13 Di Bisceglie AM, Ghalib RH, Hamzeh FM, Rustgi VK. Early virologic response after peginterferon alpha-2a plus ribavirin or peginterferon alpha-2b plus ribavirin treatment in patients with chronic hepatitis C. *J Viral Hepat* 2007; **14**: 721–9.
14 Katano Y, Kumada T, Nakano I *et al.* Comparison of biochemical safety between PEG-IFN α-2a and PEG-IFN α-2b. *Hepatogastroenterology* 2009; **56**: 485–91.
15 Shi ST, Polyak SJ, Tu H, Taylor DR, Gretch DR, Lai MM. Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. *Virology* 2002; **292**: 198–210.
16 Miyanari Y, Atsuzawa K, Usuda N *et al.* The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 2007; **9**: 1089–97.
17 Sezaki H, Suzuki F, Akuta N *et al.* An open pilot study exploring the efficacy of fluvastatin, pegylated interferon and ribavirin in patients with hepatitis C virus genotype 1b in high viral loads. *Intervirology* 2009; **52**: 43–8.
18 Hirashima N, Shimada M, Iwase H, Goto H. Double filtration plasmapheresis and peginterferon plus ribavirin combination therapy for chronic hepatitis C patients non-responded by previous interferon therapy. *Kanzo* 2009; **50**: 738–40.
19 Kim SR, Imoto S, Kudo M *et al.* Double- filtration plasmapheresis plus IFN for HCV-1b patients with non-sustained virological response to previous combination therapy: early viral dynamics. *Intervilology* 2010; **53**: 44–8.
20 Namba T, Shiba R, Yamamoto T *et al.* Successful treatment of HCV-related cryoglobulinemic glomerulonephritis with double-filtration plasmapheresis and interferon combination therapy. *Clin Exp Nephrol* 2010; **14**: 372–6.
21 Taniguchi M, Furukawa H, Shimamura T *et al.* Impact of double-filtration plasmapheresis in combination with interferon and ribavirin in living donor liver transplant recipients with hepatitis C. *Transplantation* 2006; **81**: 1747–9.
22 Miyake T, Michitaka K, Tokumoto Y *et al.* Fibrosing cholestatic hepatitis with hepatitis C virus treated by double filtration plasmapheresis and interferon plus ribavirin after liver transplantation. *Clin J Gastroenterol* 2009; **2**: 125–30.

Research Article

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Differential interferon signaling in liver lobule and portal area cells under treatment for chronic hepatitis C

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Background & Aims: The mechanisms of treatment resistance to interferon (IFN) and ribavirin (Rib) combination therapy for hepatitis C virus (HCV) infection are not known. This study aims to gain insight into these mechanisms by exploring hepatic gene expression before and during treatment.

Methods: Liver biopsy was performed in 50 patients before therapy and repeated in 30 of them 1 week after initiating combination therapy. The cells in liver lobules (CLL) and the cells in portal areas (CPA) were obtained from 12 patients using laser capture microdissection (LCM).

Results: Forty-three patients were infected with genotype 1 HCV, 20 of who were viral responders (genotype 1-Rsp) with treatment outcome of SVR or TR, while 23 were non-responders (genotype 1-nonRsp) with NR. Only seven patients were infected with genotype 2. Before treatment, the expression of *IFN and Rib-stimulated genes* (IRSGs), apoptosis-associated genes, and immune reaction gene pathways was greater in genotype 1-nonRsp than in Rsp. During treatment, IRSGs were induced in genotype 1-Rsp, but not in nonRsp. IRSG induction was irrelevant in genotype 2-Rsp and was mainly impaired in CLL but not in CPA. Pathway analysis revealed that many immune regulatory pathways were induced in CLL from genotype 1-Rsp, while growth factors related to angiogenesis and fibrogenesis were more induced in CPA from genotype 1-nonRsp.

Conclusions: Impaired IRSGs induction in CLL reduces the sensitivity to treatment for genotype 1 HCV infection. CLL and CPA in the liver might be differentially involved in treatment resistance. These findings could be useful for the improvement of therapy for HCV infection.

Keywords: HCV; IFN; LCM; Gene expression.

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Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; miRNA, micro RNA; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; HCC-B, hepatitis B-related hepatocellular carcinoma; HCC-C, hepatitis C-related hepatocellular carcinoma; OCT, optimum cutting temperature.

Introduction

A human liver infected with hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and in some instances, hepatocellular carcinoma (HCC). Although interferon (IFN) and ribavirin (Rib) combination therapy has become a popular modality for treating patients with chronic hepatitis C (CH-C), about 50% of patients relapse, particularly those with genotype 1b and high viral load [8]. The reasons for treatment failure are poorly understood. Many studies of IFN and Rib combination therapy for CH-C suggested that patients who cleared HCV viremia early during therapy tended to show favorable outcomes. On the other hand, patients who needed a longer period to clear HCV had poorer outcomes [4,7,17], and those who showed no response (no or minimal decrease in HCV-RNA) to IFN and Rib combination therapy hardly ever achieved a sustained viral response (SVR).

To elucidate the underlying mechanism of treatment resistance, expression profiles in the liver [3,6,20] and peripheral mononuclear cells (PBMC) [10,21] during IFN treatment for CH-C patients have been examined. In chronic viral hepatitis, increased numbers of immune regulatory cells infiltrate the liver. These liver-infiltrating lymphocytes (LILs) might play important roles for virus eradication and are potentially linked to treatment outcome. Previously, we selectively isolated cells in liver lobules (CLL) and cells in the portal area (CPA) from biopsy specimens using laser capture microdissection (LCM) and analyzed their gene expression profiles [11,19]. From these profile analyses, it could be inferred that the majority of CLL were hepatocytes and the majority of CPA were lymphocytes, although other cellular components such as Kupffer cells, endothelial cells, myofibroblasts, and bile duct cells co-existed as well.

To gain further insight into the mechanisms of therapy resistance, we analyzed expression profiles in CLL and CPA in addition to whole liver tissues during IFN therapy for CH-C.

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Materials and methods

Patients

Patients with CH-C were enrolled in this study at the Graduate School of Medicine, Kanazawa University Hospital, Japan, between 2001 and 2007 (Tables 1 and 2). Prior to the study, we obtained the required approvals, namely: informed consent from all participating patients and ethics approval from the ethics committee for human genome/gene analysis research at Kanazawa University Graduate School of Medical Science. Thirty patients were administered IFN-α 2b (6 MU: every day for 2 weeks, then three times a week for 22 weeks) (Schering-Plough K.K., Tokyo, Japan) and Rib (10–13 mg/kg/day) combination therapy for 24 weeks (Table 1). Twenty patients were administered Peg-IFN-α 2b and Rib combination therapy for 48 weeks (Table 2). The final outcome of the treatment was assessed at 24 weeks after cessation of the combination therapy. In addition, 10 samples of normal liver tissues obtained during surgery for metastatic liver cancer were used as controls.

We defined treatment outcomes according to the decrease in viremia as follows: sustained viral response (SVR), clearance of HCV viremia at 24 weeks after cessation of therapy; transient response (TR), no detectable HCV viremia at 24 weeks but relapse during the follow-up period; and nonresponse (NR), HCV viremia detected at the cessation of therapy. We defined a patient who achieved SVR or TR as a viral responder (Rsp) and a patient who exhibited an NR as a non-responder (nonRsp). As patient 10 stopped treatment at 5 weeks due to an adverse side effect, we grouped this patient as Rsp based on the observed viral decline within 2 weeks (Table 1).

HCV genotype was classified by the methods described by Okamoto et al. [16] Twenty-three patients were infected with genotype 1b and seven patients were infected with genotype 2 (2a; 6, 2b; 1) (Tables 1 and 2).

