Research Article

it was nearly preserved in CPA from three of five patients (Fig. 2B and C). Thus, IRSG induction in CLL could play an essential role in the eradication of the virus in genotype 1 CH-C patients.

Pathway analysis of gene expression in the livers of genotype 1-Rsp, genotype 1-nonRsp and genotype 2

To explore which signaling pathway contributed to the impaired IRSG induction, pathway comparisons between genotype 1-Rsp ($n = 20$) and genotype 1-nonRsp ($n = 23$) before treatment were performed (Table 3). Gene set comparison was analyzed based on the database of BioCarta and KEGG pathways. The Fisher and Kolmogorov–Smirnov tests were performed for statistical evaluation ($p < 0.005$) (BRB-ArrayTools). The mean probe intensities of representative genes in individual pathways are shown in Table 3. In genotype 1-nonRsp, the signaling pathways of IFN-α, apoptosis, and many of the immune pathways, such as those involved in antigen presentation, and the toll-like receptor (TRL) and Jak-STAT signaling pathways, were generally expressed at significantly higher levels before treatment than genotype 1-Rsp (Table 3 and Fig. 3). During treatment, the immune pathways were significantly up-regulated in genotype 1-Rsp, while they were not up-regulated in genotype 1-nonRsp and genotype 2 (Fig. 3, whole liver). When the CLL and CPA were analyzed separately, significant induction of these pathways was observed in CLL of genotype 1-Rsp but not of genotype 1-nonRsp and genotype 2 (Fig. 3, CLL). However, similar induction patterns were observed in CPA among genotype 1-Rsp, genotype1-nonRsp, and genotype 2 patients (Fig. 3, CPA). Thus, these immune pathways should be activated in CLL for the elimination of virus.

We then evaluated the extent of the innate immune response to treatment. The expression of 10 innate immune response genes was strongly induced in CLL from patients of genotype 1-Rsp but not from genotype 1-nonRsp and genotype 2 patients, although these genes were similarly induced in CPA among these patients (Supplementary Table 3 and Fig. 3).

To examine which signaling pathways were differentially induced during treatment, we utilized MetaCore™. MetaCore™ is more feasible for pathway analysis using a relatively low number of cases, and was therefore selected to analyze the LCM samples in this study. The network processes involving genes for which the differential expression was statistically significant ($p < 0.05$) in genotype 1 patients are shown in Fig. 4. Before treatment, many of the immune mediated pathways, such as IFN-α, cell adhesion, IFN-γ, and TCR, were up-regulated in whole liver specimens from genotype 1-nonRsp compared with Rsp. Similar

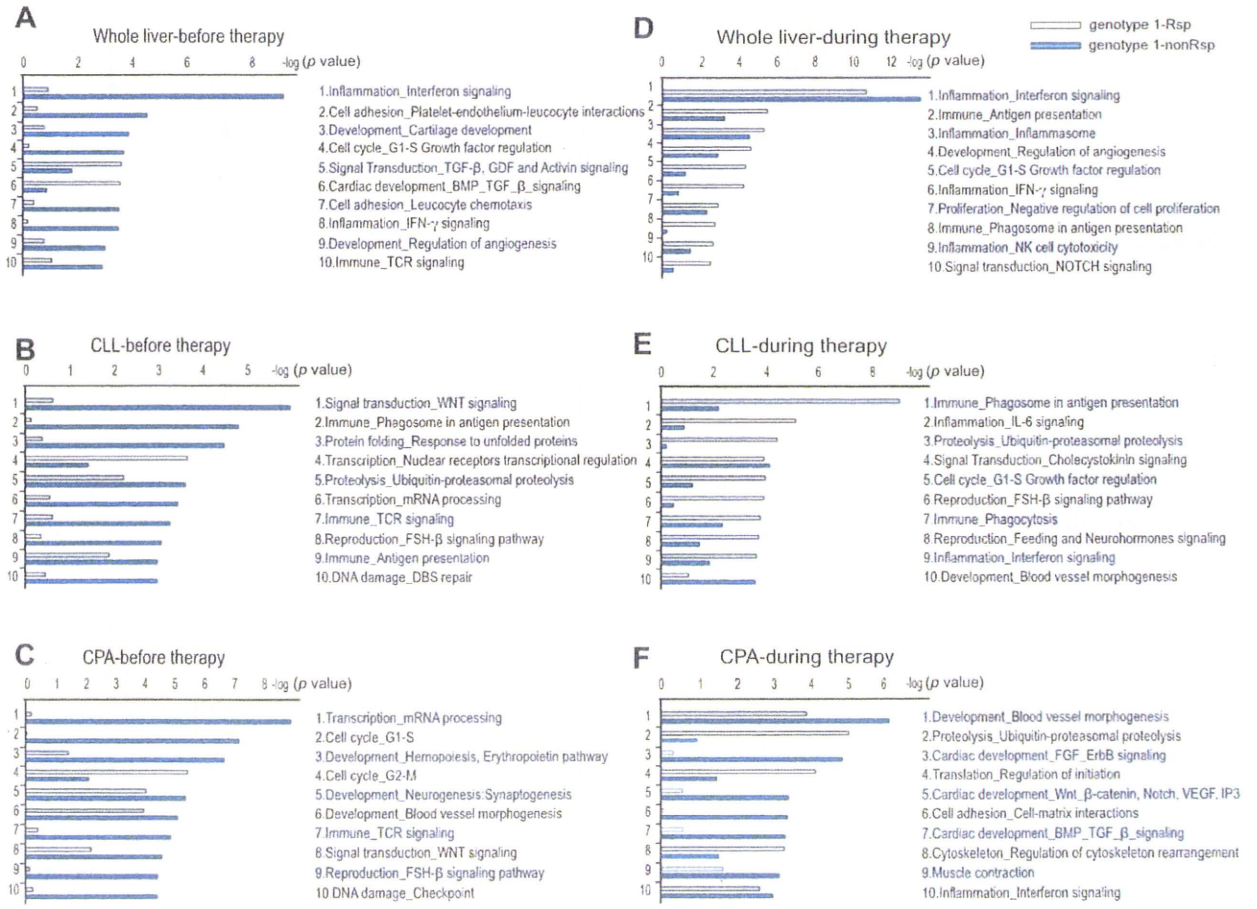

Fig. 4. Functional ontology enrichment analysis of differentially expressed genes ($p < 0.05$) using MetaCore™. GeneGo network process of differentially expressed genes between genotype 1-Rsp (white bar) and genotype 1-nonRsp (blue bar) are listed in order of decreasing statistical significance.

immune-mediated pathways were up-regulated in CLL of genotype 1-nonRsp. In CPA, many of the pathways associated with cell proliferation and DNA damage were up-regulated, reflecting the active inflammatory process in the lymphocytes of genotype 1-nonRsp (Fig. 4A–C). During treatment, many of the immune reactive pathways, such as IFN, NK cell, and antigen presenting, were induced in the whole liver and CLL specimens from genotype 1-Rsp but not in nonRsp (Fig. 4D and E). In contrast, the expression of IFN-inhibitory genes was significantly induced in CLL from nonRsp during treatment (Table 3 and Fig. 4). Interestingly, in CPA, the IFN pathway was induced in genotype 1-Rsp and nonRsp to the same degree; however, signaling pathways related to angiogenesis and fibrogenesis, such as FGF, Wnt, TGF-beta, Nocth, and VEGF signaling, were induced more in CPA from genotype 1-nonRsp than from Rsp (Figs. 3 and 4F). Thus, differential expression of signaling pathways could be observed in CLL and CPA obtained from genotype 1-Rsp and nonRsp.

Discussion

IFN and Rib combination therapy has become a commonly used modality for treating patients with CH-C, although the precise mechanism of treatment resistance is unclear. With the development of methods to quantitatively assess viral kinetics during treatment, studies were able to demonstrate that patients who cleared HCV in the early period showed favorable outcomes, whereas patients who needed a longer time to clear HCV experienced poor outcomes [4,7,17]. Thus, early clearance of virus after initiation of treatment is one of the important determinants for the complete eradication of HCV.

In this study, we analyzed gene expression from liver biopsy samples obtained before and at 1 week after initiation of treatment to investigate the precise mechanisms involved in treatment and treatment resistance. Although global gene expression profiles in the liver and PBMC during IFN treatment in a chimpanzee have been reported [12,13], the relationship between the expression profiles and clinical outcome could not be evaluated.

During the preparation of this study, two reports using a similar approach have been published [6,20]. For example, Feld et al. [6] analyzed gene expression in the livers of CH-C patients on treatment. The authors, however, compared gene expression among different patients at initiation (n = 19; 5 rapid responders, 10 slow responders, 4 naive) and during treatment (n = 11; 6 rapid responders, 5 slow responders). Because patients were not serially biopsied before and during the treatment, true treatment-related gene induction could not be evaluated. Moreover, half of the on-treatment group was administered Rib alone for three days prior to liver biopsy. In the other report, Sarasin-Filipowicz et al. [20] extensively analyzed serial liver biopsy specimens under the treatment; however, the number of the patients enrolled in their study was relatively low and heterogeneous with respect to the infected genotypes. Our study has extended their findings and provides further insights into the mechanism of IFN resistance by analyzing gene expression in CLL and CPA separately for the first time. The analysis of genotype 2 HCV also enabled us to understand the importance of the differing sensitivities to IFN between strains.

By comparing gene expression in serial liver biopsy specimens obtained at initiation and during treatment, IFN- and Rib-stimulated genes (IRSGs) in the livers of patients with CH-C could be identified (Supplementary Table 1). Our study clearly demonstrated that IRSG induction correlated with the elimination of HCV in patients with genotype 1 in accordance with previous results [6,20]. The patients who did not show a response to treatment had poor induction of IRSGs (Fig. 1A). In contrast, IRSG expression before treatment showed an opposite pattern of expression. IRSGs were induced in genotype 1-nonRsp rather than in genotype 1-Rsp. This finding was first described by Chen et al. [3] and confirmed by others [1,6,20]. Asselah et al. [1] extensively analyzed 58 curated ISGs published previously by RTD-PCR and found that three genes (IFI27, CXCL9 and IFI-6–16) were predictive of treatment outcome. However, only 12 of their 58 curated genes were also included in the 100 most up-regulated genes we observed during treatment (Supplementary Table 1). Therefore, more valuable genes for the prediction of treatment outcome might exist and our gene list could be useful for further selection of predictors of treatment outcome.

We showed that different levels of IRSG induction before treatment was associated with up-regulation of different signaling pathways, such as apoptosis and inflammatory pathways, in genotype 1-nonRsp, although histological assessment of activities and stages could not differentiate the two groups of patients. During treatment, these pathways, including the innate immune response for IFN production, were significantly induced in genotype 1-Rsp but not in genotype 1-nonRsp. The results suggest that previous up-regulation of IRSGs might be linked to impaired induction of IRSGs and contribute to poor treatment response in patients with genotype 1. Interestingly, an impaired IRSG induction was mainly noticeable in CLL, but not in CPA, and the results were confirmed by RTD-PCR (data not shown). These results suggest that IRSG induction in HCV-infected hepatocytes could play an essential role in the eradication of the genotype 1 virus in CH-C patients.

However, these scenarios did not apply in patients with genotype 2 HCV in this study. Despite the presence of active inflammation before treatment and unsatisfactory IRSG induction during treatment, these patients showed rapid responses to treatment and favorable treatment outcomes. It could be speculated that genotype 2 HCV is far more sensitive to IFN than genotype 1 HCV, and small IRSG induction might be enough to eradicate the virus. Further studies are needed to confirm these results.

