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JOURNAL OF SURGICAL RESEARCH 178 (2012) 1003–1014

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Intragraft transcriptome level of CXCL9 as biomarker of acute cellular rejection after liver transplantation

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ARTICLE INFO

Article history:
Received 2 June 2012
Received in revised form
2 June 2012
Accepted 6 July 2012
Available online 26 July 2012

Keywords:
Gene expression analysis
Microarray
Network analysis
Human
Liver biopsy
Transcriptome
Molecular biomarker
Allograft
Acute rejection
Recurrent hepatitis C

ABSTRACT

Background: Liver transplantation has been a life-saving and well-established treatment for acute liver failure and various end-stage liver diseases. However, acute cellular rejection (ACR) is one of the key factors that determine long-term graft function and survival after liver transplantation, and there are still no specific biomarkers available to monitor the alloimmune response. The aim of the present study was to identify molecular biomarkers for ACR in liver allograft.

Methods: We analyzed the gene expression profile using an oligonucleotide microarray covering 44,000 human probes in 35 liver biopsy samples after living donor liver transplant, which consisted of 13 samples with ACR (ACR group; moderate/mild, 6/7), 13 samples with other dysfunctions (non-ACR group; recurrent hepatitis C / ischemia/reperfusion injury (IRI)/ nonspecific inflammation / small-for-size syndrome, 5/4/3/1), and 9 samples without liver dysfunction (protocol group). We selected 113 informative genes based on microarray results and adopted the network analysis to visualize key modulators in ACR. We selected 6 modulators (CXCL9, GZMB, CCL19, GBP2, LAIR1, and CDC25A) and confirmed the reproducibility in 23 independent biopsy samples and investigated the response to the rejection treatment in sequential samples.

Results: Network analysis revealed the top three subnetworks, which had NF-κB, MAPK, and IFNG as central hubs. Among selected modulators, intragraft expression levels of CXCL9 mRNA was most upregulated and sensitive to alloimmune status.

Conclusion: Intragraft CXCL9 mRNA has a functionally important role in T-cell activation in liver allograft and serves as biomarker for ACR.

1. Introduction

The rapid development in the field of organ transplantation in the last few decades has made liver transplantation (LT) a life-saving and well-established treatment for acute liver failure and various end-stage liver diseases. Allograft function is the key determinant of prognosis after LT; however, it often deteriorates due to one or more reasons, such as acute cellular

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0022-4804/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2012.07.016

rejection (ACR), surgical difficulty, ischemia/reperfusion injury (IRI) [1–4], recurrent hepatitis C (RHC) [5–8], small-for-size syndrome in living donor liver transplantation [9], infection, and immunosuppressant drug toxicity [10].

ACR is one of the key factors that determine long-term graft function and survival after LT. Despite continuous improvements in immunosuppressive therapy, immune tolerance is actually not yet attainable, and ACR still occurs in 25%–40% of recipients and results in graft loss in some patients [11–13].

The current standard in graft dysfunction diagnosis after LT is histopathology of liver biopsy. However, accurate interpretation of liver biopsy necessitates expert liver transplant pathologists [14,15]. The monitoring of allograft rejection also includes laboratory tests, though they are not specific and are often elevated in other types of hepatic dysfunctions and sometimes even in stable grafts. Therefore, there is a need for more a specific and objective diagnostic marker that can monitor the immune status and could be a new therapeutic target of transplant rejection.

Currently, DNA microarray allowing high-throughput analysis of thousands of genes is frequently used in the study of organ transplantation with mouse, rat, and human materials [16–26]. Generally, when studying gene expression in clinical environments, the heterogeneity of clinical background like sex, age, comorbidity, and medications complicates conclusions regarding the underlying mechanism of rejection. However, the definition of new transcriptome sets based directly on human biopsies may provide further enhancement of this methodology.

The aim of our study is to identify the molecular biomarkers for ACR. We generalized the hypothesis that ACR is associated with differential transcriptome patterns from other cases with allograft dysfunction and characterized transcriptome patterns of ACR using high-throughput microarray. Furthermore, we visualized the molecular interaction of regulated transcriptome sets.

2. Materials and methods

2.1. *Patients and sample collection*

The study protocol was approved by the Human Subjects Review Committee of Osaka University. All study subjects provided written informed consent. Fifty-five liver transplant recipients at Osaka University Hospital (Osaka, Japan) from 2000 to 2008 were eligible for this study. In total, 67 liver biopsy specimens obtained from these 55 transplant recipients were included in this study.

Liver biopsies were obtained at the time of liver graft dysfunction and routinely according to the following schedule: at months 3 and 12 after living donor liver transplantation and thereafter once a year. Liver dysfunction represented elevated serum levels of total bilirubin (T-Bil) (>2.0 mg/dL), aspartate aminotransferase (AST) (>40 IU/L), or alanine aminotransferase (ALT) (>40 IU/L). Patients with moderate or higher ACR grade were treated with methylprednisolone (1 g intravenously) followed by steroid tapering. A follow-up control biopsy was obtained 2 wk later. Portions of the liver biopsy samples were immersed immediately in RNAlater (Qiagen, Valencia, CA), then frozen with liquid nitrogen and stored at −80°C. The remaining tissue was placed in 10% buffered formalin and routinely processed for histopathologic examination.

2.2. *Histopathologic examination of liver biopsy samples and sample classifications*

Hematoxylin–eosin-stained sections of the liver samples were examined by two independent, experienced pathologists at the University of Miami who were masked to the results of the molecular studies. ACR-labeled specimens were graded according to the Banff classification [14]. Samples from the non-ACR group showed no evidence of ACR based on Banff criteria; graft dysfunction was caused by other factors such as RHC, IRI, small-for-size syndrome, and nonspecific inflammation. Samples of the protocol group were from recipients with well-functioning grafts (T-Bil <2.0 mg/dL, AST <40 IU/L, and ALT <40 IU/L). Clinicopathologically, the tissue samples showed no evidence of ACR or other graft dysfunction.

Additional liver biopsy tissues from 21 normal donors were used as a pooled control mixture.

2.3. *Liver biopsy samples and RNA purification*

Frozen liver tissues were disrupted in TRIzol reagent (Molecular Research Center, Cincinnati, OH), using Tissue Lyzer (Cat. No. 85200; Qiagen, Haan, Germany). Total RNA was purified from the clinical samples by TRIzol reagent as described in the protocol provided by the manufacturer. The isolated RNA was quantitated by UV spectrophotometry. The quality of RNA was confirmed by 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 in the microarray study, we used a mixture of RNAs extracted from 21 normal liver samples. The RNA integrity number of all 35 samples used for microarray analysis was confirmed to be more than the cutoff value of 6.5.

2.4. *Hybridization*

A one-color microarray-based gene expression analysis system (Agilent Technologies, Santa Clara, CA) containing 44,000 genes was used, following the instructions provided by the manufacturer. Total RNA was extracted from liver biopsy tissues. The process of hybridization/washing was performed using a Gene Expression Wash Pack (Agilent Technologies) and acetonitrile (Sigma, Tokyo, Japan). The experimental protocol is available at http://www.chem.agilent.com/EN-US/PRODUCTS/Pages/default.aspx. After hybridization, the oligonucleotide microarray slides were scanned with an Agilent microarray scanner, and data were normalized using the GeneSpring GX software (Agilent Technologies) and used to generate raw image files. The background-corrected intensities were used to calculate log 2–transformed ratios. Each array was normalized using its median over the entire array. Normalization of our data included calculation of the expression level of each gene using normal liver tissue as a reference. To perform the analysis, the number of genes was reduced by filtering out genes in which >15% of the values were missing and the set of the remaining 25,454 genes was used for further data analysis.

2.5. Analysis of microarray data

To identify differentially expressed individual genes among the three groups, we subjected the log ratios to the Mann-Whitney *U* test. We selected 983 genes with statistically significant difference ($P < 0.05$) in comparisons both of ACR *versus* non-ACR group and of ACR *versus* protocol group (Fig. 1A). The expression similarity of the 983 altered genes was examined by Gene Math ver. 2.0 software (InfoCom, Austin, TX). A supervised hierarchical cluster was produced using Jaccard calculation based on the Ward method calculation.

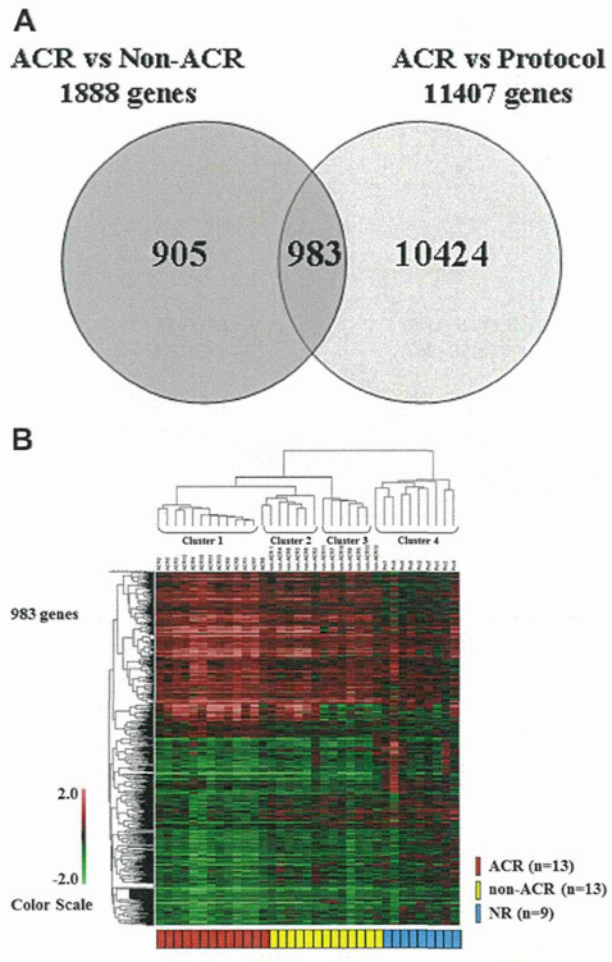

Fig. 1 – Outline of the gene selection and heat map of selected 983 genes. (A) Venn diagram represents the number of statistically significant genes among three groups. (B) A supervised hierarchical cluster was produced using a Jaccard calculation based on the Ward method calculation. Heat map represents the expression profiles of the selected 983 genes with significant differential expression when comparing ACR with protocol group and ACR with non-ACR group (Mann-Whitney test; $P < 0.05$). The intensity of each color denotes the expression levels, green for downregulated and red for upregulated. Each row represents a single gene; each column represents a single sample. The dendrogram at the left of the matrix indicates the degree of similarity among the genes examined by expression patterns. The dendrogram at the top of the matrix indicates the degree of similarity between samples. (Color version of figure is available online.)