Patient serum was aliquoted and stored at −20 °C until use. HCV-RNA was serially monitored by quantitative real-time detection (RTD)-PCR (COBAS® AmpliPrep/COBAS® TaqMan® System®) [9] before treatment, at 48 h, 2 weeks and 24 weeks after initiation of therapy and at 24 weeks after cessation of therapy.

The grading and staging of chronic hepatitis were histologically assessed according to the method described by Desmet et al. (Table 1) [5].

Table 1. Characteristics of study patients who received IFN and ribavirin combination therapy.

				ALT (IU/ml)		Liver histology				HCV-RNA (Log IU/ml)				Viral kinetics			
Pt.No.	Sex	Age (yr)	Genotype	Before therapy	During therapy	Before therapy F A	During therapy F A	LCM		Before therapy	48 h	2 wk	24 wk	1st phase delinie Log/24 h	2nd phase decline Log/week	Viral response	Outcome
1	M	48	1b	83	45	1 1	1 1	+		6.6	4.5	3.5	-	1.1	0.5	Rsp	SVR
2	M	32	1b	192	95	1 1	1 1	-		6.4	3.9	3.2	-	1.3	0.4	Rsp	SVR
3	F	50	1b	57	37	1 1	1 1	-		5.8	2.5	1.5	-	1.7	0.5	Rsp	TR
4	M	36	1b	119	117	1 1	1 1	+		6.1	4.4	4.2	+	0.9	0.1	nonRsp	NR
5	M	54	1b	82	69	1 1	1 1	-		6.6	5.1	3.9	+	0.8	0.6	nonRsp	NR
6	M	43	1b	143	116	1 1	1 1	-		6.3	4.4	4.1	+	1.0	0.2	nonRsp	NR
7	M	48	1b	33	30	1 1	1 1	+		1.5	0.0	0.0	-	>0.8	-	Rsp	SVR
8	M	52	1b	316	374	1 2	1 1	-		4.7	5.1	3.9	+	-0.2	0.6	nonRsp	NR
9	M	45	1b	112	39	1 0	2 0	-		6.2	5.1	5.7	+	0.6	-0.3	nonRsp	NR
10	M	48	1b	48	30	2 2	2 1	+		6.4	4.0	2.6	NA	1.2	0.8	Rsp	NA
11	M	52	1b	114	80	2 2	2 1	-		6.1	3.7	3.0	-	1.2	0.4	Rsp	TR
12	F	63	1b	38	30	2 1	2 1	-		5.2	4.2	4.5	+	0.5	-0.2	nonRsp	NR
13	M	58	1b	90	83	2 2	2 2	+		6.9	4.9	5.6	+	1.0	-0.4	nonRsp	NR
14	F	61	1b	87	43	2 1	2 1	+		6.5	3.9	3.7	+	1.3	0.1	nonRsp	NR
15	F	64	1b	133	111	2 1	3 2	-		6.0	4.4	3.6	+	0.8	0.4	nonRsp	NR
16	F	62	1b	251	159	3 2	3 2	-		4.8	2.7	1.5	-	1.1	0.6	Rsp	SVR
17	M	54	1b	211	205	3 2	3 2	+		6.7	0.0	0.0	-	>3.4	-	Rsp	SVR
18	F	68	1b	153	145	3 2	3 2	+		4.9	4.3	3.5	+	0.3	0.4	nonRsp	NR
19	F	69	1b	64	43	3 2	3 2	-		4.4	1.5	0.0	-	1.5	0.8	Rsp	SVR
20	M	49	1b	91	83	3 2	3 2	+		6.6	4.2	3.8	+	1.2	0.2	nonRsp	NR
21	M	55	1b	187	196	4 1	4 2	-		5.8	5.1	5.6	+	0.4	-0.3	nonRsp	NR
22	F	45	1b	113	75	4 2	3 3	-		5.7	4.2	2.7	-	0.8	0.8	Rsp	TR
23	M	60	1b	86	49	4 2	3 1	-		6.3	3.5	3.5	+	1.4	0.0	nonRsp	NR
24	F	51	2b	98	90	1 1	1 1	-		2.7	1.5	0.0	-	0.6	0.8	Rsp	SVR
25	M	37	2a	241	211	1 0	1 0	-		4.0	1.5	0.0	-	1.3	0.8	Rsp	SVR
26	F	45	2a	91	33	2 1	2 1	-		5.4	2.2	1.5	-	1.6	0.4	Rsp	TR
27	M	46	2a	101	45	2 1	2 1	+		3.6	0.0	0.0	-	>1.8	-	Rsp	SVR
28	M	54	2a	196	177	3 2	2 1	+		4.2	0.0	0.0	-	>2.1	-	Rsp	SVR
29	F	68	2a	234	135	3 1	3 2	+		4.6	3.1	0.0	-	0.8	1.7	Rsp	SVR
30	M	67	2a	155	163	4 2	4 2	-		3.9	1.5	0.0	-	1.2	0.8	Rsp	SVR

First phase decline was determined by subtracting HCV-RNA at 48 h from before therapy.
Second phase decline was determined by subtracting HCV-RNA at 2 wk from 48 h.
NA, not applicable; LCM, laser capture microdissection; ALT, alanine aminotransferase; SVR, sustained viral response; A, activity; NR, nonresponse; F, fibrosis; TR, transient response; Rsp, viral responder, patients with SVR or TR; nonRsp, non-viral responder; patients with NR; HCV-RNA was assayed by COBAS® AmpliPrep/COBAS® TaqMan® System® (Log IU/mL).

Table 2. Characteristics of patients who received Peg-IFN and ribavirin combination therapy and normal control.

Pt.No.	Sex	Age (yr)	Genotype	ALT (IU/ml)	Liver histology		HCV-RNA (Log IU/ml)				Viral response	Outcome
				Before therapy	Before therapy		Before therapy	2 wk	4 wk	24 wk		
					F	A						
1	M	57	1b	68	1	1	6.5	-	-	-	Rsp	SVR
2	F	56	1b	31	1	1	6.5	4.4	-	-	Rsp	SVR
3	M	63	1b	50	1	1	6.1	-	-	-	Rsp	SVR
4	M	44	1b	45	1	1	6.5	3.7	-	-	Rsp	SVR
5	F	51	1b	27	2	1	6.5	4.1	-	-	Rsp	SVR
6	M	58	1b	72	2	1	6.2	-	-	-	Rsp	SVR
7	M	60	1b	71	2	2	6.2	3.9	-	-	Rsp	SVR
8	F	52	1b	58	2	2	6.5	4.1	-	-	Rsp	SVR
9	F	62	1b	60	3	2	5.9	3.8	-	-	Rsp	SVR
10	M	55	1b	106	3	2	6.4	-	-	-	Rsp	SVR
11	M	30	1b	31	1	1	6.4	6.1	5.9	+	nonRsp	NR
12	F	55	1b	23	1	2	6.5	6.1	5.9	+	nonRsp	NR
13	M	58	1b	129	1	2	6.3	6.0	5.8	+	nonRsp	NR
14	M	42	1b	326	2	1	6.6	6.2	5.8	+	nonRsp	NR
15	F	61	1b	77	2	1	6.1	5.9	5.7	+	nonRsp	NR
16	F	44	1b	31	2	2	5.5	5.3	4.7	+	nonRsp	NR
17	M	51	1b	38	2	2	6.5	6.2	5.9	+	nonRsp	NR
18	F	55	1b	97	2	2	6.7	6.3	6.1	+	nonRsp	NR
19	M	59	1b	31	3	2	6.7	5.9	5.7	+	nonRsp	NR
20	F	53	1b	71	3	2	5.9	5.8	5.8	+	nonRsp	NR
21	F	51	-	18	0	0	-	-	-	-	-	-
22	F	78	-	13	0	0	-	-	-	-	-	-
23	M	75	-	20	0	0	-	-	-	-	-	-
24	M	34	-	12	0	0	-	-	-	-	-	-
25	M	64	-	30	0	0	-	-	-	-	-	-
26	M	78	-	9	0	0	-	-	-	-	-	-
27	M	53	-	19	0	0	-	-	-	-	-	-
28	F	64	-	12	0	0	-	-	-	-	-	-
29	F	60	-	20	0	0	-	-	-	-	-	-
30	M	66	-	26	0	0	-	-	-	-	-	-

SVR, sustained viral response; NR, nonresponse; Rsp, viral responder, patients with SVR or TR; nonRsp, non-viral responder; patients with NR.