We precisely analyzed the expression profiles in CLL and CPA which were obtained using the LCM method. Although IRSGs and other immune regulatory genes were similarly induced in the CPA of genotype 1-Rsp and nonRsp, more of the angiogenic- and fibrogenic-related genes were induced in CPA of genotype 1-nonRsp (Fig. 4C and F). Therefore, growth factors released from CPA might be involved in poor IRSG induction in CLL of genotype 1-nonRsp.

In summary, by comparing the hepatic gene expression in CH-C patients with different treatment outcomes, we identified a gene expression signature characteristic of IFN resistance. Our study is very important for two reasons: first, it will help in the development of new therapeutic strategies, and second, we have identified many of the genes found to be up-regulated between genotype 1-Rsp and nonRsp, which encode molecules secreted

in serum (cytokines). Therefore, the study represents a logical functional approach for the development of serum markers as predictors of response to treatment [2]. The precise mechanisms underlying these findings should be clarified further in future studies.

Conflict of interest

The authors who have taken part in this study do not have a relationship with the manufactureres of the drugs involved either in the past or present and did not recieve fundig from the manufactureres to carry out their research. The authors received support from the Japanese Society of Gastroenterology and Ministry of Helath, Labour and Welfare.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.04.036.

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Differential gene expression profiling in blood from patients with digestive system cancers

Masao Honda [a,b], Yoshio Sakai [a], Taro Yamashita [a], Tatsuya Yamashita [a], Akito Sakai [a], Eishiro Mizukoshi [a], Yasunari Nakamoto [a], Isamu Tatsumi [a], Yoshitaka Miyazaki [a], Hiroshi Tanno [a], Shuichi Kaneko [a,*], Hokuriku Liver Study Group [1]

[a] Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Japan
[b] Department of Advanced Medical Technology, Graduate School of Health Medicine, Kanazawa University, Kanazawa, Japan

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ABSTRACT

To develop a non-invasive and sensitive diagnostic test for cancer using peripheral blood, we evaluated gene expression profiling of blood obtained from patients with cancer of the digestive system and normal subjects. The expression profiles of blood-derived total RNA obtained from 39 cancer patients (11 colon cancer, 14 gastric cancer, and 14 pancreatic cancer) was clearly different from those obtained from 15 normal subjects. By comparing the gene expression profiles of cancer patients and normal subjects, 25 cancer-differentiating genes ($p < 5.0 \times 10^{-6}$ and fold differences >3) were identified and an "expression index" deduced from the expression values of these genes differentiated the validation cohort (11 colon cancer, 8 gastric cancer, 18 pancreatic cancer, and 15 normal subjects) into cancer patients and normal subjects with 100% (37/37) and 87% (13/15) accuracy, respectively. Although, the expression profiles were not clearly different between the cancer patients, some characteristic genes were identified according to the stage and species of the cancer. Interestingly, many immune-related genes such as antigen presenting, cell cycle accelerating, and apoptosis- and stress-inducing genes were up-regulated in cancer patients, reflecting the active turnover of immune regulatory cells in cancer patients. These results showed the potential relevance of peripheral blood gene expression profiling for the development of new diagnostic examination tools for cancer patients.

1. Introduction

Cancer of the digestive system is one of the most common forms of cancer worldwide. The early detection of cancer enables the administration of therapy and the subsequent prolongation of overall survival; however, the detection of early-stage cancer is difficult, and patients with general symptoms are likely to have advanced-stage cancer. Particularly, in pancreatic cancer [1,2], early diagnosis is extremely difficult despite the development of modern imaging technology such as ultrasonography or computed tomography. Even though the recent development of chemotherapy combined with molecular target drugs has improved the survival rate of patients with advanced cancer, the therapeutic benefit of this treatment is limited [1].

Peripheral blood in patients includes a variety of immune regulatory cells such as leukocytes and lymphocytes that are essential players in the host immune defense system. These cells respond to various abnormal conditions such as viral infection, metabolic disease, and cancer [3–12]. We previously reported that the expression profiles of peripheral blood mononuclear cells (PBMCs) from patients with hepatocellular carcinoma (HCC) differed significantly from those of patients without HCC ($p < 0.0005$) [8]. The results also suggest that the gene expression profile of blood may be useful as a clinical surrogate biomarker for HCC assessment.

In this study, we extended our previous findings to the diagnosis of cancer of the digestive system, including gastric cancer, colorectal cancer, and pancreatic cancer. We identified clear differences in the gene expression profiles of cancer patients and normal subjects, suggesting the potential diagnostic relevance of gene expression signatures from blood samples for cancer of the digestive system.

Abbreviations: AUC, area under the curve; BMI, body mass index; CA 19-9, carbohydrate antigen (CA) 19-9; CEA, carcinoembryonic antigen; HCC, hepatocellular carcinoma; HSC, hematopoietic stem cell; IFN, interferon; NPV, negative predictive value; PBMC, peripheral blood mononuclear cell; PPV, positive predictive value; ROC, receiver operating characteristic; SVM, support vector machine.

* Corresponding author. Address: Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Takara-Machi 13-1, Kanazawa 920-8641, Japan. Fax: +81 76 234 4250.
E-mail address: skaneko@m-kanazawa.jp (S. Kaneko).

[1] Participating investigators are listed in Appendix A.

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2. Material and methods

2.1. Patients and blood samples

We enrolled 76 patients with cancer of the digestive system, including 22 patients with colon cancer, 22 patients with gastric cancer, and 32 patients with pancreatic cancer at the Graduate School of Medicine, Kanazawa University Hospital and its related hospitals, Japan from 2008 to 2009 (Table 1). Blood samples were obtained from patients following their diagnosis with cancer of the digestive system. The age- and sex-matched control samples were obtained from 30 healthy volunteers who received health screening examinations (Table 1). Informed consent was obtained from all patients, and ethics approval for this study was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science. The cancer patients and normal subjects were randomly divided into the training ($n = 54$) and validation ($n = 52$) cohorts according to their entry number. There were no significant differences in age, sex, body mass index (BMI), and habits between the cancer patients and normal subjects (Table 1).

2.2. RNA extraction from blood

Blood samples collected in PAXgene Blood RNA tubes (BD, NJ, USA) were incubated and stored according to the manufacturer's instructions. Total RNA was isolated after thawing the samples at room temperature using the PAXgene Blood RNA System kit (Qiagen, CA, USA) following the manufacturer's instructions. The quality of purified RNA was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA).

2.3. Microarray and data analysis

Cy-3-labeled cRNA was synthesized from 300 ng of total RNA using the Quick Amp Labeling kit, One-Color (Agilent Technologies, CA, USA) and purified using an RNeasy column (Qiagen). After checking the quality of the RNA using an Agilent 2100 Bioanalyzer, the RNA was hybridized to 4 × 44 K Whole Human Genome Microarray (Agilent Technologies, CA, USA). The microarray slide was incubated in a hybridization oven at 65 °C for 17 h, washed, and then scanned using a DNA Microarray Scanner, Model G2505B (Agilent Technologies, CA, USA). All procedures from the labeling to the scanning were performed according to the manufacturer's instructions (Agilent Technologies, CA, USA). The scanned data of each slide were extracted using Feature Extraction software (Agilent Technologies).

Gene expression analysis was carried out using GeneSpring GX software (Agilent Technologies). Each measurement was divided by the 75th percentile of all measurements in that sample at per chip normalization. Hierarchical clustering was generated using the Pearson correlation similarity metric and the average or complete linkage clustering algorithm. Welch's t-test with Benjamini and Hochberg's false discovery rate were used to identify the genes that were differentially expressed in the patients of each category.

2.4. Class prediction analysis and calculation of the expression index

Building and running prediction models were performed using GeneSpring GX software (Agilent Technologies). Models were generated for the statistically extracted genes from the training cohort using a support vector machine (SVM) algorithm.

In addition to the supervised learning methods, we calculated an "expression index" that was used for class prediction analysis. Logistic regression analysis to predict cancer patients and normal subjects was performed using the individual gene expression values. The gene expression cut-off values were determined using a receiver operating characteristic (ROC) curve. If the expression value of a gene exceeded the cut-off value, the index was scored as "1," and if the expression value of a gene was not beyond the cut-off value, then the index was scored as "0." The total index was calculated and designated as the "expression index." The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the expression index for the prediction of cancer patients and normal subjects were further evaluated using the training and variation cohorts.

2.5. Pathway analysis of the expression data

The pathway analysis of the differentially expressed genes was performed using the MetaCore software suite (GeneGo, MI, USA), a unique, curated database of human protein–protein and protein–DNA interactions, transcription factors, and signaling, metabolic, and bioactive molecules. Differentially expressed genes were analyzed by GeneGo annotation, and categories of differentially expressed genes are shown by their frequency; moreover, possible networks of differentially expressed genes were created according to the direct interaction relation program of MetaCore.

2.6. Statistical analysis

The Mann–Whitney U-test was used to analyze continuous variables. Chi-squared and Fisher's exact tests were used to analyze categorical data. Multivariate logistic analysis was performed using a stepwise logistic regression model. A p-value of less than 0.05 was considered significant. Statistical analyses were performed using JMP8 for Windows (SAS Institute, NC, USA).

3. Results

3.1. Clinical characteristic of patients enrolled in this study

The clinical characteristics of the patients enrolled in this study are shown in Table 1. The training cohort included 39 patients with cancer of the digestive system (11 colon cancer, 14 gastric cancer, and 14 pancreatic cancer) and 15 normal subjects. The validation cohort included 37 patients with cancer of the digestive system (11 colon cancer, 8 gastric cancer, and 18 pancreatic cancer) and 15 normal subjects. There were no statistical differences in age, gender, habits, BMI (kg/m^2), and blood cell count between the cancer patients and the normal subjects in the training and validation cohorts. The majority of the advanced-stage cancer was observed in the pancreatic cancer patients in both cohorts. The serum levels of CA 19-9 were significantly higher in patients with pancreatic cancer than in those with gastric or colon cancers in the validation cohort (Table 2).

3.2. Hierarchical clustering analysis

The results from the unsupervised hierarchical cluster analysis of the training cohort using an average linkage clustering algorithm based on the 23,278 expressed genes are shown in Fig. 1A. Interestingly, the expression profiles in the blood obtained from cancer patients and normal subjects were clearly different, except in one normal subject. There was no clear clustering within the cancer patients; however, patients with pancreatic cancer or advanced-stage cancer associated with distant metastasis or vascular

Table 1
Clinical characteristics of patients.

