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.

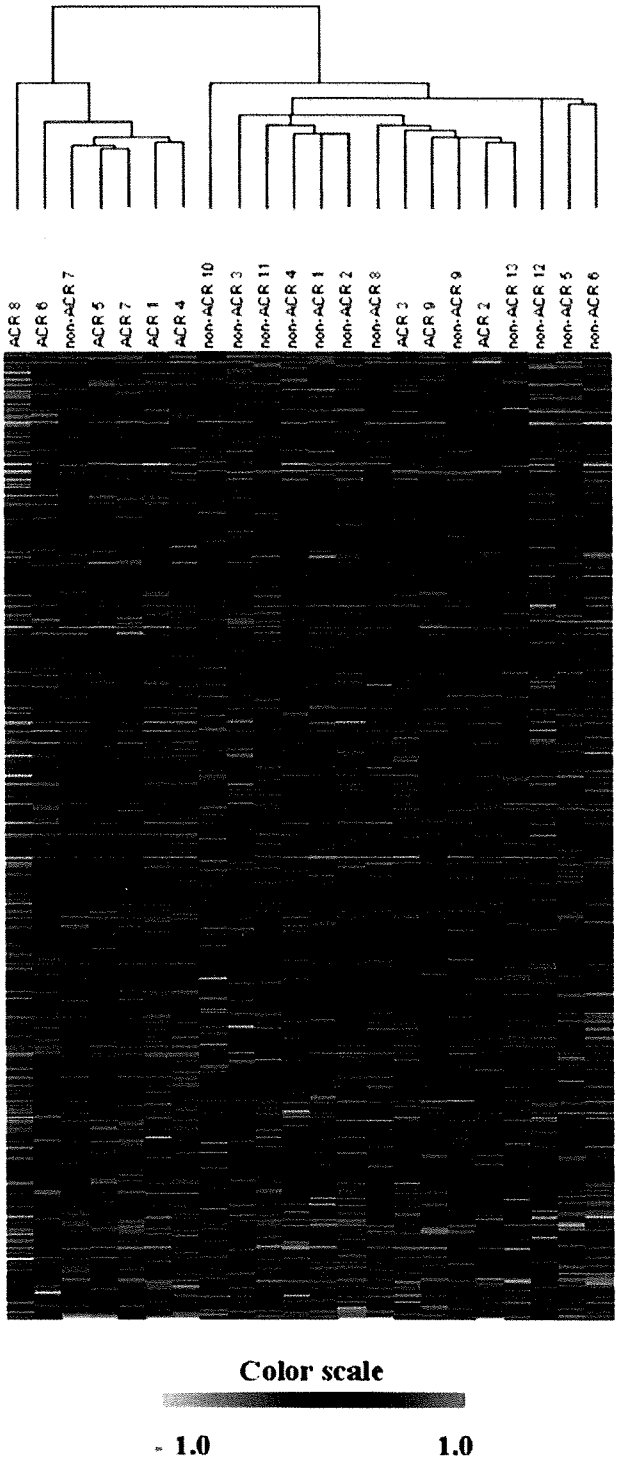

Figure 1. Unsupervised hierarchical clustering of gene expression profiles. Hierarchical cluster analysis with Euclidean distance was based on 14,475 full genes. The dendrogram in the unsupervised clustering demonstrated separation of the ACR group and non-ACR group, except for 4 samples. This pattern 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

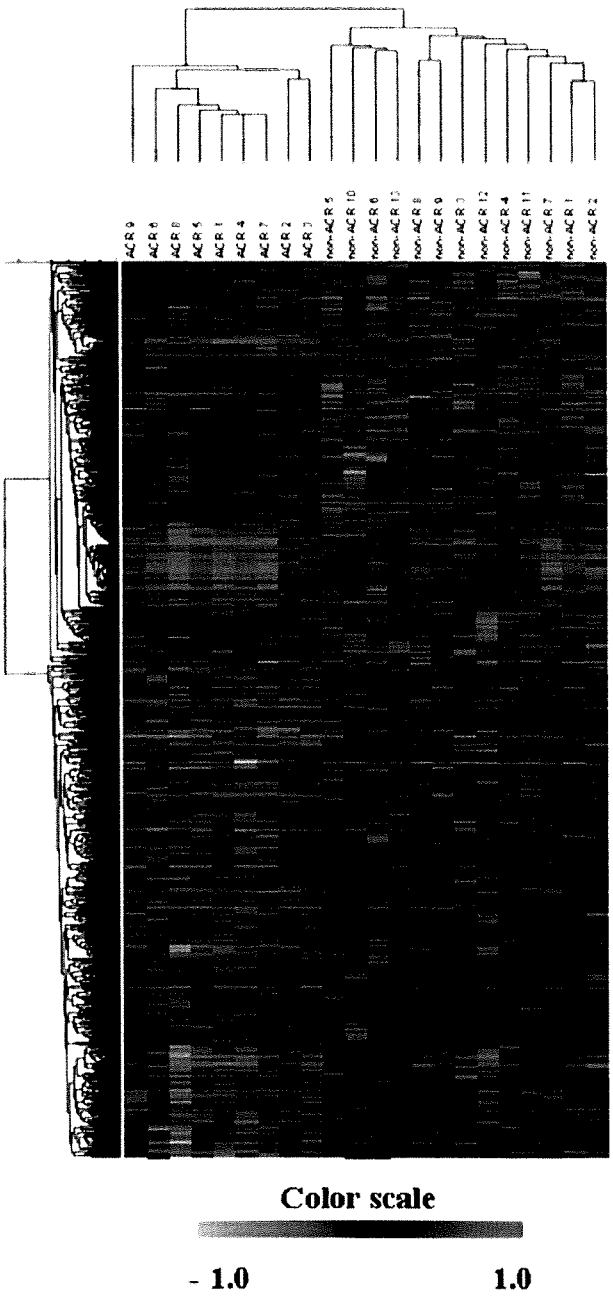

Figure 2. Gene expression pattern with selected 2206 genes. Cluster analysis was performed with Pearson correlation analysis based on 2206 differentially expressed genes. Patients could be divided into 2 groups. The molecular analysis was in agreement with the clinical diagnosis. Samples were sorted 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-