Preparation of liver tissue samples

Liver biopsy samples were taken from all the patients at around 1 week before treatment and at 1 week after starting therapy (Fig. 1A). The biopsy samples were divided into three parts: the first part was immersed in formalin for histological assessment, the second was immediately frozen in liquid nitrogen tank for future RNA isolation, and the final part was frozen in OCT compound for LCM analysis and stored at −80 °C until use. As a control, a liver tissue sample was surgically obtained from a patient who showed no clinical signs of hepatitis and was analyzed as described previously [11].

CLL and CPA were isolated by LCM using a CRI-337 (Cell Robotics, Albuquerque, NM, USA) (Supplementary Fig. 1) from the liver biopsy specimens frozen in OCT compound. The detailed procedure for LCM is described in the Supplementary materials and methods and was performed as previously described [11,19].

RNA isolation and Affymetrix gene chip analysis

Total RNA in each liver biopsy specimen was isolated using the RNAqueous® kit (Ambion, Austin, TX, USA). Total RNA in the specimens frozen for LCM was isolated with a carrier nucleic acid (20 ng poly C) using RNAqueous®-Micro (Ambion). The quality of the isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer (Palo Alto, CA, USA). Aliquots of total RNA (50 ng) isolated from the liver biopsy specimens were subjected to amplification with the WT-Ovation™ Pico RNA Amplification System (NuGen, San Carlos, CA, USA) as recommended by the manufacturer. About 10 μg of cDNA was amplified from 50 ng total RNA, and 5 μg of cDNA was used for fragmentation and biotin labeling using the FL-Ovation™ cDNA Biotin Module V2 (NuGen) as recommended by the manufacturer. The biotin-labeled cDNA was suspended in 220 μl of hybridization cocktail (NuGen), and 200 μl was used for the hybridization. Half of the total RNA isolated from the LCM specimens was amplified twice with the TargetAmp™ 2-Round Aminoallyl-aRNA Amplification Kit 1.0 (EPICENTRE, Madison, WI, USA). Twenty-five micrograms of amplified antisense RNA were used for biotin labeling according to the manufacturer's protocol *Biotin-X-X-NHS* (provided by EPICENTRE). The biotin-labeled aRNA was suspended in 300 μl of hybridization cocktail (Affymetrix Inc., Santa Clara, CA, USA), and 200 μl was used for the hybridization with the Affymetrix Human 133 Plus 2.0 microarray chip 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 with the GeneChip® Operating Software 1.4 (GCOS) (Affymetrix). All the expression data were deposited in Gene Expression Omunibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) (NCBI) and the accession ID is GSM 425,995. The experimental procedure is described in detail in the Supplementary materials and methods.

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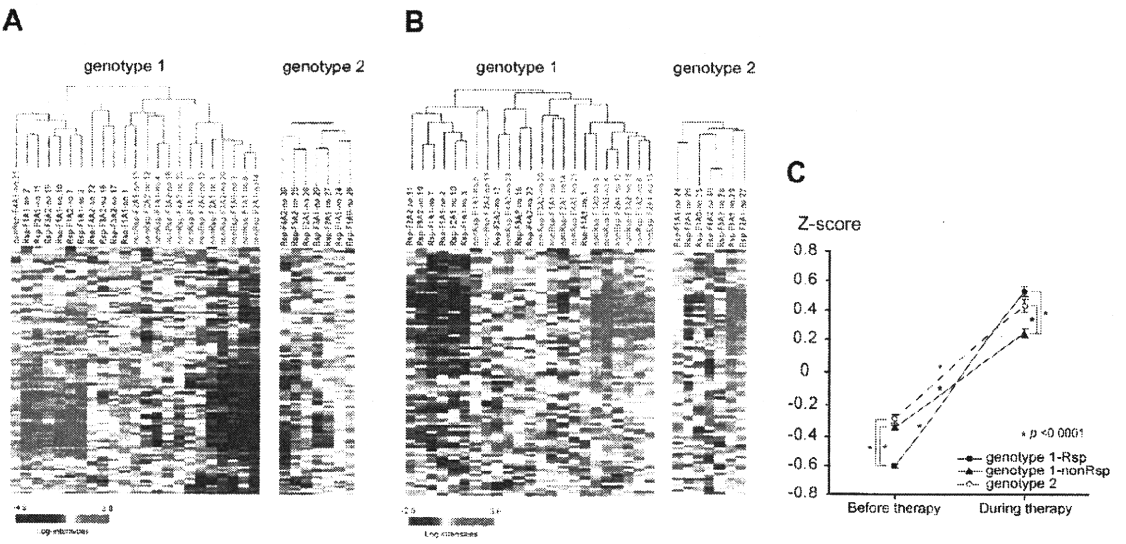

Fig. 1. (A) Hierarchical clustering of expression in genotype 1 and genotype 2 patients during treatment according to fold induction of IRSGs. (B) Hierarchical clustering of expression in genotype 1 and genotype 2 patients before treatment. (C) Serial changes in standardized expression values (Z-score) of IRSGs from genotype 1-Rsp, genotype 1-nonRsp, and genotype 2 patients before and during treatment.

Statistical and pathway analysis of gene chip data

Statistical analysis and hierarchical clustering were performed by BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.htm). A class comparison tool based on univariate or paired *t*-tests was used to find differentially expressed genes (p <0.005). To confirm statistical significance, 2000 random permutations were performed, and all of the *t*-tests were re-computed for each gene. The gene set comparison was analyzed using the BioCarta and the KEGG pathway data bases. The Fisher and Kolmogorov–Smirnov tests were performed for statistical evaluation (p <0.005) (BRB-ArrayTools). Functional ontology enrichment analysis was performed to compare the Gene Ontology (GO) process distribution of differentially expressed genes (p <0.05) using MetaCore™ (GeneGo, St. Joseph, MI, USA).

For the comparison of standardized expression values among different pathway groups, standard units (*Z*-score) of each gene expression value were calculated as:

$$Z_i = \frac{X_i - X_m}{S}$$

where X_i is the raw expression value, X_m is the mean of the expression values in the pathway, and S is the standard deviation of the expression values.

The standard units in each pathway were expressed as mean ± SEM. A *P*-value of less than 0.05 was considered significant. Multivariate analysis was performed using a logistic regression model with a stepwise method using JMP7 for Windows (SAS Institute, Cary, NC, USA).

Quantitative real-time detection (RTD)-PCR

We performed quantitative real-time detection PCR (RTD)-PCR using TaqMan Universal Master Mix (PE Applied Biosystems, CA). Primer pairs and probes for Mx1, IFI44 and IFITM1, and GAPDH were obtained from TaqMan assay reagents library (Applied Biosystems, CA).