Clinical category	Training cohort ($n = 54$)					Validation cohort ($n = 52$)				
	Patients with digestive cancer			Normal	p-value	Patients with digestive cancer			Normal	p-value
	Colon ($n = 11$)	Gastric ($n = 14$)	Pancreatic ($n = 14$)	($n = 15$)		Colon ($n = 11$)	Gastric ($n = 8$)	Pancreatic ($n = 18$)	($n = 15$)	
Age	68.8 ± 8.3	66.7 ± 12.7	68.2 ± 8.2	62.4 ± 4.8	N.S.	70.1 ± 9.3	68.9 ± 7.3	66.7 ± 13.8	62.2 ± 5.9	N.S.
Gender										
Male	10	7	9	7	N.S.	9	4	10	6	N.S.
Female	1	6	5	8		2	4	8	9	
BMI (>25 m^2/kg)	19.9 ± 3.2	22.2 ± 3.3	19.5 ± 3.1	22.6 ± 2.2	N.S.	22.5 ± 5.0	24.0 ± 2.4	22.0 ± 4.1	22.5 ± 2.4	N.S.
Clinical stage										
0 or I	3	6	0	–	C vs. P: 0.002	4	7	0	–	C vs. G: 0.009
II	2	0	0	–	G vs. P: 0.002	2	1	3	–	C vs. P: 0.03
III	3	2	1	–	(0–II vs. III–IV)	4	0	3	–	G vs. P: 0.001
IV	3	6	13	–		1	0	12	–	(0–II vs. III–IV)
Laboratory data										
WBC ($\times 10^3$)	6.62 ± 2.2	6.72 ± 2.6	6.77 ± 2.5	5.95 ± 1.9	N.S.	6.05 ± 1.7	6.60 ± 1.3	5.64 ± 1.9	5.85 ± 3.0	N.S.
RBC ($\times 10^6$)	393 ± 54	414 ± 50	417 ± 70	441 ± 37	N.S.	415 ± 76	411 ± 65	417 ± 69	451 ± 120	N.S.
Hb (g/dL)	11.1 ± 2.8	12.5 ± 2.8	12.9 ± 2.0	13.5 ± 1.4	N.S.	12.3 ± 3.2	12.1 ± 3.5	12.6 ± 2.3	13.1 ± 0.7	N.S.
Tumor marker										
CEA (>5 ng/mL)										
Mean ± SD	442 ± 1433	120 ± 450	98 ± 273	2 ± 0.8	N.S.	47 ± 124	10 ± 23	9 ± 15	2 ± 0.8	N.S.
CA 19-9 (>37 U/mL)										
Mean ± SD	6011 ± 1988	1169 ± 4263	86,867 ± 257,340	2 ± 1.6	N.S.	47 ± 96	21 ± 30	1714 ± 2473	2.2 ± 1.6	P vs. N: 0.009 P vs. C: 0.02 P vs. G: 0.04
Habits										
Alcohol	0	1	1	0	N.S.	1	0	0	0	N.S.
Smoking	0	0	0	0	N.S.	0	1	2	0	N.S.

Alcohol: history of alcohol intake more than 60 g/day; Smoking: history of smoking more than 400 Brinkman index. Data are expressed as mean ± SD. C: colon cancer; G: gastric cancer; P: pancreatic cancer; N.S.: not significant.

Table 2
Class prediction analysis by supervised learning method based on the support vector machine (SVM).

Clinical category	Subgroup	Total no. of classes	No. of cases misclassified	Mean percent of correct classification	No. of differentially expressed genes	
					$p < 0.05$, fold > 2	$p < 5.0 \times 10^{-6}$, fold > 3
Normal vs. cancer	Normal	15	2 (1)*	87 (93)*	1348	25
	Cancer	39	1 (0)*	97 (100)*		
Age	⩾65	30	–	–	0	0
	<65	24	–	–		
Stage	0–II	11	2	82	45	0
	III–IV	28	3	89		
Colon + gastric vs. pancreatic	Gastric + colon	25	2	92	44	0
	Pancreatic	14	4	71		
Colon vs. gastric	Gastric	14	–	–	0	0
	Colon	11	–	–		

( )*: no. of cases misclassified and mean percent of correct classification using 25 genes ($p < 5.0 \times 10^{-6}$, fold > 3).

invasion were likely to be clustered together (Fig. 1A). We performed class prediction analysis using a supervised learning method based on the SVM algorithm to confirm these findings. Using the statistical values ($p < 0.05$) and fold differences (>2) as filtering criteria, 1348 genes were identified that differentiated cancer patients from normal subjects (cancer-differentiating genes) (Table 2). Similarly, 45 genes were identified that differentiated patients with advanced-stage cancer (stages III–IV) from early-stage cancer (stages 0–II) (stage-differentiating genes) (Supplementary Table 2), and 44 genes were identified that differentiated patients with gastric or colon cancers from those with pancreatic cancer (GI tract/pancreas-differentiating genes) (Table 2) (Supplementary Table 3). No significant differences were identified in gene expression between the patients with different ages (⩾65 yr and <65 yr), and between patients with gastric or colon cancers (Table 2). We observed a high prediction capacity for the cancer-differentiating genes (87–97% accuracy), while the predictive value of stage- and GI tract/pancreas-differentiating genes was not sufficient (71–89% accuracy) (Table 2). Hierarchical clustering using more strict selection criteria ($p < 5.0 \times 10^{-6}$ and fold differences >3) identified 25 cancer-differentiating genes (Fig. 1B), confirming the clear differentiation of cancer patients and normal subjects. Hierarchical clustering using 45 stage- and 44 GI tract/pancreas-differentiating genes is shown in Fig. 1C. Within the cancer patients, gastric or colon cancer was differentiated from pancreatic cancer, and advanced-stage cancer associated with metastasis or vascular invasion was roughly differentiated from early-stage cancer (Fig. 1C).

3.3. Calculation of the expression index

To apply these findings to clinical and practical settings, we calculated the expression index in individual cases. Logistic regression analysis of cancer patients and normal subjects was performed using the individual expression values of the 25 cancer-differentiating genes. The cut-off value of gene expression was determined from the ROC curve. The individual distribution of the expression values of the 25 genes in the training cohort patients is shown in Supplementary Fig. 1. Eleven genes were up-regulated in cancer patients, while 14 genes were down-regulated. We standardized each expression value using the following approach: if the expression value exceeded the cut-off value, the expression value was counted "1," and if the expression value was less than the cut-off value, the expression value was counted as "0." The hierarchical clustering of the training cohort patients using the standardized expression values is shown in Fig. 2; we observed clearer clustering of the cancer patients and normal subjects for these values. For statistical evaluation, a total expression score was calculated and designated as the "expression index," where high expression index values could indicate patients with cancer. The cut-off value of the expression index was determined by an ROC curve, and the sensitivity, specificity, PPV, and NPV of the expression index are shown in Table 3. The distribution of the expression index in patients is shown in Fig. 3A. The results demonstrated the high sensitivity, specificity, PPV, and NPV of the expression index for predicting cancer patients and normal subjects in the training and validation cohorts. The predictive values of the 44 stage- and 45 GI tract/pancreas-differentiating genes in the training cohort were fair (70–100%); however, they were not sufficient in the validation cohort (59–84%) (Table 3 and Fig. 3B, C).

3.4. Pathway analysis

To examine which signaling pathways were differentially expressed in blood from cancer patients, we performed pathway analysis of the 841 differentially expressed genes ($p < 5.0 \times 10^{-5}$ and fold differences >1.7) using MetaCore software (GeneGo). Interestingly, many of the immune-related genes, such as antigen presenting, cell cycle accelerating, and apoptosis- and stress-inducing genes, were up-regulated in cancer patients, while development-related genes, such as tissue remodeling and hedgehog signaling, were down-regulated (Supplementary Fig. 2). We generated the possible network processes of the differentially expressed genes according to the direct interaction algorithm (Supplementary Fig. 3). Interestingly, many p53 target genes were up-regulated in association with the induction of caspase-3, suggesting the presence of cell cycle regulation and the induction of apoptosis. Interestingly, stem cell-related and differentiation genes such as Oct-3/4 and Oct-1 were down-regulated, suggesting the impaired differentiation of immune regulatory cells. Therefore, the expression profile may reflect the active immune reaction and the decreased pluripotency or repertoire of immune regulatory cells in cancer patients.

With regard to the stage-differentiating genes, it is interesting to note that a larger number of interferon-stimulated genes were up-regulated in advanced-stage cancer than in early-stage cancer (Supplementary Table 2). With regard to the GI tract/pancreas-differentiating genes, a larger number of G-protein-related genes were up-regulated in pancreatic cancer patients (Supplementary Table 3). These differences may reflect the possible interaction between tumor cells and tumor-infiltrating lymphocytes.

4. Discussion

Detection of cancer of the digestive system using peripheral blood is an attractive diagnostic method because of its simplicity

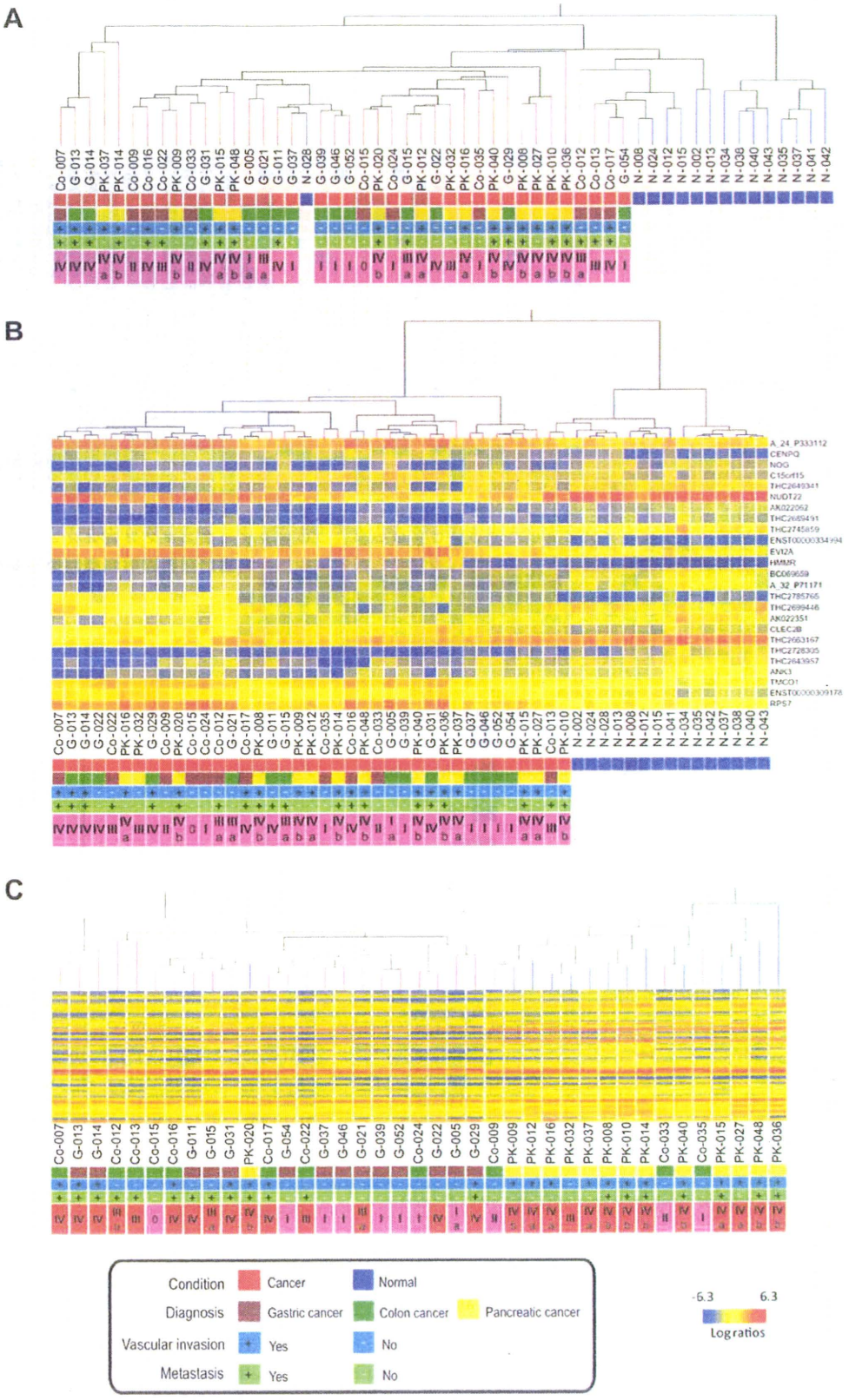

Fig. 1. (A) Hierarchical clustering analysis of 54 training cohort samples based on the expression levels of 23,278 genes. (B) Hierarchical clustering analysis of 54 training cohort samples based on the expression levels of 25 cancer-differentiating genes ($p < 5.0 \times 10^{-6}$ and fold differences >3). (C) Hierarchical clustering analysis of 54 training cohort samples based on the expression levels of 45 stage- and 44 GI tract/pancreas-differentiating genes ($p < 0.05$ and fold differences >2).