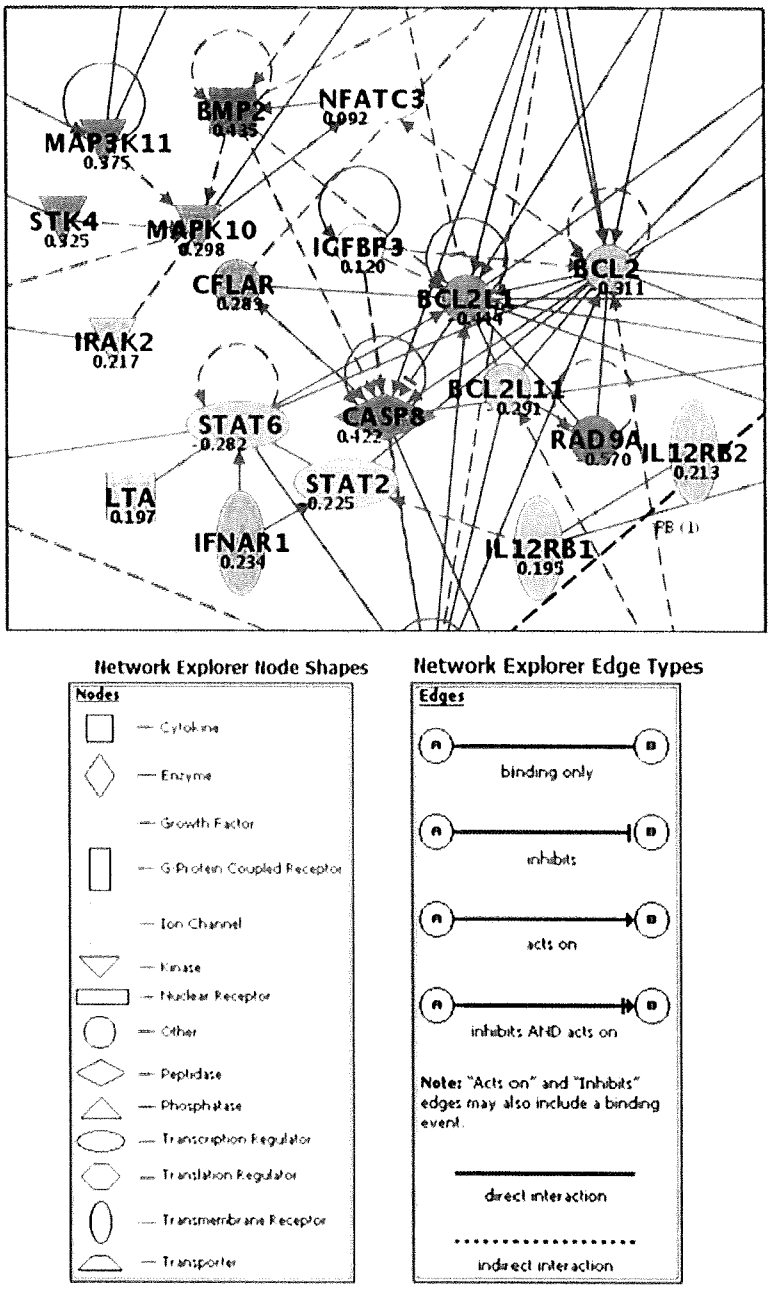

Figure 3. IPA. The data set containing 2206 genes was used as the starting point for generating biological networks. IPA identified 75 biological networks. Nodes represent genes, their shape represents the functional class of the gene product, and the edges indicate the biological relationships between 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-

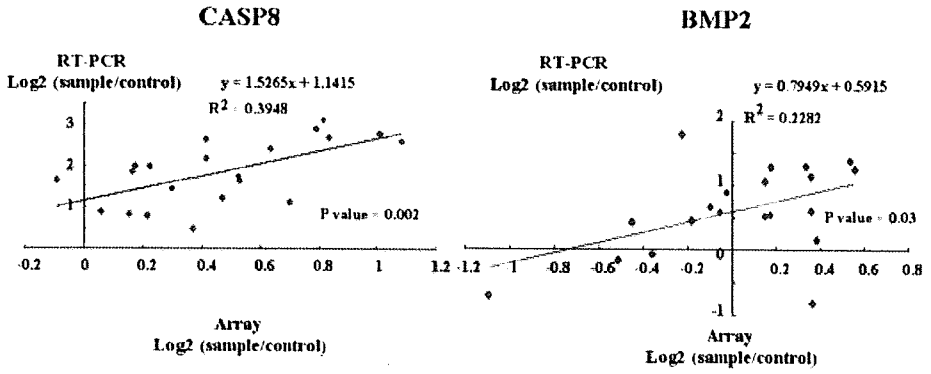

Figure 4. RT-PCR verification of the microarray results. The gene expression levels were first normalized to glyceraldehyde-3-phosphate dehydrogenase of the same samples and rescaled with respect to the control (donor liver). The expression level was calculated as the logarithm of each sample/control.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