Results

Serial changes in HCV-RNA after initiation of IFN-α 2b and Rib combination therapy

Serial changes in HCV-RNA were monitored at 48 h, 2 weeks, and 24 weeks after the initiation of therapy (Table 1). The biphasic viral decline after the initiation of IFN therapy has been characterized [14,15,18]. We calculated the first phase decline by comparing viral load before therapy and after 48 h, and the second phase decline by comparing viral load after 48 h and 2 weeks (Table 1) [14,15,18]. Both the first and the second phase declines could be associated with treatment outcome and interestingly, viral responders (Rsp) who achieved SVR or TR showed more than a 1-log drop of first phase decline (Log/24 h) and more than a 0.3-log drop of second phase decline (Log/w) (Table 1). In contrast, non-responders (nonRsp) who exhibited NR failed to meet the criteria. The first phase decline of Rsp and nonRsp were 1.38 ± 0.65 log/24 h and 0.77 ± 0.44 log/24 h (p = 0.005), respectively. The second phase decline of Rsp and nonRsp were 0.71 ± 0.34 log/w and 0.11 ± 0.34 log/w (p = 0.0001), respectively. Therefore, the classification of Rsp or nonRsp according to the treatment outcome might be feasible based on the viral kinetic responses to IFN. All but one patient infected with genotype 2 HCV eliminated the virus within 2 weeks. There were no significant differences in the degree of histological activity or staging, nor in the sex, age, or alanine aminotransferase (ALT) level among these patients (Table 1). The amount of HCV-RNA was significantly lower in genotype 2 patients (4.06 ± 0.32 log IU/ml) than in genotype 1 patients (5.70 ± 1.10 log IU/ml) (Table 1).

Identification of IFN-α 2b plus Rib-induced genes in the livers of patients with chronic hepatitis C infection

To identify the genes induced in the liver by combination treatment with IFN-α 2b plus Rib, the gene expression profiles from samples taken around 1 week before and 1 week after initiation of therapy were compared. The pairwise *t*-test comparison showed that 798 genes were up-regulated and 220 genes were down-regulated significantly (p <0.005). The 100 most up-regulated genes according to p values were selected; these are listed in Supplementary Table 1. Many of the interferon-stimulated

genes (ISGs), such as Myxovirus (influenza virus) resistance 1 (MX), 2′,5′-oligoadenylate synthetase (OAS), chemokine (C–C motif) ligand 8 (CCL8), and interferon alpha-inducible protein 27 (IFI 27), were significantly induced (Supplementary Table 1). We designated these genes as *IFN and Rib-stimulated genes* (IRSGs) and analyzed them further.

Hepatic gene expression and responsiveness to IFN-α 2b and Rib combination therapy

To investigate the relationship between hepatic gene expression and responsiveness to treatment, we applied nonsupervised learning methods, hierarchical clustering analysis using all the expressed genes (n = 34,988) from samples taken before and 1 week after initiation of therapy. While hierarchical clustering analysis did not form clusters when done for all patients, it formed two clusters – Rsp and nonRsp – when performed within genotype 1 patient (data not shown).

Fold changes in expression in the 100 most up-regulated IRSGs, before and during therapy, were calculated and subjected to hierarchical clustering, and this clearly differentiated Rsp, which exhibited higher IRSGs induction, from nonRsp, as shown in Fig. 1A and Supplementary Table 1. Despite the rapid virus decline in genotype 2 patients, IRSG induction was not so evident in these patients.

Unexpectedly, the hierarchical clustering of IRSG expression in samples taken before treatment showed a reverse pattern of gene expression (Fig. 2B): IRSG induction was significantly higher in nonRsp than in Rsp. Upon treatment, the expression of IRSGs was more induced in Rsp than in nonRsp (Fig. 1C).

The findings were confirmed in patients who were administered Peg-IFN-α 2b and Rib combination therapy (Table 2). IRSG expression was induced in CH-C infected livers and substantially up-regulated in nonRsp compared with Rsp (Supplementary Fig. 1). Multivariate logistic analysis including age, sex, fibrosis stage, activity, HCV-RNA, genotype, treatment regime, ALT and

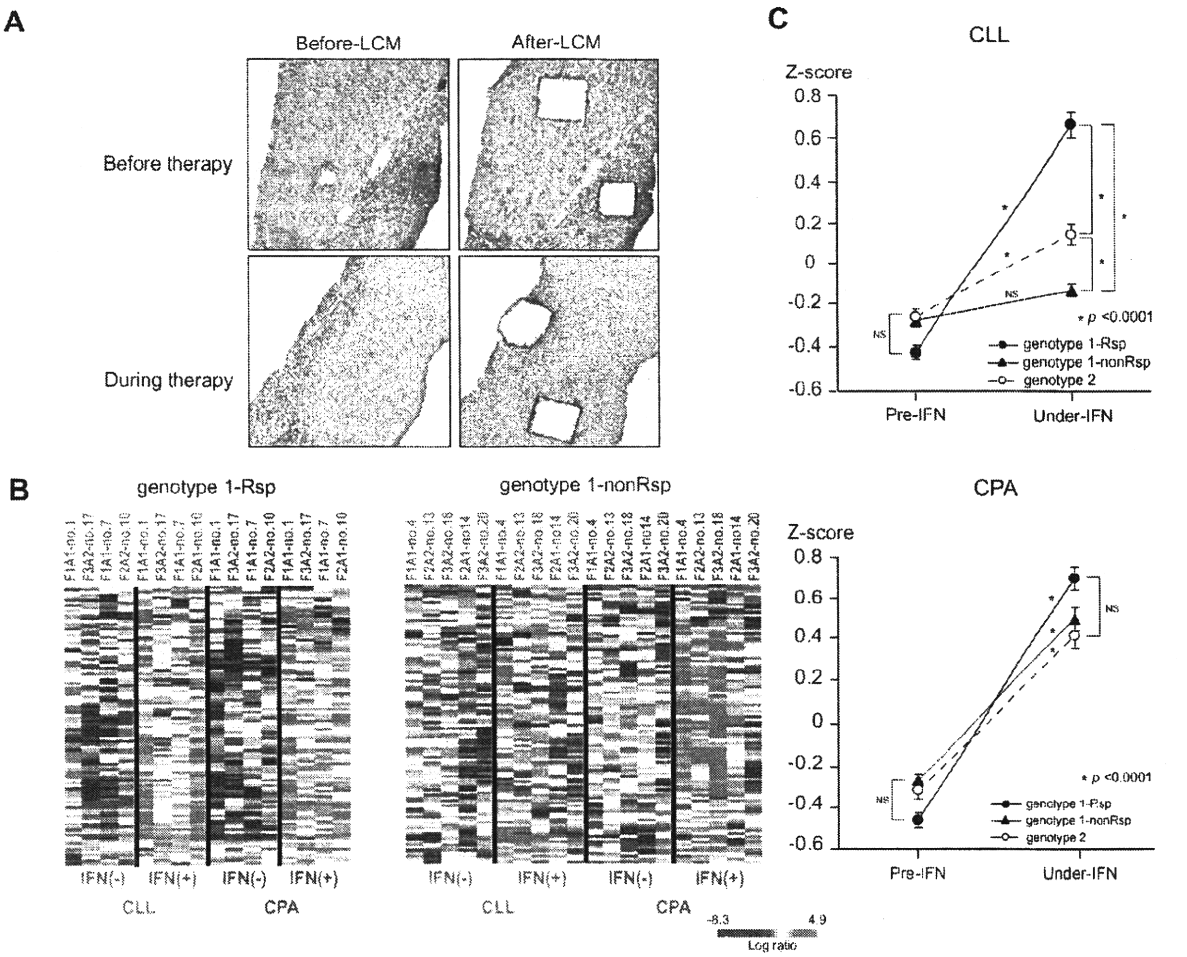

Fig. 2. (A) LCM of liver biopsy samples before and during treatment. (B) Heat map of gene expression of IRSGs in CLL and CPA before and during treatment. (C) Serial changes in standardized expression values (Z-score) of IRSGs in CLL and CPA from genotype 1-Rsp, genotype 1-nonRsp, and genotype 2 patients before and during treatment.

