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The feasibility of using biopsy samples from esophageal cancer for comprehensive gene expression profiling

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Abstract. Advanced esophageal cancer has been recently treated by multimodal therapy including preoperative chemotherapy or chemoradiotherapy and surgery. A biopsy sample provides a valuable specimen for understanding the biological characteristics of individual esophageal cancer. Pretreatment prediction of the response to chemotherapy or radiotherapy based on biological characteristics using biopsy samples is a desirable goal. In using biopsy samples for molecular analysis, there are two problems; the proportion of cancer cells and the intratumor heterogeneity. This study was conducted to investigate the feasibility of using endoscopic biopsy samples of esophageal squamous cell cancer (ESCC) for comprehensive gene expression profiling (GEP). Comprehensive GEP was performed in 40 bulky ESCC specimens and 10 normal esophageal epithelial specimens from patients who underwent esophageal resection and 52 endoscopic ESCC biopsy samples from 26 patients (two samples per one patient). Unsupervised hierarchical cluster analysis showed distinct profiles between the bulky ESCC specimens and normal epithelial specimens. Also, unsupervised hierarchical cluster analysis revealed distinct profiles between the biopsy ESCC samples and normal epithelial specimens. Moreover, a couple of biopsy samples taken from different locations of the same tumor were closely clustered together. That is, biopsy ESCC samples were distinguished from normal esophageal epithelial specimens and the intratumor heterogeneity of GEP was smaller than intertumor heterogeneity. GEP using biopsy ESCC samples is feasible and has the potential to represent the biological properties.

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Key words: esophageal cancer, endoscopic biopsy sample, gene expression profiling, intratumor heterogeneity

Introduction

Advanced esophageal cancer, which has a poor prognosis, has been previously treated by multimodal therapy including preoperative chemotherapy or chemoradio therapy and surgery (1,2). Neoadjuvant therapy has been shown to improve the prognosis of responders. On the other hand, non-responders not only suffer from side effects but also lose precious time to take advantage of other possible treatments (3,4). Therefore, pretreatment prediction of the response to chemo- or radiotherapy is one of the most desirable goals in clinical practice, but pretreatment clinocopathological factors are unable to predict the response and there is no reliable method. Biological characteristics of a tumor are important factors affecting the malignant potential and sensitivity to chemotherapy or radiotherapy.

A pretreatment biopsy sample provides a valuable specimen for understanding the biological characteristics of individual esophageal cancer. Molecular analyses, such as RT-PCR and immunohistochemistry, of pretreatment endoscopic biopsy samples of esophageal cancer have been performed to understand the biological characteristics of esophageal cancer (5-7). However, only one gene or a few genes have been addressed in these studies. Multiple genetic alterations are involved in the development and progression of esophageal cancer and these aberrations may affect the expression of a large number of genes (8,9) and numerous molecular pathways may contribute to the sensitivity of chemotherapy or radiotherapy. Gene expression profiling (GEP) allows assessment of expression of thousands of genes simultaneously and is one of the powerful tools for understanding the biological characteristics of each tumor. In fact, this approach has already been used to identify genes that could serve as molecular markers of cancer classification and

outcome prediction (10-14). In esophageal cancer, GEP using surgical resection samples has been performed (15-17). However, these results can be used only in the selection of post operative adjuvant therapy or follow-up schedules. To apply the results of GEP to therapeutic planning of esophageal cancer in clinical practice, pretreatment endoscopic biopsy samples should be analyzed. Recently, GEP using not only surgically resection samples but also biopsy samples has been successfully performed (18-21).

Endoscopic biopsy samples are usually small and morphologically esophageal cancer often displays intratumor macroscopic and microscopic heterogeneity. If the biopsy samples used for molecular analysis contain no or few cancer cells, it would not represent the biological characteristics of a tumor. If the gene expression of samples taken from different locations in the same tumor is drastically different, biological classification based on molecular analysis of biopsy samples may not be suitable. That is, in using biopsy samples for molecular analysis, there are two problems; the proportion of cancer cells and the intratumor heterogeneity of gene expression. However, there have been few studies addressing these problems (7).

This study investigated whether biopsy ESCC samples can be distinguished from normal esophageal epithelial specimens by GEP and assessed the intratumor heterogeneity of GEP by analyzing a couple of biopsy samples taken from different locations of the same tumor.

Materials and methods

Patients and clinical samples. Esophageal squamous cell cancer (ESCC) samples were obtained from 40 patients and normal esophageal epithelial specimens from 10 patients who underwent a surgical resection. The clinicopathological characteristics of the resected ESCC specimens are listed in Table I. Normal esophageal epithelial specimens were collected from the area normally stained by the Lugol dye. In addition, a couple of endoscopic biopsy samples of ESCC were obtained from 26 patients and assayed separately (Fig. 1). The clinicopathological characteristics of the biopsy ESCC specimens are listed in Table II. None of the patients received either chemotherapy or radiotherapy before the surgery or endoscopy. Tissue specimens were disrupted in RNAlater (Ambion, Austin, TX) and stored at 4˚C for 1-2 h, then at -80˚C until use. For each biopsy specimen, an adjacent cancer tissue biopsy was given to a pathologist for assessing the presence of cancer and its histology. Routine hematoxylin and eosin- (H&E) stained slides were used. All aspects of this study protocol were performed according to the ethical guidelines set by the committee of the three Ministries of the Japanese Government and a signed consent form was obtained from each subject.

Cellular composition of the biopsy specimens. The cellular composition of biopsy specimens was determined by an evaluation of the cell squares in the H&E-stained slides using light microscopy. These results were recorded as percentages. A total 110 biopsy samples from 45 ESCC patients, partly including patients enrolled in the microarray analysis, were analyzed.

Table I. The clinicopathological characteristics of the resection ESCC specimens.

Characteristics	No. of patients
Gender	
Male/Female	030/10
Age	
Median	64
Tumor location[a]	
Cervical esophagus	1
Upper thoracic esophagus	7
Middle thoracic esophagus	17
Lower thoracic esophagus	15
Pathological T category[a]	
pT1	6
pT2	3
pT3	26
pT4	5
Pathological N category[a]	
pN0	9
pN1	19
pM1(LYM)	12
Pathological disease stage[a]	
pStage I	2
pStage II	11
pStage III	13
pStage IV	14

[a]According to TNM classification.

Extraction and quality assessment of RNA. Total RNA was purified from clinical samples utilizing TRIzol reagent (Invitrogen, San Diego, CA) as described in the accompanying protocol. The integrity of RNA was assessed by Agilent 2100 Bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNA with intact 18S and 28S ribosomal RNA was used for the subsequent analysis. For control reference, 15 RNA samples from normal esophageal epithelial specimens were mixed.

Preparation of fluorescent-labeled aRNA targets and hybridization. The extracted RNA samples were amplified with T7 RNA polymerase using Amino Allyl MessageAmp™ aRNA kit (Ambion) according to the manufacturer's protocol. The quality of each Amino Allyl-aRNA sample was checked by Agilent 2100 Bioanalyzer. Five μg of control and experimental aRNA samples were labeled with Cy3 and Cy5, respectively, mixed and hybridized on an oligonucleotide microarray covering 30,000 human probes (AceGene Human 30K; DNA Chip Research Inc. and Hitachi Software Engineering Co., Ltd., Yokohama Japan). The experimental protocol is available at http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf. Thereafter, the microarrays were scanned using the ScanArray 4000 (GSI Lumonics, Billerica, MA).

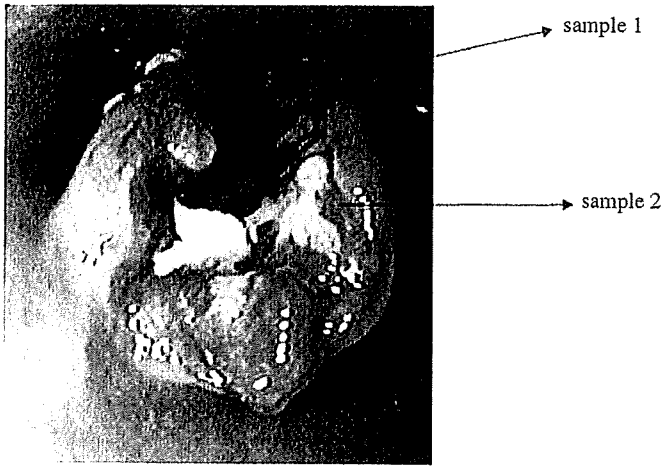

Figure 1. Sampling method for the biopsy ESCC specimens. A couple of biopsy samples were collected from each patient during a routine endoscopic examination.

Table II. The clinicopathological characteristics of the biopsy ESCC specimens.

Characteristics	No. of patients
Gender	
Male/Female	19/7
Age	
Median	67
Tumor location[a]	
Upper thoracic esophagus	11
Middle thoracic esophagus	11
Lower thoracic esophagus	4
Pretherapeutic clinical T category[a]	
cT1	1
cT2	5
cT3	14
cT4	6
Pretherapeutic clinical N category[a]	
cN0	0
cN1	13
cM1(LYM)	13
Pretherapeutic clinical stage[a]	
cStage I	0
cStage II	2
cStage III	9
cStage IV	15

[a]According to TNM classification.

Analysis of microarray data. Signal values were calculated by DNASISArray software (Hitachi Software Inc. Tokyo, Japan). Following background subtraction, data with low signal intensities were excluded from additional investigation. In each sample, the Cy5/Cy3 ratio values were log-transformed and global equalization to remove a deviation of the signal intensity between whole Cy3- and Cy5-fluorescence was performed by subtracting a median of all log(Cy5/Cy3) values from each log(Cy5/Cy3) value. Genes with missing values in >10% of the samples were excluded from further analysis. Hierarchical cluster analysis (HCA) with Euclidean distance as a similarity coefficient and Ward as a clustering algorithm was performed using GeneMath 2.0 software (Applied Maths, Inc., Austin, TX).