2.6. Pathway analysis

We adopted the Ingenuity Pathway Analysis version 3.1 (IPA) software (Ingenuity System, Mountain View, CA) (http://www.Ingenuity.com) as in our previous report [26]. IPA queries the Ingenuity Pathway Knowledge Base for interactions between selected genes and then generates a set of networks. The IPA also computes a score for each network according to the fit of the user's set of significant genes. This score was used as the cutoff for identifying significantly affected gene networks.

2.7. Quantitative analysis by real-time polymerase chain reaction

For verification of microarray results, several genes (*CXCL9*, *LAIR1*, *GZMB*, and *CCL19*) were analyzed by quantitative real-time RT-PCR (qRT-PCR) using the original pooled samples. In addition to this verification, we evaluated the utility of the genes in focus (*CXCL9*, *GZMB*, *CCL19*, *LAIR1*, *GBP2*, and *CDC25A*) by qRT-PCR using another group of 23 liver biopsy samples. We examined the correlation of these target genes with the clinical course after rejection treatment. All primers were designed using the web-based software Primer3 (version 0.9, Whitehead Research Institute; http://primer3.sourceforge.net/) (Supplemental Table, available online at www.JournalofSurgicalResearch.com). Total RNA (1 μg) from each pooled sample was subjected to reverse transcription and complementary DNA was generated using the Reverse Transcription System (Promega, Madison, WI). The expression of target mRNA was quantified using real-time thermal cycler LightCycler and detection system (Roche Diagnostics R&D, Mannheim, Germany). The expression values of genes were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the same samples.

2.8. Statistical analysis

Data were expressed as median and range values. Differences were tested by the exact χ^2 test or Mann-Whitney *U* test, and correlation between two variables was analyzed using the Pearson correlation coefficient. All differences were considered statistically significant at P value less than 0.05. Cutoff values for diagnosis were ascertained using the receiver operating characteristic curve, and the sensitivity and specificity were calculated for each cutoff value.

The Statistical Package for Social Sciences statistical software (SPSS for Windows 8.0J; SPSS Inc, Chicago, IL) was used for all analyses.

3. Results

3.1. Clinicopathologic features of transplant recipients and liver biopsies

A total of 58 liver biopsy samples obtained from 55 patients were enrolled in this study. Based on the classification described in

Table 1 – Clinicopathologic information on patients who underwent microarray.

Category	ACR group ($n = 13$)	Non-ACR group ($n = 13$)	Protocol ($n = 9$)	P value
Age (y), median (range)	50 (19–63)	55 (29–65)	55 (26–69)	0.04
Sex: M/F	8/5	9/4	6/3	N.S.
Days after transplantation, median (range)	10 (7–314)	15 (4–1384)	357 (96–1860)	N.S.
Primary disease				N.S.
HCV	6	9	3	
HBV	3	2	2	
Others	4	2	4	
Rejection grade				
Moderate	6	0	0	
Mild	7	0	0	
Other dysfunction				
Recurrent hepatitis C	0	5	0	
IRI	0	4	0	
Small-for-size syndrome	0	1	0	
Nonspecific	0	3	0	
Immunosuppressant				N.S.
FK-based	7	4	6	
CyA-based	6	9	3	
Steroid addition	6	3	3	
Serum trough level, median (range)				
FK (ng/mL)	9.3 (4.7–12.9)	8.3 (4.5–12.9)	9.3 (5–11.1)	N.S.
CyA (ng/mL)	294 (82–424)	235 (158–362)	138 (70–220)	N.S.
Laboratory data, median (range)				
T-Bil (mg/dL)	8.1 (0.5–26.8)	4.8 (0.7–15.9)	0.8 (0.4–1.8)	N.S.
AST (IU/L)	43 (24–396)	45 (11–169)	15 (8–33)	N.S.
ALT (IU/L)	86 (32–334)	64 (11–124)	9 (3–27)	N.S.

CyA = cyclosporine; FK = tacrolimus; N.S. = not significant; IRI = ischemia/reperfusion injury.

Table 2 – Clinicopathologic information on patients who underwent RT-PCR.

Category	ACR group ($n = 7$)	Non-ACR group ($n = 16$)	P value
Age (y), median (range)	47 (31–65)	47 (19–66)	N.S.
Sex: M/F	3/4	9/7	N.S.
Days after transplantation, median (range)	34 (7–78)	89 (11–1299)	0.03
Primary disease			N.S.
HCV	2	6	
HBV	0	2	
Others	5	8	
Rejection grade			
Moderate	2	0	
Mild	5	0	
Other dysfunction			
Recurrent hepatitis C	0	5	
Recurrent hepatitis B	0	1	
Bile duct obstruction	0	4	
Nonspecific	0	4	
IRI	0	1	
Outflow block	0	1	
Immunosuppressant			
FK-based	7	9	
CyA-based	0	7	
Steroid addition	3	5	
Serum trough level, median (range)			
FK (ng/mL)	8.5 (4.4–12.4)	13.5 (3.9–23.3)	N.S.
CyA (ng/mL)		173 (66–259)	
Laboratory data, median (range)			
T-Bil (mg/dL)	2.2 (0.3–28.7)	0.5 (0.4–42.4)	N.S.
AST (IU/L)	53 (24–151)	55 (16–155)	N.S.
ALT (IU/L)	70 (34–338)	64.5 (16–160)	N.S.

CyA = cyclosporine; FK = tacrolimus; N.S. = not significant; IRI = ischemia/reperfusion injury.

Table 3 – Selected 113 informative genes list.

Biological process	UniGene ID	Gene name	Gene symbol	Relative fold ACR/non-ACR	P value
Immume response ($n = 21$)	Hs.77367	Chemokine (C-X-C motif) ligand 9	*CXCL9*	6.4	0.007
	Hs.504048	CD3d molecule, delta (CD3-TCR complex)	*CD3D*	4.0	0.004
	Hs.50002	Chemokine (C-C motif) ligand 19	*CCL19*	3.6	0.029
	Hs.85258	CD8a molecule	*CD8A*	3.0	0.026
	Hs.355307	CD27 molecule	*CD27*	2.2	0.011
	Hs.272409	T-box 21	*TBX21*	2.2	0.011
	Hs.409925	Guanylate binding protein 4	*GBP4*	2.2	0.043
	Hs.534956	Fc fragment of IgG, high-affinity Ib, receptor (CD64)	*FCGR1B*	2.2	0.020
	Hs.511794	Chemokine (C-C motif) receptor 2	*CCR2*	1.9	0.048
	Hs.520048	Major histocompatibility complex, class II, DR alpha	*HLA-DRA*	1.9	0.029
	Hs.591967	Interleukin 18 binding protein	*IL18BP*	1.8	0.026
	Hs.54403	C-type lectin domain family 10, member A	*CLEC10A*	1.8	0.026
	Hs.128065	Cathepsin C	*CTSC*	1.7	0.038
	Hs.73797	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 6	*EDG6*	1.7	0.048
	Hs.386567	Guanylate binding protein 2, interferon-inducible	*GBP2*	1.7	0.043
	Hs.572535	Leukocyte-associated immunoglobulin-like receptor 1	*LAIR1*	1.6	0.010
	Hs.433300	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	*FCER1G*	1.6	0.038
	Hs.376208	Lymphotoxin beta (TNF superfamily, member 3)	*LTB*	1.6	0.026
	Hs.631592	Leukocyte immunoglobulin-like receptor, subfamily A (with T M domain), member 5	*LILRA5*	1.6	0.038
	Hs.415067	Coronin, actin binding protein, 1A	*CORO1A*	1.6	0.038
	Hs.369438	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	*ETS1*	1.5	0.007
T cell activation ($n = 5$)	Hs.2259	CD3g molecule, gamma (CD3-TCR complex)	*CD3G*	3.8	0.017
	Hs.405667	CD8b molecule	*CD8B*	3.7	0.022
	Hs.590883	Signal-regulatory protein, gamma	*SIRPG*	3.6	0.013
	Hs.523500	CD2 molecule	*CD2*	3.0	0.043
	Hs.470627	Lymphocyte-specific protein tyrosine kinase	*LCK*	2.6	0.006
Natural killer cell –mediated cytotoxicity ($n = 2$)	Hs.210546	Interleukin 21 receptor	*IL21R*	2.1	0.026
	Hs.636480	Tubulin, beta	*TUBB*	1.5	0.004
Defense response ($n = 3$)	Hs.74647	T cell receptor alpha locus	*TRA@*	3.6	0.008
	Hs.243564	CD48 molecule	*CD48*	1.8	0.038
	Hs.155975	Protein tyrosine phosphatase, receptor type, C-associated protein	*PTPRCAP*	1.8	0.004
Signal transduction ($n = 7$)	Hs.549152	G protein–coupled receptor 171	*GPR171*	1.8	0.026
	Hs.531619	Egf-like module containing, mucin-like, hormone receptor-like 2	*EMR2*	1.8	0.033
	Hs.517601	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	*RAC2*	1.7	0.026
	Hs.440898	Ficolin (collagen/fibrinogen domain containing) 1	*FCN1*	1.7	0.048
	Hs.302435	Centaurin, gamma 1	*CENTG1*	1.5	0.043
	Hs.22065	CDC42 small effector 1	*CDC42SE1*	1.5	0.007
	Hs.348500	Vasoactive intestinal peptide receptor 1	*VIPR1*	0.7	0.017
Cell proliferation, cell differentiation, cell cycle ($n = 8$)	Hs.8878	Kinesin family member 11	*KIF11*	2.2	0.046
	Hs.103527	SH2 domain protein 2A	*SH2D2A*	2.0	0.029
	Hs.523660	Signaling lymphocytic activation molecule family member 1	*SLAMF1*	1.8	0.010
	Hs.437705	Cell division cycle 25 homolog A (*S. cerevisiae*)	*CDC25A*	1.8	0.017
	Hs.70327	Cysteine-rich protein 1 (intestinal)	*CRIP1*	1.6	0.038
	Hs.21331	Zwilch, kinetochore associated, homolog (*Drosophila*)	*ZWILCH*	1.5	0.022
	Hs.508999	Protein kinase D1	*PRKD1*	0.7	0.004
	Hs.231655	Obscurin, cytoskeletal calmodulin and titin-interacting Rho GEF	*OBSCN*	0.7	0.043