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expression pattern of IRSGs (up or down) of the 50 patients before treatment showed that genotype 2 (p <0.0001, Odds = 4×10^7) and down-regulated IRSGs (p <0.0001, Odds = 71.2) are significant variables associated with SVR.

Gene expression analysis in cells in liver lobules (CLL) and portal area (CPA)

To explore these findings in more detail, we examined the gene expression profiles of CLL and CPA that had been isolated separately from whole liver biopsy specimens of 12 patients, using the LCM method before and during treatment (Fig. 2A). The representative differentially expressed genes between CLL and CPA are shown in Supplementary Tables 2-1 and 2-2. In CLL, liver-specific proteins and enzymes, such as cytochrome P450, apolipoprotein, and transferrin, were all expressed. In CPA, cytokines, chemokines and lymphocyte surface markers, such as chemokine (C–X–C motif) receptor 4, interleukin-7 receptor and CD83 antigen, were all expressed (Supplementary Tables 2-1 and 2-2). The results confirmed our previous speculation that cells from the lobular area were mostly of hepatocyte origin and that those from the portal area were mostly of liver-infiltrating lymphocyte origin [11,19].

IRSG expression in CLL and CPA from genotype 1-Rsp and non-Rsp is shown in Fig. 2B. In genotype 1-Rsp, IRSG expression was significantly induced in both CLL and CPA by the treatment (Fig. 2B and C). On the other hand, in genotype 1-nonRsp and genotype 2, IRSG induction was impaired especially in CLL, while

Table 3. Up- and down-regulated pathways by gene set comparison between Rsp and nonRsp of genotype 1 patients before therapy (BRB-array tool).

Pathway	No. of genes	LS p value	KS p value	Representative Genes	Mean probe intensity of representative genes		
					Rsp (n = 20)	nonRsp (n = 23)	Normal (n = 10)
Up-regulated in slow viral drop							
IFN alpha signaling pathway	21	0.00001	0.00300	STAT1	1608	3117	686
				IRF9	1249	1842	614
				IFNAR2	1892	1988	903
Apoptotic Signaling in Response to DNA Damage	55	0.00001	0.07974	CASP3	675	870	426
				CASP7	1165	1510	1264
				CASP9	355	403	264
				TP53	1465	1797	1028
Toll-like receptor signaling pathway	150	0.00006	0.06659	CXCL10	1922	3979	193
				CXCL11	176	321	51
				MYD88	1022	1372	723
				TIRAP	582	722	447
Wnt signal pathway	55	0.00009	0.16058	EIF2AK2	664	1190	484
				CCND1	2439	3558	1162
				APC	143	186	154
				PIK3R1	1570	1906	682
Antigen processing and presentation	139	0.00117	0.00091	TAP2	169	317	93
				HLA-A	11005	14726	6221
				HLA-B	13144	17942	6823
				HLA-C	1937	3993	783
Jak-STAT signaling pathway	220	0.00180	0.13154	STAT2	716	1065	274
				IL28RA	390	544	204
				IL10RB	398	506	338
Down-regulated in slow viral drop							
Metabolism of xenobiotics by cytochrome P450	98	0.00018	0.00082	CYP3A4	15219	10118	19256
				CYP2E1	29129	24549	30929
				AKR1C4	6126	4898	6671
Fatty acid metabolism	88	0.00480	0.05373	ACADL	826	687	785
				ALDH2	18325	16337	21844
				HSD17B4	9619	8807	10653
				ACAD11	6858	6238	8279
				ACOX1	6988	5862	8279

No. of genes, the number of genes comprising the pathway, Rsp, viral responder, patients with SVR or TR; nonRsp, non-viral responder; patients with NR.

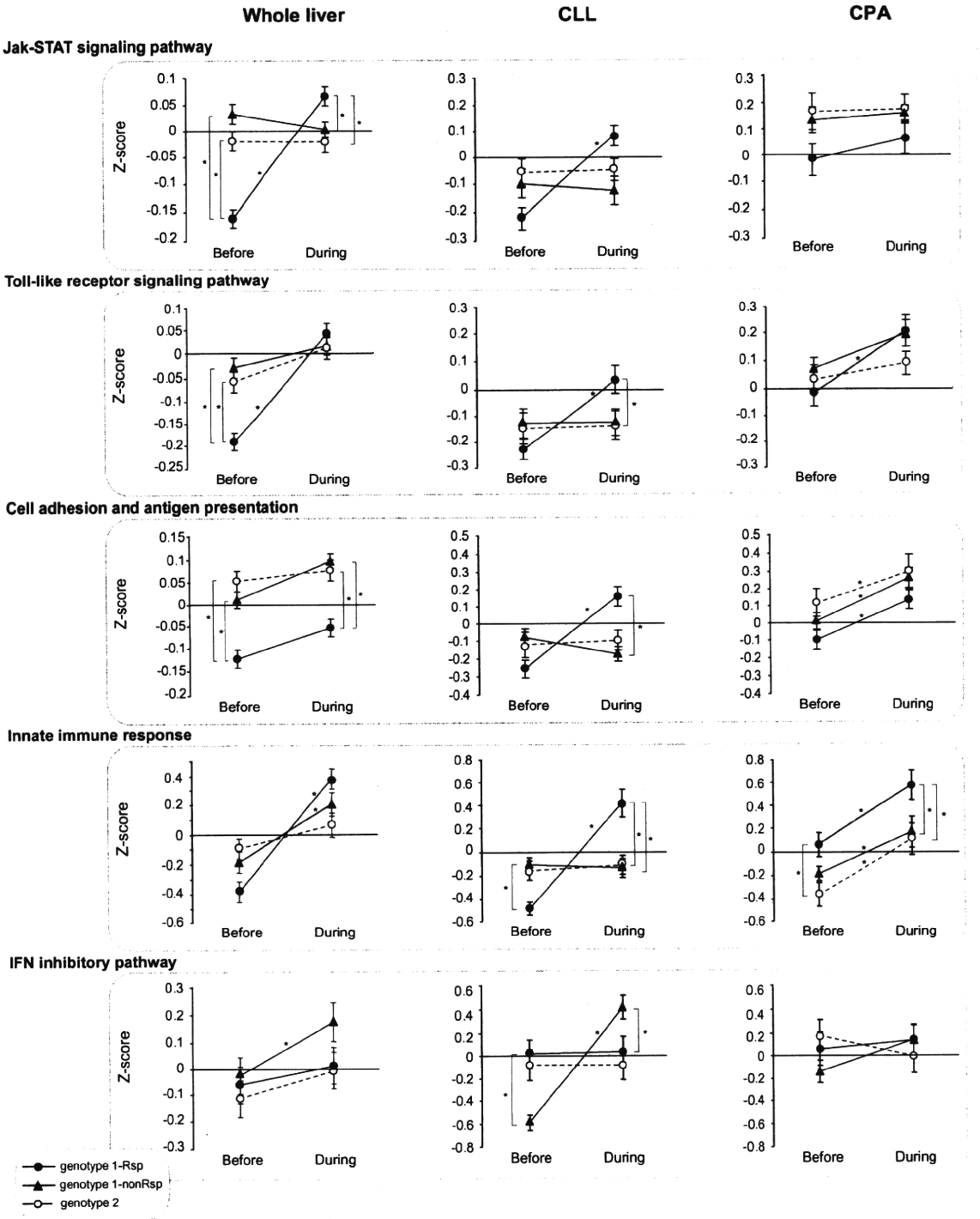

Fig. 3. Serial changes in standardized expression values (*Z*-score) of differentially expressed pathways from genotype 1-Rsp, genotype 1-nonRsp, and genotype 2 patients before and during treatment in whole liver, CLL, and CPA.

