compact) type HCC and four had the moderately differentiated pseudo-glandular type. In patients 5 and 7, the background disease was alcoholic cirrhosis. All others had HBV-related chronic hepatitis.

Preparation of Total RNA. Human 3.1 k cDNA clones were obtained from The Human Stromal Cells cDNA Bank at Kyungpook National University. Total RNA was isolated from liver tissues by a modified

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guanidine isothiocynate-phenol/chloroform extraction method using a MicroRNA isolation kit (Stratagene). After adding the denaturation solution and β-mercaptoethanol, tissue samples were homogenized with a Dounce homogenizer and the homogenate was transferred to a new tube. The homogenate was mixed with 0.1 vol of 2 M sodium acetate and an equal volume of a phenol, chloroform, and isoamylalchol mixture and then placed on ice for 15 min. Samples were centrifuged at 13,000 rpm for 15 min at 4 °C, and then the aqueous phase containing RNA was carefully transferred into a new tube.

RNA Amplification Using in Vitro Transcription Method. Messenger RNA was mixed with T7-Oligo (dT) primer and was heated at 70 °C for 10 min. First strand cDNA synthesis was carried out by icing the mixture and adding the first strand buffer, DTT and dNTPs. The mixture was prewarmed at 42 °C for 2 min, and Superscript II reverse transcriptase was added and incubated at 42 °C for 50 min. The resulting first strand product was then mixed with second strand buffer, dNTP, *E. coli* DNA ligase, *E. coli* DNA polymerase, and *E. coli* RNase H, and the mixture was incubated at 16 °C for 2 h. T4 DNA polymerase was then added, followed by incubation at 16 °C for 5 min. The double-stranded cDNA product was then purified with phenol/chloroform extraction, resuspended in DEPC-treated water, and amplified using the Ampliscribe T7 transcription kit, according to the manufacturer's protocol. The quality and quantity of the double-stranded cDNA was checked by measurement of absorbance at 260 and 280 nm with a UV spectrophotometer (Pharmacia Biotech).

Preparation of Fluorescent DNA Probes from mRNA. Reverse transcription was used to label mRNA extracted from HCC and nontumor liver tissue with either fluorescent Cy3- or Cy5-dCTP. The fluorescent targets were hybridized to the microarrays under stringent conditions, and laser excitation of the microarrays yielded a characteristic emission spectrum that was measured with a confocal laser microscope. Data from the hybridization experiment were viewed as a normalized ratio (Cy5/Cy3).

Normalization. Normalization of cDNA microarray data is needed to reduce or minimize systematic variations. Several normalization techniques have been developed in recent years to minimize the system variation and fluctuation (*16*, *17*). Here, we used a slight modification of the normalization method proposed by Yang et al. (*17*). Instead of producing a ($\log R$ vs $\log G$) plot, where R and G denote the measured fluorescent intensities for the red and green dyes, respectively, we used an MA plot in which $M = \log_2(R/G)$ is plotted against $A = \log_2(RG)$, since this more accurately reflects the intensity-dependent pattern of M. Then, the normalized fold difference was obtained by $M_{\text{nor}} = M - f(M,A)$, where $f(M,A)$ is a locally weighted linear regression model (*18*). This locally weighted regression (LOESS) fitting gives a basis for zero fold difference between the intensities of the two dyes and is robust enough to resist the effect of differentially expressed patterns. Therefore, after normalization, we were able to more confidently identify differentially expressed genes.

Fisher's Discriminant Analysis (FDA). FDA finds the best line that separates two predefined classes by determining the best set of discriminant vectors. In particular, when compared to principal component analysis (PCA), FDA is more efficient from a classification viewpoint, since it uses data with class identification, whereas PCA does not. The difference between PCA and FDA can be represented by the following two generalized eigenvalue problems, respectively:

$$\mathbf{S}_{\text{T}}\mathbf{v}_i = \lambda_i \mathbf{v}_i \tag{1}$$

$$\mathbf{S}_{\text{W}}\mathbf{w}_i = \gamma_i \mathbf{S}_{\text{B}}\mathbf{w}_i, \tag{2}$$

Here, $\mathbf{S}_{\text{T}}$ is total scatter, $\mathbf{S}_{\text{W}}$ is within-class scatter, and $\mathbf{S}_{\text{B}}$ is between-class scatter, and they satisfy $\mathbf{S}_{\text{T}} = \mathbf{S}_{\text{W}} + \mathbf{S}_{\text{B}}$. Also, $(\lambda_i,\mathbf{v}_i)$ and $(\gamma_i,\mathbf{w}_i)$ are eigenvalue-eigenvector pairs of PCA and FDA, respectively. PCA provides the orthogonal vector yielding the maximum total scatter, whereas FDA provides discriminant vectors yielding the maximum ratio of between-class scatter to within-class scatter. A proper number of transforming vectors $\mathbf{w}_i$ can be determined by various criteria such as Akaike information criterion (AIC), minimum description length (MDL) criterion (*19*), or cross-validation. There exist $k - 1$ eigenvectors for k predefined classes, which are obtained from eq 2. The FDA of histological subtypes of HCC is calculated by the abovementioned procedure. However, it may require enormous computation time and causes a singularity problem when the discriminant function includes all variables (genes). Therefore, we used gene selection (discussed below) to choose 59 informative genes for FDA calculations (Figure 1). For more details, refer to Cho et al. (*11*) and Duda et al. (*20*).