(continued on next page)

Table 3 – (continued)

Biological process	UniGene ID	Gene name	Gene symbol	Relative fold ACR/non-ACR	P value
Transcription ($n = 5$)	Hs.555947	Lymphoid enhancer-binding factor 1	*LEF1*	2.6	0.011
	Hs.509964	Basic leucine zipper transcription factor, ATF-like	*BATF*	2.5	0.017
	Hs.160673	Ras homolog gene family, member H	*RHOH*	1.8	0.043
	Hs.171426	Nuclear receptor coactivator 7	*NCOA7*	0.6	0.048
	Hs.76171	CCAAT/enhancer binding protein (C/EBP), alpha	*CEBPA*	0.6	0.026
Apoptosis ($n = 3$)	Hs.1051	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	*GZMB*	2.7	0.020
	Hs.648101	Caspase recruitment domain family, member 11	*CARD11*	1.7	0.038
	Hs.165607	Hypothetical protein FLJ25416	*FLJ25416*	1.6	0.022
Oxidation reduction ($n = 2$)	Hs.567547	Aspartate beta-hydroxylase domain containing 2	*ASPHD2*	1.7	0.022
	Hs.1360	Cytochrome P450, family 2, subfamily B, polypeptide 6	*CYP2B6*	0.6	0.015
Transport ($n = 2$)	Hs.80658	Uncoupling protein 2 (mitochondrial, proton carrier)	*UCP2*	1.8	0.011
	Hs.418167	Albumin	*ALB*	1.6	0.011
Others ($n = 28$)	Hs.421750	V-set and immunoglobulin domain containing 9	*VSIG9*	3.8	0.007
	Hs.622865	T cell receptor beta variable 5-4	*TRBV5-4*	3.1	0.010
	Hs.402773	Protein tyrosine phosphatase, non-receptor type 7	*PTPN7*	2.4	0.010
	Hs.531776	Lectin, galactoside-binding, soluble, 2 (galectin 2)	*LGALS2*	2.2	0.033
	Hs.276770	CD52 molecule	*CD52*	2.0	0.010
	Hs.16291	Adhesion molecule, interacts with CXADR antigen 1	*AMICA1*	2.0	0.033
	Hs.1183	Dual specificity phosphatase 2	*DUSP2*	2.0	0.022
	Hs.534331	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	*NUDT1*	1.9	0.026
	Hs.37617	Myosin IG	*MYO1G*	1.8	0.010
	Hs.468274	Solute carrier family 8 (sodium/calcium exchanger), member 1	*SLC8A1*	1.7	0.007
	Hs.468840	Pleckstrin	*PLEK*	1.7	0.038
	Hs.396393	Ubiquitin-conjugating enzyme E2S	*UBE2S*	1.7	0.048
	Hs.591166	LIM domain containing 2	*LIMD2*	1.7	0.017
	Hs.56294	RAB33A, member RAS oncogene family	*RAB33A*	1.6	0.043
	Hs.409065	Flap structure-specific endonuclease 1	*FEN1*	1.6	0.038
	Hs.177926	Exonuclease NEF-sp	*LOC81691*	1.6	0.038
	Hs.381220	Hypothetical protein LOC441168	*RP1-93H18.5*	1.6	0.038
	Hs.61469	Chromosome X open reading frame 9	*CDorf9*	1.5	0.029
	Hs.474991	Coiled-coil domain containing 134	*CCDC134*	1.5	0.015
	Hs.444933	Phospholipase B1	*PLB1*	1.5	0.022
	Hs.501200	Regulator of G-protein signaling 10	*RGS10*	1.5	0.038
	Hs.210377	Coiled-coil domain containing 38	*CCDC38*	0.6	0.029
	Hs.651259	ORM1-like 1 (*S. cerevisiae*)	*ORMDL1*	0.6	0.022
	Hs.24684	Arrestin domain containing 3	*ARRDC3*	0.6	0.038
	Hs.579264	Leucine rich repeat containing 48	*LRRC48*	0.6	0.006
	Hs.387671	Tudor domain containing 10	*TDRD10*	0.6	0.033
	Hs.575083	UDP glucuronosyltransferase 2 family, polypeptide B17	*UGT2B17*	0.6	0.043
	Hs.522640	Proprotein convertase subtilisin/kexin type 1 inhibitor	*PCSK1N*	0.5	0.043

the materials and methods section, the 35-sample set used in the microarray analysis consisted of 13 samples of the ACR group, 13 of the non-ACR group, and 9 of the protocol group. Nine of 13 patients of the ACR group underwent liver biopsy after treatment of the ACR. The validation group of 23 samples consisted of 7 samples classified as the ACR group and 16 as the non-ACR group. The most common reason for liver biopsy was elevation of liver enzymes (44/58, 76%), and 14 samples were obtained as protocol biopsies (14/58, 24%). Acute rejection episodes were confirmed by histologic findings and response to rejection therapy. The median time from liver transplantation to biopsy was 2.5 mo (0.1–25 mo) for all patients. Most patients were treated with a combination of calcineurin inhibitor, corticosteroids, and mycophenolate mofetil (30 of the 58 were on mycophenolate mofetil and 20 of 58 received maintenance corticosteroids). We verified that the pathologic diagnosis matched the clinical course of the patients. All patients in the ACR group received anti-rejection therapy with steroid pulse or an increase in the dose of maintenance immunosuppression, and all showed recovery of liver function. The clinical characteristics of the patients who underwent microarray ($n = 35$) and RT-PCR ($n = 23$) studies are summarized in Tables 1 and 2.

3.2. *Differential gene expression profiles of human liver grafts*

Two-dimensional hierarchical cluster allows verification of the presence of differential expression patterns consisting of samples with rejection compared with other dysfunctional and well-functioning samples (Fig. 1B). This approach yielded a good separation of each event (ACR/non-ACR/protocol), except for two samples (ACR6/non-ACR12), and revealed predominant gene expression patterns for the ACR. Interestingly, five RHC cases included in the non-ACR group (non-ACR1–5) had the more similar expression pattern to ACR than any other samples.

3.3. *Relative overexpression of top 86 genes*

Of the 983 genes, 504 showed upregulated expression and 479 showed downregulated expression in the ACR group, relative to the non-ACR group. The top 113 relatively overexpressed and underexpressed genes (fold change >1.5) from the 983 genes in the ACR group were subjected to further analysis. Of these 113 genes, 86 genes are listed in Table 3 (the 27 unknown genes are excluded). Most of the upregulated genes are associated with antigen presenting cells (e.g., *CD52*, *HLA-DRA*), cytotoxic T cells (e.g., *CD8a*, *CD8b*, *LCK*, *GZMB*), or interferon gamma (IFNG) induction (e.g., *CXCL9*, *GBP2*, *GBP4*, *CCL19*). On the other hand, the expression of several genes involved in glucocorticoid receptor signaling (*CEBPA*, *VIPR1*) and drug metabolism (*CYP2B6*) was relatively low in the ACR group (Table 3).

3.4. *Identification of biologically relevant networks and potential key genes highly expressed during ACR*

We used the Ingenuity Pathway analysis to categorize the canonical pathways associated with the selected 113 genes. The top 10 canonical pathways that were identified as significant pathways are listed in Table 4. These findings support the clinicomorphologic impression of increased alloreactivity.

We also simultaneously carried out network analysis to visualize the molecular interaction of the same informative 113 gene set. A total of 90 genes were mapped to the Ingenuity database, and seven genetic subnetworks were identified; these networks were ranked by the score on a *P* value calculation. The top three subnetworks with the highest score (score ≥34) are shown in Figure 2A–C. In network 1, NF-κB formed a subnetwork as the central hub, connecting to the upregulated focus genes associated with T cell receptor signaling (*CD3*, *CD3D*, *CD3G*, *CD8*, *CD8A*, *CD8B*, *LCK*, *TCR*, and *TRA@*), T cell trafficking cytokine (*CXCL9* and *CCL19*), and IFNG production (*GBP2* and *IL18BP*) (Fig. 2A). Network 2 comprised several inferred genes (ERK, interferon alpha, Jnk, MAPK, P38MAPK, and TGF beta) associated with IL-12 signaling and production in macrophages, which were interestingly linked to the upregulated focus genes (*GZMB*, *TBX21*, and *CDC25A*) expressed in activated T cells (Fig. 2B). Network 3, comprising IFNG as the central hub, contained mainly upregulated focus genes associated with natural killer cell signaling (*LAIR1*, *RAC2*) (Fig. 2C). We selected the most upregulated genes (*CXCL9*, *GZMB*, and *CCL19*) in each subnetwork for further analysis with the neighboring genes (*GBP2*, *CDC25A*, and *LAIR1*), which were linked to the central hub in each subnetwork as well as the former targets.

Table 4 – List of top canonical pathways for 113 informative genes.