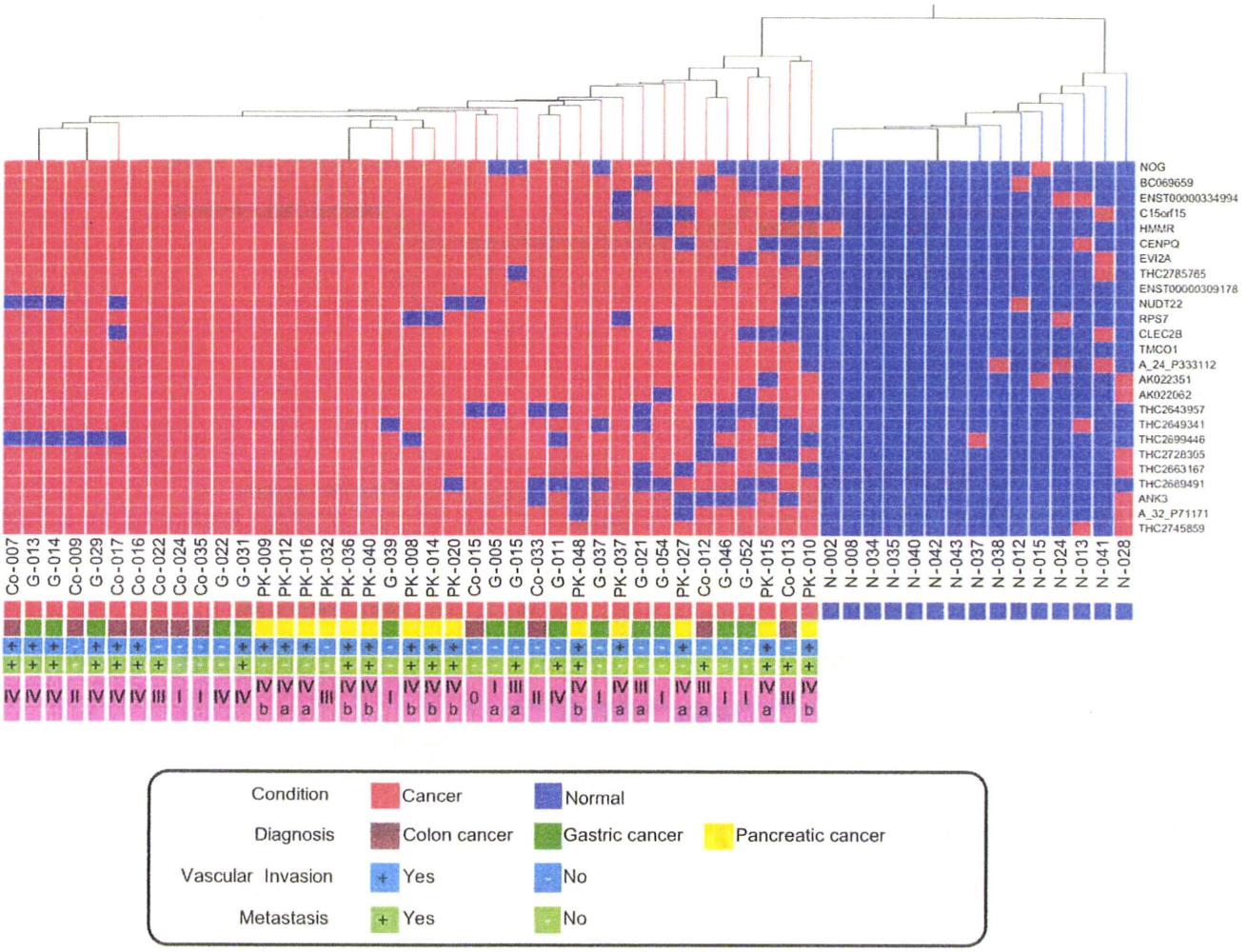

Fig. 2. Hierarchical clustering analysis of 54 training cohort samples based on the standardized expression level (0 or 1).

Table 3
Sensitivity, specificity, PPV, and NPV of the expression index.

Prediction category	No. of genes	Expression index cut-off	Training//validation	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
Cancer (vs. normal)	25	14	Training	100 (39/39)	100 (15/15)	100 (39/39)	100 (15/15)	1
			Validation	100 (37/37)	87 (13/15)	95 (37/39)	100 (13/13)	0.99
Stages III–IV (vs. 0–II)	45	18	Training	96 (27/28)	82 (9/11)	93 (27/29)	90 (9/10)	0.94
			Validation	80 (16/20)	59 (10/17)	70 (16/23)	71 (10/14)	0.69
Colon + gastric (vs. pancreatic)	44	28	Training	76 (19/25)	100 (14/14)	100 (19/19)	70 (14/20)	0.95
			Validation	84 (16/19)	65 (11/17)	73 (16/22)	79 (11/14)	0.78

PPV: positive predictive value; NPV: negative predictive value; AUC: area under the curve.

and non-invasive nature. For the detection of early-stage cancer of the digestive system, endoscopic examinations of the stomach and colon or imaging studies, such as abdominal ultrasonography or computed tomography, should be performed periodically; however, these examinations are expensive and the patients suffer from high levels of stress during these examination. Although, serological tumor markers such as CEA and CA 19-9 have been utilized for the diagnosis of cancer of the digestive system, these tumor markers have a low sensitivity and specificity [13,14].

Peripheral blood in patients includes a variety of immune regulatory cells that respond to various abnormal conditions such as viral infection, metabolic disease, and cancer. Recent emerging reports including ours [5,6,8] support the possibility that the gene expression profiling of peripheral blood could be a useful surrogate biomarker [3,4,7,9–12].

In this study, we evaluated gene expression profiling of blood obtained from patients with cancer of various digestive system including gastric cancer, colon cancer and pancreatic cancer that have not been characterized systematically. To our knowledge, this is the first report to find a common gene set for the diagnosis of cancer with the digestive system. The identified gene set could be useful for the screening of patients with cancer of the digestive system. The gene expression profiles of peripheral blood from cancer patients were clearly different from those in normal subjects

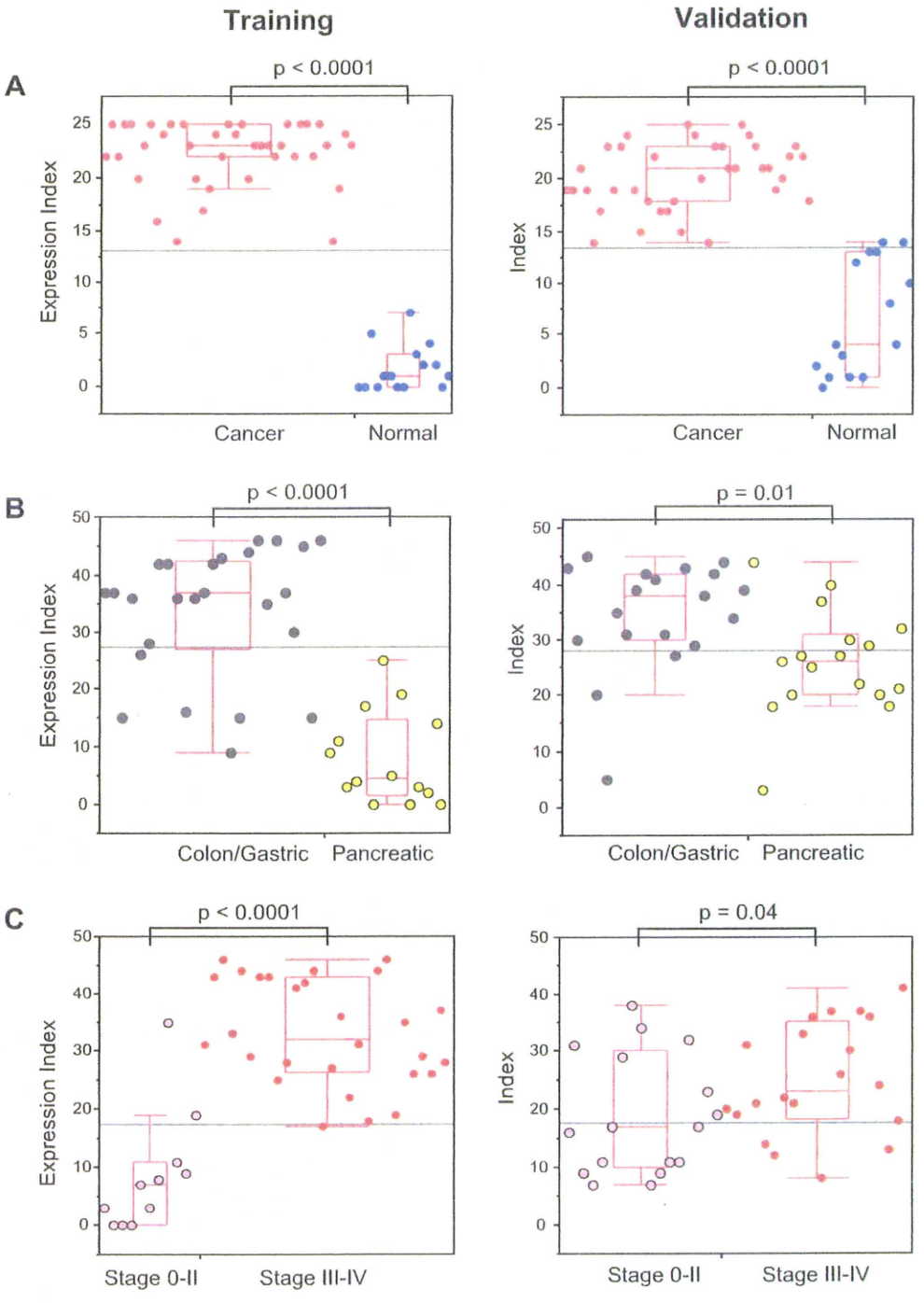

Fig. 3. (A) Calculated expression index of 25 cancer-differentiating genes in the training and validation cohorts. (B) Calculated expression index of 44 GI tract/pancreas-differentiating genes in the training and validation cohorts. (C) Calculated expression index of 45 stage-differentiating genes in the training and validation cohorts.

(Fig. 1A). We identified 1348 cancer-differentiating genes using the filtering criteria of $p < 0.05$ and fold differences >2, and 25 more strictly selected genes using the filtering criteria of $p < 5.0 \times 10^{-6}$ and fold differences >3 in the training cohort. Hierarchical clustering using the unsupervised learning method clearly differentiated cancer patients and normal subjects in the validation cohort using the 25 selected genes (data not shown). The supervised learning method based on the SVM using the 25 cancer-differentiating genes predicted the cancer patients in the training cohort with an accuracy of 93–100% (Table 2) and 77–100% in the validation cohort (data not shown). Thus, unsupervised and supervised learning methods successfully identified the cancer patients in the training and validation cohorts with a high accuracy. Importantly, there were no obvious differences in the clinical backgrounds of the cancer patients and normal subjects in the training and validation cohorts, except for the serum CA 19-9 levels in the pancreatic cancer patients of the validation cohort (Table 1).