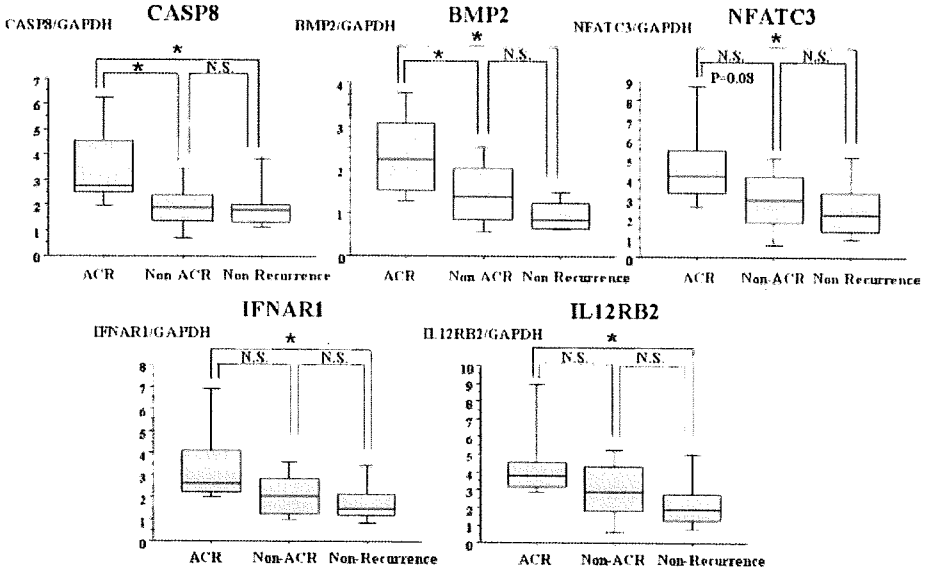

Figure 5. Evaluation of 5 representative genes using 32 liver biopsy samples from the validation group. The gene expression levels were normalized to GAPDH of the same samples. RT-PCR data of each group were compared with the Mann-Whitney test. In these box-and-whisker plots, lines within the boxes represent 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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ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

Prevention of Severe Pelvic Abscess Formation Following Extended Radical Surgery for Locally Recurrent Rectal Cancer

Mamoru Uemura, MD, PhD, Masataka Ikeda, MD, PhD, Mitsugu Sekimoto, MD, PhD, Naotsugu Haraguchi, MD, PhD, Tsunekazu Mizushima, MD, PhD, Hirofumi Yamamoto, MD, PhD, Ichiro Takemasa, MD, PhD, Hideshi Ishii, MD, PhD, and Masaki Mori, MD, PhD

Department of Surgery, Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan

ABSTRACT

Background. For treatment of locally recurrent rectal cancer (LRRC), extended radical surgery is sometimes required to obtain a negative margin. Such surgery is often associated with severe postoperative pelvic abscess (PA) formation. The aim of this study was to determine the effects of reconstructive surgery using a large rectus abdominis myocutaneous (RAM) flap and anal preservation surgery on the incidence of severe PA.

Method. Between February 1998 and June 2008, 44 patients underwent extended surgery for LRRC. Patients were divided into the pre-2004 group ($n = 15$) and the post-2004 group ($n = 29$). To reduce the risk of infections, we modified the surgical approach after 2004 to include a larger volume of RAM flap (modified RAM flap) and implemented anal preservation surgery.

Results. The overall incidence of severe PA was significantly lower in the post-2004 group [6 of 29 (21%)] than the pre-2004 group [9 of 15 (60%), $P = 0.017$]. The incidence of severe PA was lower in the anal preservation group [1 of 12 (8.3%)] compared with those who did not undergo such surgery [14 of 32 (44%), $P = 0.035$]. Modified RAM flap reduced the incidence of severe PA, albeit insignificantly (pre-2004 group: 57%, post-2004 group: 23%). All three patients who underwent anal preservation and modified RAM flap reconstruction did not develop severe PA. Multiple logistic analysis identified no anal preservation (Odds ratio [OR] = 10.6) and performing of sacrectomy (OR = 20.0) as risk factors for severe PA.

Conclusion. Anal preservation surgery is an effective measure against the development of severe PA after radical resection of LRRC.

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M. Ikeda, MD, PhD
e-mail: mikeda@gesurg.med.osaka-u.ac.jp

Local recurrence of rectal cancer (LRRC) is a formidable problem after surgery for primary advanced rectal cancer. The reported incidence of LRRC ranges between 5% and 30% after curative resection.[1,2] The prognosis of these patients is usually poor in terms of survival and quality of life. Since 20%–50% of these patients have local recurrence in the absence of distant metastasis, surgical intervention is one of the best treatment choices for cure.[1,3] However, extended radical surgery including sacrectomy is required to obtain negative surgical margin.[4–8] Pelvic abscess (PA) frequently develops after such radical surgeries with large pelvic defect and could cause severe infection and septicemia. Jimenez et al. reported that the incidence of severe PA after total pelvic exenteration for colorectal cancer was 20%.[9] Furthermore, Moriya et al. reported that pelvic sepsis occurred in 39% of patients who underwent total pelvic exenteration with distal sacrectomy for LRRC.[6]

Several reports indicate that pelvic reconstruction after extended pelvic surgery using vertical rectus abdominis myocutaneous (RAM) flap is useful in preventing various perineal wound complications.[10–14] To reduce both pelvic defect and wound dehiscence, we have used various techniques with the aim of preserving the anal region, especially in cases with sufficient distal surgical margin. To our knowledge, there are no reports on the oncological criteria of anal preservation. The purpose of this study was to determine the impact of RAM flap reconstruction and anal preservation on prevention of severe perineal wound complications after resection of LRRC.