Pathway	*P* value
T cell receptor signaling	4.79E-08
Cytotoxic T lymphocyte–mediated apoptosis of target cells	8.69E-06
Calcium-induced T lymphocyte apoptosis	1.27E-05
Natural killer cell signaling	2.20E-04
CD28 signaling in T helper cells	2.87E-04
CCR5 signaling in macrophages	3.96E-04
CTLA4 signaling in cytotoxic T lymphocytes	9.55E-04
SAPK/JNK signaling	1.30E-03
Role of NFAT in regulation of the immune response	1.87E-03
iCOS-iCOSL signaling in T helper cells	1.92E-03

3.5. *Verification of microarray data*

We performed qRT-PCR for four genes (*CXCL9*, *GZMB*, *CDC25A*, and *LAIR1*) to verify our microarray data, using unamplified total RNA originally extracted from 35 liver tissues. The data of qRT-PCR validation confirmed the conclusion from the microarray data ($P < 0.05$, Fig. 3A).

3.6. *Validation of six representative genes*

The RT-PCR studies on six selected genes (*CXCL9*, *GBP2*, *GZMB*, *CDC25A*, *CCL19*, and *LAIR1*) were extended to the 23 additional samples (ACR/non-ACR; 6/17). With the exception of the *GZMB*, 5 of 6 genes (*CXCL9*, *CCL19*, *LAIR1*, *GBP2*, and *CDC25A*) were significantly upregulated in the ACR group compared with the non-ACR group ($P < 0.05$, Fig. 3B). There were no significant differences in *GZMB* between the groups, though *GZMB* tended to be upregulated in the ACR group.

3.7. *Correlation with the clinical course after rejection treatment*

In order to investigate the response to the rejection treatment, we compared the expression level of the six target genes in the samples obtained before and after rejection treatment. Nine liver biopsy samples after rejection treatment were obtained from 9 out of 13 patients, who were nominated for the original ACR group in the microarray study. All patients except one were treated with steroid pulse (8/9, 89%). The clinicopathologic features of all patients improved after the treatment, and the treatment significantly reduced the expression levels of *CXCL9*, *LAIR1*, and *CCL19*. There were no significant differences in the expression levels of *GZMB*, *CDC25A*, and *GBP2* between before and after treatment, though these levels tended to diminish after treatment, except for *CDC25A* (Fig. 3C).

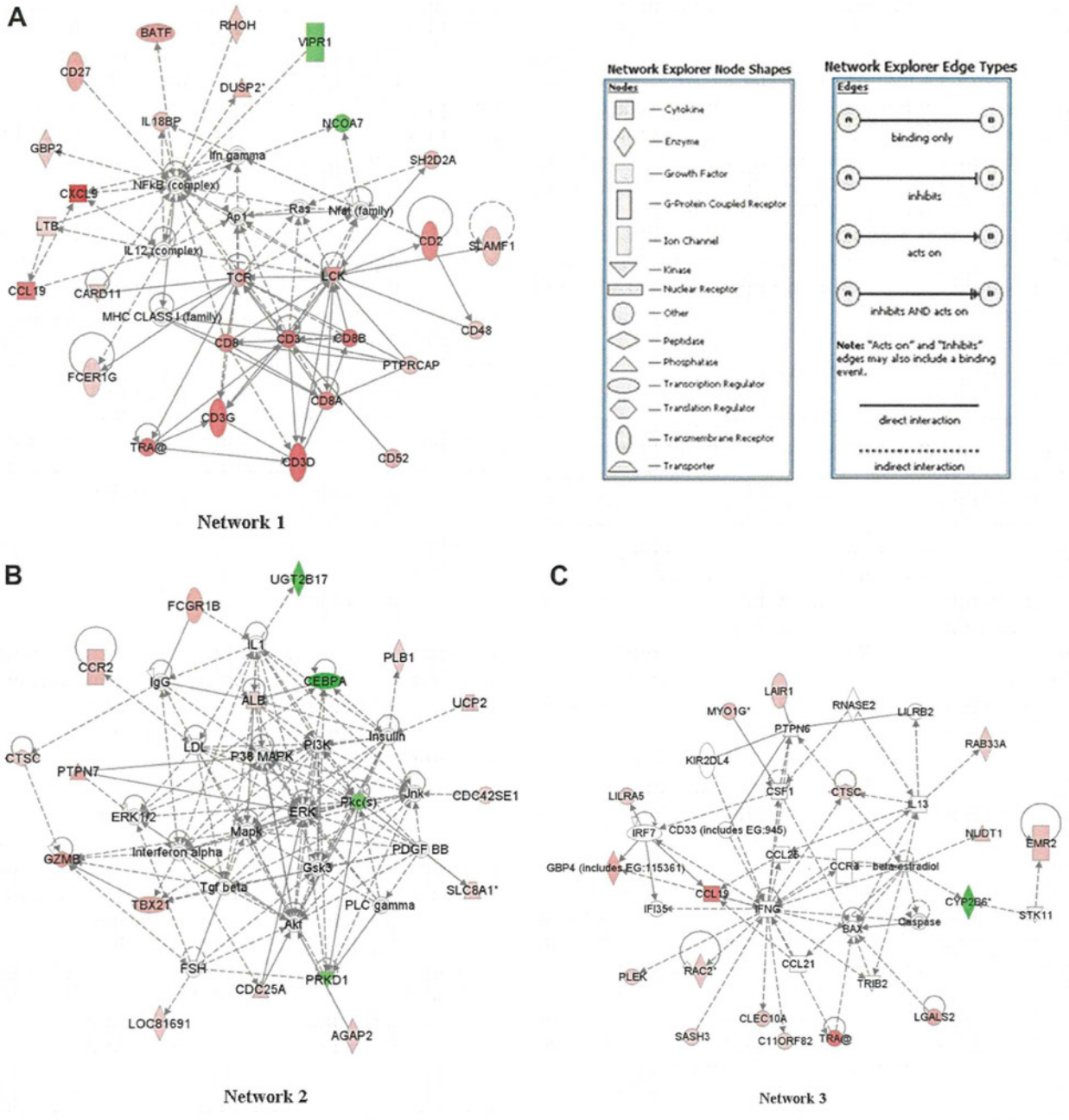

Fig. 2 – Network analysis. The data set containing 113 genes was used as the starting point for generating biological networks. IPA identified seven biological networks. Shown are the top three subnetworks with the highest score (A–C). Nodes represent genes, with their shape representing the functional class of the gene product, while the edges indicate the biological relationships between the nodes. Color concentration indicates the intensity of expression (fold changes of ACR group relative to non-ACR group: red, overexpression; green, underexpression; noncolored, interconnecting molecules). (Color version of figure is available online.)

3.8. *Predictive values of mRNA levels of CXCL9 and LAIR1 in liver grafts*

We made focus on the molecules which had significant differences between before and after rejection treatment and analyzed the cutoff points for CXCL9 and LAIR1 in the liver graft that yielded the highest combination of sensitivity and specificity with respect to distinguishing patients with ACR from those with other dysfunctions or stable graft. We carried out a conventional receiver operating characteristic curve based on qRT-PCR results (n = 58; original/validated, 35/23). Our analysis showed that ACR can be diagnosed using the levels of CXCL9/GAPDH and LAIR1/GAPDH in the liver graft (CXCL9: cutoff 1.5, sensitivity 70%, specificity 71%, accuracy