For more practical and clinical usage, we calculated the conventional "expression index" and utilized it for the prediction of cancer patients. The expression index was based on the individual expres-

sion values (see Sections 2 and 3) and the cut-off value was determined by the ROC curve generated from the logistic regression analysis. The sensitivity, specificity, PPV (%), and NPV (%) of the expression index for the 25 cancer-differentiating genes were well tolerated for the prediction of cancer patients and normal subjects in the training and validation cohorts (Table 3 and Fig. 3). Multivariate analysis using the expression index, CA 19-9, CEA, age, and sex in the validation cohort indicated that the expression index was the only independent variable associated with cancer patients ($p < 0.001$, odds = 3.0×10^5/score). Thus, the expression index is practically useful for the identification of cancer patients with digestive system.

By using the same strategy, we identified 45 stage-differentiating genes (Supplementary Table 2) and 44 GI tract/pancreas-differentiating genes (Supplementary Table 3). Although, the predictive performance of these genes was less efficient, the results suggest that the expression profiles may be different according to the stage and species of the cancer.

What causes these differences in the expression profiles of blood from cancer patients? Previously, we examined the gene expression profiles of PBMCs obtained from patients with or without HCC and showed that the expression profiles of PBMC from patients with HCC differed significantly from those of patients without HCC [8]. Interestingly, the gene expression profiles of the redox status, cell cycle, and proteasome system, along with immunologic genes were up-regulated in PBMCs from patients with HCC, suggesting the regulation of anticancer immunity. Importantly, these genes were also up-regulated in HCC-infiltrating mononuclear inflammatory cells, implying that local anticancer immunity may be reflected in the peripheral gene expression signature. In this study, it was also found that many immune-related genes, such as antigen presenting, cell cycle accelerating, and apoptosis- and stress-inducing genes, were up-regulated in cancer patients, reflecting the presence of an active immune reaction in cancer patients. Interestingly, the expression of many differentiation-related genes such as Oct-3/4 and Oct-1 was down-regulated, suggesting that the differentiation of immune cells was impaired. These may represent a characteristic immune feature of cancer and reflect the impaired immune system of cancer patients. Although, we did not analyze regional tumor-infiltrating mononuclear inflammatory cells in this study, a similar reaction may occur in the local tumor lesion.

In addition to the cancer-differentiating genes, there could be characteristic genes that reflect the stage and species of the cancer. It is interesting to note that more interferon-stimulated genes were up-regulated in advanced-stage cancers. A recent study reported that interferon (INF)-α activated dormant hematopoietic stem cells (HSCs) and sensitize these cells to 5-fluoro-uracil exposure. In contrast, HSCs chronically activated by INF-α are functionally compromised. Therefore, the up-regulation of IFN signaling in advanced-stage cancer reflects the refractory state of the differentiation of immune regulatory cells. Although, the specificity of these genes was not sufficient, the detailed diagnosis of cancer of the digestive system may be possible by generating a decision tree (Supplementary Fig. 4).

In conclusion, we demonstrated a distinct gene expression profile of blood from cancer patients of the digestive system compared to healthy individuals, and showed the potential diagnostic values of these differences for clinical usage. Further studies should be performed to validate these findings in detail and identify the fundamental mechanisms underlying this phenomenon.

Conflict of interest

None.

Appendix A

The Hokuriku Liver Study Group (HLSG) is composed of the following members: Drs. Takashi Kagaya, Kuniaki Arai, Kaheita Kakinoki, Kazunori Kawaguchi, Kazuya Kitamura, Hajime Takatori, Hajime Sunakosaka (Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa); Drs. Touru Nakahama, Shinji Kamiyamamoto, (Kurobe City Hospital, Kurobe, Toyama); Dr. Yasuhiro Takemori (Toyama Rosai Hospital, Uozu, Toyama); Dr. Hikaru Oguri (Koseiren Namerikawa Hospital, Namerikawa, Toyama); Drs. Yatsugi Noda, Hidero Ogino (Toyama Prefectural Central Hospital, Toyama, Toyama); Drs. Yoshinobu Hinoue, Keiji Minouchi (Toyama City Hospital, Toyama, Toyama); Dr. Nobuyuki Hirai (Koseiren Takaoka Hospital, Takaoka, Toyama); Drs. Tatsuho Sugimoto, Koji Adachi (Tonami General Hospital, Tonami, Toyama); Dr. Yuichi Nakamura (Noto General Hospital, Nanao, Ishikawa); Drs. Masashi Unoura, Ryuhei Nishino (Public Hakui Hospital, Hakui, Ishikawa); Drs. Hideo Morimoto, Hajime Ohta (National Hospital Organization Kanazawa Medical Center, Kanazawa, Ishikawa); Dr. Hirokazu Tsuji (Kanazawa Municipal Hospital, Kanazawa, Ishikawa); Drs. Akira Iwata, Shuichi Terasaki (Kanazawa Red Cross Hospital, Kanazawa, Ishikawa); Drs. Tokio Wakabayashi, Yukihiro Shirota (Saiseikai Kanazawa Hospital, Kanazawa, Ishikawa); Drs. Takeshi Urabe, Hiroshi Kawai (Public Central Hospital of Matto Ishikawa, Hakusan, Ishikawa); Dr. Yasutsugu Mizuno (Nomi Municipal Hospital, Nomi, Ishikawa); Dr. Shoni Kameda (Komatsu Municipal Hospital, Komatsu, Kanazawa); Drs. Hirotoshi Miyamori, Uichiro Fuchizaki (Keiju Medical Center, Nanao, Ishikawa); Dr. Haruhiko Shyugo (Kanazawa Arimatsu Hospital, Kanazawa, Ishikawa); Dr. Hideki Osaka (Yawata Medical Center, Komatsu, Ishikawa); Dr. Eiki Matsushita (Kahoku Central Hospital, Tsubata, Ishikawa); Dr. Yasuhiro Katou (Katou Hospital, Komatsu, Ishikawa); Drs. Nobuyoshi Tanaka, Kazuo Notsumata (Fukuiken Saiseikai Hospital, Fukui, Fukui); Dr. Mikio Kumagai (Kumagai Clinic, Tsuruga, Fukui); Dr. Manabu Yoneshima (Municipal Tsuruga Hospital, Tsuruga, Fukui).

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.123.

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Hepatic ISG Expression Is Associated With Genetic Variation in Interleukin 28B and the Outcome of IFN Therapy for Chronic Hepatitis C

MASAO HONDA,*,‡ AKITO SAKAI,* TATSUYA YAMASHITA,* YASUNARI NAKAMOTO,* EISHIRO MIZUKOSHI,* YOSHIO SAKAI,* TARO YAMASHITA,* MIKIKO NAKAMURA,* TAKAYOSHI SHIRASAKI,‡ KATSUHISA HORIMOTO,§ YASUHITO TANAKA,‖ KATSUSHI TOKUNAGA,¶ MASASHI MIZOKAMI,# SHUICHI KANEKO,* and the Hokuriku Liver Study Group

*Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa; ‡Department of Advanced Medical Technology, Kanazawa University Graduate School of Health Medicine, Kanazawa; §Biological Network Team, Computational Biology Research Center, National Institute of Advanced Industrial Science and Technology, Tokyo; ‖Department of Virology and Liver Unit, Nagoya City University Graduate School of Medicine, Nagoya; ¶Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo; and #Research Center for Hepatitis and Immunology, Kohnodai Hospital, National Center for Global Health and Medicine, Ichikawa, Japan

See related article, Younossi and Stepanova, on page 718 in *CGH*.

BACKGROUND & AIMS: Multiple viral and host factors are related to the treatment response to pegylated-interferon and ribavirin combination therapy; however, the clinical relevance and relationship of these factors have not yet been fully evaluated. **METHODS:** We studied 168 patients with chronic hepatitis C who received pegylated-interferon and ribavirin combination therapy. Gene expression profiles in the livers of 91 patients were analyzed using an Affymetrix genechip (Affymetrix, Santa Clara, CA). The expression of interferon-stimulated genes (ISGs) was evaluated in all samples by real-time polymerase chain reaction. Genetic variation in interleukin 28B (IL28B; rs8099917) was determined in 91 patients. **RESULTS:** Gene expression profiling of the liver differentiated patients into 2 groups: patients with up-regulated ISGs and patients with down-regulated ISGs. A high proportion of patients with no response to treatment was found in the up-regulated ISGs group (P = .002). Multivariate logistic regression analysis showed that ISGs (<3.5) (odds ratio [OR], 16.2; $P < .001$), fibrosis stage (F1-F2) (OR, 4.18; P = .003), and ISDR mutation (≥2) (OR, 5.09; P = .003) were strongly associated with the viral response. The IL28B polymorphism of 91 patients showed that 66% were major homozygotes (TT), 30% were heterozygotes (TG), and 4% were minor homozygotes (GG). Interestingly, hepatic ISGs were associated with the IL28B polymorphism (OR, 18.1; $P < .001$), and its expression was significantly higher in patients with the minor genotype (TG or GG) than in those with the major genotype (TT). **CONCLUSIONS: The expression of hepatic ISGs is strongly associated with treatment response and genetic variation of IL28B. The differential role of host and viral factors as predicting factors may also be present.**

Keywords: Pegylated Interferon, Ribavirin; Gene Expression; Single Nucleotide Polymorphism.

A human liver infected with hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and, in some instances, hepatocellular carcinoma.[1] Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C); approximately 50% of patients usually relapse, particularly those with HCV genotype 1b and a high viral load.[2,3] Therefore, it is beneficial to predict the response of patients with the 1b genotype and a high viral load to pegylated-IFN (Peg-IFN) and RBV combination therapy before starting treatment because therapy can be long, costly, and have many adverse effects. Amino acid (aa) substitutions in the interferon sensitivity determining region (ISDR), located in the HCV nonstructural region 5A, are useful for predicting the response of patients with genotype 1b to IFN therapy.[4] However, viral factors alone do not sufficiently predict the outcome of treatment in every case.[5]

In addition to viral factors, hepatic gene expression before and during IFN treatment has been examined to determine host factors associated with the response to treatment.[6,7] Interferon-stimulated genes (ISGs) up-regulated in the liver prior to treatment might be related to the poor induction of ISGs and the impaired eradication of HCV during treatment.[6-9] This may be because the ISGs have already been maximally induced before treat-

Abbreviations used in this paper: aa, amino acid; AST, aspartate aminotransferase; cDNA, complementary DNA; CH-C, chronic hepatitis C; Down-ISGs, down-regulated ISGs; EVR, early virologic response; GWAS, genome-wide association studies; HCV, hepatitis C virus; IFN, interferon; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IL, interleukin; IL28B, interleukin 28B; ISDR, interferon sensitivity determining region; ISGs, interferon stimulated genes; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); NR, no response; Peg, pegylated; RBV, ribavirin; ROC, receiver operating characteristic; RTD, real-time detection; PCR, polymerase chain reaction; RTD-PCR, real-time detection-polymerase chain reaction; SNP, single nucleotide polymorphism; SVR, sustained viral response; TR, transient response; Up-ISGs, up-regulated ISGs.

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ment. However, the clinical relevance of the expression of ISGs as predictive factors for the outcome of treatment has not yet been fully evaluated.

In parallel to gene expression analysis, genome-wide association studies (GWAS) have been used to identify loci associated with the response to treatment; genetic variation in interleukin 28B (IL28B) was found to predict hepatitis C treatment-induced viral clearance.[10–12]

In this study, with a relatively large cohort of CH-C patients treated with Peg-IFN and RBV, we validated the clinical relevance of the expression of hepatic ISGs as predictive factors for the outcome of treatment. In addition, we demonstrated that the expression of hepatic ISGs was closely related to genetic variation in IL28B.