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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

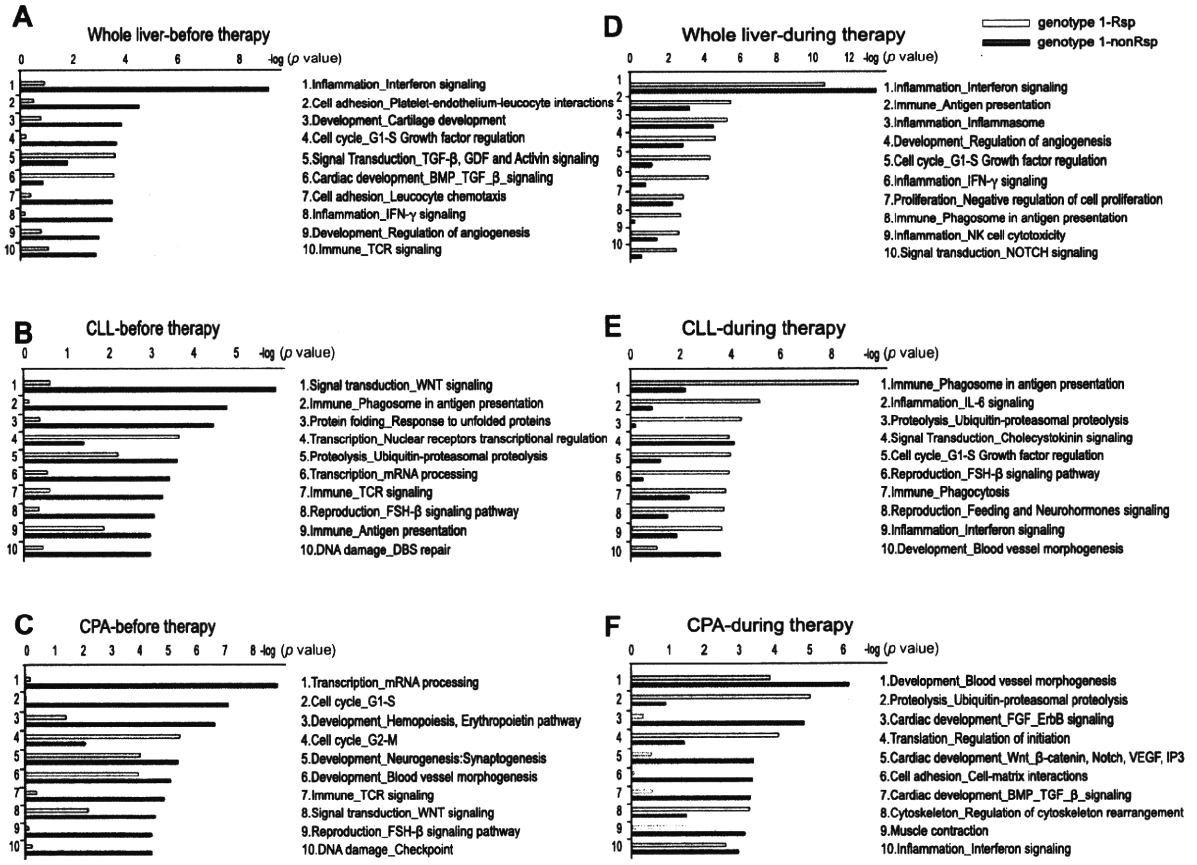

Fig. 4. Functional ontology enrichment analysis of differentially expressed genes ($p < 0.05$) using MetaCore™. GeneGo network process of differentially expressed genes between genotype 1-Rsp (white bar) and genotype 1-nonRsp (blue bar) are listed in order of decreasing statistical significance.

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

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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.

References

[1] Asselah T, Bieche I, Narguet S, Sabbagh A, Laurendeau I, Ripault MP, et al. Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C. Gut 2008;57:516–524.
[2] Asselah T, Bieche I, Sabbagh A, Bedossa P, Moreau R, Valla D, et al. Gene expression and hepatitis C virus infection. Gut 2009;58:846–858.
[3] Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, et al. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology 2005;128:1437–1444.
[4] Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 2003;38:645–652.
[5] Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19: 1513–1520.
[6] Feld JJ, Nanda S, Huang Y, Chen W, Cam M, Pusek SN, et al. Hepatic gene expression during treatment with peginterferon and ribavirin: identifying molecular pathways for treatment response. Hepatology 2007;46: 1548–1563.
[7] Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Goncales Jr FL, et al. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a (40 KDa)/ribavirin. J Hepatol 2005;43: 425–433.
[8] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.
[9] Germer JJ, Harmsen WS, Mandrekar JN, Mitchell PS, Yao JD. Evaluation of the COBAS TaqMan HCV test with automated sample processing using the MagNA pure LC instrument. J Clin Microbiol 2005;43:293–298.
[10] He XS, Ji X, Hale MB, Cheung R, Ahmed A, Guo Y, et al. Global transcriptional response to interferon is a determinant of HCV treatment outcome and is modified by race. Hepatology 2006;44:352–359.
[11] Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Kaneko S. Different signaling pathways in the livers of patients with chronic hepatitis B or chronic hepatitis C. Hepatology 2006;44:1122–1138.
[12] Huang Y, Feld JJ, Sapp RK, Nanda S, Lin JH, Blatt LM, et al. Defective hepatic response to interferon and activation of suppressor of cytokine signaling 3 in chronic hepatitis C. Gastroenterology 2007;132:733–744.
[13] Lanford RE, Guerra B, Bigger CB, Lee H, Chavez D, Brasky KM. Lack of response to exogenous interferon-alpha in the liver of chimpanzees chronically infected with hepatitis C virus. Hepatology 2007;46: 999–1008.
[14] Layden TJ, Layden JE, Reddy KR, Levy-Drummer RS, Poulakos J, Neumann AU. Induction therapy with consensus interferon (CIFN) does not improve sustained virologic response in chronic hepatitis C. J Viral Hepat 2002;9:334–339.
[15] Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, et al. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science 1998;282 (5386):103–107.
[16] Okamoto H, Tokita H, Sakamoto M, Horikita M, Kojima M, Iizuka H, et al. Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. J Gen Virol 1993;74: 2385–2390.
[17] Payan C, Pivert A, Morand P, Fafi-Kremer S, Carrat F, Pol S, et al. Rapid and early virological response to chronic hepatitis C treatment with IFN alpha2b or PEG-IFN alpha2b plus ribavirin in HIV/HCV co-infected patients. Gut 2007;56:1111–1116.
[18] Rosen HR, Ribeiro RR, Weinberger L, Wolf S, Chung M, Gretch DR, et al. Early hepatitis C viral kinetics correlate with long-term outcome in patients receiving high dose induction followed by combination interferon and ribavirin therapy. J Hepatol 2002;37:124–130.
[19] Sakai Y, Honda M, Fujinaga H, Tatsumi I, Mizukoshi E, Nakamoto Y, et al. Common transcriptional signature of tumor-infiltrating mononuclear inflammatory cells and peripheral blood mononuclear cells in hepatocellular carcinoma patients. Cancer Res 2008;68:10267–10279.
[20] Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci USA 2008;105:7034–7039.
[21] Younossi ZM, Baranova A, Afendy A, Collantes R, Stepanova M, Manyam G, et al. Early gene expression profiles of patients with chronic hepatitis C treated with pegylated interferon-alfa and ribavirin. Hepatology 2009;49:763–774.

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

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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.