PATIENTS AND METHODS

Between February 1998 and June 2008, 59 patients underwent resection for LRRC. Among them, 44 patients underwent extended surgery for curative intent. In this study, extended rectal surgery is defined as resection of LRRC with resection of adjacent organs such as urogenital organs and sacral bone. The patients included 32 males (73%) and 12 females (27%), with a median age of 59 years (range, 28–74 years). Patient data including age, gender, height, weight, smoking habit, history of diabetes, preoperative albumin level, preoperative hemoglobin level, the American Society of Anesthesiologists (ASA) score as determined by the anesthesiology team, the use of preoperative chemoradiotherapy, procedures performed, duration of operation, perioperative transfusion, intraoperative contamination, and nature of the primary surgery were examined.

In this study, severe PA was defined as: (1) infection in the pelvis with large wound dehiscence lasting more than 8 weeks, (2) infection in pelvis that needs not only drainage but also reoperation because of large dead space (larger than a fist size) with large wound break down was left after drainage, and (3) infection in the pelvis that resulted in septicemia. PA was defined as mild and small abscess when it did not fulfill the criteria of severe PA; it required drainage by interventional radiology or direct drainage through the wound dehiscence, but did not require reoperation. Severe PA was clinically diagnosed by the confirmation of computed tomography (CT) scan and/or purulent drainage. Perineal wound infection was defined as infection at the site of surgical incision and abbreviated as SSI (superficial or deep) of perineal or sacral wound, as defined by the National Nosocomial Infection Surveillance system.[15] The criteria for superficial incisional SSI were an infection that occurred at the incision site within 30 days after surgery involving only the skin and subcutaneous tissue, and at least one of the following: purulent discharge from the incision; an organism isolated from a culture of fluid from the incision; incisional pain, tenderness, localized swelling, redness, or heat; and opening of the wound. The criteria for deep incisional SSI were an infection that occurred within 30 days after surgery involving the muscle and fascial layers but not organ space, and at least one of the following: purulent discharge from the deep incision, an incision that spontaneously dehisced or was deliberately opened by a surgeon in the presence of signs, and symptoms of infection described previously. Superficial and deep incisional SSIs were combined under the same diagnosis of incisional SSI because it was difficult to determine the exact type in some cases, which might have led to misclassification.

We examined the relationship between surgical procedure and wound complications by dividing patients into the pre-2004 group (1998–2004) and the post-2004 group (2004–2008) for the following reasons. After 2004, in order to reduce the risk of severe infections after resection, we introduced radical changes to the strategy of resection for LRRC: (1) increasing the volume of the RAM flap and (2) aggressive efforts to preserve the anal region. Before 2004, we did not include preservation of the anal region routinely in our management because it was technically demanding in terms of securing a negative surgical margin.

Technique of RAM Flap Reconstruction

Before scheduling the surgical procedure, blood flow in the inferior epigastric artery was examined by ultrasonography to confirm adequate flap blood flow. The right rectus muscle was usually chosen to allow for colostomy through the left rectus muscle. The RAM flap was designed over the rectus abdominis muscle, including the overlying fat and skin. In the post-2004 group of patients, we increased the volume of the flap by retrieving the fat and muscle from a wider area. The flap was fully mobilized with the inferior epigastric artery pedicle. The rectus abdominis muscle was used for filling the pelvic defect, and the attached skin was used for the reconstruction of the perineal defect when required. These procedures are shown in Fig. 1.

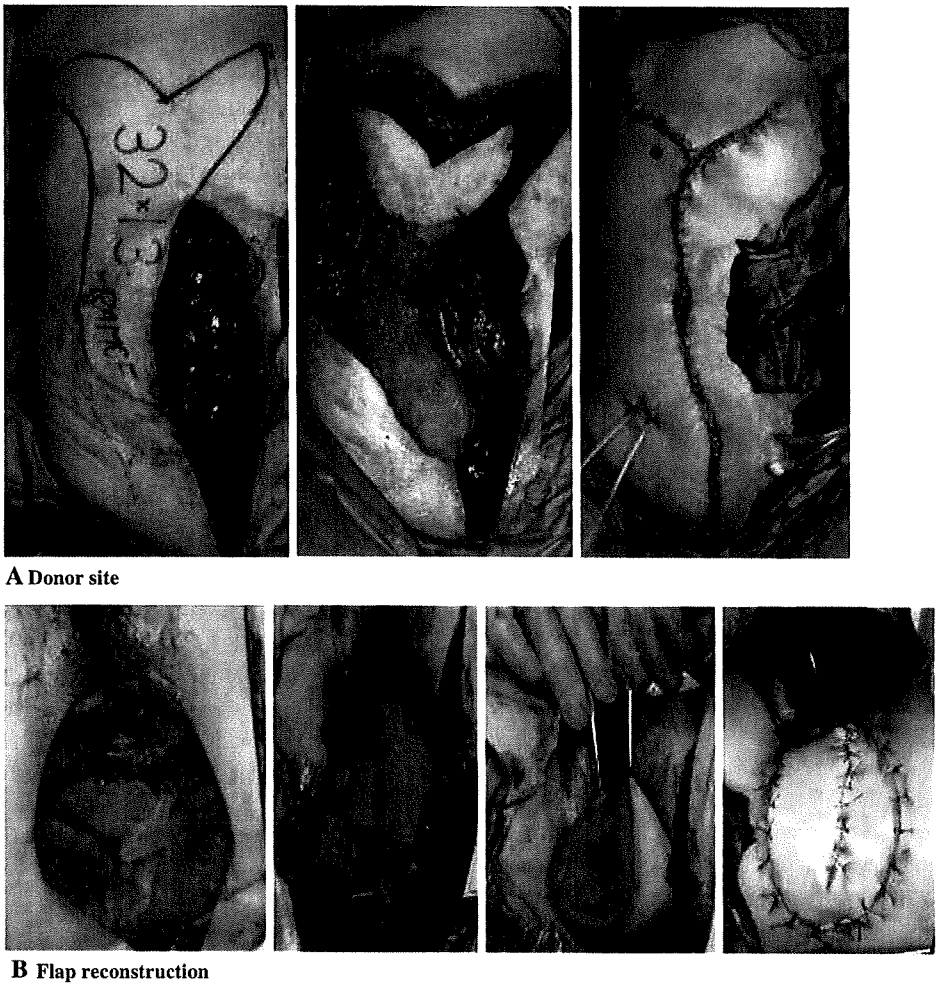
The decision whether or not to use a RAM flap was based on several factors. In other words, there were no strict or definitive guidelines for the use of such flap. Generally speaking, the decision to use a flap depended on the size of the pelvic defect and the volume of the rectus abdominis muscle. The final decision was left to two or more plastic surgeons and two rectal surgeons.