Materials and Methods

Patients

We enrolled 168 patients with CH-C at the Graduate School of Medicine, Kanazawa University Hospital and its related hospitals, Japan (Table 1, Supplementary Table 1). The cohort included 92 men and 76 women, ranging from 21 to 73 years of age, who were registered prospectively in 2005 and 2007. All patients had HCV

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR		NR		Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
No. of patients	n = 125		n = 43			—	
Age and sex							
Age, *y*	57	(30–72)	56	(30–73)	.927	—	
Sex (M vs F)	68 vs 57		24 vs 19		.872	—	
Liver factors							
F stage (F1-2 vs F3-4)	95 vs 30		20 vs 23		.001	4.18 (1.61–11.5)	.003
A grade (A0-1 vs A2-3)	68 vs 57		19 vs 24		.248	—	
ISGs (Mx, IFI44, IFIT1)							
(<3.5 vs ≥3.5)	103 vs 22		12 vs 31		<.001	16.2 (6.21–47.8)	<.001
Laboratory parameters							
HCV-RNA (*KIU/mL*)	2300	(126–5000)	1930	(140–5000)	.725	—	
BMI (kg/m^2)	23.2	(16.3–34.7)	23.4	(19.5–40.6)	.439	—	.107
AST (*IU/L*)	46	(18–258)	64	(21–283)	.017	—	
ALT (*IU/L*)	60	(16–376)	82	(18–345)	.052	—	
γ-GTP (*IU/L*)	36	(4–367)	75	(26–392)	<.001	—	
WBC ($/mm^3$)	4800	(2100–11,100)	4800	(2500–8200)	.551	—	
Hb (*g/dL*)	14	(9.3–16.6)	14.4	(11.2–17.2)	.099	—	
PLT ($\times 10^4/mm^3$)	15.7	(7–39.4)	15.2	(7.6–27.8)	.378	—	
TG (*mg/dL*)	98	(30–323)	116	(45–276)	.058	—	
T-Chol (*mg/dL*)	167	(90–237)	160	(81–214)	.680	—	
LDL-Chol (*mg/dL*)	82	(36–134)	73	(29–123)	.019	—	
HDL-Chol (*mg/dL*)	42	(20–71)	47	(18–82)	.098	—	
FBS (*mg/dL*)	94	(60–291)	96	(67–196)	.139	—	
Insulin (*μU/mL*)	6.6	(0.7–23.7)	6.8	(2–23.7)	.039	—	
HOMA-IR	1.2	(0.3–11.7)	1.2	(0.4–7.2)	.697	—	
Viral factors							
ISDR mutations ≤1 vs ≥2	80 vs 44		34 vs 9		.070	5.09 (1.69–17.8)	.003
Treatment factors							
Total dose administered							
Peg-IFN (*μg*)	3840	(960–7200)	3840	(1920–2880)	.916	—	
RBV (*g*)	202	(134–336)	202	(36–336)	.531	—	
Achieved administration rate							
Peg-IFN (%)							
≥80%	84		28		.975	—	
<80%	42		14				
RBV (%)							
≥80%	76		24		.745	—	
<80%	50		18				
Achievement of EVR	101/125 (81%)		0/43 (0%)		<.001	—	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; EVR, early virologic response; γ-GTP, γ-glutamyl transpeptidase; ISDR, interferon sensitivity determining region; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); WBC, leukocytes; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; RBV, ribavirin; PLT, platelets; TG, triglycerides; TR, transient response; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; FBS, fasting blood sugar; CI, confidence interval.

genotype 1b and high viral loads (≥100K IU/mL) measured by quantitative Cobas Amplicor assays (Roche Diagnostics Co Ltd, Tokyo, Japan). All patients had undergone liver biopsy before combination therapy. Exclusion criteria for patients not eligible for Peg-IFN and RBV combination therapy were as follows: (1) pregnant women or women of childbearing potential, nursing mothers, or male patients whose partner might become pregnant; (2) patients with hepatocellular carcinoma; (3) patients with serious complications in the heart, kidneys, or lungs; (4) patients with autoimmune diseases, such as autoimmune hepatitis, and primary biliary cirrhosis; and (5) patients infected with the hepatitis B virus. Informed consent was obtained from all patients, and ethics approval for the study was obtained from the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science.

All patients were administered Peg-IFN-α 2b (Schering-Plough KK, Tokyo, Japan) and RBV combination therapy for 48 weeks. Peg-IFN was given in weekly doses and adjusted to body weight according to the manufacturer's instructions (45 kg or less, 60 μg/dose; 46-60 kg, 80 μg/dose; 61-75 kg, 100 μg/dose; 76-90 kg, 120 μg/dose; and 91 kg or more, 150 μg/dose). Similarly, RBV (Schering-Plough KK) was administered in daily doses adjusted to body weight according to the manufacturer's instructions (60 kg or less, 600 mg/day; 61-80 kg, 800 mg/day; and 81 kg or more, 1000 mg/day).

The final outcome of treatment was assessed 24 weeks after the cessation of combination therapy. We defined treatment outcomes according to the decrease in viremia as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapsed during the follow-up period; and no response (NR), HCV viremia detected at the cessation of therapy. An early virologic response (EVR) (complete EVR) was defined as undetectable HCV-RNA in the serum by 12 weeks. HCV genotypes were determined according to the method of Okamoto et al. Serum HCV RNA was determined using qualitative and quantitative COBAS Amplicor assays (Roche Diagnostics Co, Ltd, Tokyo, Japan). The grading and staging of chronic hepatitis were histologically assessed according to the method of Desmet et al (Table 1).[13]

Preparation of Liver Tissue Samples

Liver biopsy samples were taken from all patients before treatment. The biopsy samples were divided into 2 parts: the first part was immersed in formalin for histologic assessment, and the second was immediately immersed in RNAlater (QIAGEN, Valencia, CA) for RNA isolation. Liver tissue RNA was isolated using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. Isolated RNA was stored at −70°C until use.

Affymetrix Genechip Analysis

The quality of the isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer (Agilent, Santa Clara, CA). Aliquots of total RNA (50 ng) isolated from the liver biopsy specimens were subjected to amplification using the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA) according to the manufacturer's instructions. Approximately 10 μg of complementary DNA (cDNA) was amplified from 50 ng of total RNA, and 5 μg of cDNA was used for fragmentation and biotin labeling using the FL-Ovation cDNA Biotin Module V2 (NuGen) according to the manufacturer's instructions. Biotin-labeled cDNA was suspended in 220 μL of hybridization cocktail (NuGen), and 200 μL was used for hybridization to the Affymetrix Human 133U Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) containing 54,675 probes. After stringent washing, the microarray chips were stained with streptavidin-phycoerythrin, and probe hybridization was determined using a GeneChip Scanner 3000 (Affymetrix). Data files (CEL) were obtained using the GeneChip Operating Software 1.4 (Affymetrix).

Hierarchical Clustering and Pathway Analysis of Genechip Data

Genechip data analysis was performed using BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.htm). The data were log transferred, normalized, centered, and applied to the average linkage hierarchical clustering with centered correlation.

For genechip analysis, we selected 37 representative ISGs. Hepatic gene expression profiling was obtained from 30 CH-C patients before and 1 week after the initiation of IFN and RBV combination therapy and the 100 most up-regulated genes were selected (submitted for publication). ISGs were suppressed in patients with a rapid viral response and up-regulated in patients with a slow viral response before treatment. Using the 100 treatment-induced genes, we evaluated hepatic gene expression in 30 patients before treatment. Hierarchical clustering analysis showed that a cluster of 37 ISGs was up-regulated in patients with a slow viral response.

Pathway analysis was performed using MetaCore (GeneGo, St. Joseph, MI). Functional ontology enrichment analysis was performed to compare the gene ontology process distribution of differentially expressed genes ($P < .01$).

Quantitative Real-time Detection-Polymerase Chain Reaction

We performed quantitative real-time detection (RTD)-polymerase chain reaction (PCR) (RTD-PCR) using TaqMan Universal Master Mix (PE Applied Biosystems, Carlsbad, CA). Primer pairs and probes for myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse) (Mx1), 2′-5′-oligoadenylate synthelase 3 (OAS3), interferon-induced protein 44 (IFI44),

interferon-induced protein 44-like (IFI44L), 2′-5′-oligoadenylate synthetase 2 (OAS2), ubiquitin specific peptidase 18 (USP18), radical S-adenosyl methionine domain containing 2 (RSAD2), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon induced with helicase C domain 1 (IFIH1), XIAP associated factor 1 (XAF1), cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial (CMPK2), epithelial stromal interaction 1 (EPSTI1), hect domain and RLD 6 (HERC6), poly (ADP-ribose) polymerase family, member 9 (PARP9), phospholipid scramblase 1 (PLSCR1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from the TaqMan assay reagents library. Primer pairs and probes for IL28B were designed as previously described.[12] The standard curve was obtained in every assay using the RNA obtained from a normal liver.[14,15] The expression values were normalized by GAPDH, and normalized values indicate the relative fold expression to a normal liver.

Amino Acid Substitutions of ISDR in the Nonstructural 5A Region

The nucleotide sequence of ISDR in the nonstructural 5A region was determined by direct sequencing of PCR amplified materials.[4] Mutant-type ISDR was defined as containing 2 or more aa substitutions.

Genetic Variation of IL28B Polymorphism

A single nucleotide polymorphism (SNP) of IL28B was evaluated in 91 patients whose hepatic gene expression profiling was obtained. We genotyped 32 patients using Affymetrix Genome-Wide Human SNP Array 6.0 as previously described.[12] The results for rs8105790, rs11881222, rs8099917, and rs7248668 were retrieved from a database to evaluate the association of these SNPs. rs12979860 was determined by direct sequencing, and rs8099917 was determined using TaqMan Pre-Designed SNP Genotyping Assays (PE Applied Biosystems) as recommended by the manufacturer.

Statistical Analysis

The Mann-Whitney *U* test was used to analyze continuous variables. Fisher exact test and χ^2 test were used for the analysis of categorical data. The overall plausibility of the treatment response groups was assessed using Fisher C statistic (Supplementary Table 2).[16,17] C is defined by $C = -2 \Sigma \ln(p_i)$, where p_i is the probability (*P* value) of each independent statement (clinical factors). C follows a χ^2 distribution with 2k degrees of freedom, k being the number of independent statements (clinical factors).[16] A nonsignificant C value means that the treatment response in the 2 groups was not statistically independent.

Multivariate analysis was performed using a stepwise logistic regression model. Each cut-off point for the continuous variables was decided by analysis of the receiver operating characteristic (ROC) curve. A *P* value of less than .05 was considered significant. Statistical analyses were performed using JMP7 for Windows (SAS Institute, Cary, NC).

Results

Response Rate and Clinical Characteristics

The clinical characteristics of the patients are shown in Table 1 and Supplementary Table 1. All of the patients were infected with HCV genotype 1b and had a high viral load (>100K IU/mL). No patients were coinfected with the hepatitis B virus (HBV). The intention-to-treat analysis showed that SVR, TR, and NR were observed in 70 (42%), 55 (33%), and 43 (25%) patients, respectively (Supplementary Table 1). Before comparing patients with 3 different responses, the overall plausibility of the treatment response groups was assessed using Fisher C statistic. Fisher C statistic utilizes the *P* values obtained by comparing pretreatment factors including age, gender, liver factors, laboratory parameters, and viral factors. Because the SVR and TR groups could not be defined as different, they were grouped together and compared with NR (Table 1, Supplementary Table 2).