Gene Selection. Proper gene selection is crucial for successful identification of HCC histological subtypes and better understanding of the underlying causes of HCC. To determine the histological subtype-specific genes, we used Wilks' λ ratio to assess the discrimination power of individual genes. Here, the Wilks' λ represents the ratio of the within-group sum of squares to the total sum of squares:

$$\Lambda = \frac{\mathbf{S}_{\text{W}}}{\mathbf{S}_{\text{T}}} \tag{3}$$

The above equation can be transformed as follows (*21*):

$$F = \frac{(1-\Lambda)}{\Lambda}\frac{(n-c)}{c-1} \sim \text{F}_{\alpha}(c-1, n-c) \tag{4}$$

where n and c denote the number of samples and the known number of classes.

Informative genes are determined by F-distribution using the level of significance ($\alpha = 0.003$). However, this test is not a good measurement for gene selection because it can be degraded by spike-like noise. To avoid this unusual situation, we introduced a sample leave-one-out procedure (*9*). After the sample leave-one-out procedure, we were able to obtain informative genes with an inherent level of significance. On the basis of those, we constructed the FDA classifier and evaluated it using two test samples.

Support Vector Machine (SVM). The linear discriminant introduced above presents linear decision boundaries for pattern classification. As a generalized concept, SVM produces nonlinear boundaries by generating linear boundaries in the high feature space formed by nonlinear transformation. SVM finds function parameters to realize the maximal margin hyperplane construction. Here, not all training data samples are used to define the resulting decision boundary in finding the maximal margin. Model-based classifiers such as neural networks and fuzzy inference systems use all training data samples to construct a classifier. On the other hand, a decision function in SVM is built on the support vectors

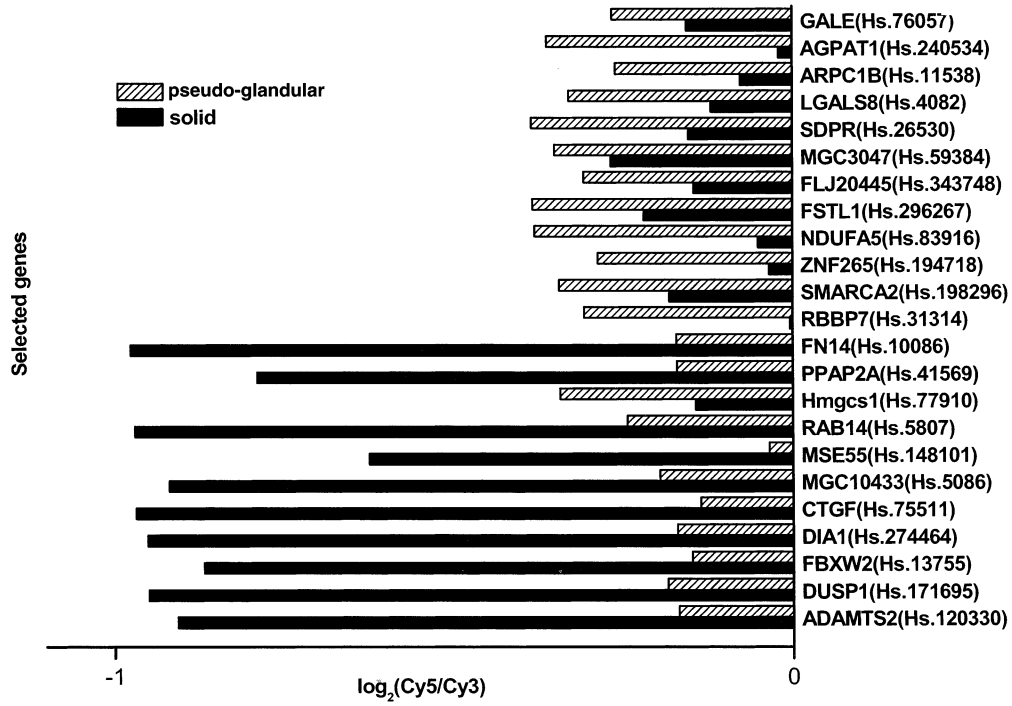

Figure 1. Expression profiles of important parts of the selected genes.

that are a small fraction of training data samples, and so it is not affected by other samples. SVM is trained by two sequential steps: extension to feature space and function building. If the raw input dimension is nonlinearly transformed into a feature space of sufficiently high dimension, the patterns are linearly separable (Cover's theorem). In this respect, if raw data patterns are not linearly separable, a proper nonlinear transformation of raw space into a higher feature space is required. Then, a decision boundary in the feature space is constructed on the basis of support vectors maximizing the margin hyperplane. The SVM solution corresponds to structural risk minimization, thus making the learning procedure deterministic, not stochastic (*22*). For a more detailed description, refer to refs 23−26.