Up- or down-regulated genes. Commonly up-regulated genes were defined when their expression levels were 2-fold or more against the control reference in at least 50% of the samples. In addition, commonly down-regulated genes were defined when their expression levels were half-fold or less against the control reference in at least 50% of the samples.

RT-PCR. To verify our microarray data, RT-PCR was performed for two of the commonly up-regulated genes (*MMP9* and *SPARC*). Total RNA (2 μg) from eight biopsy ESCC specimens and control reference (mixture of fifteen RNAs from normal esophageal epithelial specimens) was used for the reverse-transcription reaction with oligo-(dT) primer, using the Reverse Transcription System (Promega, Madison, WI). PCR was performed in a 25 μl reaction mixture containing 1 μl of cDNA template, 0.2 mmol/l of each primer and 1 unit of Taq DNA Polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA), as follows; one cycle of 95°C for 12 min, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1.5 min, followed by 72°C for 10 min. The primers were designed by using Web-based Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). GAPDH was also amplified as a marker to confirm the amounts of cDNA generated from each sample. PCR products were visualized with ethidium bromide following separation by electrophoresis on 2% agarose gel.

Results

Cellular composition of biopsy specimens. The mean percentage of tumor cells and stromal cells were 46 and 26% with a standard deviation of 20 and 16, respectively.

Total RNA yield from biopsy specimens. The average and minimum volume of total RNA from one biopsy sample was 17.7 and 2.2 μg, respectively. The quality of all the extracted RNAs was sufficient for comprehensive GEP with intact 18S and 28S ribosomal RNA.

Gene expression profiling between resection ESCC specimens and normal esophageal epithelial specimens and between biopsy ESCC specimens and normal esophageal epithelial specimens. First, GEP was compared between resection ESCC specimens and normal esophageal epithelial specimens. After gene processing described previously, 18,718 genes were used for further analysis. Unsupervised HCA using all 18,718 genes showed distinct profiles between the two groups (Fig. 2). All resection ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP.

Secondly, GEP was compared between biopsy ESCC specimens and normal esophageal epithelial specimens. After gene processing, 18,734 genes were used for further analysis.

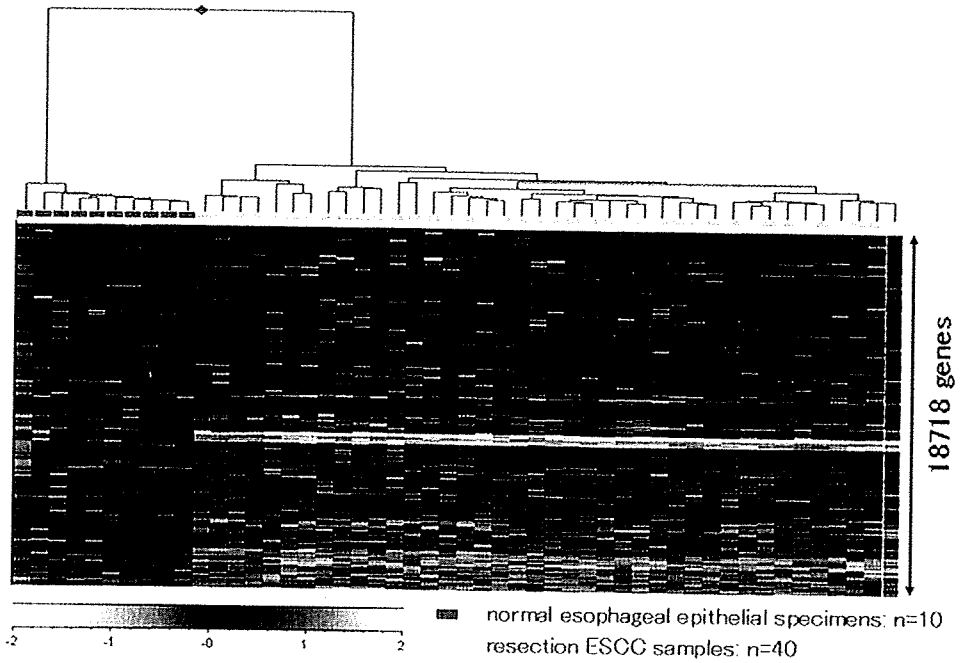

Figure 2. Hierarchical cluster analysis with 18,718 genes in 40 resection ESCC samples and 10 normal esophageal epithelial specimens. The rows and columns represent genes and samples, respectively. The color scale at the bottom indicates the relative expression levels in terms of standard deviations from the median.

Unsupervised HCA using all 18,734 genes showed distinct profiles between the two groups (Fig. 3). Almost all biopsy ESCC specimens except one specimen were distinguished from normal esophageal epithelial specimens by GEP. Moreover, a couple of biopsy samples taken from different locations of the same tumor were closely clustered together, except one case.

Commonly up- or down-regulated genes in resection and biopsy ESCC specimens. A total of 129 and 136 commonly up-regulated genes were identified in the resection and biopsy ESCC specimens, respectively and 85 genes (~65%) were overlapped in both groups. In addition, 518 and 506 commonly down-regulated genes were identified in resection and biopsy ESCC specimens, respectively and 444 genes (~85%) were overlapped in both groups. To confirm the microarray data, RT-PCR was performed for two of the commonly up-regulated genes (*MMP9* and *SPARC*) in eight biopsy samples. These genes have been reported to be associated with progression of esophageal cancer (22,23). The expression patterns of RT-PCR closely agreed with those of the microarray in both genes (Fig. 4).

Discussion

To understand the biological characteristics of individual esophageal cancer, molecular analysis of pretreatment endoscopic biopsy samples have been performed. Miyata *et al* performed immunohistochemical analysis of six molecules in pre radiation biopsy samples. The sensitivity of radiation therapy was significantly correlated with *p53* and *CDC25B* expression (6). Langer *et al* investigated expression of 12 molecules in pretreatment biopsy samples using a real-time RT-PCR analysis and compared the histological effect to cisplatin and 5-fluorouracil chemotherapy. *MTHFR*, *caldesmon* and *MRP1* were significantly associated with the response (7). However, it is clear that several genes will not define the biological characteristics of individual tumors. The properties of each tumor are likely to reflect the functions of all gene products. Therefore, multiple markers will be needed to adequately define the sensitivity of tumors to chemotherapy or radiotherapy and GEP, which can assess the expression of thousands of genes, will likely to be a suitable approach. This is a feasibility study of using biopsy samples in comprehensive GEP for future clinical application.

Cancer tissues consist of mixed populations of cancer cells and stromal cells, such as fibroblasts, infiltrating lymphocytes and endothelial cells. GEP of cancer is currently based on two main methods of RNA preparation; whole tissue RNA extraction and laser capture microdissection (LCM). LCM certainly can improve tissue sampling and achieve homogeneity of the tumor tissue. However, stromal elements play multiple roles in tumor growth and progression and also contribute to tumor response to chemotherapy or radiotherapy (24-26). The biological characteristics of a tumor are considered to be reflected by both the cancer cells and stromal cells, so whole tissues of the specimens were analyzed.

Endoscopic ESCC biopsy samples are small, so it is very difficult to assess the proportion of cancer cells by investigating a part of a sample. In the H&E-stained slides, the average ratio of cancer cells and stromal cells of biopsy specimens was 46 and 26%, respectively, although these samples were different from those actually used in the microarray analysis. If the biopsy sample using comprehensive GEP is composed of mostly normal cells, it would not represent the biological characteristics of a tumor. First, this study confirmed that resection ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP. Then, it verified that biopsy ESCC specimens were distinguished from normal esophageal epithelial specimens by GEP. In this study, the

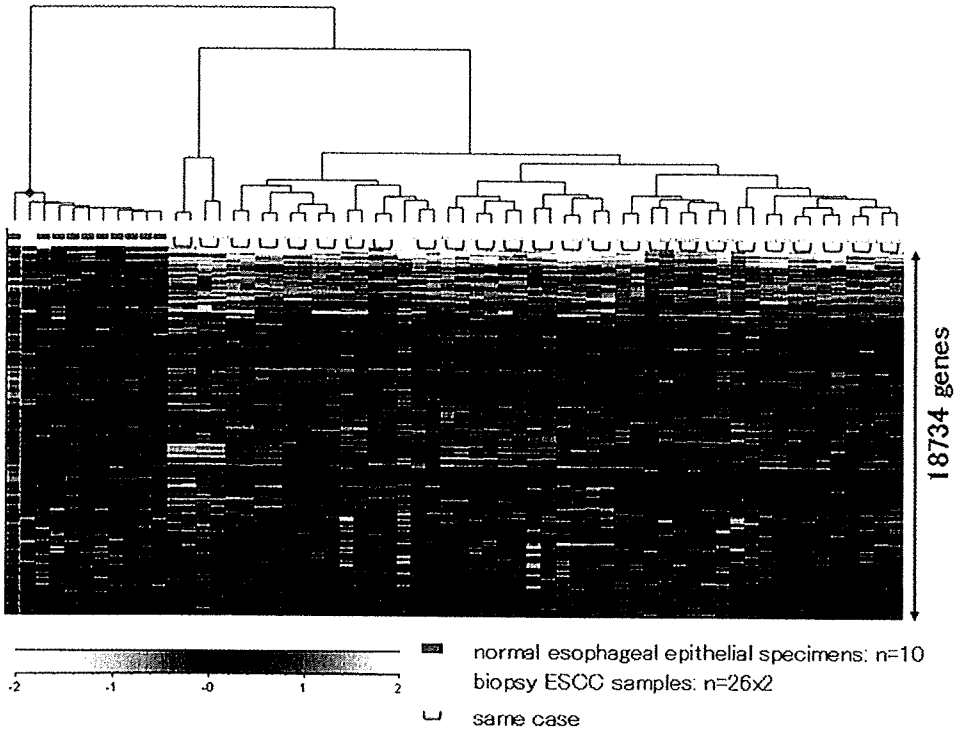

Figure 3. Hierarchical cluster analysis with 18,734 genes in 52 biopsy ESCC samples and 10 normal esophageal epithelial specimens. The rows and columns represent genes and samples, respectively. The color scale at the bottom indicates the relative expression levels in terms of standard deviations from the median.