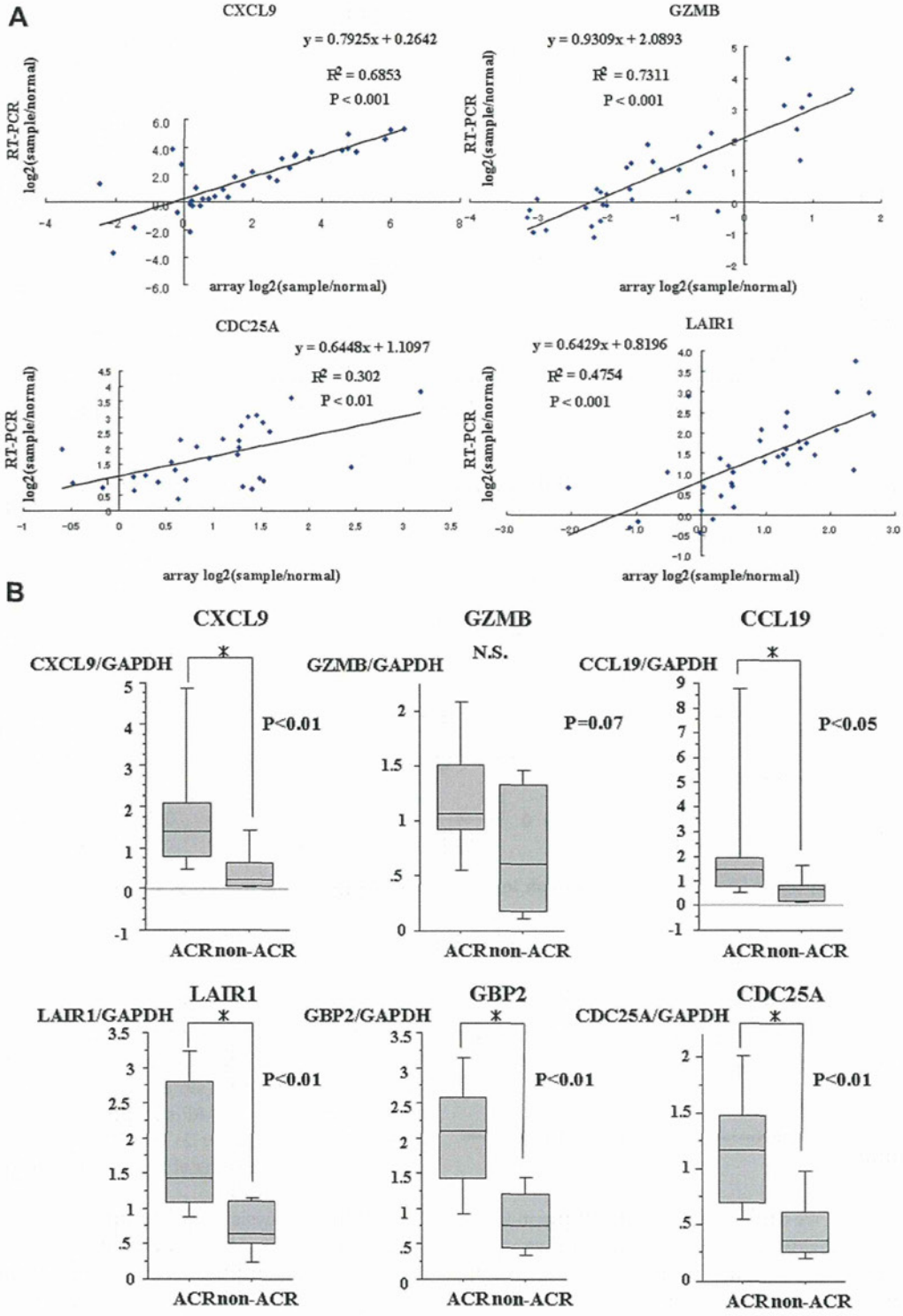

Fig. 3 – Validation of representative genes. qRT-PCR was performed for representative genes to verify microarray data. The gene expression levels were normalized to GAPDH of the same samples. (A) qRT-PCR was performed for four genes (*CXCL9*, *GZMB*, *CDC25A*, and *LAIR1*) to verify microarray data using the original pooled samples. The gene expression levels were rescaled relative to the control (donor liver). The expression level was calculated as the logarithm of each sample/control. *CXCL9* = chemokine (C-X-C motif) ligand 9; *GZMB* = granzyme B; *CDC25A* = cell division cycle 25 homolog A; *LAIR1* = leukocyte-associated immunoglobulin-like receptor 1. (B) The reproducibility of six representative genes was evaluated using an additional 23 liver biopsy samples. 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. $^{*}P < 0.05$.

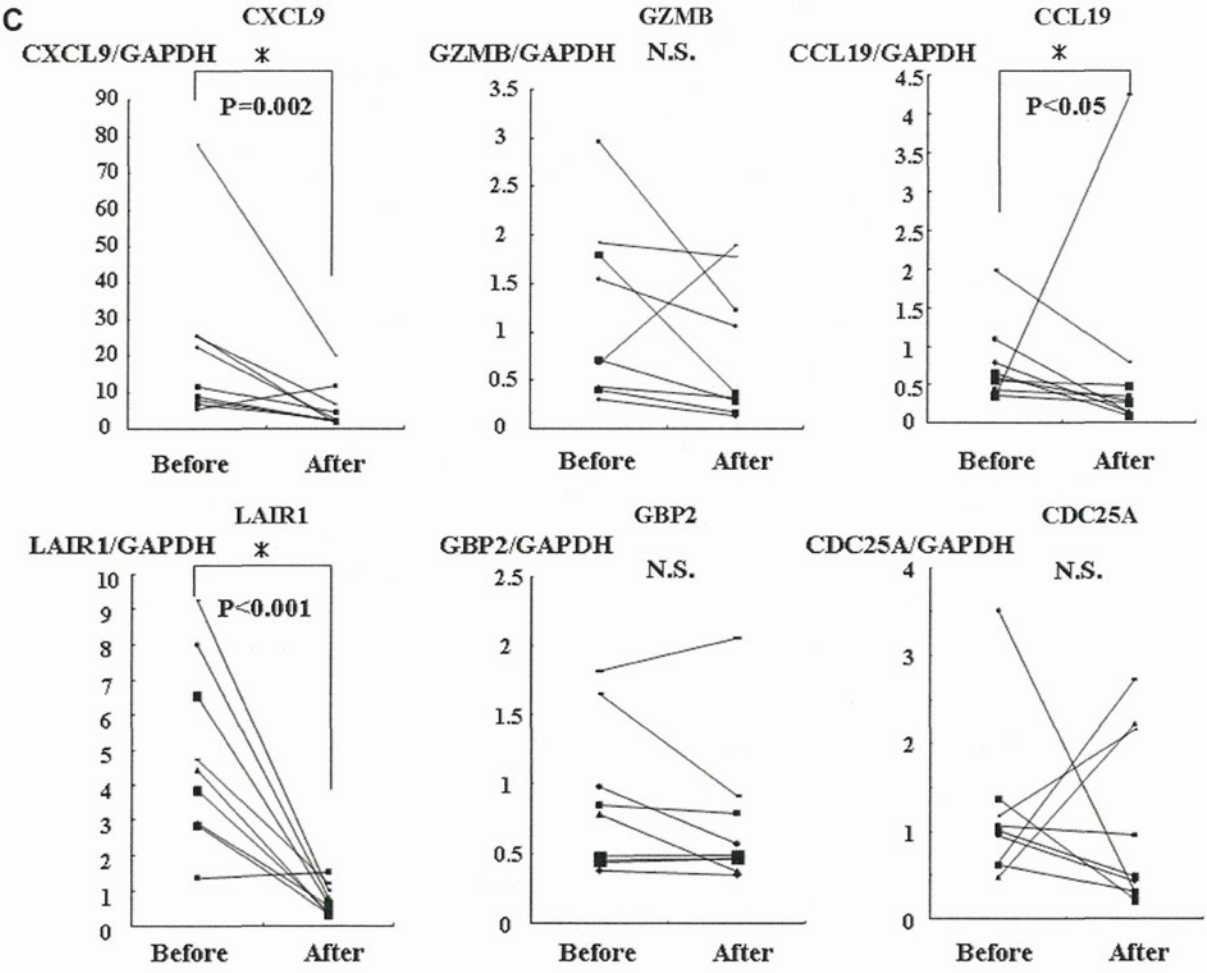

Fig. 3 – (*continued*) (C) Comparison of the expression level of the six target genes in liver graft before and after rejection treatment.

71%, LAIR1: cutoff 2.0, sensitivity 70%, specificity 68%, accuracy 69%) (Supplemental Fig., available online at www.JournalofSurgicalResearch.com).

4. Discussion

In the present study, we compared the gene expression levels in matched sets of the ACR, non-ACR, and protocol groups. A total of 983 genes were identified as specific target genes for ACR, which showed transcriptome changes in liver graft after LT. Two-dimensional supervised clustering based on the selected 983 genes showed interesting findings. Thirty-five samples, which were subsequently analyzed by the microarray study, including several samples obtained from the same recipients collected at different times (ACR 4/protocol 9, ACR 11/non-ACR 3, and ACR 13/non-ACR 5), were correctly separated into different clusters according to the event, not to their own characteristics (Fig. 1B). This result indicates that these 983 genes exactly reflect each event, not baseline cellular characteristics. The dendrogram approximately divided the samples into four groups. The first branch separated the samples into functional and dysfunctional grafts. The non-ACR group was further divided into two small clusters; cluster 2 had more similar expression patterns to cluster 1 rather than cluster 3. All samples with RHC (non-ACR 1–5) were sorted into cluster 2. These results suggested that the gene expression patterns of RHC cases were relatively similar to those of ACR cases.

Network analysis revealed the top three subnetworks, which had NF-κB, MAPK, and IFNG as central hubs. It has been known that the activations of these molecules were key events in T-cell activation. We selected six modulators from the top three subnetworks and evaluated the reproducibility of and correlation with the clinical course. Among the six selected modulators, CXCL9 was the relatively most upregulated during ACR compared with the non-ACR group (relative fold = 6.4, $P = 0.007$), and its expression level correlated well with the clinical course. CXCL9 is a chemokine of the CXC subfamily and is thought to be involved in T-cell trafficking. CCL19 also plays an important role in T- and B-cell migration [27–30]. Both CXCL9 and CCL19 molecules regulated by IFNG were significantly associated with the selected subnetwork

(Fig. 2A and C). These networks appeared to highlight the importance of CXCL9 and CCL19 in the selective recruitment of activated/effector T cells into the graft and the importance of high local production of IFNG by effector T cells. T-bet (TBX21) is a Th1-specific transcription factor that controls the expression of IFNG, IL-2, and IL-12 production [31]. The overexpression of Th1-associated molecules (T-bet, GZMB, and IFNG) might reflect a shift in the Th1/Th2 balance, with a preferential tilt toward Th1 during ACR, and demonstrate that Th1-predominant infiltration promotes cytotoxic T lymphocyte activation and GZMB-mediated graft injury is induced by cytotoxic T-lymphocyte activation (Fig. 2B). Our findings are also in agreement with studies demonstrating that Th1 cells may be predominantly associated with ACR [32].

Another network also contained several molecules known to modulate B-cell signaling, like CD27, RAC2, and CARD11, and natural killer cell signaling, like Fc fragment of IgE (FCERG), leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), and lymphocyte-specific protein tyrosine kinase (LCK) (Fig. 2C). These results appear to reflect the association with not only T cell–mediated but also B cell–mediated immune response during ACR [17]. On the other hand, LAIR1, which is found on peripheral blood mononuclear cells, acts as an inhibitory receptor for antigen-specific effector T cells and downregulates T cell functions. LAIR1 also modulates cytokine production and suppresses the immune response [33,34]. Upregulation of this gene might reflect an attempt at neutralization of host defenses within the rejecting graft milieu.

In our evaluation of samples collected before and after treatment for ACR, CXCL9, LAIR1, and CCL19 perceptively reflected the clinical features, whereas GBP2 and CDC25A did not. This difference may be explained by the notion that markers of immune activation (CXCL9, LAIR1, and CCL19) should appear early in the rejection process, like cytokine secretion from antigen presenting cells, while markers representative of effector pathways (GZMB and GBP2) or local cell cycle (CDC25A) can be expected to appear in later stages [35].