Results and Discussion

We used two methods, FDA and SVM, to classify microarray data from tissue samples of either solid or pseudo-glandular types of HCC. Prior to this, we used Wilks' λ values to determine the HCC subtype-specific genes and applied an F-test to the gene selection. Then we performed FDA with the selected genes and guarded against misclassification through leave-one-out cross validation. Figure 2 shows discriminant scores of the training and test (prediction) sets. In the training set, patients 1−4 (solid HCC) had negative scores, whereas patients 5−8 (pseudo-glandular HCC) had positive scores, showing that we could easily discriminate between solid and pseudo-glandular tumors using FDA with the microarray data. In the prediction set, patients 9 and 10 were correctly classified as solid types. More patient data could allow determination of credible and robust transformation axes, but with only four samples for each case, we had some difficulty in finding consistent estimates for the transformation axes. However, we were clearly able to differentiate between the two different types of HCC with our technique. Similar results were obtained using SVM. Figures 3 and 4 depict classification results in the three-dimensional space spanned by the first three scores and the two-dimensional space spanned by the first two scores, respectively. As shown in these figures, patients 9 and 10 were closer to the cluster of patients 1−4, resulting in their classification as solid types.

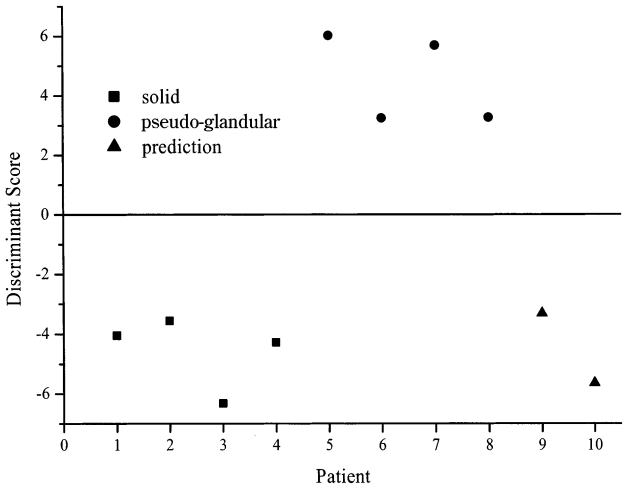

Figure 2. Discrimination result of the subtypes of HCC using FDA.

Cytogenetic Effects of the Selected Genes in HCC. We mainly focused on selecting genes associated with induction of tumor-related morphogenic processes, since our goal was to construct a biologically meaningful classifier that could reveal histological divergence. Classifying HCCs according to histological morphology is clinically relevant in understanding the tumor's type-specific characteristics, although it is not a critical problem for surgical treatments. Hepatic cells alter their gene expression patterns to overcome various extrinsic and intrinsic stresses and maintain homeostasis. In general, most carcinogenesis in liver is associated with the following steps: cell injury by exposure to various extrinsic and intrinsic stresses, inflammation, activation of extracellular matrix (ECM)-producing cells, overproduction of ECM, and irreversible cellular damage (apoptosis or carcinogenesis). This progression leads to

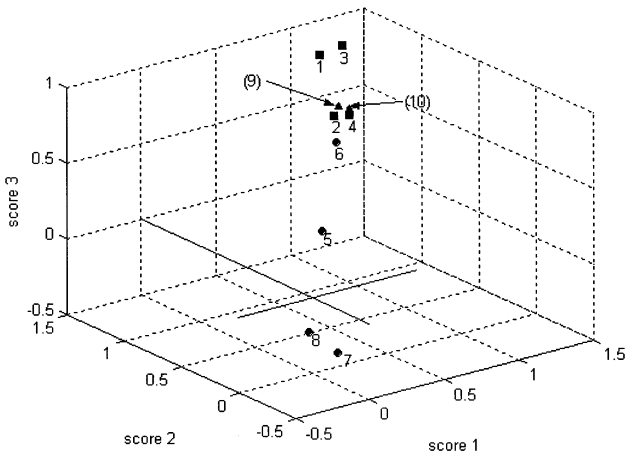

Figure 3. Discrimination result of the subtypes of HCC in the three-dimensional space spanned by the first three discrimination vectors using SVM: (■) solid type, (●) pseudo-glandular type, (▲) prediction.