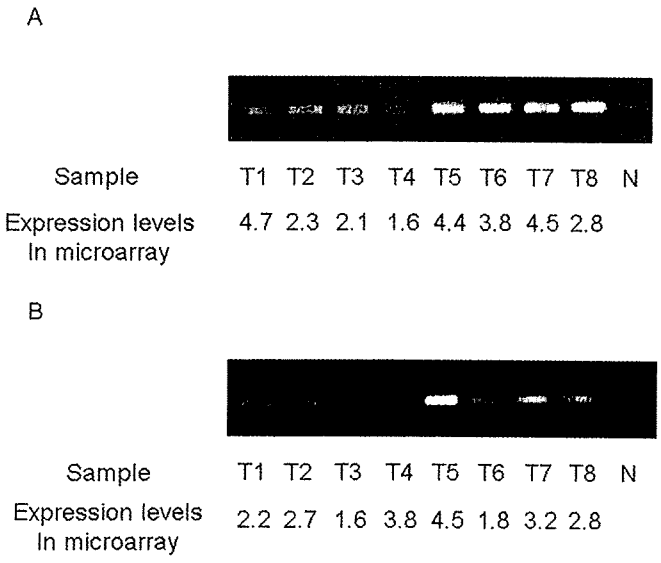

Figure 4. The expression patterns of RT-PCR. (A) *SPARC* and (B) *MMP9*. T1-T8, biopsy cancer sample. N, control reference.

reason that one biopsy specimen could not be distinguished from the normal esophageal epithelial specimens, perhaps, was that there was a low proportion of cancer cells due to a sampling error, though it was possibly adequate for the diagnosis of cancer by microscopy.

There is histopathological heterogeneity in esophageal cancer, as in other solid tumors. The influence of such morphological intratumor heterogeneity on GEP is not clear, though the heterogeneity is detected in individual genes (4,27,28). Therefore, the difference of GEP between biopsy samples taken from different locations in the same tumor should be elucidated. In the current study, a couple of endoscopic biopsy samples obtained from the same case were closely clustered together in almost all cases. This result means that the intratumor heterogeneity of GEP is smaller than intertumor heterogeneity in the superficial position of esophageal cancer and one biopsy specimen may represent GEP of the superficial position of esophageal cancer. Concerning the intratumor heterogeneity of GEP in other solid tumors, the degree of GEP variability within gastric cancer samples isolated from resection specimens of same patient was remarkably low (29). In surgically resected soft tissue sarcomas, the average intratumor distance was considerably shorter than the intertumor distance and intratumor heterogeneity seems to have only a small impact on the variability of GEP (30,31). In endoscopic biopsy samples of colorectal cancer, the intratumor heterogeneity of GEP is smaller than intertumor heterogeneity (21). The findings of these studies are consistent with the current results. It is not clear how many biopsy samples from one patient are sufficient to assess the intratumor heterogeneity. In normal rectal epithelial specimens, two biopsy samples per person are recommended for microarray analysis based on the variation in gene expression data within a person (32). In cervical cancer, although the majority of genes are expressed relatively uniformly, a subset of genes can be expressed quite variably within a single patient. Genes which have a wide variation within a single patient require several biopsies, sometimes >10 biopsies, based on a statistical analysis. However, the optimum number of biopsies cannot be chosen based on statistical reasoning alone, because in the clinical practice, the feasibility of taking many biopsies from one patient is a restrictive factor (33).

The question remains as to whether an endoscopic biopsy ESCC sample reflects the characteristics of the whole tumor. In breast cancer, by comparing GEP of tissue samples with the same cases of FNAB samples, the differences are looked closer (34). Komori *et al* reported that an endoscopic biopsy sample of colorectal cancer might give an accurate picture of the GEP in the whole tumor (21). In this study, although biopsy samples and resection samples were taken from different patients, ~65% of the commonly up-regulated genes and 85% of the commonly down-regulated genes were overlapped. This result indicates that the GEP of endoscopic biopsy samples of ESCC may potentially represent the GEP of whole tumors.

In summary, comprehensive GEP using biopsy ESCC specimens is feasible and has the potential to represent the biological properties of ESCC. Further studies with comprehensive GEP using biopsy samples would provide a novel prediction system of neoadjuvant chemotherapy or chemoradiotherapy for ESCC.

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LIVER TRANSPLANTATION 15:1738-1749, 2009

ORIGINAL ARTICLE

Differential Transcriptome Patterns for Acute Cellular Rejection in Recipients with Recurrent Hepatitis C After Liver Transplantation

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Histopathological evaluation of the liver via biopsy remains the standard procedure for the diagnosis of both acute cellular rejection (ACR) and recurrent hepatitis C (RHC) after liver transplantation. Nevertheless, it is often difficult to diagnose ACR in hepatitis C virus–positive recipients because of changes in common and overlapping with RHC. The aim of this study was to identify potential target genes for ACR in recipients with RHC. We analyzed 22 liver biopsy samples obtained from 21 hepatitis C virus–positive recipients. The clinicopathological diagnosis based on biopsy examination was ACR-predominant with superimposed RHC in 9 samples (ACR group) and RHC without ACR (non-ACR group) in 13. Using oligonucleotide microarrays, we compared the transcriptional changes in the 2 groups and selected 2206 genes that were significantly modulated in ACR. We analyzed the regulatory networks in ACR with Ingenuity Pathway Analysis software, and we confirmed with quantitative real-time polymerase chain reaction the reproducibility of caspase 8, apoptosis-related cysteine peptidase and bone morphogenetic protein 2 up-regulation in another group of validation samples, representing 2 genes from the core network as the target genes for ACR. Our results demonstrated novel transcriptome patterns for ACR with concurrent RHC that were distinct from those of recipients with only RHC, suggesting that gene expression profiling may be useful in the diagnosis of ACR in recipients with hepatitis C. *Liver Transpl 15:1738-1749, 2009.* © 2009 AASLD.

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Although the rapid development of potent immunosuppressants in the last decades has enabled liver transplantation (LT) to be a well-established treatment for various end-stage liver diseases and acute liver failure, immune tolerance is not yet attainable, and acute cellular rejection (ACR) remains a common problem after LT. Despite continuous improvements in immunosuppressive therapy, ACR still occurs in 25% to 40% of

Additional Supporting Information may be found in the online version of this article.

Abbreviations: ACR, acute cellular rejection; AST, aspartate aminotransferase; BCL2, B cell lymphoma 2; BMP2, bone morphogenetic protein 2; CASP8, caspase 8, apoptosis-related cysteine peptidase; CCC, cholangiocellular carcinoma; CFLAR, caspase 8 and Fas-associated protein with death domain–like apoptosis regulator; CyA, cyclosporine A; FK, tacrolimus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFNAR1, interferon (alpha, beta, and omega) receptor 1; IGFBP3, insulin-like growth factor binding protein 3; IL12RB, interleukin 12 receptor beta; IPA, Ingenuity Pathway Analysis; IRAK2, interleukin 1 receptor-associated kinase 2; LC, liver cirrhosis; LT, liver transplantation; LTA, lymphotoxin α; MAP3K11, mitogen-activated protein kinase kinase kinase 11; MAPK10, mitogen-activated protein kinase 10; MMF, mycophenolate mofetil; NFAT, nuclear factor of activated T cells; NFATC3, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3; NS, not significant; qRT-PCR, quantitative real-time polymerase chain reaction; RAD9A, RAD9 homolog A; RHC, recurrent hepatitis C; RT-PCR, real-time polymerase chain reaction; STAT, signal transducer and activator of transcription; STK4, serine/threonine kinase 4; T-Bil, total bilirubin.

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recipients and results in graft loss in some patients.[1-3] In addition, there is often a need to reduce the dose of immunosuppressive drugs to suboptimal levels, especially after the development of infection, renal dysfunction, and/or drug toxicity, and this may facilitate the development of ACR.

Currently, hepatitis C is the most common indication for LT, accounting for 40% to 50% of all cases. However, graft survival in hepatitis C virus (HCV)–infected recipients is worse than graft survival in patients with all other indications, except for malignancy, because of the high recurrence rate and aggressive features of HCV.[4] HCV recurrence is apparent histologically in 40% to 60% of these recipients within the first postoperative year.[1,5] The progression of recurrent hepatitis C (RHC) often exhibits an accelerated course, leading to cirrhosis in 20% of the patients within 5 years and in as many as 50% within 10 years after LT.[6-8]

ACR and RHC can demonstrate similar clinical features, such as worsening liver function tests, and the histomorphology of liver biopsy samples can reveal overlapping features in the 2 entities. However, the treatments of the 2 complications can be quite different. HCV-positive recipients who develop ACR need increased and/or different immunosuppression to blunt the alloimmune response, whereas reduced immunosuppression, sometimes with antiviral therapies (eg, interferon), benefits the host that has RHC. An inaccurate diagnosis can result in therapy that may actually promote the process in question because the mechanisms mediating ACR and RHC are distinct.[6,9,10]

In this context, the diagnosis of ACR and differentiation from RHC remain a challenge in managing transplant recipients with HCV. Although liver biopsy is the most common technique to evaluate both ACR and RHC, the accurate interpretation of liver biopsy samples necessitates skilled and expert LT pathologists, and there may be disagreement even among experienced pathologists.[11] Thus, in practice, it can be difficult to distinguish ACR from RHC histopathologically in HCV-positive transplant recipients. Therefore, there is a need to find novel biomarkers expressed distinctly in ACR to assist in establishing a correct diagnosis.