CXCL9 is produced by dendritic cells, B cells, and macrophages and binds the receptor CXCR3. In the transplant of other organs, it has been reported that its ligands play prominent roles in the recruitment of effector T cells into allograft and the utility of anti-CXCL9 therapy for ACR [36,37]. Rotondi *et al.* already assessed the utility of serum level CXCL9 in evaluating risk of graft failure prior to transplantation [38]. The other study demonstrated that the measurement of urinary levels of CXCL9 is useful to predict ACR onset [39]. Our results also suggested that the elevation of intragraft CXCL9 mRNA was typical to ACR and could be a therapeutic target of ACR after LT. However, such results need to be confirmed in a larger randomized study with higher statistical power.

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 pathologic diagnosis and basic analyses were performed at two different sites, with each site masked to the information held by the other; and (2) the reproducibility of the six selected genes' expressions was confirmed on the basis of our analysis of the validation of liver biopsy samples.

Another limitation of this study is sample selection. The clinical issue is whether molecular biomarkers could discriminate ACR from other causes of changes in liver tests during the early posttransplant course. But we eliminated the early postoperative period to minimize the influence of preoperative and early postoperative conditions such as surgical stress. In order to state the clinical utility of selected candidates as markers, we need to monitor their expression during the early postoperative course.

In conclusion, we reported in the present study an intragraft gene expression pattern in ACR compared with other causes of graft dysfunctions after liver transplantation. We identified intragraft CXCL9 mRNA as potential molecular biomarkers for the diagnosis of ACR and monitoring of allograft response to rejection treatment.

Acknowledgment

This study was partly supported by Astellas Pharma Inc, Tokyo, Japan.

Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.jss.2012.07.016.

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Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2012.07129.x

HEPATOLOGY

Interleukin-28B single nucleotide polymorphism of donors and recipients can predict viral response to pegylated interferon/ribavirin therapy in patients with recurrent hepatitis C after living donor liver transplantation

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Key words
core, hepatitis C virus, interferon sensitivity-determining region, interleukin-28B, liver transplantation.

Accepted for publication 12 March 2012.

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Abstract

Background and Aim: Interleukin-28B (*IL28B*) single nucleotide polymorphism (SNP) influences viral response (VR) to interferon (IFN) therapy in patients with hepatitis C. We studied the relationship between VR and the *IL28B* polymorphism (rs8099917) in patients on long-term pegylated IFN plus ribavirin (PEGIFN/RBV) therapy for recurrent hepatitis C after living-donor liver transplantation (LDLT).

Methods: Thirty-five patients with recurrent hepatitis C after LDLT were treated with PEGIFN/RBV. We evaluated the effect of *IL28B* SNP on the outcome in 20 patients infected with hepatitis C virus genotype 1 who completed IFN therapy.

Results: The sustained VR (SVR) rate was 54% (19/35) for all patients; 46% (13/28) for genotype 1. The SVR rate of donors' TT group (major genotype) was higher than that of donors' TG + GG group (minor genotype) (73% *vs* 20%), while that of recipients' TT group was similar to that of recipients' TG + GG group (64% *vs* 50%). With regard to the combined effect of donors' and recipients' *IL28B* SNP, the SVR rates of TT : TT (donors' : recipients'), TT : TG + GG, TG + GG : any group were 81%, 50%, and 20%, respectively. The VR rate of TT : TT, TT : TG + GG and TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, 20%, and those at the end of treatment were 100%, 50%, 20%, respectively. The multivariate analysis identified *IL28B* of donors : recipients (TT : TT) as the only independent determinant of SVR (odds ratio 15.0, $P = 0.035$).

Conclusion: Measurement of donors' and recipients' *IL28B* SNP can predict the response to PEGIFN/RBV therapy, and the donors' *IL28B* SNP might be a more significant predictor than that of the recipients.

Introduction

Hepatitis C virus (HCV) has infected 170 million people worldwide, and such infection sometimes progresses to liver cirrhosis and/or hepatocellular carcinoma.[1] The current treatment for patients infected with HCV genotype 1 (HCV-1) is the combination of pegylated interferon-α and ribavirin (PEGIFN/RBV) for 48 weeks.[2] However, this treatment results in sustained viral response (SVR) in only approximately 50% of patients with HCV-1 infection.

In a recent genome-wide association study, a single nucleotide polymorphism (SNP) upstream of the interleukin (IL)-28B (*IL28B*) gene on chromosome 19, coding for IFN-λ-3, was found to be strongly associated with SVR rate in treatment-adherent HCV-1 patients.[3–8] The G nucleotide of rs8099917 was associated with a poor response to treatment (minor allele), whereas a T nucleotide was found to be associated with a fair response to treatment (major allele) in Japanese patients.

HCV-related end-stage liver disease is currently the leading indication for liver transplantation (LT). However, the outcome of LT for patients with HCV-related liver disease has been less satisfactory than those with HCV-negative liver disease.[9–15] HCV recurrence is universal after LT with accelerated progression of liver fibrosis. Approximately 20–25% of HCV-positive

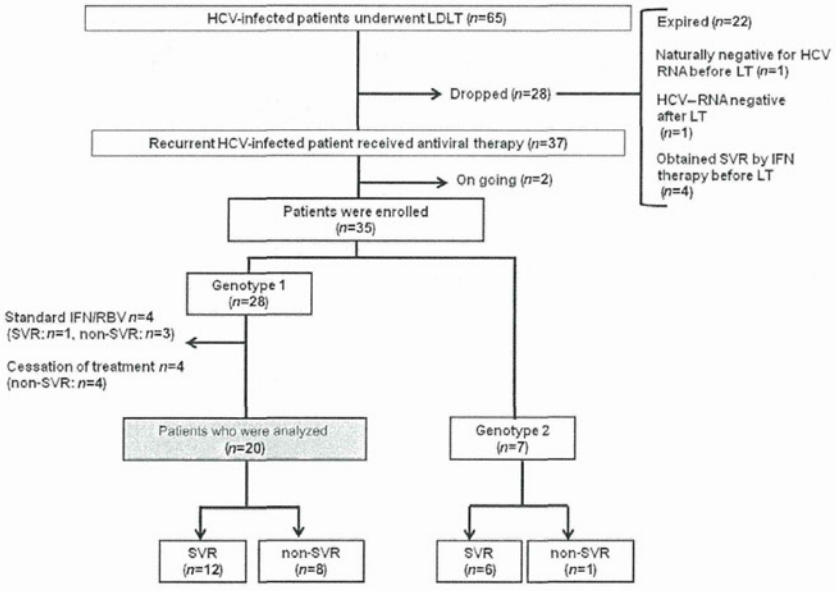

Figure 1 Flow diagram of patient recruitment. HCV, hepatitis C virus; IFN, interferon; LDLT, living-donor liver transplantation; LT, liver transplantation; RBV, ribavirin; SVR, sustained viral response.

patients develop cirrhosis within 5 years after LT, and approximately 50% within 10 years.[13,16,17] LT recipients with recurrent HCV are treated with a combination of PEGIFN/RBV for 48 weeks. However, eradication with IFN therapy after LT is hampered by the use of immunosuppressive agents, anemia, frequent side-effects, and the need to discontinue or reduce therapy. The outcome of PEGIFN/RBV antiviral therapy after LT is poor, with the SVR rate ranging from 10% to 30% for HCV-1-infected patients.[18–24]

However, Fukuhara *et al.*[8] reported that in patients with recurrent HCV infection after LT, combination analyses of SNP of *IL28B* in both the donor and recipient tissues and mutations in HCV–RNA allow the prediction of SVR to PEGIFN/RBV therapy.

We reported previously the effectiveness of the treatment of recipients with PEGIFN/RBV until HCV–RNA reaches undetectable levels, followed by continuation of treatment for at least 48 weeks (i.e. long-term IFN therapy).[25] Others also reported SVR rates of 34% and 50% under the same treatment, respectively.[26,27]

In the present study, we analyzed the viral response to long-term PEGIFN/RBV therapy in patients according to the major and minor genotypes of the polymorphic *IL28B* gene.

Methods

Patients. Sixty-five patients underwent living-donor LT (LDLT) for HCV-related end-stage liver disease between 2000 and January 2011. Among them, 22 patients died before the start of therapy, one was naturally negative for HCV–RNA before LT, one did not become positive for HCV–RNA after LDLT, and four obtained SVR by IFN therapy before LT, thus leaving 37 patients treated with IFN therapy at our institution. Of these, two patients are currently continuing antiviral therapy. A total of 35 patients were enrolled in this retrospective study.

There were 28 patients with HCV-1, and seven with HCV-2. The data of eight of the 28 patients with HCV-1 were excluded from the analysis due to the use of standard IFN/RBV in four patients, and cessation due to side-effects in four patients. Thus, the study included 20 patients with HCV-1 (Fig. 1).

Protocol of antiviral therapy. Patients received PEGIFN-α-2b subcutaneously once weekly combined with RBV (200 mg/day). The dose of the latter was increased to 800 mg/day in a stepwise manner, according to individual tolerance within the first 12 weeks of therapy. The combination PEGIFN/RBV therapy was continued for more than 48 weeks after the disappearance of serum HCV–RNA. At the end of the active treatment, patients were followed for another 24 weeks without treatment. In patients who remained positive for HCV–RNA in spite of treatment for more than 48 weeks, PEGIFN was switched to PEGIFN-α-2a, and treatment was continued as described earlier.

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the local ethics committees of all participating centers. Written, informed consent was obtained from all participating patients.

Assessment of therapy efficacy. HCV–RNA levels were measured using one of several reverse transcription–polymerase chain reaction (RT–PCR)-based methods (*Taq*Man RT–PCR test) at weeks 4, 8, and 12, and thereafter every 4 weeks of treatment, and at 24 weeks after the cessation of therapy.