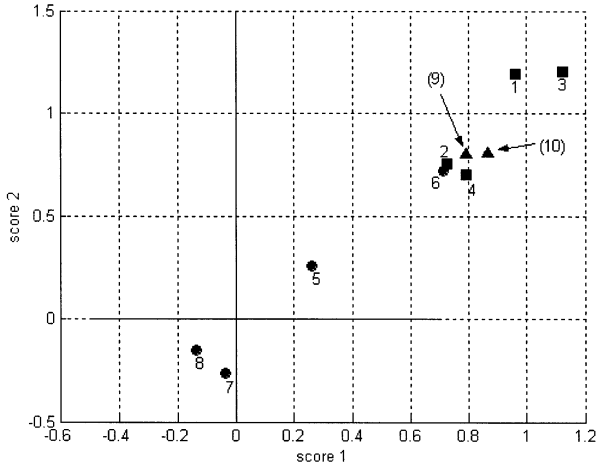

Figure 4. Discrimination result of the subtypes of HCC in the two-dimensional space spanned by the first two discrimination vectors using SVM: (■) solid type, (●) pseudo-glandular type, (▲) prediction.

remarkable changes in the ECM proteins of the stroma of HCC (*27*). Therefore, it is reasonable to hypothesize that some of the genes relevant to the classification of HCC will be related to both the regulation of cellular stress response and synthesis of ECM, since HCC mainly occurs after fibrosis (*28*). In that regards, we examined DUSP1 (Hs.171695), as it is known to reflect the presence of myofibroblasts in breast cancer stroma (*29*). Dual specificity phosphatase 1, the product of DUSP1, suppresses the activation of MAP kinase by dephosphorylating MAP kinase erk2 on both thr-183 and tyr-185 and plays an important role in the regulation of cell cycle and signaling pathways mediated by MAP kinase (*30*, *31*). ADAMTS2 (Hs.120330) encodes a disintegrin and metalloproteinase with thrombospondin motifs-2, the latter being one member of the extracellular matrix protease family. This protease is needed to assemble collagen fibrils in the ECM (*32*). Feng et al. (*33*) reported that Fn14 (Hs.10086), which encodes type I transmembrane protein Fn14, is overexpressed in many HCC specimens. The continuous expression of this protein decreases cellular adhesion of ECM with fibronectin or vitronectin (*34*), which may account for histopathological differences of cellular growth and migration (motility) in HCC.

Next, we investigated whether a divergence in ECM synthesizing or cellular growth genes might reflect the morphological type of HCC. Hadari et al. (*35*) has shown that LGALS8 (Hs.4082, galectin 8) is related to cell adhesion, cell growth regulation, inflammation, apoptosis, and metastasis by interacting with several intergrins. FSTL1 (Hs.296267) binds heparin by secretion and modulates the action of some growth factors on cell proliferation and differentiation. The follistatin-related protein 1 encoded by FSTL1 may function as a negative regulator of cell growth (*36*). CTGF (Hs.75511) encodes a connective tissue growth factor that is well-known to promote fibrosis during pathogenesis and wound healing in liver (*37*) and may be involved in regulating normal and neoplastic cell growth related to the action of tumor growth factor β (*38*). In addition to the abovementioned genes, many other genes may be involved in the discrimination between solid and pseudo-glandular tumors, including genes involved in such mechanisms as fibrillar synthesis of specific types of collagen (Hs.5086: collagen alpha 1(III) chain, Hs.59384: collagen alpha 1(I) chain precursor), vesicular protein traffic as a member RAS oncogene family (Hs.5807: RAB14), galactose metabolism (Hs.76057: Galectin-8), and so on. Consequently, the selected type-specific genes included several informative ones that can mirror the principal cellular phenomena in the various HCC serotypes, such as extrinsic or intrinsic stress response, cellular signaling, ECM-related fibrogenesis, inflammation, proliferation and differentiation, and cellular adhesion to the ECM. This attempt to identify important genes on the basis of the molecular monitoring may one day lead to new therapeutic methods for overcoming HCC by altering type-specific mechanisms.

Conclusion

The purpose of this study was to identify genes that may be used to identify and predict two histological subtypes of hepatocellular carcinoma, solid and pseudo-glandular. We created a systematic approach for the revelation of the biological phenomena involved in the divergence of HCC with gene expression profiles of 10 HCC patients using cDNA microarray analysis. On the basis of normalized data, we identified a number of genes that could be used to discriminate the histological subtypes. The two classification methods, FDA and SVM, were used to evaluate the selected genes, and we found that they were able to effectively discriminate and predict the histological subtypes. Therefore, these methods and genes may be used as markers for classification of these two HCC subtypes and may provide insight into the cytogenetics of HCC, leading to new, improved therapies.

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