We hypothesized that ACR is associated with differential transcriptome patterns and used human whole gene oligonucleotide microarrays to find novel markers for ACR. In this study, we used liver biopsy samples from LT recipients to determine the transcriptome patterns in patients with considerable ACR associated with histological features suggestive of either RHC overlapping with ACR or RHC alone. In previous studies,[12-17] significant gene sets were selected by analysis based on the gene expression intensity and functional category, but it remains difficult to detect useful biomarkers for ACR. We adopted Ingenuity Pathway Analysis (IPA) software, which is designed for the analysis of known genome-wide interactions, to clarify the molecular changes between the different cases and to assist in finding genetic markers for ACR.

PATIENTS AND METHODS

Study Sites and Internal Review Board Approval

This study was performed collaboratively between the Department of Surgery and Clinical Oncology of Osaka University Medical School (gene array analysis; principal investigator: Morito Monden) and the Miami Transplant Institute of the University of Miami (clinicopathological diagnosis and sample collection; principal investigator: Tomoaki Kato). The study protocol was approved by the Human Subjects Review Committee of Osaka University and the Internal Review Board of the University of Miami.

Patients and Biopsy Specimens

Twenty-two liver biopsy samples were used in the microarray study. These specimens were obtained from 21 HCV-positive transplant recipients who underwent deceased donor LT at the University of Miami/Jackson Memorial Hospital (Miami, FL) from 1995 to 2004. In addition, we used another group of 32 liver biopsy samples obtained from 25 HCV-positive transplant recipients at Osaka University and the University of Miami who underwent LT between 1995 and 2008 to validate the results of the microarray analysis. All biopsy samples were obtained more than 1 month after LT; the early postoperative period was avoided to minimize the influence of preoperative and early postoperative conditions such as surgical stress. The biopsy samples were obtained when the patients had liver dysfunction, with all changes in immunosuppressive or antiviral therapy being recorded. We defined *liver dysfunction* as elevated total bilirubin, aspartate aminotransferase, and alanine aminotransferase levels (total bilirubin > 2.0 mg/dL, aspartate aminotransferase > 40 IU/L, or alanine aminotransferase > 40). Portions of the liver biopsy samples were immediately immersed in RNAlater (Qiagen, Valencia, CA) and then were frozen with liquid nitrogen and stored at −80°C. The remaining tissue was placed in 10% buffered formalin and routinely processed for histology.

Histopathological Examination of the Liver Biopsy Samples

Hematoxylin and eosin–stained sections of the 54 samples were examined by 2 independent experienced pathologists (P.R. and H.T.) at the University of Miami who were blinded to the clinical information. ACR-labeled specimens were graded according to the Banff classification,[18-20] and the inflammatory grade and fibrosis stage for RHC were scored with the Histological activity index (HAI) system.[21] After the evaluation of these biopsy samples, prior to the gene profiling analysis, the investigators followed the patients and confirmed that those pathological diagnoses matched the clinical course of the patients. On the basis of the clinicopathological assessment, patients were assigned into 3 groups:

1. The ACR group: mild (rejection activity index = 4-5) or moderate (rejection activity index = 6-7) ACR on the basis of the Banff criteria and an inflammatory grade of hepatitis greater than G1. These patients were pathologically diagnosed as ACR-predominant with superimposed RHC.
2. The non-ACR group: no evidence of ACR (rejection activity index = 0-2) on the basis of the Banff criteria and an inflammatory grade of hepatitis greater than G1.
3. The nonrecurrence group: no evidence of ACR and RHC. Tissue samples from 21 normal livers were used as the control samples.

RNA Isolation

RNA isolation and the following experiments were performed at Osaka University, where all research team members were blinded to the clinicopathological data from the University of Miami. Frozen liver tissues were disrupted in TRIzol reagent (Molecular Research Center, Cincinnati, OH) with a tissue lyzer (catalog number 85200, Qiagen, Haan, Germany). Total RNA was purified from clinical samples by TRIzol reagent as described in the protocol provided by the manufacturer. The isolated RNA was quantitated and assessed for purity by UV spectrophotometry. The quality of RNA was confirmed with an Agilent 2100 bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNA with intact 18S and 28S RNA was used for subsequent analysis. As a control reference, we used a mixture of RNAs extracted from 21 normal liver samples collected at Osaka University. The RNA integrity numbers of all 22 samples used for microarray analysis were confirmed to be more than the cutoff value of 6.5 (Supporting Table 1)

Hybridization

We used an oligonucleotide microarray covering human whole genes (AceGene Human 30k, DNA Chip Research, Inc., and Hitachi Software Engineering Co., Yokohama, Japan). The extracted RNA samples were amplified with T7 RNA polymerase with an Amino Allyl MessageAmp antisense RNA kit (Ambion, Austin, TX) according to the protocol provided by the manufacturer. The quality of each amino allyl antisense RNA sample was checked with the Agilent 2100 bioanalyzer. Next, 5-μg control and experimental antisense RNA samples were labeled with Cy3 and Cy5, respectively, mixed, and hybridized on an oligonucleotide microarray covering 30,000 human probes. The experimental protocol is available at http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf. Then, the microarrays were scanned with ScanArray 4000 (GSI Lumonics, Billerica, MA).

Analysis of the Microarray Data

Signal values were calculated with DNASIS Array version 2.6 (Hitachi Software, Inc., Tokyo, Japan). After background subtraction, data with low signal intensities were excluded from additional analysis. In each sample, the values of the Cy5/Cy3 ratio were log-transformed. Global equalization to remove a deviation of the signal intensity between whole Cy3 and Cy5 fluorescence was performed by subtraction of the median of all $\log_2$(Cy5/Cy3) values from each $\log_2$(Cy5/Cy3) value. Genes with missing values in more than 15% of the samples were excluded from further analysis. After normalization, using Gene Math 2.0 (InfoCom, Austin, TX), we performed hierarchical clustering analysis to generate fundamental gene expression patterns inherent in the massive data sets. This cluster analysis was performed with an unsupervised data set to avoid bias based on preclassification by sample type.

Functional Network Analysis

We adopted IPA version 3.1 (Ingenuity System, Mountain View, CA) to clarify the molecular changes between different cases and to assist in finding genetic markers for ACR. IPA is a Web-delivered application that allows visualization of significantly relevant networks based on our experimental data. Differences between the 2 groups were analyzed with the Mann-Whitney test. We selected 2206 differentially expressed genes at the $P < 0.05$ significance level and uploaded a tab-delimited text file into the online software. These submitted genes were used as the starting point for generating biological networks. IPA then computed a score for each network according to the fit of the user's set of significant genes. A score of 2 indicated a 1 in 100 chance that the focus genes were together in a network because of random chance. This score was used as the cutoff for identifying significantly affected gene networks.

Quantitative Analysis by Real-Time Polymerase Chain Reaction (RT-PCR)

For verification of the microarray results, quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the original pooled samples. In addition to this verification, we evaluated the utility of the genes in focus [caspase 8, apoptosis-related cysteine peptidase (CASP8); bone morphogenetic protein 2 (BMP2); nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3 (NFATC3); interferon (alpha, beta, and omega) receptor 1 (IFNAR1); and interleukin 12 receptor beta 2 (IL12RB2)] by qRT-PCR with the 32 validation samples. All primers were designed with the Web-based software PRIMER3 (version 0.9, White Head Research Institute; see http://primer3.sourceforge.net; Table 1.). Total RNA (1 μg) from each pooled sample was subjected to reverse transcription, and complementary DNA was generated with a reverse-transcription system (Promega, Madison, WI). The complementary DNA was synthesized with avian myeloblastosis virus reverse transcriptase according to the protocol provided by the supplier (Promega). In the next step, 1 μg of RNA was mixed with reverse-transcription reaction reagents with oligodeoxythymidylic acid primer.

TABLE 1. Polymerase Chain Reaction Primers Used in Quantitative Real-Time Polymerase Chain Reaction

Gene Name	Gene Symbol	Left Primer	Right Primer
Caspase 8, apoptosis-related cysteine peptidase	CASP8	CACCATCCTGACTGAAGTGAA	CAGAGCGAGATTCTGTCTCAAA
Bone morphogenetic protein 2	BMP2	AAACCTGCAACAGCCAACTC	TTGTTTCTCCTCCAAGTGGG
Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3	NFATC3	TCAGGAGTTCAAGACCAGCC	CGATCTTAGCTCCCTGCAAC
Interferon (alpha, beta, and omega) receptor 1	IFNAR1	GCAGGAGAATCGCTTGAAA	AGTCCGGTGACAGGCTTT
Interleukin 12 receptor beta 2	IL12RB2	ACTGGAGCCTCAGCACATCT	AGCCTCACCACTCAGAGCAT
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	CAACTACATGGTTTACATGTTC	GCCAGTGGACTCCACGAC

The reverse-transcription reaction was performed at 42°C for 90 minutes, and this was followed by heating at 95°C for 5 minutes; it was then placed on ice for 5 minutes before frozen storage.