Eleven patients with NR discontinued the therapy after 24 weeks because of an insufficient effect, namely, serum HCV-RNA was still detectable at this time. The remaining patients completed 48 weeks of Peg-IFN and RBV combination therapy. The administration rate of Peg-IFN with 80% or more was achieved in 67% of patients, and the administration rate of RBV with 80% or more was achieved in 60% of patients (Table 1).

Analysis of Hepatic Gene Expression

Prior to treatment, 91 of 168 patients (Supplementary Table 3) were randomly selected, and their hepatic gene expression was determined using Affymetrix genechip analysis.

Hierarchical clustering using 37 representative ISGs (see Materials and Methods) demonstrated 2 clear clusters of patients: one was a group composed of patients with up-regulated ISGs (Up-ISGs), and the other was a group consisting of patients with down-regulated ISGs (Down-ISGs) (Figure 1). In patients with Up-ISGs, 21 (49%) showed NR, whereas 8 (17%) patients with Down-ISGs showed NR ($P = .002$). In contrast, 14 (33%) patients with Up-ISGs showed SVR, whereas 27 (56%) patients with Down-ISGs showed SVR ($P = .03$). There were no significant differences in the frequency of advanced stages of liver fibrosis (F3-F4) between patients with Up-ISGs and patients with Down-ISGs (18 [42%] and 17 [35%], respectively, $P = .664$). These data indicated that the up-regulation of ISGs in the liver before treatment was strongly associated with resistance to IFN treatment.

Host and Viral Factors Associated With the Response to Combination Therapy

To evaluate the multiple host and viral factors associated with the response to Peg-IFN and RBV combination therapy in all patients, univariate and multivariate analyses were performed. To assess the expression of hepatic ISGs, 15 genes (Mx1, OAS3, IFI44, IFI44L, OAS2,

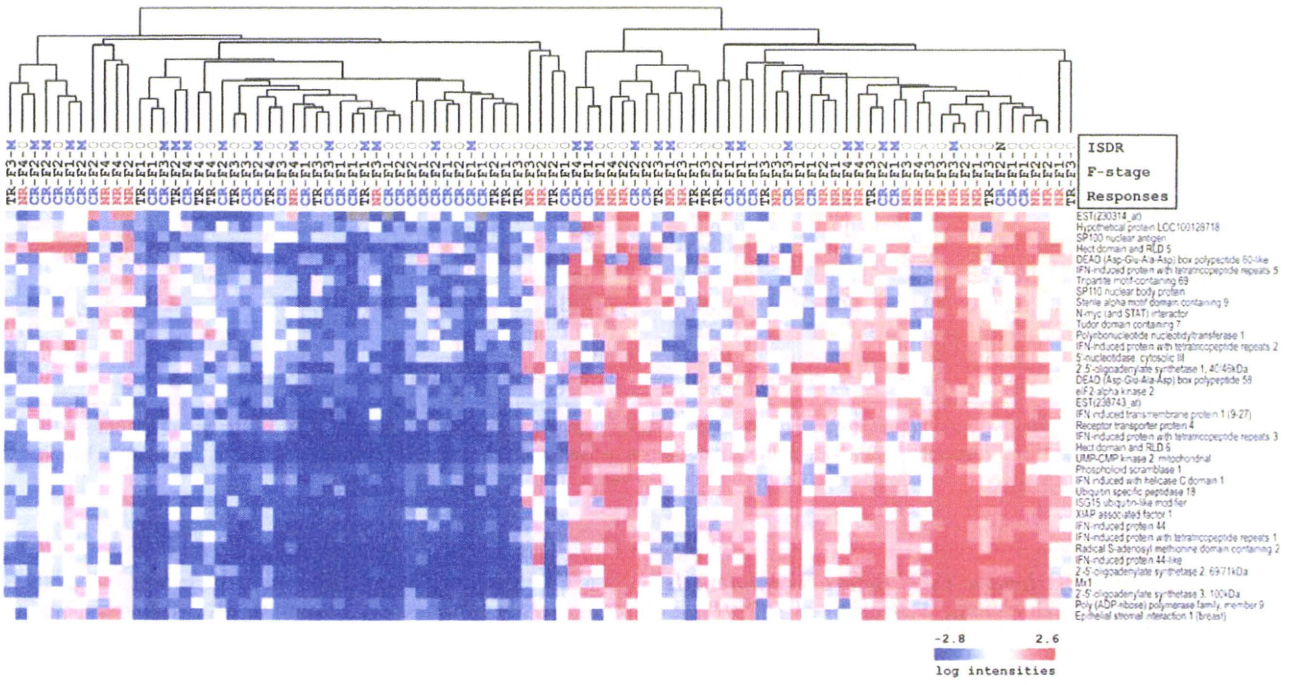

Figure 1. Hierarchical clustering analysis of 91 patients using 37 representative ISGs. Responses to therapy (SVR, TR, and NR), fibrosis stage (F1–F4), and status; ISDR mutations are also shown. ISDR mutation ≥2 = M, ≤1 = 0.

USP18, RSAD2, IFIT1, IFIH1, XAF1, CMPK2, EPSTI1, HERC6, PARP9, and PLSCR1) out of 37 representative ISGs were selected for their expression values of probe intensity, and their expression was confirmed in liver tissue obtained from 168 patients by RTD-PCR. Although there were significant correlations of their expression with each other, except RARP9 and PLSCR1 (Supplementary Table 4), the dynamic range of gene expression was high for 3 genes, namely, Mx1, IFI44, and IFIT1 (Supplementary Figure 1*A*). We averaged the expression values of Mx1, IFI44, and IFIT1 and used them for further study.

When we compared patients with SVR+TR and NR, the fibrosis stage of the liver ($P = .001$), expression of hepatic ISGs ($P < .001$), aspartate aminotransferase (AST) serum level ($P = .017$), γ-glutamyl transpeptidase (γ-GTP) ($P < .001$), low-density lipoprotein cholestrol (LDL-Chol) ($P = .019$), and insulin (μU/mL) ($P = .039$) were significantly different prior to treatment (Table 1). For treatment factors, the total dose and administration of IFN and RBV were not significantly different between these 2 groups. EVR was observed in 101 (81%) patients, and the proportion was significantly different ($P < .001$) between patients with SVR+TR and NR (Table 1).

Regression analysis of pretreatment factors showed a strong correlation among γ-GTP, alanine aminotransferase (ALT), and aspartate aminotransferase (AST); and homeostasis model assessment-insulin resistance (HOMA-IR), fasting blood sugar, and insulin; and total cholesterol (T-Chol), high-density lipoprotein cholestrol (HDL-Chol), and LDL-Chol (data not shown). We selected fibrosis stage, ISGs, HCV-RNA, ISDR mutation, and body mass index (BMI) as factors for multivariate analysis. Stepwise multivariate logistic regression analysis was performed using the selected factors. From the ROC curve, we set the cut-off value for the expression of ISGs as 3.5 (Supplementary Figure 1*B*). The results showed that expression of hepatic ISGs (<3.5), fibrosis stage (F1-F2), and ISDR mutation (≥2) were significant pretreatment factors contributing to SVR+TR (Table 1).

Clinical Parameters Associated With the Expression of Hepatic ISGs

Univariate and multivariate analyses revealed that the expression of hepatic ISGs was a strong predictor of the treatment outcome for SVR+TR patients. We next examined which clinical parameters were associated with the expression of hepatic ISGs (Table 2). Univariate analysis showed that the expression of ISGs was strongly correlated with the serum levels of γ-GTP ($P < .001$) and AST ($P < .001$) and weakly correlated with HCV-RNA, fasting blood sugar, insulin, HOMA-IR, triglyceride (TG), and LDL-Chol. Multivariate analysis showed that γ-GTP ($P < .001$), HCV-RNA ($P < .001$), and LDL-Chol ($P = .048$) were significantly associated with hepatic ISGs. Noticeably, the expression of ISGs was negatively correlated with HCV-RNA in SVR+TR patients ($P = .009$), whereas this correlation was not evident in NR patients ($P = .298$) (Table 2, Supplementary Figure 2). These results may indicate that endogenous ISGs suppress HCV in SVR+TR patients, whereas they are not active in NR patients.

Table 2. Clinical Factors Associated With Expression of Hepatic Interferon-Stimulated Genes

	Univariate analysis				Multivariate analysis			
Clinical factor	β	95% CI		P value	β	95% CI		P value
AST (*IU/L*)	0.274	0.13	0.42	<.001	—	—	—	
γ-GTP (*IU/L*)	0.326	0.18	0.47	<.001	0.288	0.14	0.43	<.001
HCV-RNA (*KIU/mL*)	−0.170	−3.19	−0.02	.025	−0.255	−0.40	−0.11	<.001
SVR+TR	−0.237	−0.32	−0.05	.009	—	—	—	
NR	−0.168	−0.57	0.18	.298	—	—	—	
FBS (*mg/dL*)	0.182	0.03	0.35	.021	—	—	—	
Insulin (*μU/mL*)	0.190	0.03	0.34	.016	—	—	—	
HOMA-IR	0.181	0.03	0.33	.017	—	—	—	.073
TG (*mg/dL*)	0.201	0.05	0.35	.011	—	—	—	.089
LDL-Chol (*mg/dL*)	−0.177	−0.33	−0.02	.025	−0.143	−0.28	0.00	.048

γ-GTP, γ-glutamyl transpeptidase; AST, aspartate aminotransferase; FBS, fasting blood sugar; TG, triglycerides; TR, transient response; NR, no response; SVR, sustained viral response; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-chol, low-density lipoprotein cholesterol; CI, confidence interval; β, β coefficient; CI, confidence interval.

Expression of Hepatic ISGs Before Treatment Is Associated With Genetic Variation of IL28B

Recently, a GWAS successfully identified the genomic locus associated with the treatment response to Peg-IFN and RVB combination therapy for CH-C. Genetic variation in IL28B predicts HCV treatment-induced viral clearance.[11,12] We determined the genetic variation in IL28B of 32 patients[12] (Table 3). The SNPs rs8105790, rs11881222, rs8099917, and rs7248668 had a significant association with treatment response (odds ratio: 24.7–27.1, $P = 1.84 \times 10^{-30}$–2.68×10^{-32}). These SNPs are located in block 2 of the IL28B haplotype and show significant linkage disequilibrium in the HapMap data.[12] Ge et al[11] reported a different SNP (rs12979860) that was located between rs11881222 and rs8099917. The nucleotide sequence of rs12979860 was determined by direct sequencing, and the results are shown in Table 3. There was a strong association of rs12979860 and the other 4 SNPs indicating that this SNP was located within the same haplotype block. We confirmed these findings in multiple samples from Japanese patients (data not shown).

We selected rs8099917 for further study and evaluated it using TaqMan Pre-Designed SNP Genotyping Assays. The G nucleotide of rs8099917 was associated with a poor response to treatment (minor allele), whereas the T was associated with a fair response to treatment (major allele).[12] Out of 91 patients (Supplementary Table 3), the proportion of major homozygotes (TT), heterozygotes (TG), and minor homozygotes (GG) were 66% (60/91), 30% (27/91), and 4% (4/91), respectively (Table 4); 86% (51/60) of the major genotype (TT) patients had SVR or TR, whereas 65% (20/31) with the minor genotypes (TG or GG) had NR ($P < .001$).