Criteria for Anal Preservation

To successfully preserve the anal region, careful preoperative and intraoperative assessments are crucial. When the LRRC was $\geq$ 4.0 cm (the most distal resection line was just above the internal sphincter level) from the anal verge, the anal region could be preserved. The use of this criterion allowed us to obtain optimal distal margin in all cases.

Independent Variables in Univariate and Multivariate Analysis

Patient age (< 60, $\geq$60 years), body mass index (BMI) (<25, $\geq$25 kg/m^2), preoperative serum albumin level ($\leq$3.6, >3.6 g/dL), ASA score ($\leq$2, $\geq$3), duration of operation (<910, $\geq$910 min), and intraoperative blood loss (<5200, $\geq$5200 mL) were evaluated as categorical variables. Anemia was defined as a preoperative hemoglobin



A Donor site

B Flap reconstruction

FIG. 1 Intraoperative photographs showing the technique of RAM flap construction

level <11.0 g/dL. Other variables were categorical variables and are presented in the Results.

As for smoking cessation, we chose the cut-off point based on the following reason. Ideally, patients should refrain from smoking for at least 4 weeks prior to surgery because 4 weeks of abstinence from smoking reduces the incidence of wound infections after surgery, and the Centers for Disease Control and Prevention (CDC) recommends smoking cessation at least 30 days prior to surgery.[15,16]

Statistical Analysis

Continuous data were expressed as median and range. Statistical analysis was performed using the χ^2 test or Fisher exact test for categorical data and Mann-Whitney *U* test for nonparametric data. Independent variables with a *P* value < 0.1 in the univariate analysis were entered into a multivariate logistic regression model. Anal preservation is one of the most important factors (another one is technical modifying of RAM flap reconstruction) by which the classification of pre/post-2004 was decided. So we believe it is inappropriate to include it in the multivariate analysis because of the problems of both multicolinearity and interpretability in the multivariate fitting. A *P* value less than .05 was considered significant. All statistical analyses were conducted using StatView 5.0 J (SAS Institute Inc, Cary, NC).

RESULTS

Surgical Procedure

Among the 44 patients, 25 underwent total pelvic exenteration, while 7 patients received abdominoperineal resection, 9 low anterior resection, and 3 patients underwent other procedures (with resection of part of the urogenital system). Sacral bone resection was concomitantly performed

TABLE 1 Surgical procedures

Surgical procedure	Pre-2004 group $n = 15$	Post-2004 group $n = 29$
Low anterior resection		
Without sacrectomy	0	1
With sacrectomy	1	7
Abdominoperineal resection		
Without sacrectomy	1	2
With sacrectomy	1	3
Total pelvic exenteration		
Without sacrectomy	1	6
With sacrectomy	9	9
Others (with resection of urogenital organs)		
Without sacrectomy	1	0
With sacrectomy	1	1
Sacral resection	12/15	19/29

in 31 patients(70%) (Table 1). There were no significant differences in surgical procedures between the pre-2004 and post-2004 groups.

Patient Characteristics

Table 2 lists the characteristics of patients of the two groups. There were no significant differences in age, gender, operation time, intraoperative blood loss, tumor diameter, RAM flap reconstruction of defect, and concomitant sacrectomy between the two groups. The percentages of patients who received preoperative chemoradiation therapy ($P < 0.0001$) and anal preservation ($P = 0.0352$) were significantly higher in the post-2004 group. Almost all patients who received anal preservation were from the post-2004 group. However, there was no significant difference in the median distance between the lower border of the tumor and the anal verge between the two groups [pre-2004 group: 7 cm, post-2004 group: 4.5 cm, $P = 0.1719$].

Wound Complications

For the entire group of 44 patients, 33 (75%) developed postoperative wound complications. Among the affected patients, 15 (34%) had severe PA. There were no significant differences in the incidence of postoperative abdominal wound complications, perineal wound complications, and PA formation between the two groups (Table 3). Despite the larger number of patients who received preoperative chemoradiotherapy, the incidence of severe PA was significantly lower in the post-2004 group than the pre-2004 group ($P = 0.0174$).

Impact of Anal Preservation

The anal region was preserved in 12 patients. The incidence of severe PA was lower in the anal preservation group: 1 of 12 (8.3%) vs 14 of 32 (44%), $P = 0.035$ (Table 4).

Impact of RAM Flap

A total of 20 patients received RAM flap reconstruction. Among these, 7 patients were of the pre-2004 group and 13 of the post-2004 group. Preoperative chemoradiotherapy was provided to 9 patients of the post-2004 group but to none of patients of the pre-2004 group ($P = 0.0047$). Although there was no significant difference in the incidence of severe PA between the two groups, the number of patients who developed this complication postoperatively was lower in the post-2004 group [pre-2004 group: 4 of 7 (57%), post-2004 group: 3 of 13 (23%), $P = 0.17$]. All three patients of the post-2004 group who developed severe PA received preoperative chemoradiotherapy.