The expression of target messenger RNA was quantified with a real-time thermal cycler (LightCycler) and detection system (Roche Diagnostics, Mannheim, Germany) with LightCycler DNA Master SYBR Green I (Boehringer Mannheim, Mannheim, Germany). Briefly, a 20-μL reaction volume containing 2 μL of complementary DNA and 0.2 μmol/L of each primer was applied to a glass capillary. The analysis was performed with the LightCycler analysis software (Roche Diagnostics). The expression values of genes were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase of the same samples and rescaled with respect to the control (donor liver). The relative value of each group was calculated from the median of each sample and then $-\log_2$-transformed.

Statistical Analysis

Data were expressed as median and range values. Differences were tested by the exact chi-square test or Mann-Whitney U test, and correlations between 2 variables were analyzed with Pearson's correlation coefficient. All differences were considered statistically significant at a P value less than 0.05.

RESULTS

Clinicopathological Features of the Transplant Recipients and Liver Biopsy Samples

On the basis of the classification described in the Patients and Methods section, the set of 22 samples used in the microarray analysis consisted of 9 samples belonging to the ACR group and 13 samples belonging to the non-ACR group. The other validation group of 32 samples from 25 HCV-positive recipients consisted of 8 samples belonging to the ACR group, 14 samples belonging to the non-ACR group, and 10 samples belonging to the nonrecurrence group, which showed no evidence of ACR or recurrence of HCV. The most common reasons for liver biopsy were elevation of liver enzymes (46/54, 85%), and 8 samples from the nonrecurrence group were obtained because of monitoring during the tapering of basal immunosuppression or interferon therapy (8/54, 15%). All biopsy samples of the ACR and non-ACR groups had evidence of RHC with a similar background inflammatory grade greater than 1. Acute rejection episodes were confirmed by histological findings and responses to antirejection therapy. The clinical characteristics of both groups are summarized in Tables 2 and 3.The median time of all patients from LT to biopsy was 13.4 months (0.9-111.4). All patients received a calcineurin inhibitor with corticosteroids, mycophenolate mofetil, and/or rapamycin. Thirty-nine percent of the subjects (21/54) were on mycophenolate mofetil, and 24% of the subjects (13/54) received a maintenance dose of a steroid. We verified that the pathological diagnosis matched the clinical course of the patients. All patients of the ACR group received rejection therapy with a steroid pulse, or the dose of maintenance immunosuppression was increased; all showed recovery of liver function. Patients of the non-ACR group did not receive rejection therapy but were treated instead with interferon and/or ribavirin. There were no significant differences in age, sex, clinical course, immune suppression, serum total bilirubin, serum alanine aminotransferase, or primary liver disease between the ACR and non-ACR groups (Tables 2 and 3).

DNA Microarray Analyses of the ACR and Non-ACR Groups

After normalization, we identified 14,475 genes. We used hierarchical clustering to generate fundamental gene expression patterns inherent in the massive data sets (Fig. 1). The dendrogram demonstrated separation of the ACR group and non-ACR group, except for 4 samples. It is possible that the massive data set could reflect various clinical features. Unsupervised clustering analysis misclassified 4 of the 22 samples. Although this study included 2 samples from the same patient (ACR, 8; non-ACR, 6), unsupervised clustering successfully classified these samples into different groups on the basis of ACR events. This pattern implied the possibility that ACR is an important factor in gene expres-

TABLE 2. Clinicopathological Features of the Patients Whose 22 Original Samples Were Subjected to Microarray Analysis

Factor	Variable	ACR (n = 9)	Non-ACR (n = 13)	*P* Value
Age (years)		54 (43-64)	50 (38-71)	NS
Sex	Male	8	11	NS
	Female	1	2	
Clinical course (days)		507 (209-3343)	473 (46-1760)	NS
Primary disease	HCV/LC	6	8	NS
	HCV/HCC	3	3	
	HCV/CCC	0	1	
	HCV/HBV	0	1	
Immunosuppression	FK-based	9	13	NS
	Steroid addition	1	4	
	MMF addition	4	4	
	Rapamycin addition	1	0	
ACR grade	Moderate	1		
	Mild	8		
Rejection therapy	Steroid pulse	1		
	FK dose up	8		
Inflammatory grade	G0/G1/G2/G3	0/1/4/4	0/6/6/1	NS
Fibrosis stage	S0/S1/S2/S3	2/0/6/1	5/4/0/4	<0.01
T-Bil (mg/dL)		1.0 (0.8-15)	1.1 (0.4-19.3)	NS
AST (U/L)		64 (29-521)	74 (28-365)	NS

Abbreviations: ACR, acute cellular rejection; AST, aspartate aminotransferase; CCC, cholangiocellular carcinoma; FK, tacrolimus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, liver cirrhosis; MMF, mycophenolate mofetil; NS, not significant; T-Bil, total bilirubin.

TABLE 3. Clinicopathological Features of the Patients from Whom the 32 Validation Biopsy Samples Were Taken

Factor	Variable	ACR (n = 8)	Non-ACR (n = 14)	Nonrecurrence (n = 10)	*P* Value
Age (year)		57 (42-65)	54 (42-69)	55 (46-66)	NS
Sex	Male	3	10	7	NS
	Female	5	4	3	
Clinical course (days)		202 (72-1378)	563 (28-2030)	360 (35-1173)	NS
Primary disease	HCV/LC	5	3	8	<0.05
	HCV/HCC	3	11	2	
Immunosuppression	FK-based	5	11	10	NS
	CyA-based	3	3	0	
	Steroid addition	3	1	4	
	MMF addition	4	5	4	
ACR grade	Moderate	1			
	Mild	8			
Rejection therapy	Steroid pulse	1			
	FK dose up	8			
Inflammatory grade	G0/G1/G2/G3	0/5/3/0	0/10/3/1	10/0/0/0	NS*
Fibrosis stage	S0/S1/S2/S3	0/6/2/0	2/5/6/1	6/3/1/0	<0.05*
T-Bil (mg/dL)		1.0 (0.3-14.7)	1.0 (0.5-33.5)	1.0 (0.5-21.0)	NS*
AST (U/L)		98.5	66.0	23.0	NS*

Abbreviations: ACR, acute cellular rejection; AST, aspartate aminotransferase; CyA, cyclosporine A; FK, tacrolimus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, liver cirrhosis; MMF, mycophenolate mofetil; NS, not significant; T-Bil, total bilirubin.
*There were statistically significant differences between the ACR group and non-ACR group.

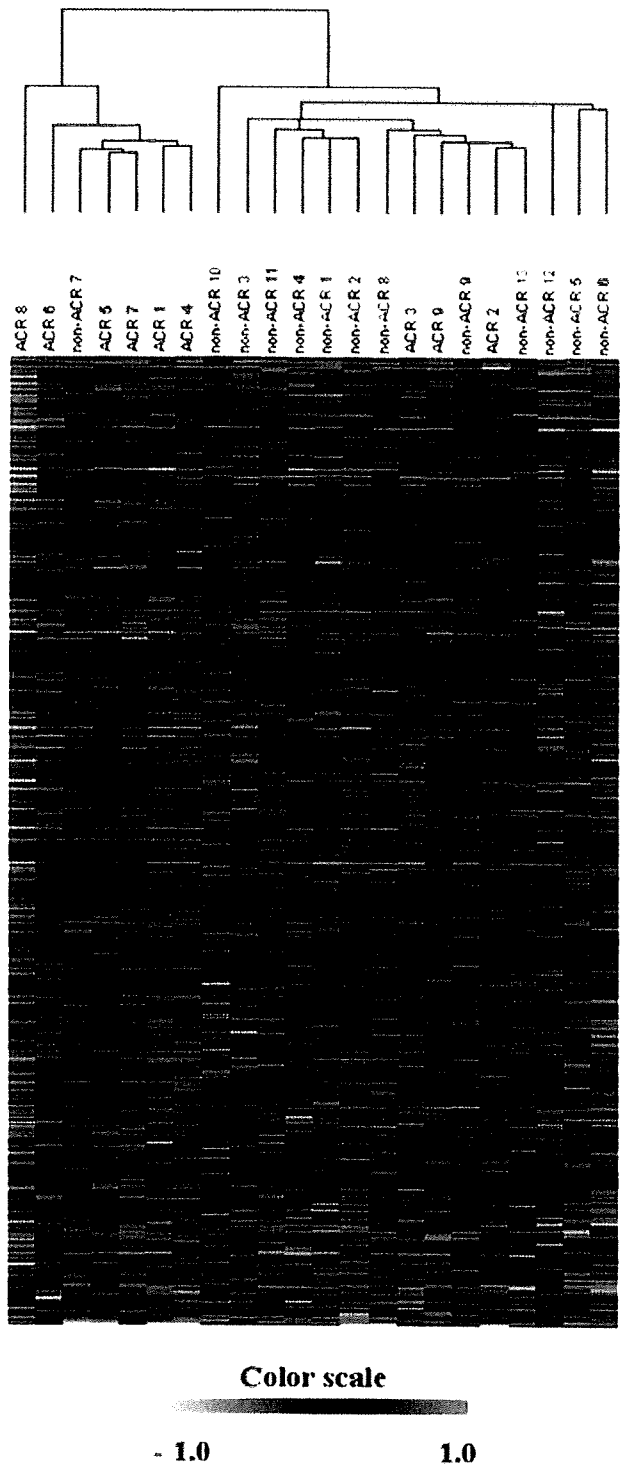

Figure 1. Unsupervised hierarchical clustering of gene expression profiles. Hierarchical cluster analysis with Euclidean distance was based on 14,475 full genes. The dendrogram in the unsupervised clustering demonstrated separation of the ACR group and non-ACR group, except for 4 samples. This pattern implied possible biological or clinical relevance inherent in the massive data sets. Red and green indicate the relative extents of gene overexpression and underexpression, respectively, with darker colors indicating larger differences. Abbreviation: ACR, acute cellular rejection.

sion profiles in the liver in comparison with other biological and clinical variables.