SNP genotyping and quality control. Because the two reported significant *IL28B* SNP (rs8099917 and rs12979860) are in strong linkage disequilibrium, we examined only rs8099917 in this study. Some samples obtained from patients with HCV-1 were determined using the Illumina HumanHap610-Quad Genotyping BeadChip (San Diego, CA, USA), whereas the remaining samples were genotyped using the Invader assay (Third Wave Technologies, Madison, WI, USA), as described previously.[28,29]

Table 1 Characteristics of 20 patients with recurrent hepatitis C genotype 1 after living-donor liver transplantation

Age (years)†	58 (44–70)
Sex (male/female)	15/5
Body mass index (kg/m²)†	24.3 (18.8–42.2)
Viral load at therapy (LogIU/mL)†	6.6 (4.9–7.8)
Time from transplantation to therapy (months)†	4 (1–41)
No. mutations in the ISDR (0–1/2–5)	12/8
HCV core70 region (mutant/wild)	12/8
HCV core 91 region (mutant/wild)	10/10
Donors' *IL28B* genotype TT/TG + GG	15/5
Recipients' *IL28B* genotype TT/TG + GG	14/6
Combination of donors' and recipients' *IL28B* genotype (TT : TT/TT : TG + GG/TG + GG : TT/TG + GG : TG + GG)	11/4/3/2
Immunosuppression (tacrolimus/cyclosporine)	16/4
Adherence to PEGIFN ≥ 70/< 70 (%)†	11/9
Adherence to RBV ≥ 50/< 50 (%)†	8/12

†Values are median (range). HCV, hepatitis C virus; *IL28B*, interleukin-28B; ISDR, interferon sensitivity-determining region; PEGIFN, pegylated interferon; RBV, ribavirin.

Analysis of the nucleotide sequences of the core and non-structural 5A regions. The amino acid (aa) substitutions at aa 70 and aa 91 of the HCV core region and mutation at the IFN sensitivity-determining region were analyzed in the non-structural 5A region of HCV by the direct sequencing method, as described previously by our group.[25,30,31] Samples after LT were used.

Statistical analysis. Non-parametric tests (χ^2-test and Fisher's exact probability tests) were used to compare the characteristics of the groups. Univariate logistic regression analysis was used to determine those factors that significantly contributed to early viral dynamics. The odds ratios and 95% confidence intervals were also calculated. All *P*-values < 0.05 using two-tailed tests were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) in the univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Statistical analyses were performed using PASW 18 statistical software (SPSS, Chicago, IL, USA).

Results

Patient characteristics. Table 1 shows the baseline characteristics of the 20 patients with recurrent hepatitis C after LT who completed PEGIFN/RBV treatment. The median age of the patients (15 males and 5 females) was 58 years, and the median body mass index was 24.3. The median latency between transplantation and the initiation of antiviral therapy was 4 months. The median pretreatment serum HCV–RNA viral load was 6.6 LogIU/mL. The *IL28B* genotype (rs8099917) of the donors was TT in 15 patients, and TG + GG in five patients, whereas that of the recipients was TT in 14, and TG + GG in six. Immunosuppressive therapy included tacrolimus in 16, and cyclosporine in four.

Efficacy and tolerance of IFN therapy and side-effects. Figure 1 shows the effects of IFN therapy according to genotype. The SVR rate was 54.2% (19/35) for all patients. Among the patients infected with HCV-1, one of eight patients who were treated with mono-IFN/RBV or ceased treatment had SVR. Twelve of 20 patients with HCV-1 who completed IFN therapy achieved SVR. Thus, the SVR rate was 46.4% (13/28) for those with HCV-1, and 85.7% (6/7) with HCV-2. In patients with HCV-1, four ceased IFN therapy due to adverse effects. These included general fatigue in one, rejection in two, and cerebral hemorrhage in one patient.

Relationship between IL28B and viral response in patients infected with HCV genotype 1. Data or eight of 28 patients with HCV-1 were excluded from the analysis due to standard-IFN plus RBV in four patients, and the cessation of IFN therapy due to adverse effects in four patients. Thus, the data of 20 patients with HCV-1 were available for the analysis of *IL28B*.

In the donors, the SVR rate of the TT group (73.3% [$n = 11/15$]) was higher than that of the TG + GG group (20% [$n = 1/5$], $P = 0.053$, Fig. 2a). In the recipients, the SVR rate of the TT group (64.2% [$n = 9/14$]) was similar to that of the TG + GG group (50% [$n = 3/6$]) (Fig. 2b). The SVR rate of the TT : TT group (donors' *IL28B* : recipients' *IL28B*) was 81.8% ($n = 9/11$), which was higher than the SVR rate of the TT : TG + GG group (50% [$n = 2/4$], Fig. 2c). The SVR rate of the TG + GG : any group (donors' *IL28B* : recipients' *IL28B* of either TT or TG + GG) was 20% ($n = 1/5$), which was lowest among the three groups. There was significant difference between the SVR of the TT : TT group and TG + GG : any group ($P = 0.036$). We also analyzed the viral response (VR) rate according to the combination of donors' and recipients' *IL28B*. The VR rates of TT : TT, TT : TG + GG, TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, and 20%; and those at the end of treatment were 100%, 50%, and 20%, respectively. The VR rate of the TT : TT group was 63.6% ($n = 7/11$), which was higher than the VR rate of the TG + GG : any group (0% [$n = 0/5$]) at 24 weeks. The VR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the VR rate of the TG + GG : any group (20% [$n = 1/5$]) at the end of treatment. The SVR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the SVR rate of the TG + GG : any group (20%, $n = 1/5$) at 24 weeks at the end of treatment (Fig. 3).

Analysis of factors associated with SVR in HCV-1 patients with recurrent hepatitis C. The univariate analysis identified three parameters that correlated with SVR either significantly or marginally: the combination of donors' and recipients' *IL28B* (TT : TT $P = 0.037$), donors' *IL28B* (TT genotype; $P = 0.053$), and adherence to RBV therapy (≥ 50; $P = 0.076$, Table 2). The combination of donors' and recipients' *IL28B* (TT : TT genotype) and adherence to RBV (> 50; $P = 0.076$) were entered into the multiple logistic regression analysis to identify significant independent predictive factors. The multivariate analysis identified the combination of donors' and recipients' *IL28B* (TT : TT) as the only significant and independent factor that influenced the SVR: (odds ratio: 15.0, 95% CI: 1.2–185.1, $P = 0.035$).

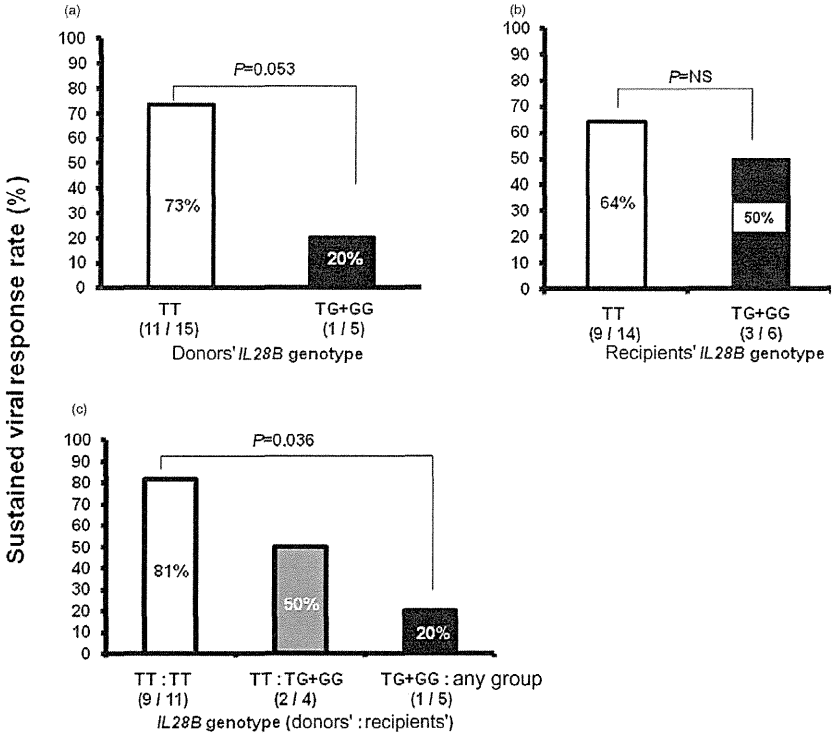

Figure 2 Sustained viral response rates according to (a) donors' interleukin-28B (*IL28B*), (b) recipients' *IL28B*, and (c) donors' and recipients' *IL28B* in patients infected with hepatitis C virus genotype 1. TT : TT group (donors' *IL28B* TT: recipients' *IL28B* TT), TT : TG + GG group (donors' *IL28B* TT: recipients' *IL28B* TG + GG), TG + GG : any group (donors' *IL28B* TG + GG: recipients' *IL28B* either TT or TG + GG). NS, not significant.

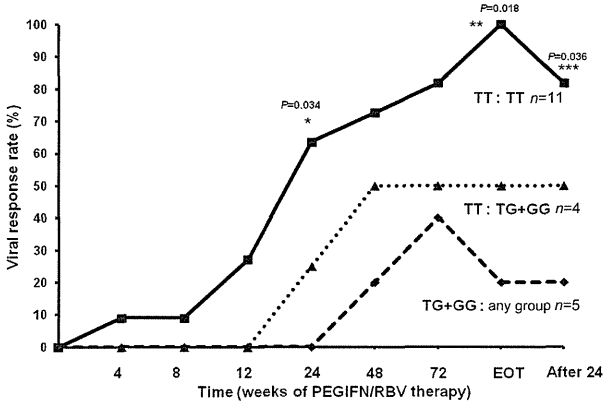

Figure 3 Viral response rates according to donors' and recipients' interleukin-28B (*IL28B*) genotyping. TT : TT group (donors' *IL28B* TT: recipients' *IL28B* TT), TT : TG + GG group (donors' *IL28B* TT: recipients' *IL28B* TG + GG), TG + GG : any group (donors' *IL28B* TG + GG: recipients' *IL28B* either TT or TG + GG). *Viral rate (VR) of the TT : TT group was 63.6% ($n = 7/11$), which was higher than the VR rate of the TG + GG : any group (0%, $n = 0/5$) at 24 weeks. **VR rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the VR rate of the TG + GG : any group (20%, $n = 1/5$) at the end of treatment (EOT). ***Sustained VR (SVR) rate of the TT : TT group was 100% ($n = 11/11$), which was higher than the SVR rate of the TG + GG : any group (20%, $n = 1/5$) at 24 weeks at the EOT. PEGIFN, pegylated interferon; RBV, ribavirin.