TABLE 2 Patients' characteristics

Factor	Pre-2004 group ($n = 15$)	Post-2004 group ($n = 29$)	P value
Age (years)	60 (47–74)	58 (28–70)	0.2706
Sex (male:female)	12:3	20:9	0.5000
Operation time (min)	855 (512–1,025)	915 (440–1,350)	0.3727
Intraoperative blood loss (mL)	5,580 (1,100–13,340)	5,060 (2,000–17,300)	0.7011
Tumor diameter (cm)	4.6 (2.4–6.5)	4.6 (2.0–7.0)	0.7544
Preservation of the anal region (yes:no)	1:14	11:18	0.0352
Flap reconstruction of defect (yes:no)	11:4	24:5	0.4640
Sacrectomy (yes:no)	12:3	19:10	0.4884
Preoperative chemoradiotherapy (yes:no)	1:14	21:8	<0.0001

TABLE 3 Summary of wound complications in the pre-2004 and post-2004 groups

Wound complication	Pre-2004 group ($n = 15$)	Post-2004 group ($n = 29$)	P value
None	3	8	0.7222
Abdominal wound	3	2	0.3187
Perineal wound	3	12	0.1948
Pelvic abscess	1	5	0.6467
Severe pelvic abscess	9 (60%)	6 (21%)	0.0174

TABLE 4 Perineal wound infection, severe pelvic abscess and preservation of the anal region

	Anal preservation (+) $n = 12$	Anal preservation (−) $n = 32$	P value
Perineal wound infection (+)	4	11	>0.9999
Pelvic abscess (+)	2	4	0.6577
Severe pelvic abscess (+)	1 (8.3%)	14 (44%)	0.0352

Results of Univariate Analysis

The development of severe AP did not correlate with any of the variables related to patients' characteristics (Table 5). However, smokers tended to develop severe PA ($P < 0.1$). Table 6 shows the association between surgical characteristics and severe PA. Preservation of the anal region and sacrectomy were significantly associated with a high incidence of severe PA.

Results of Multivariate Analysis

Univariate parameters with a P value less than .1 were entered into multivariate analysis. Preservation of the anal region and sacrectomy were identified as two independent and significant determinants of development of severe PA (Table 7). Smoking was also an independent factor for severe PA, although with a marginal P value ($P = 0.0547$).

Other Complications

None of the patients in the post-2004 group developed ventral hernia postoperatively, compared with 1 of the pre-2004 group (6.7%, $P = 0.34$). Table 8 lists all other complications encountered in these patients, in addition to wound complications. There were no significant differences between the two groups with respect to the incidence of these complications.

TABLE 5 Patients' characteristics and severe pelvic abscess

Variable	n	Severe pelvic abscess (+) %	P value
Sex			0.171
Female	12	16.7	
Male	32	40.6	
Age (years)			0.525
<60	23	30.4	
≥60	21	38.1	
BMI (kg/m^2)			0.646
<25	38	16.7	
≥25	6	36.8	
Diabetes mellitus			0.540
+	2	0	
−	42	35.7	
Albumin (g/dL)			0.525
≤3.6	21	30.1	
>3.6	23	30.4	
Anemia			0.695
+	9	22.2	
−	35	30.4	
Smoking habit			0.071
Smoking/cessation <1 month	12	58.3	
None/cessation ≥1 month	32	25	
ASA score			0.646
1, 2	39	35.6	
≥3	5	20	

DISCUSSION

Resection of LRRC is a high-risk procedure. SSI such as severe PA is a potentially fatal complication.[6,9] Prevention of severe PA following extended radical surgeries for LRRC is very important. Since curability is the most important issue in such invasive surgery, we started preoperative chemoradiotherapy since 2004 with the aim of preventing local re-recurrence and expansion of operative indications for LRRC. However, preoperative radiation is an independent risk factor for SSI in rectal surgery.[17] Therefore, we started to preserve the anal region and to enlarge the volume of RAM flap to reduce the risk of severe infections after resection. Before 2004, we did not attempt to preserve the anal region on a routine basis because we thought it was technically demanding and we could not confirm its oncological feasibility. Histopathological examination of the resected specimens of patients of the pre-2004 group led us to design a safe procedure for preservation of the anal region by using the criteria listed in the Patients and Methods. We could not make definitive conclusions because the numbers of patients were small,

TABLE 6 Surgical characteristics and severe pelvic abscess

Variable	*n*	Severe pelvic abscess (+) %	*P* value
Preoperative chemoradiotherapy			>0.9999
+	22	31.8	
−	22	36.4	
Sacrectomy			0.033
+	31	45.2	
−	13	7.7	
Flap reconstruction of defect			>0.9999
+	35	34.3	
−	9	33.3	
RAM flap reconstruction of defect			0.1738
Pre-2004 group	7	57.1	
Post-2004 group	13	23.1	
Preservation of the anal region			0.035
+	23	8.3	
−	32	43.4	
In the pre- or post-2004 group			0.0174
Pre-2004 group	15	60.0	
Post-2004 group	29	20.7	
Operation time (min)			0.525
<910	22	27.3	
≥910	22	40.1	
Intraoperative blood loss (mL)			0.202
<5200	22	22.7	
≥5200	22	45.5	
Blood transfusion			>0.9999
+	42	33.3	
−	2	50	
Intraoperative contamination			>0.9999
+	3	33.3	
−	41	34.1	
Region of primary rectal cancer			0.736
Rb	31	32.3	
Others	13	38.5	

TABLE 7 Results of multivariate analysis

Variable	OR	95%CI	*P* value
Preservation of the anal region (−)	10.6	1.0–109.9	0.0463
Sacrectomy (+)	20.0	1.7–236.3	0.0173
Smoking habit (−)	6.4	0.9–42.6	0.0547

though there was no significant difference in the local re-recurrence rate between with/without anal preservation groups [with anal preservation group: 3of 12 (25%), without anal preservation group: 12 of 32 (37.5%), $P = 0.50$]. There is, however, a need to confirm that anal preservation is a safe oncological procedure.