Differences between the 2 groups were analyzed with the Mann-Whitney test. We selected 2206 differentially expressed genes at the $P < 0.05$ significance level, and reclustering was performed with these genes. The supervised approach yielded a clear separation of both groups and revealed predominant gene expression patterns for the ACR group (Fig. 2).

Relative Overexpression of the Top 126 Genes in ACR

Of the 2206 genes, 992 showed up-regulated expression and 1214 showed down-regulated expression in the ACR group versus the non-ACR group. The top 126 relatively overexpressed genes (fold change > 1.25) from the 992 up-regulated genes in the ACR group are listed in Supporting Table 2. The biological processes corresponding to these 126 up-regulated genes based on Gene Ontology (see http://www.geneontology.org) included signal transduction (16 genes), inflammatory and immune response and antigen presentation (15 genes), cell cycle and cell differentiation (13 gene), cell adhesion (12 genes), metabolism (11 genes), regulation of transcription (10 genes), apoptosis and cell death (7 genes), transport (7 genes), and others (31 genes).

Pathway Analysis of the Regulated Genes in ACR

Network analysis was conducted for 2206 genes. Their gene accession numbers were imported into the IPA software. A total of 1561 genes were mapped to the Ingenuity database, and 75 genetic networks were identified, which were ranked by a score. The score is the probability that a collection of genes equal to or greater than the number in a network can be achieved by chance alone. Eight networks with a score > 15 that most significantly affected ACR are listed in Table 4. These networks were related to pathways involved in cell death, immunological disease, hematological system development and function, gene expression, drug metabolism, cell cycle, cellular movement, and DNA replication.

In the next step, using all 75 networks, we constructed biological interactions and focused on the core part of the merged network (Fig. 3). The pathway shown in Fig. 3 promotes mainly the modulation of several genes associated with apoptosis, antiapoptosis, cell death, G1/S checkpoint regulation, and immune response.

Verification of the Microarray Data

We selected 5 up-regulated representative genes (IFNAR1, NFATC3, IL12RB2, CASP8, and BMP2) from the core network shown in Fig. 3 because this network seemed to reflect most significantly the molecular changes between each event. Using unamplified total

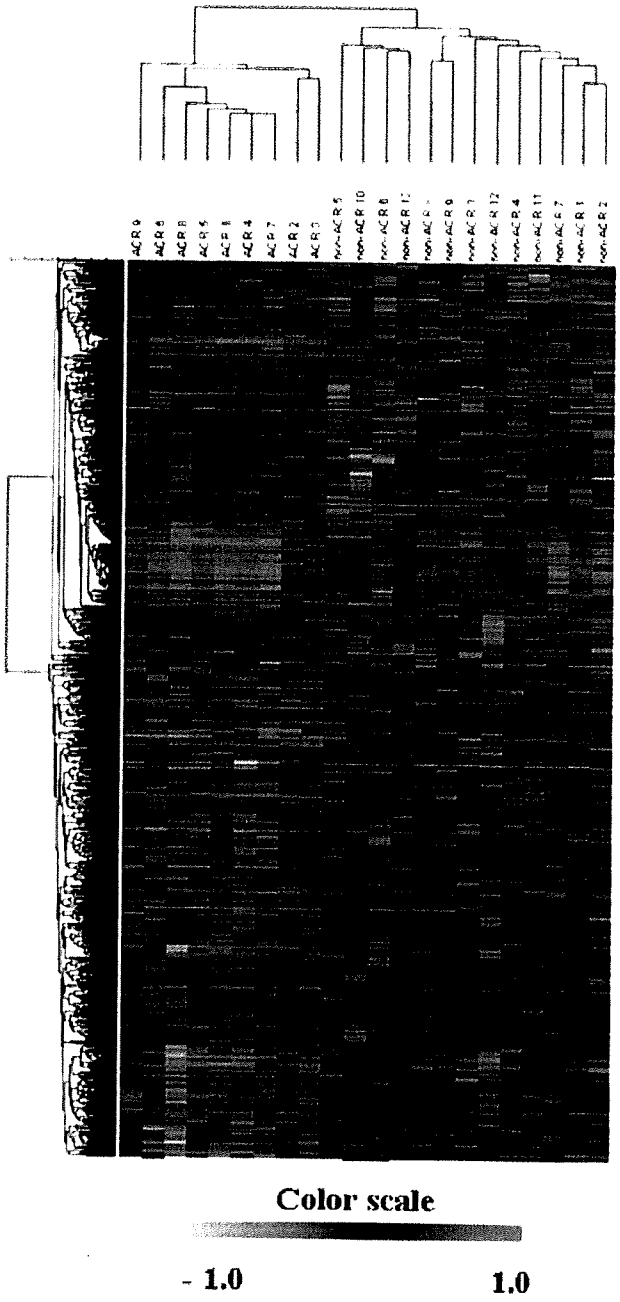

Figure 2. Gene expression pattern with selected 2206 genes. Cluster analysis was performed with Pearson correlation analysis based on 2206 differentially expressed genes. Patients could be divided into 2 groups. The molecular analysis was in agreement with the clinical diagnosis. Samples were sorted on the basis of the corresponding rejection or nonrejection, rather than the grade of hepatitis. Abbreviation: ACR, acute cellular rejection.

RNA originally extracted from 22 liver tissue samples, we performed qRT-PCR for 2 genes (CASP8 and BMP2) to verify our microarray data. The data of qRT-PCR validation confirmed the impression from the microarray data ($P < 0.05$; Fig. 4).

Evaluation of the 5 Representative Genes with the 32 Validation Biopsy Samples

The RT-PCR studies of these 5 representative genes were extended to the 32 validation samples. These 5 genes (CASP8, BMP2, NFATC3, IFNAR1, and IL12RB2) play important roles in immunological function and the induction of apoptosis. Among the 5 genes, CASP8 and BMP2 were significantly up-regulated in the ACR group versus the non-ACR group and nonrecurrence group ($P < 0.05$; Fig. 5). Furthermore, NFATC3 tended to be up-regulated in the ACR group, but there were no significant differences in IFNAR1 and IL12RB2 between the groups.

DISCUSSION

Recent studies have yielded a number of gene expression profiles that appear to be associated with outcomes in other types of solid organ transplantation.[13-17] For example, using human protocol biopsy samples, Scherer et al.[14] identified gene expression patterns that were highly prognostic for the development of renal chronic allograft rejection. Furthermore, Flechner et al.[15] used microarray analysis of peripheral blood lymphocytes and identified distinct gene expression profiles for both biopsy samples and peripheral blood that correlated with renal transplant outcome. In LT, using a 6400 oligonucleotide microarray, Sreekumar et al.[12] found 40 differentially regulated genes in ACR versus RHC from protocol liver biopsy samples on postoperative day 21. Despite such extensive research worldwide, there are no confirmative data yet that indicate that ACR has distinct gene expression profiling in cases that amalgamate RHC after LT.

In the present study, we applied microarray analysis to 9 ACR-predominant cases with RHC and 13 cases with RHC only. Despite some clinical and pathological similarities between the 2 groups (ACR and non-ACR groups), our results showed that they displayed distinct transcriptome (messenger RNA) profiles (Fig. 2). We identified 2206 genes with expression levels significantly different between the 2 groups and later detected genes associated with ACR in samples with both ACR and RHC (Fig. 2). The selected 2206 genes clustered the samples into 2 main groups, one that included all samples of the ACR group and another that included only the non-ACR group. Samples were sorted on the basis of the corresponding rejection or nonrejection and not on the basis of the hepatitis grade. This finding supports the possibility that gene expression profiling could be a useful and sensitive adjunct assay for ACR complementary to liver biopsy.

The threshold used for statistical significance was the traditional $P < 0.05$ level. However, we expected the presence of about 100 false positive genes in every 1000 analyzed data. We paid attention to genes that were up-regulated by more than 1.25-fold because we consider the use of this threshold to be an inadequate control for the false positives. Furthermore, we adopted