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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

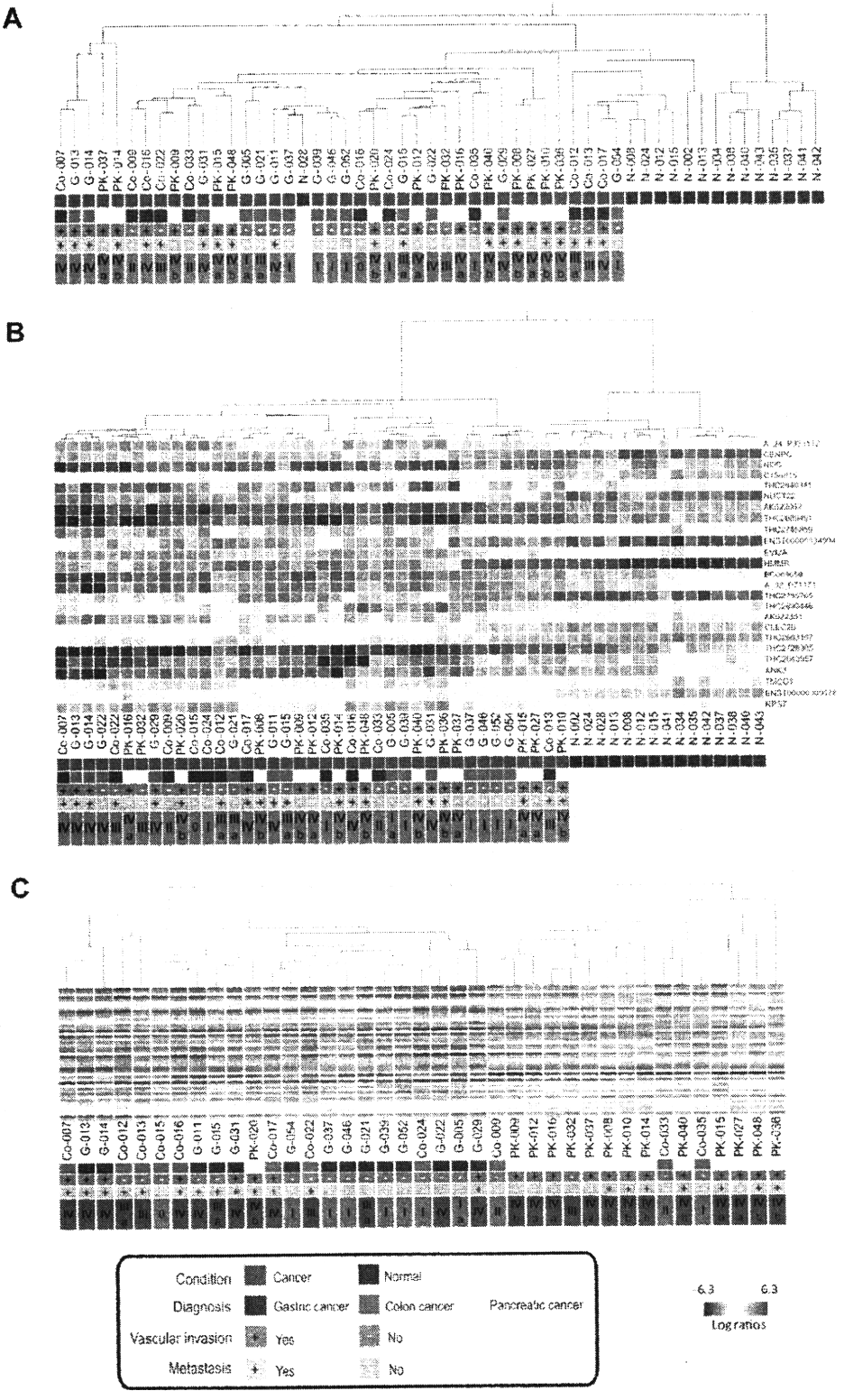

Fig. 1. (A) Hierarchical clustering analysis of 54 training cohort samples based on the expression levels of 23,278 genes. (B) Hierarchical clustering analysis of 54 training cohort samples based on the expression levels of 25 cancer-differentiating genes ($p < 5.0 \times 10^{-6}$ and fold differences >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).

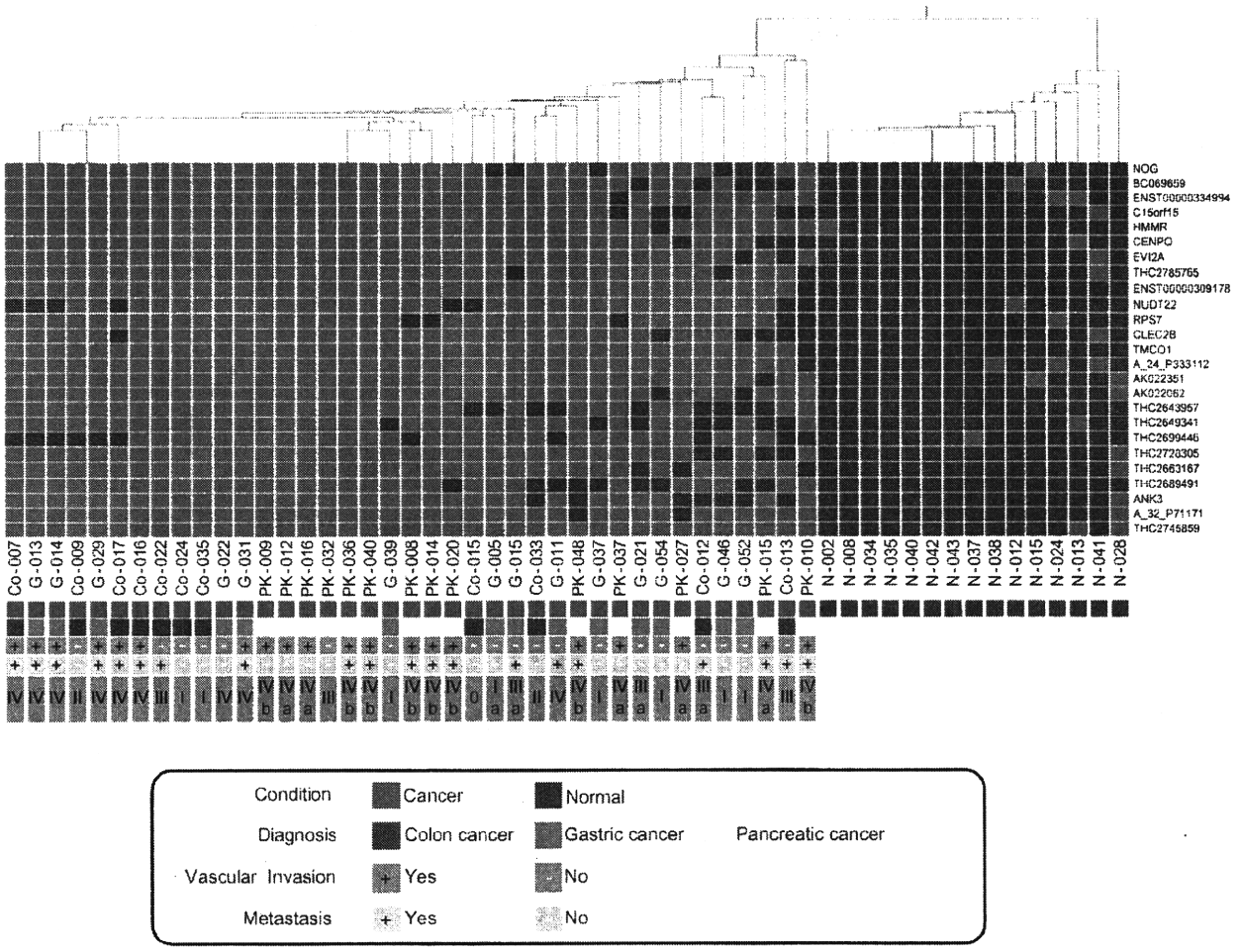

Fig. 2. Hierarchical clustering analysis of 54 training cohort samples based on the standardized expression level (0 or 1).