Multivariate analysis identified anal preservation and sacrectomy as independent factors that influence the incidence of severe PA. These results indicate that large pelvic defect is one of the main factors that contributes to the development of severe PA, and thus anal preservation is a recommended procedure when applicable.

Before 2004, reconstruction was performed using RAM flaps for patients with large pelvic defects. However, in some patients, the volume of RAM flap was too small to fill up the defect resulting, in PA. Accordingly, we started to increase the volume of the RAM flap in subsequent years to resolve this problem. To assess the effect of this modification, we compared the incidence of severe PA in the pre-2004 and post-2004 groups with RAM flap reconstruction. There was no significant difference between the two groups, but the incidence of severe PA was lower in the post-2004 group compared with the pre-2004 group. Most patients of the post-2004 group received preoperative chemoradiotherapy, though it is a known risk factor for postoperative infections. Despite this unfavorable background, the incidence of severe PA was lower in the post-2004 group. Several reports indicated that pelvic reconstruction after extensive pelvic surgery using vertical RAM flap can reduce perineal wound complications.[10–14] Furthermore, in the present study population, none of the patients suffered from the aftereffects of pelvic reconstruction using the RAM flap. Taking this into consideration, modified RAM flap reconstruction and anal preservation are reasonable techniques to prevent severe PA. Thus, aggressive reconstruction using a RAM flap is preferable when pelvic defect is large, but only introducing modified RAM flap reconstruction is sometimes insufficient to prevent severe PA as our data had shown. The need for adding not only modified RAM flap but also other techniques is suggested. However, to confirm the positive effect of RAM flap on prevention of pelvic defect, there is a need to establish standards for indication of RAM flap. Further studies are needed in large population samples to make definitive conclusions on the modified RAM flap reconstruction.

There is little or no information on the usefulness of anal preservation in the prevention of severe PA after resection of LRRC. Reconstruction by RAM flap is a common technique to reduce dead space after resection, but the procedure is sometimes inadequate to completely fill the space. Our approach was that adequate preservation of the anal region could help reduce the incidence of postoperative complications. Accordingly, we introduced anal preservation procedure to reduce the incidence of postoperative severe PA formation. The results showed that anal preservation surgery significantly reduced the incidence of severe pelvic abscess. Analysis of patients who received preoperative chemoradiotherapy ($n = 22$) showed that none of the patients who later underwent anal preservation

TABLE 8 Postoperative complications other than wound infection

Complication	Pre-2004 group ($n = 15$)	Post-2004 group ($n = 29$)	P value
Urinary tract infection	2 (13%)	1 (3.5%)	0.2643
Ventral hernia	1 (6.7%)	0	0.3409
Postoperative hemorrhage	2 (13%)	2 (6.9%)	0.5962
Bowel obstruction	1 (6.7%)	5 (17%)	0.6467

surgery developed severe PA, while 58% of those who did not developed this problem ($P = 0.0053$). These results emphasize the importance of not only RAM flap but also anal preservation surgery in reducing the risk of severe PA especially in patients who receive preoperative chemoradiotherapy.

In conclusion, anal preservation surgery reduce the likelihood of severe PA after resection of LRRC. Anal preservation surgery based on precise preoperational and intraoperational assessments is recommended aggressive surgical treatment for patients with LRRC. It is possible that the lack of difference is the result of the small population of the present study. Further studies are needed in larger population samples to determine the overall effect of preoperative chemoradiotherapy, reconstructive surgery using a large RAM flap and anal preservation surgery on the survival rate of patients with LRRC.

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治療法の進歩

大腸癌に対する化学療法，分子標的治療

Chemotherapy and molecular targeting therapeutics for colorectal cancer

植村　守 *UEMURA Mamoru*
山本　浩文 *YAMAMOTO Hirofumi*
原口　直紹 *HARAGUCHI Naotsugu*
竹政伊知朗 *TAKEMASA Ichiro*
水島　恒和 *MIZUSHIMA Tsunekazu*
池田　正孝 *IKEDA Masataka*
石井　秀始 *ISHII Hideshi*
関本　貢嗣** *SEKIMOTO Mitsugu*
土岐祐一郎* *DOKI Yuichiro*
森　正樹* *MORI Masaki*

特集

消化管疾患－診断と治療の進歩 Key words 多剤併用 分子標的薬 バイオマーカー KRAS

現在大腸癌の治療に利用される化学療法剤としては，5-fluorouracil(5-FU)，L-OHP(oxaliplatin)，CPT-11(irinotecan)，l-LV(leucovorine)の4剤が主であり，通常l-LV(leucovorine)は5-FUのbiological modulatorとしてセットで使用される(表1)．なかでも5-FUは1957年に開発されて以来，中心的な薬剤として使われている．さらにCPT-11，L-OHPなど新規抗癌剤が加わり，多くの臨床試験の結果から5-FU/LVはCPT-11やL-OHPと組み合わせて多剤併用療法(FOLFOX，FOLFILI，IFL)として利用されるようになった．