TABLE 4. Genetic Networks with High Scores in the Acute Cellular Rejection Group

Network	Gene Symbols in Ingenuity Network*	Score	Focus Genes	Top Functions
1	**AKAP11**, **ALOX15**, BAG3, BAX, BCAP31, BCL2, BCL2L1, BCL2L11, **CASP8**, CASP9, CD177, **CFLAR**, CSF3, **FFAR3**, **IFNAR1**, **IGFBP3**, **IL12RB1**, **LTA**, PAX4, **POU4F1**, PPP1CA, **PPP1R8**, **PPP1R3A**, PPP1R9B, PRF1, **PVRL1**, RAD9A, SPHK2, STAT2, STAT6, TEGT, TNFRSF25, TNFSF13, **TNK2**, WWOX	38	35	Cell death, hematological disease, and immunological disease
2	APBB1, APLP2, APOH, CPB2, CTSD, DNAJB9, **ENAH**, F7, F9, F10, F11, FGA, FGG, **FLNB**, **FN1**, GP1BA, HPN, LGALS3BP, LPA, **MEP1A**, MTCP1, PCBP2, PCBP1 (includes EG:5093), PLG, PROC, PROCR, PROS1, **PRPF40A**, PRTN3, SERPINA5, SERPINA10, SERPINC1, SERPINF2, SH2D3A, **TCERG1**	38	35	Hematological system development and function, organismal functions, and hematological disease
3	ALG5, ARID3B, **ARID4A**, **CBX5**, **CDK9**, **CXADR**, **DNMT3L**, **EPHA8**, **GPS2**, **GUCA2A**, **HDAC3**, HDAC4, HDAC5, HEXIM1, **HOXB4**, **KLF6**, LDB3, MAP3K3, MEF2D, MORF4L1 (includes EG:10933), MYOD1, NR4A1, PGRMC1, PML, **RARA**, **RB1**, **SEPT4**, SOX7, SOX15, TDG, THAP7, **TNNI2**, **TRIM24**, ZBTB16, **ZNF638**	38	35	Gene expression, cancer, and gastrointestinal disease
4	**ADCY2**, ANAPC5, ANAPC11, **BMP2**, **BRAF**, **CDC27**, **ESR1**, GJB1, GREB1, HSP90AB1, **IKBKE**, IRAK1, **IRAK2**, MAP1S, **MAP3K11**, **MAPK10**, **MED28**, **MLLT7**, MTA3, **NF2**, **NFATC3**, NPR1, **PCQAP**, **PELI2**, **PGR**, **PPID**, PPP5C, RASSF1, **S100G**, **STIP1**, **STK4**, TAT, UBE2L6, **UBE3A**, UNC45A	38	35	Drug metabolism, endocrine system development and function, and lipid metabolism
5	ADRA1D, APOC3, ATF4, ATP5G2, CEBPA, **CEBPB**, CSDE1, DCC, DSG3, EDF1, EEF1A2, FOSB, FOSL2, GH1, GLUD1, HAMP, KLK8, LR8, LST1, NDRG2, **NFKBIZ**, **NFYC**, ORM1, PRDX3, SECTM1, **SNPH**, SOD1, **SOX4**, SULT1A3, **TAF2**, **TAF11**, THRSP, **TRIB3**, **USP33**, ZNF587	38	35	Gene expression, connective tissue development and function, and cellular development
6	**ANP32A**, **BIRC5**, CCNE1, CLU, **COL6A1**, **CUL3**, DAD1, DHCR7, EDN3 (includes EG:1908), **EGR1**, **ELK3**, **ERBB3**, **FOXM1**, G3BP, GAD1, GAS1, GHR, IDH1, IER2, **IGF2**, INSL3, **LAMA4**, MATK, MBOAT5, MLXIPL, **NRG1**, PFDN6, PFKFB1, PIK3R3, **PITX2**, **PKLR**, PNMT, RNF103, TNXB, **TOB2**	38	35	Cell cycle, developmental disorder, and cellular growth and proliferation
7	ACO1, AR, ARF1, **ARF4**, ARFIP2, C3, C1S, CALM2, CD151, CFH, CTSB, **DDEF1**, DDEF2, **DPYSL2**, DPYSL5, DRD4, FOXO3A (includes EG:2309), GAPDH, GIPC1, **GORASP1**, **ITGA6**, JRK, K-ALPHA-1, KCNJ9, MARCKS, PGAM1, PIAS3, PKN1, PLD2, RBP4, RPL26, RPLP1, RPS9, **TCF7L2**, TMED10	38	35	Cellular movement, posttranslational modification, and cancer
8	CDC25A, CYB5A, DERL1, EPOR, ERCC1, ERCC4, FADS2, **GLB1**, H2AFX, IL4R, **JAK3**, **MAX**, MID1, MID2, MXD4, MYCT1, N-PAC, NGLY1, POT1, PPP2CB, PPP2CBP, PPP2R4, PPP2R1A, PPP2R1B (includes EG:5519), PPP2R2C, PPP2R5C, RAD52, RCC1 (includes EG:1104), RNF17, SELS, SYVN1, TERF2, **UBE4B**, VCP, **WRN (includes EG:7486)**	15	22	DNA replication, recombination, and repair, cell cycle, and protein degradation

*The listed genes are included in the selected 2206 genes, except for the underlined genes; bold text is used for up-regulated genes, and regular text is used for down-regulated genes.

IPA to clarify the molecular changes in the different cases and to identify genetic markers for ACR.

The biological processes of the top 126 genes, which showed up-regulation of more than 1.25-fold in the ACR group versus the non-ACR group, are listed in Supporting Table 2. They were notably associated with the signal transduction and immune response categories; this finding supports the clinicomorphological impression of increased alloreactivity.

We detected the relative overexpression of genes associated with major histocompatibility complex class 1, lymphocytes, and other inflammatory cell activity. Mitogen-activated protein kinase-activated protein kinase 2 and ribosomal protein S6 kinase (90 kDa) polypeptide 1 encode a member of the Ser/Thr protein kinase family, which is associated with the mitogen-activated protein kinase signaling pathway. Mitogen-activated protein kinase signaling pathways are known to be essential for T cell regulation through the induction of activator protein 1 activation.[22] Human leukocyte antigen F, which is expressed on the surface of T and B cells, is considered a candidate serum marker of acute rejection in human LT.[23] Fc fragment of immunoglobulin G low affinity IIa receptor (CD32), which is expressed on macrophages and neutrophils, has been reported also as a potentially useful marker for risk of rejection in renal transplantation.[24] Chemokine (C-C motif) receptor 3 is overexpressed in eosinophils, ba-

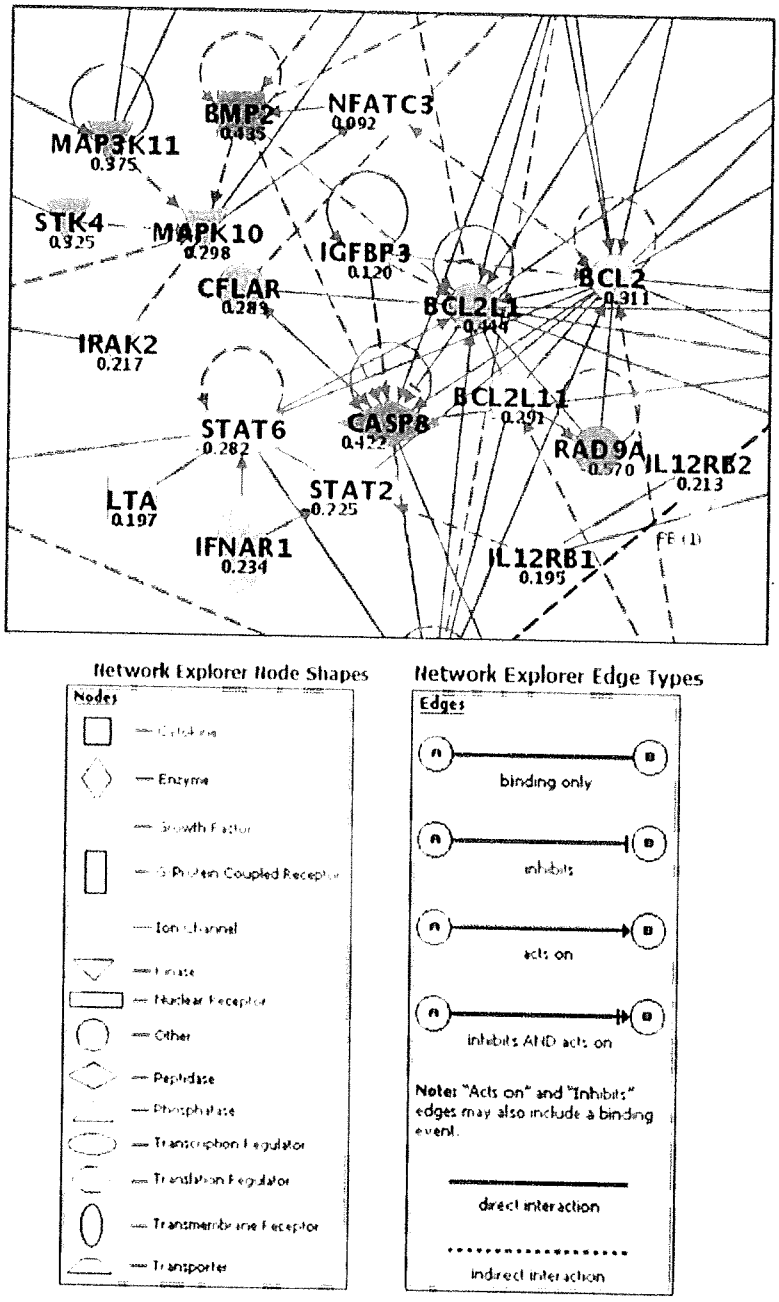

Figure 3. IPA. The data set containing 2206 genes was used as the starting point for generating biological networks. IPA identified 75 biological networks. Nodes represent genes, their shape represents the functional class of the gene product, and the edges indicate the biological relationships between the nodes. The color concentration indicates the intensity of expression, with red for overexpression and green for underexpression (relative fold change versus non-ACR). Abbreviations: ACR, acute cellular rejection; BCL2, B cell lymphoma 2; BMP2, bone morphogenetic protein 2; CASP8, caspase 8, apoptosis-related cysteine peptidase; CFLAR, caspase 8 and Fas-associated protein with death domain–like apoptosis regulator; IFNAR1, interferon (alpha, beta, and omega) receptor 1; IGFBP3, insulin-like growth factor binding protein 3; IL12RB, interleukin 12 receptor beta; IPA, Ingenuity Pathway Analysis; IRAK2, interleukin 1 receptor-associated kinase 2; LTA, lymphotoxin α; MAP3K11, mitogen-activated protein kinase kinase kinase 11; MAPK10, mitogen-activated protein kinase 10; NFATC3, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3; RAD9A, RAD9 homolog A; STAT, signal transducer and activator of transcription; STK4, serine/threonine kinase 4.

sophils, T helper 1, and T helper 2 cells. This receptor contributes to the accumulation and activation of eosinophils and other inflammatory cells. Natural cytotoxicity triggering receptor 1 and SLAM family member 7, which are closely associated with natural killer cell activation,[25,26] were also relatively overexpressed. Up-