Interestingly, hepatic gene expression profiles revealed that patients with the minor genotype showed higher expression of hepatic ISGs, whereas patients with the major genotype showed lower expression of hepatic ISGs (Figures 2 and 3). To examine further the relationship of the genetic variation in IL28B and its expression levels, we evaluated the expression of IL28B in the liver by RTD-PCR (Figure 3). IL28B expression was approximately 10-fold less than the expression of ISGs. Although IL28B expression tended to be higher in some patients with the major genotype, there was no significant difference in IL28B expression in the liver between the major and minor genotypes (Figure 3*A*). Nevertheless, the expression of ISGs was clearly high in patients with the minor genotype ($P < .0001$) (Figure 3*B*). IL28 activates signal tranducers and activators of transcription 1 (STAT1) through downstream signaling from a heterodimeric class II cytokine receptor that consists of IL-10 receptor β (IL-10Rβ) and IL-28 receptor α (IL-28Rα).[18,19] Therefore, we examined the correlation between the expression of IL28B and ISGs. IL28B expression correlated with the expression of ISGs ($r = 0.44$, $P < .001$); however, the correlation was different according to the SNP genotype. We observed a steep-slope correlation for the minor genotype and a slow-slope correlation for the major genotype (Figure 3*C* and *D*). Interestingly, 4 minor homozygotic (GG) patients showed a steeper correlation than the heterozygotes (TG) (Figure 3*D*). Thus, the IL28B polymorphism might differentially regulate the expression of ISGs in the liver, leading to the different treatment outcomes.

We performed univariate and multivariate analyses to identify the clinical factors associated with the major and minor genotypes (Table 4). Univariate analysis showed that higher hepatic ISGs and lower body mass index were significantly associated with the minor genotype; however, multivariate analysis showed that only hepatic ISGs (≥3.5) were associated with the minor genotype ($P < .001$; OR, 18.1; 95% confidence interval: 3.95–113). We further compared the predictive capacity of multivariate models using the expression of hepatic ISGs (<3.5 vs ≥3.5) or the IL28B genotype (major vs minor) (Supplementary Table 6). The predictive performance and fitness of the multivariate model using the IL28B genotype was superior to that using the expression of hepatic ISGs. However, when these factors were included in the same model, the expression of hepatic ISGs was still useful for the predictive model independent of the IL28B genotype (Supplementary Table 6).

Table 3. Clinical Characteristics of 32 Patients Genotyped by GWAS and 5 SNPs in Strong Linkage Disequilibrium With IL28B,[11] Including rs12979860

Patient No.	Response	Age, y	Sex	F stage	ISGs	IL28B	RefSNP (chr pos) Minor allele	rs8105790 (44424341) C	rs11881222 (44426763) G	rs12979860 (44430627) T	rs8099917 (44435005) G	rs7248668 (44435661) A
1	SVR	42	M	1	4.20	83.8		TT	AA	CC	TT	GG
2	SVR	59	M	1	2.62	45.5		TT	AA	CC	TT	GG
3	SVR	41	F	1	1.54	1.3		TT	AA	CC	TT	GG
4	TR	57	M	1	3.18	21.7		TT	AA	CC	TT	GG
5	TR	68	F	1	1.43	20.3		TT	AA	CC	TT	GG
6	SVR	44	M	1	0.97	4.6		TT	AA	CC	TT	GG
7	SVR	61	M	2	2.15	6.1		TT	AA	CC	TT	GG
8	SVR	50	M	2	3.25	66.4		TT	AA	CC	TT	GG
9	SVR	49	M	2	1.25	ND		TT	AA	CC	TT	GG
10	TR	59	F	2	1.29	17.4		TT	AA	CC	TT	GG
11	SVR	48	F	2	1.00	90.2		TT	AA	CC	TT	GG
12	TR	65	F	2	2.86	36.4		TT	AA	CC	TT	GG
13	NR	34	M	3	0.82	17.8		TT	AA	CC	TT	GG
14	SVR	55	M	3	0.83	13.8		TT	AA	CC	TT	GG
15	TR	68	M	3	0.75	20.6		TT	AA	CC	TT	GG
16	SVR	64	M	3	0.94	15.7		TT	AA	CC	TT	GG
17	SVR	67	F	3	1.50	25.7		TT	AA	CC	TT	GG
18	SVR	48	M	4	1.69	7.9		TT	AA	CC	TT	GG
19	NR	66	F	1	4.57	16.5		TC	AG	CT	TG	GA
20	SVR	52	F	1	5.23	29.3		TC	AG	CT	TG	GA
21	NR	55	F	1	8.25	57.2		TC	AG	CT	TG	GA
22	SVR	49	F	1	5.36	ND		TC	AG	CT	TG	GA
23	TR	44	M	1	2.08	7.0		TC	AG	CT	TG	GA
24	NR	63	M	1	2.77	10.5		TC	AG	CT	TG	GA
25	NR	61	F	2	3.98	39.1		TC	AG	CT	TG	GA
26	NR	42	M	2	4.89	5.9		TC	AG	CT	TG	GA
27	SVR	49	M	3	3.31	6.9		TC	AG	CT	TG	GA
28	TR	71	F	3	5.53	27.3		TC	AG	CT	TG	GA
29	TR	63	M	3	3.40	33.5		TC	AG	CT	TG	GA
30	NR	70	F	3	4.78	8.1		TC	AG	CT	TG	GA
31	TR	62	F	3	3.53	14.0		TC	AG	CT	TG	GA
32	NR	56	M	4	7.37	30.8		CC	GG	TT	GG	AA

NOTE. The Pearson correlation of the r^2 estimates for adjacent pairs; rs8099917 vs rs8105790, rs8099917 vs rs11881222, rs8099917 vs rs12979860, and rs8099917 vs rs7248668 = 0.99, 0.99, 0.98, and 0.97, respectively.
IL28B, interleukin 28B; GWAS, genome-wide association studies; ISGs, interferon stimulated genes; SNP, single nucleotide polymorphism; SVR, sustained viral response; TR, transient response; NR, no response; M, male; F, female.

To examine further the different hepatic gene expression of patients with the major or minor genotypes, pathway analysis of differentially expressed genes between the 2 groups was performed. By comparing the expression of hepatic genes between patients with the major and minor genotypes, 1359 differentially expressed genes were identified ($P < .01$; 711 genes were up-regulated with the minor genotype, and 648 genes were up-regulated with the major genotype). Pathway analysis of these genes demonstrated that signaling pathways related to interferon action, apoptosis, and Wnt signaling were up-regulated in the liver of patients with the minor genotype, whereas B-cell-, dendritic cell-, and natural killer cell-related genes were up-regulated in the liver of patients with the major genotype (Supplementary Figure 3). These results suggest that IL28B may be involved in innate and adaptive immune responses and that different antiviral signaling pathways might be involved in the liver of patients with different SNPs.

Discussion

Multiple viral and host factors may be related to the treatment response to Peg-IFN and RBV combination therapy. For the viral factors, a higher number of aa substitutions in the ISDR of nonstructural 5A region was strongly associated with a favorable response to IFN-α monotherapy in patients with genotype-1 HCV.[4]

Besides viral factors, host factors such as age, gender, fibrotic stage of the liver, and the presence of steatosis and insulin resistance were associated with the treatment outcome.[20] Analysis of hepatic gene expression demonstrated that the up-regulation of ISGs in the liver before treatment may be related to a poor treatment response.[6-9] To reveal the underlying mechanism of treatment resistance, 2 reports compared gene expression profiling in the liver before and during therapy and showed that patients with up-regulated ISGs in the liver prior to treatment failed to further induce ISGs following the ad-

Table 4. Comparison of Clinical Factors Between Patients With Major (TT) and Minor (TG+GG) Alleles

Clinical category	TT		TG+GG		Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
No. of patients	n = 60		n = 31			—	
Treatment response							
SVR+TR vs NR	51 vs 9		11 vs 20		<.001	—	
Age and gender							
Age, *y*	56	(30–69)	56	(30–71)	.843	—	
Sex (M vs F)	39 vs 21		19 vs 12		.518	—	
Liver factors							
F stage (F1-2 vs F3-4)	36 vs 24		23 vs 17		.905	—	
A grade (A0-1 vs A2-3)	27 vs 33		20 vs 11		.075	—	
ISGs (Mx, IFI44, IFIT1)							
(<3.5 vs ≥3.5)	46 vs 14		5 vs 26		<.001	18.1 (3.95–113)	<.001
Laboratory parameters							
HCV-RNA (*KIU/mL*)	2055	(160–5000)	1970	(126–5000)	.602	—	
BMI (kg/m^2)	24.5	(16.3–40.5)	22.9	(19.1–26.6)	.006	—	.077
AST (*IU/L*)	59	(22–258)	54	(21–283)	.227	—	
ALT (*IU/L*)	75	(24–376)	60	(18–236)	.077	—	
γ-GTP (*IU/L*)	61	(4–392)	53	(20–229)	.517	—	.167
WBC ($/mm^3$)	4450	(2100–11,100)	4600	(2500–8200)	.947	—	
Hb (*g/dL*)	14.2	(11.4–16.7)	14.5	(11.2–17.2)	.606	—	
PLT ($\times 10^4/mm^3$)	15.4	(7–39.4)	16.2	(9.2–27.7)	.832	—	
TG (*mg/dL*)	98	(58–248)	131	(30–303)	.053	—	.055
T-Chol (*mg/dL*)	172	(115–222)	168	(129–237)	.910	—	
LDL-Chol (*mg/dL*)	84	(42–123)	69	(51–107)	.052	—	.055
HDL-Chol (*mg/dL*)	44	(18–72)	45	(29–77)	.218	—	
FBS (*mg/dL*)	95	(59–291)	96	(66–206)	.849	—	
Insulin (*μU/mL*)	7.5	(0.7–23.2)	9.2	(2–23.2)	.195	—	
HOMA-IR	1.3	(0.3–11.7)	1.2	(0.4–9.6)	.339	—	
Viral factors							
ISDR mutations (≤1 vs ≥2)	38 vs 22		23 vs 7		.194	—	.083
Treatment factors							
Total dose administrated							
Peg-IFN (*μg*)	3960	(1500–7200)	3840	(1920–5760)	.377	—	
RBV (*g*)	203	(26–336)	201	(106–268)	.777	—	
Achieved administration rate							
Peg-IFN (%)							
≥80%	41		17		.207	—	
<80%	19		14				
RBV (%)							
≥80%	34		19		.671	—	
<80%	26		12				
Achievement of EVR	40/60 (62%)		9/31 (29%)		<.001	—	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; EVR, early virologic response; γ-GTP, γ-glutamyl transpeptidase; ISDR, interferon sensitivity determining region; Mx1, myxovirus (influenza virus) resistance 1 interferon-inducible protein p78 (mouse); WBC, leukocytes; HOMA-IR, homeostasis model assessment of insulin resistance; Hb, hemoglobin; RBV, ribavirin; PLT, platelets; TG, triglycerides; TR, transient response; T-chol, total cholesterol; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol; FBS, fasting blood sugar; CI, confidence interval.

ministration of IFN and could not eliminate HCV.[6,7] We performed a similar analysis and observed that these findings were more evident in liver lobular cells than in infiltrating lymphocytes in the portal area (submitted for publication). Thus, both viral and host factors might be closely related to the treatment response to Peg-IFN and RBV combination therapy. However, the clinical relevance and relationships of these factors have not been fully evaluated. In this study, we validated the clinical significance of the expression of hepatic ISGs on treatment outcome using a relatively large cohort of patients and compared its significance with other viral and host factors. To compare the patients with SVR, TR, and NR, we assessed the overall plausibility of each group using Fisher C statistic,[16] and patients with SVR and TR were grouped together for further analysis.

We examined hepatic gene expression in 91 of 168 patients using the Affymetrix genechip. Expression profiling using 37 representative ISGs (see Materials and Methods), which were selected from gene expression profiling comparing pretreatment and under treatment liver, differentiated 2 groups of