さらに分子標的モノクローナル抗体が導入されることにより，抗腫瘍効果と延命効果の著しい向上が獲得されてきた(図1)．大腸癌に対する分子標的治療薬としてすでに臨床効果の認められているものは，抗EGFR(epidermal growth factor receptor)抗体と抗VEGF(vascular endothelial growth factor)抗体のふたつが代表的であるが，これらの分子標的治療薬も多くの臨床試験の結果，従来の多剤併用療法との組み合わせで臨床の場に登場した．

各薬剤の作用機序と薬物動態

大腸癌に対する化学療法剤や分子標的治療剤などの抗悪性腫瘍剤のなかで，代表的なもの(表1)について以下にその作用機序と薬理動態について解説する．

1. フッ化ピリミジン系抗癌剤

1) 5-FU

2大作用機序として，DNA合成阻害とRNA機能障害があげられる．5-FUは最終的な活性代謝物であるフルオロデオキシウリジン一リン酸(5-fluorodeoxyuridine monophosphate；FdUM)またはフルオロウリジン三リン酸(5-fluorouridine triphosphate；5-FUTP)まで代謝されて抗腫瘍効果を発揮する．

同化代謝(作用系)では，主にオロチン酸ホスホリボシルトランスフェラーゼ(orotate phosphori-

大阪大学大学院医学系研究科外科学講座消化器外科学 *教授 **准教授

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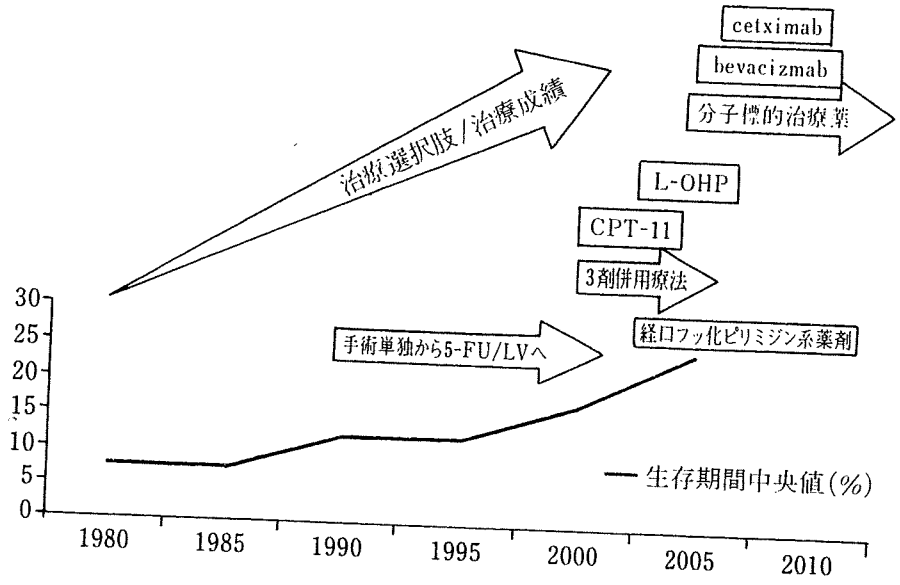

図1 大腸癌における治療方法の変遷

表1 消化器癌に対する主な抗悪性腫瘍の分類

化学療法薬
1．ピリミジン代謝拮抗薬
5-Furuorouracil (5-FU)
Tegafur/Oteracil/Gimeracil (S-1)
Capecitabin
Tegafur-Uracil (UFT)
2．プラチナ化合物
Oxaliplatin (L-OHP)
3．I型トポイソメラーゼ阻害薬(カンプトテシン類)
Irinotecamn hydrochloride (CPT-11)
分子標的薬(抗体9
1．抗VEGF抗体 Bevacizumab (Avastin®)
2．抗EGFRモノクローナル抗体(Cetuximab：Erbitux®)
その他
Leucovorine (LV)

bosyltransferase；OPRT)により，FdUMなどの活性代謝物が生成される．

DNA合成阻害は，活性代謝物のFdUMPがDNA合成経路のデオキシウリジン一リン酸(deoxyuridine monophosphate；dUMP)からデオキシチミジン一リン酸(deoxythymidine monophosphate；dTMP)への代謝に必要なチミジル酸合成酵素(thymidylate synthase；TS)の活性を抑制し，その結果としてデオキシチミジン三リン酸(deoxythymidine triphosphate；dTTP)が枯渇しDNA合成が阻害されることによる．このTS阻害は，FdUMPが活性型葉酸である5,10メチレンテトラヒドロ葉酸(5,10-CH2-THF)の存在下でTSとTernary complex(ターナリーコンプレックス：三者複合体)を形成することにより増強される．

RNA機能障害は，5-FUからフルオロウリジン三リン酸(5-fluorouridine triphosphate；5-FUTP)が生成され，F(フッ素)がついた5-FUTPがウリジン三リン酸(Uridine triphosphate；UTP)の代わりにRNAに取り込まれ，Fが結合しているために，正常なRNA機能を障害することによる．

5-FUの異化代謝(分解系)では，主にジヒドロピリミジンデヒドロゲナーゼ(dihydropyrimidine dehydrogenase；DPD)により分解されF-β-Alaとなり尿中に排泄される．このF-β-Alaが神経毒性あるいは，手足症候群を引き起こす．

上述のように，5-FUがDNA合成阻害とRNA機能障害を持っているが，この機序はリボヌクレオシドリダクターゼ(Ribonucleotide reductase；RNR)の飽和により機序が変化すると考えられている．5-FUを少量持続型で投与すると，RNRが飽和しないためにFdUMPを継続的に生成するので，主にDNA合成を阻害すると考えられる．一方，5-FUを大量間歇型で投与すると，RNRが飽和するために，FUTP生成が増大する．したがっ