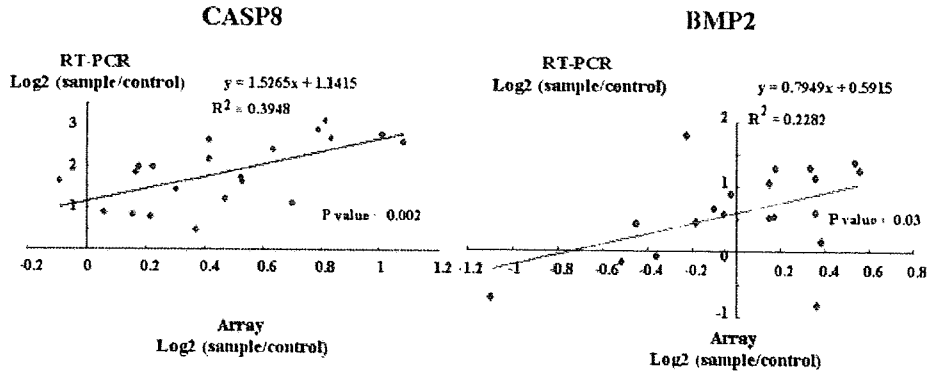

Figure 4. RT-PCR verification of the microarray results. The gene expression levels were first normalized to glyceraldehyde-3-phosphate dehydrogenase of the same samples and rescaled with respect to the control (donor liver). The expression level was calculated as the logarithm of each sample/control. Abbreviations: BMP2, bone morphogenetic protein 2; CASP8, caspase 8, apoptosis-related cysteine peptidase; RT-PCR, real-time polymerase chain reaction.

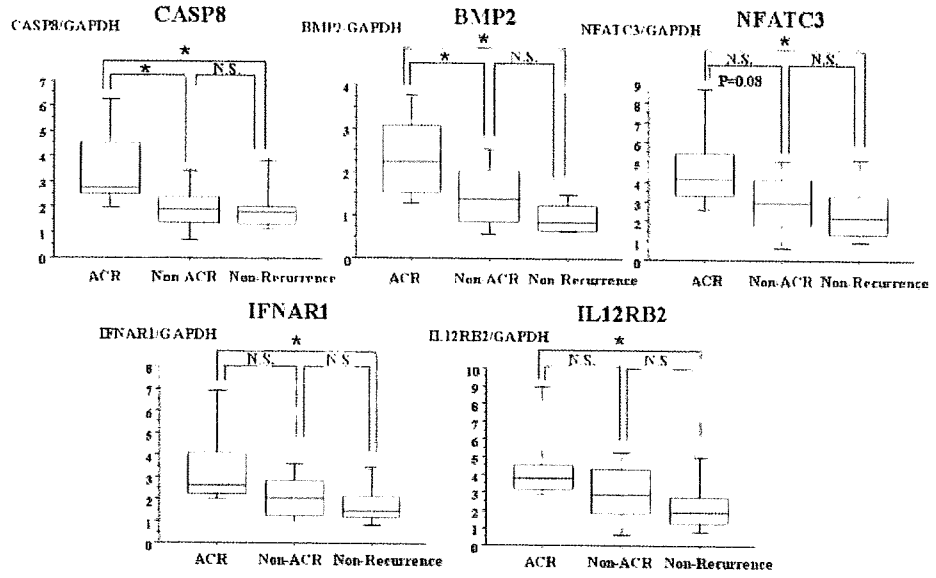

Figure 5. Evaluation of 5 representative genes using 32 liver biopsy samples from the validation group. The gene expression levels were normalized to GAPDH of the same samples. RT-PCR data of each group were compared with the Mann-Whitney test. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. *P < 0.05. Abbreviations: ACR, acute cellular rejection; BMP2, bone morphogenetic protein 2; CASP8, caspase 8, apoptosis-related cysteine peptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFNAR1, interferon (alpha, beta, and omega) receptor 1; IL12RB2, interleukin 12 receptor beta 2; NFATC3, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3; NS, not significant; RT-PCR, real-time polymerase chain reaction.

regulation of these genes reflects infiltration of lymphocytes, macrophages, eosinophils, and natural killer cells to the liver graft as well as the host immune status during ACR. This finding supports the notion that ACR is characterized by antigen-triggered T cell activation and subsequent migration of other inflammatory cells.

Although the *P* values and fold changes help us to recognize the selected genes, they do not provide a better understanding of molecular interactions. It is difficult to extract new biological insights from high-throughput genomic studies because of difficulties in recognizing and evaluating relevant biological processes from a large amount of experimental data. Therefore, the integrated analyses should be applied to understand inclusively the correlations among 2206 genes.

We used the IPA tool to categorize our microarray results. The pathways represented by the selected 2206 genes included 75 subnetworks, and we focused on the high-score subnetworks listed in Table 4. On the basis of these networks, we detected simultaneous up-regulation of lymphotoxin α (tumor necrosis factor superfamily, member 1), CASP8 (apoptotic pathway), caspase 8 and Fas-associated protein with death domain–like apoptosis regulator (CFLAR; apoptotic pathway), IFNAR1 (interferon signaling), retinoblastoma 1 (cell cy-

cle: G1/S checkpoint regulation), NFATC3 (T cell receptor signaling), BMP2 (transforming growth factor β signaling and nuclear factor kappa B signaling), CCAAT/enhancer binding protein (C/EBP) beta (interleukin 6 signaling), and ubiquitin specific peptidase 33 (protein ubiquitination pathway), which implied an extensive pathway interrelationship in the development of ACR in the liver.

Using all 75 subnetworks, we constructed biological interactions and focused specifically on the core network (Fig. 3). We considered this part to be the most attractive in the merged complicated network for discriminating ACR, and accordingly, we paid attention to 5 genes (IFNAR1, IL12RB2, NFATC3, BMP2, and CASP8) located in the core network as the target genes for ACR.

IFNAR1 encodes a type I membrane protein that forms 1 of the 2 chains of the receptor for interferon α and interferon β. Type I interferons are potent immunomodulatory cytokines that enhance the expression of major histocompatibility complex class I antigens, T cell cytotoxicity, and natural killer cell activity, all of which are implicated in graft rejection.[27-29] The expression of IL12RB2 is up-regulated by interferon γ in T helper 1 cells and plays a role in T helper 1 cell differentiation.[30] Interferon γ–secreting T helper 1 cells contribute to the induction of allograft rejection; thus, IL12RB2 is thought to be an important effector of ACR.

Our analysis showed relative overexpression of NFATC3 in ACR. NFATC3 is a member of the NFATC family of transcription factors, which play pivotal roles in the development and function of the immune system. Their pathway is tightly regulated by calcium-dependent phosphatase calcineurin and is a target of the immunosuppressive drugs cyclosporine A and tacrolimus.[31] These 2 agents block the phosphatase activity of calcineurin and consequently inhibit dephosphorylation and nuclear localization of nuclear factor of activated T cells (NFAT) proteins. BMP2 gene expression is induced by activated calcineurin/NFAT and is suppressed by a calcineurin inhibitor.[32] However, our results showed relative overexpression of NFATC3 and BMP-2 in the ACR group, suggesting a lack of effect of calcineurin inhibitors. This finding may be related to known individual variations in calcineurin inhibitors and a lack of correlation between trough drug levels and susceptibility to ACR. In this respect, it remains unknown whether high NFATC3 and BMP2 levels reflect the effectiveness of calcineurin inhibitors and whether they are associated with an increased risk of ACR.

Our results showed relative down-regulation of antiapoptotic genes of the B cell lymphoma 2 (bcl-2) family (BCL2, BCL2L1, and BCL2L11) in ACR versus the non-ACR group. On the other hand, genes involved in the induction of apoptosis (CASP8, CFLAR, and lymphotoxin α) were relatively up-regulated during ACR. These results support the notion that induction of apoptosis is one of the major components of ACR. In this respect, Sreekumar et al.[12] commented that apoptosis, in addition to T cell activation, is mechanically more important in ACR than in the recurrence of HCV on the basis of their microarray results.

We evaluated the reproducibility of the selected 5 genes (IFNAR1, IL12RB2, NFATC3, BMP2, and CASP8) in the validation samples. The expression levels of CASP8 and BMP2 determined by qRT-PCR were significantly higher in the ACR group than the non-ACR and nonrecurrence groups. The intragraft expression of the other 3 selected genes (IFNAR1, IL12RB2, and NFATC3) tended to be higher in ACR than non-ACR. These 5 selected genes might provide important clues about the biological mechanisms of ACR and also may be useful for the detection ACR in recipients with HCV infection. However, determining which of the candidate genes contributes to ACR will require further experimental approaches.

One of the limitations of this study is the sample size. Although bias in the interpretation of liver biopsy samples could be high because of the small number of liver biopsy samples, the results of our study are still valid for the following reasons: (1) the clinicopathological diagnosis and basic analyses were performed at 2 different sites, with each site blinded to the information held by the other, and (2) the differential profiles of the 5 selected genes were also considered to be potential predictors of ACR in HCV-positive recipients on the basis of our analysis of the validation liver biopsy samples.

Another limitation of this study is the patients' background. The study population consisted of HCV-positive recipients; therefore, the results reflect the distinguished profile between ACR with RHC and RHC alone and do not represent the pure ACR event. To generalize the results to patients without RHC, further research using a non-HCV population is necessary.

In conclusion, microarray analysis demonstrated a distinct gene expression profile in association with ACR. The application of this technology to the study of ACR may lead to the development of an adjunct diagnostic tool and novel biomarkers for monitoring immunosuppression and treatment outcomes. Furthermore, these studies provide new insights into the molecular mechanisms underlying ACR in recipients who also experience RHC.

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