Discussion

The SVR rate has improved since the introduction of PEGIFN/RBV for patients who undergo LT for HCV-related end-stage liver disease. The current estimated SVR rate for LT patients with a history of HCV-1 infection is 30–50%.[21–24,26,27] These results are much better than those reported in the 1990s and early 2000s; however, more than half of recipients still suffer from recurrent chronic hepatitis C.

Although many studies have determined the predictive factors of the viral response for PEGIFN/RBV among patients with chronic hepatitis C, recent molecular biological analyses and genome-wide analyses of the human genome have identified genetic variations of *IL28B* and amino-acid substitution of HCV core 70 as the most significant predictive factors for IFN response.[3–5,32,33] *IL28B* encodes a cytokine distantly related to type I IFN and the IL-10 family. It has been reported that the expression level of the *IL28* gene in peripheral blood mononuclear cells is significantly lower in individuals with minor alleles than in individuals with major alleles.[5]

Several studies have determined the predictive factors for the viral response to PEGIFN/RBV in patients with recurrent post-LT hepatitis C viral infection, and recent molecular and genome wide analyses of the human genome have demonstrated that genetic variation of *IL28B* is the most significant predictive factor of the response to IFN.[8,34–37] In the present study, we examined whether the same factors can also predict the response to PEGIFN/RBV in LT recipients. Several groups have reported that recipients' and donors' *IL28B* influenced the SVR to PEGIFN/RBV in patients with recurrent hepatitis C after LT.[8,36,37] Furthermore, others

Table 2 Univariate analysis of factors associated with sustained viralresponse (SVR) during interferon therapy in genotype 1 patients with recurrent hepatitis C

	SVR ($n = 12$)	Non-SVR ($n = 8$)	*P*-value
Age (years)†	60 (44–69)	57 (47–65)	0.48
Sex (male/female)	10/2	5/3	0.3
Body mass index (kg/m^2)†	24.1 (21.4–26.5)	24.2 (18.9–42.2)	0.4
Viral load at therapy (LogIU/mL)†	6.3 (5.8–6.6)	6.6 (5.9–7.2)	0.52
Time from transplantation to therapy (months)†	4 (1–41)	3 (1–6)	1.7
No. mutations in the ISDR (0–1/2–5)	7/5	5/3	1.0
HCV core70 region (mutant/wild)	7/5	5/3	1.0
HCV core 91 region (mutant/wild)	7/5	3/5	0.6
Donors' *IL28B* genotype TT/TG + GG	11/1	4/4	0.053
Recipients' *IL28B* genotype TT/TG + GG	9/3	5/3	0.6
Donors' and recipients' *IL28B* genotype TT : TT/others	9/3	2/6	0.037
Immunosuppression (tacrolimus/cyclosporine)	9/3	7/1	1.0
Adherence to PEGIFN ≥ 70/< 70 (%)†	8/4	3/5	0.3
Adherence to RBV ≥ 50/< 50 (%)†	7/5	1/7	0.076

†Values are median (range). HCV, hepatitis C virus; *IL28B*, interleukin-28B; ISDR, interferon sensitivity-determining region; PEGIFN, pegylated interferon; RBV, ribavirin.

reported that donors' *IL28B* influenced the SVR in patients treated with PEGIFN/RBV for recurrent hepatitis C after LT,[34] and that recipients' *IL28B* influenced the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C.[35,36]

The results of the present study indicate that both donors' and recipients' *IL28B* influence the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C. Both recipients' and donors' *IL28B* influenced the SVR to PEGIFN/RBV in recurrent hepatitis C after LT; however it is not clear whether the recipients' or donors' *IL28B* influenced the SVR to PEGIFN/RBV.

However, the donors' *IL28B* might have influenced the SVR to PEGIFN/RBV in patients with recurrent post-LT hepatitis C more than the recipients' *IL28B*. This conclusion is based on the following results: although the SVR rate of the TT group (64.2%) was similar to that of the TG + GG group (50%), according to the recipients' *IL28B*, the SVR rate of the TT group (73.3%) was higher than that of the TG + GG group (20%), according to the donors' *IL28B*. Furthermore, the VR rates of TT : TT, TT : TG + GG, TG + GG : any group at 12 weeks were 28%, 0%, and 0%; those at 48 weeks were 70%, 50%, and 20%; and those at the end of treatment were 100%, 50%, and 20%, respectively. That is, the time to VR of the TG + GG : any group was the latest among the three groups. Lange *et al.* reported that donors' *IL28B* influenced the SVR in patients treated with PEGIFN/RBV for recurrent hepatitis C after LT.[34] In this regard, Hiraga *et al.*[38] reported that IFN-stimulated gene expression levels in mice livers measured at 2 weeks after IFN treatment were significantly higher in mice transplanted with donor human hepatocytes (*IL28B*; TT) than from donor (*IL28B*; TG + GG) mice. Furthermore, previous studies reported that the expression level of IFN- λ-3, coded for the *IL28B* gene, was higher in hepatocytes than hematopoietic cells.[39]

However, we demonstrated the feasibility of treatment of LT recipients with PEGIFN/RBV until HCV–RNA reached undetectable levels, followed by the continuation of treatment for at least 48 weeks (i.e. long-term IFN therapy). In fact, the SVR rate (50%) of the recipients' *IL28B* TG + GG group was higher than that reported by others[8] (SVR rate: 11%). Furthermore, the SVR rate (81%) of the combination of donors' and recipients' *IL28B* (TT : TT) group was higher than that reported by Fukuhara *et al.*[8] (SVR rate: 56%). However, the SVR rate of the donors' *IL28B* TG + GG group (SVR rate: 20%) was similar to that reported by Fukuhara *et al.*[8] (SVR rate: 9%). We believe that the treatment of LT recipients with PEGIFN/RBV until HCV–RNA reaches undetectable levels, followed by the continuation of treatment for at least 48 weeks, is not useful for donors with *IL28B* TG + GG.

In Japan, LDLT is more common than orthotopic LT. In finding a suitable donor, it is better to select a donor with TT of the *IL28B* gene than a TG or GG donor. In conclusion, our results demonstrated the suitability of donors with the TT *IL28B* genotype, and that long-term PEGIFN/RBV therapy seems useful for recipients of LDLT who develop recurrent hepatitis C after transplantation.

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Official Journal of the American Association for the Study of Liver Diseases

Severe Necroinflammatory Reaction Caused by Natural Killer Cell-Mediated Fas/Fas Ligand Interaction and Dendritic Cells in Human Hepatocyte Chimeric Mouse

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The necroinflammatory reaction plays a central role in hepatitis B virus (HBV) elimination. Cluster of differentiation (CD)8-positive cytotoxic T lymphocytes (CTLs) are thought to be a main player in the elimination of infected cells, and a recent report suggests that natural killer (NK) cells also play an important role. Here, we demonstrate the elimination of HBV-infected hepatocytes by NK cells and dendritic cells (DCs) using urokinase-type plasminogen activator/severe combined immunodeficiency mice, in which the livers were highly repopulated with human hepatocytes. After establishing HBV infection, we injected human peripheral blood mononuclear cells (PBMCs) into the mice and analyzed liver pathology and infiltrating human immune cells with flow cytometry. Severe hepatocyte degeneration was observed only in HBV-infected mice transplanted with human PBMCs. We provide the first direct evidence that massive liver cell death can be caused by Fas/Fas ligand (FasL) interaction provided by NK cells activated by DCs. Treatment of mice with anti-Fas antibody completely prevented severe hepatocyte degeneration. Furthermore, severe hepatocyte death can be prevented by depletion of DCs, whereas depletion of CD8-positive CTLs did not disturb the development of massive liver cell apoptosis. ***Conclusion:*** **Our findings provide the first direct evidence that DC-activated NK cells induce massive HBV-infected hepatocyte degeneration through the Fas/FasL system and may indicate new therapeutic implications for acute severe/fulminant hepatitis B.** (HEPATOLOGY 2012;56:555-566)

Between 4% and 32% of fulminant hepatitis cases, characterized by acute massive hepatocyte degeneration and subsequent development of hepatic encephalopathy and liver failure, are caused by acute hepatitis B virus (HBV) infection.[1] Host[2] and viral factors[3] may influence the development of fulminant hepatitis, but these factors have not been fully elucidated.

Innate and adaptive immunity both play a role in the elimination of viral infections. In the innate immune response, cytoplasmic and membrane-bound receptors recognize viruses and induce interferon (IFN)-β production, which, in turn, up-regulates IFN-α and induces an antiviral state in surrounding cells.[4] In the adaptive immune response, viruses are recognized by dendritic cells (DCs), which activate cluster of differentiation (CD)8-positive T cells to reduce viral replication through cytolytic[5] and noncytolytic mechanisms.[6] The role of immune cells, especially HBV-specific cytotoxic T lymphocytes (CTLs), is crucial in the

Abbreviations: APC, allophycocyanin; asialo GM1, ganglio-N-tetraosylceramide; CD, cluster of differentiation; CHB, chronic hepatitis B; CTLs, cytotoxic T lymphocytes; DC, dendritic cell; FasL, Fas ligand; FHB, fulminant hepatitis B; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HLA, human leukocyte antigen; HSA, human serum albumin; IFN, interferon; IP, intraperitoneally; ISG, interferon-stimulated gene; mAb, monoclonal antibody; mDC, myeloid DC; mRNA, messenger RNA; NK, natural killer; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; pDC, plasmacytoid DC; SCID, severe combined immunodeficiency; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; uPA, urokinase-type plasminogen activator.

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Received August 16, 2011; accepted February 4, 2012.

This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor, Health, and Welfare.