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

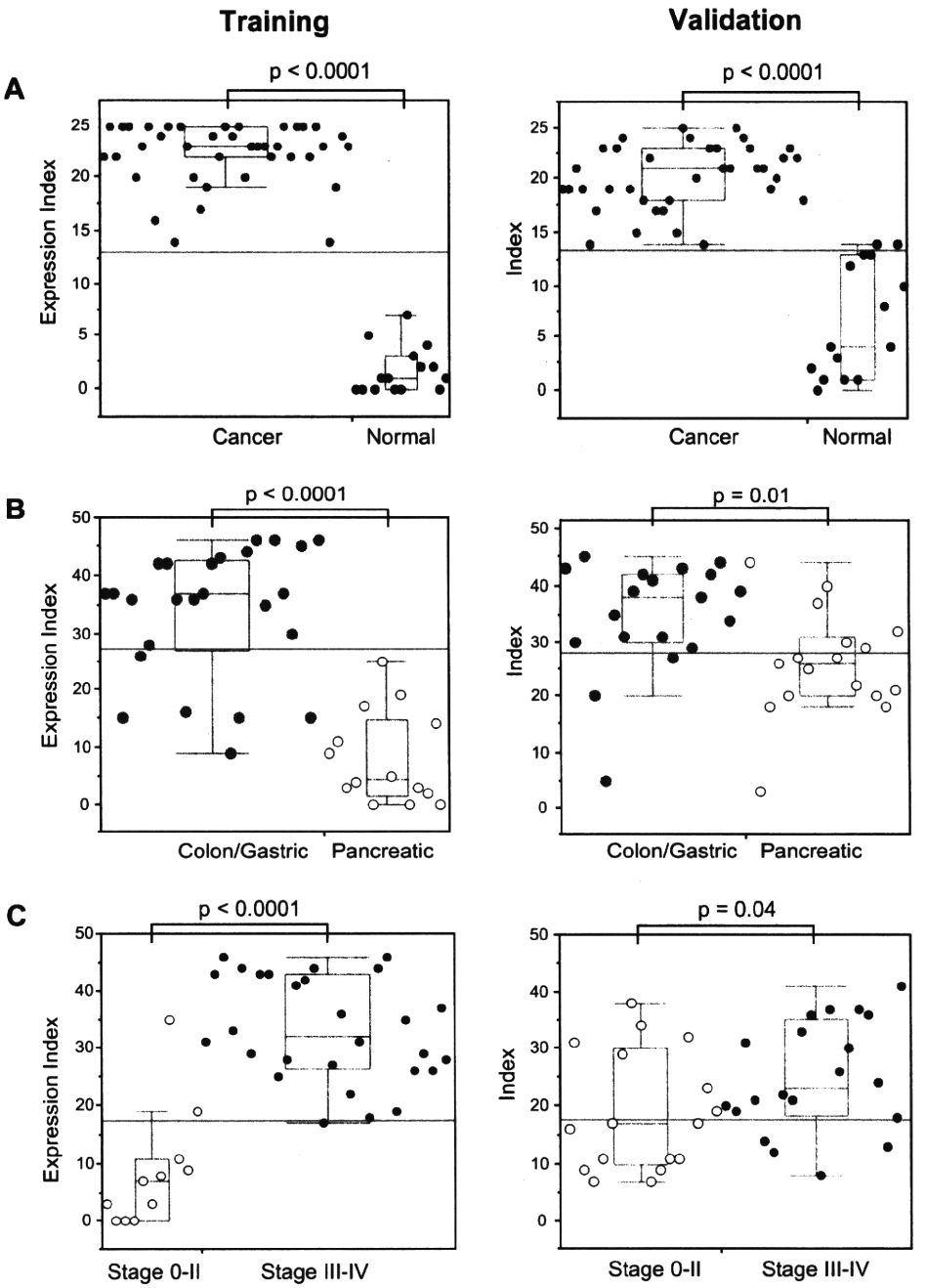

Fig. 3. (A) Calculated expression index of 25 cancer-differentiating genes in the training and validation cohorts. (B) Calculated expression index of 44 GI tract/pancreas-differentiating genes in the training and validation cohorts. (C) Calculated expression index of 45 stage-differentiating genes in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

References

[1] X. Liu, J. Li, Y. Tian, P. Xu, X. Chen, K. Xie, Z. Qiu, Y. Wang, D. Zhang, F. Wolf, C. Li, Q. Huang, Enhanced pancreatic cancer gene therapy by combination of adenoviral vector expressing c-erb-B2 (Her-2/neu)-targeted immunotoxin with a replication-competent adenovirus or etoposide, Hum. Gene Ther. 21 (2004) 157–170.
[2] G. Schneider, J.T. Siveke, F. Eckel, R.M. Schmid, Pancreatic cancer: basic and clinical aspects, Gastroenterology 128 (2005) 1606–1625.
[3] M.E. Burczynski, N.C. Twine, G. Dukart, B. Marshall, M. Hidalgo, W.M. Stadler, T. Logan, J. Dutcher, G. Hudes, W.L. Trepicchio, A. Strahs, F. Immermann, D.K. Slonim, A.J. Dorner, Transcriptional profiles in peripheral blood mononuclear cells prognostic of clinical outcomes in patients with advanced renal cell carcinoma, Clin. Cancer Res. 11 (2005) 1181–1189.
[4] I. Osman, D.F. Bajorin, T.T. Sun, H. Zhong, D. Douglas, J. Scattergood, R. Zheng, M. Han, K.W. Marshall, C.C. Liew, Novel blood biomarkers of human urinary bladder cancer, Clin. Cancer Res. 12 (2006) 3374–3380.
[5] M. Tateno, M. Honda, T. Kawamura, H. Honda, S. Kaneko, Expression profiling of peripheral-blood mononuclear cells from patients with chronic hepatitis C undergoing interferon therapy, J. Infect. Dis. 195 (2007) 255–267.
[6] T. Takamura, M. Honda, Y. Sakai, H. Ando, A. Shimizu, T. Ota, M. Sakurai, H. Misu, S. Kurita, N. Matsuzawa-Nagata, M. Uchikata, S. Nakamura, R. Matoba, M. Tanino, K. Matsubara, S. Kaneko, Gene expression profiles in peripheral blood mononuclear cells reflect the pathophysiology of type 2 diabetes, Biochem. Biophys. Res. Commun. 361 (2007) 379–384.
[7] M. Han, C.T. Liew, H.W. Zhang, S. Chao, R. Zheng, K.T. Yip, Z.Y. Song, H.M. Li, X.P. Geng, L.X. Zhu, J.J. Lin, K.W. Marshall, C.C. Liew, Novel blood-based, five-gene biomarker set for the detection of colorectal cancer, Clin. Cancer Res. 14 (2008) 455–460.

[8] Y. Sakai, M. Honda, H. Fujinaga, I. Tatsumi, E. Mizukoshi, Y. Nakamoto, S. Kaneko, Common transcriptional signature of tumor-infiltrating mononuclear inflammatory cells and peripheral blood mononuclear cells in hepatocellular carcinoma patients, Cancer Res. 68 (2008) 10267–10279.
[9] M.K. Showe, A. Vachani, A.V. Kossenkov, M. Yousef, C. Nichols, E.V. Nikonova, C. Chang, J. Kucharczuk, B. Tran, E. Wakeam, T.A. Yie, D. Speicher, W.N. Rom, S. Albelda, L.C. Showe, Gene expression profiles in peripheral blood mononuclear cells can distinguish patients with non-small cell lung cancer from patients with nonmalignant lung disease, Cancer Res. 69 (2009) 9202–9210.
[10] H. Huang, X. Dong, M.X. Kang, B. Xu, Y. Chen, B. Zhang, J. Chen, Q.P. Xie, Y.L. Wu, Novel blood biomarkers of pancreatic cancer-associated diabetes mellitus identified by peripheral blood-based gene expression profiles, Am. J. Gastroenterol. 105 (2010) 1661–1669.
[11] B. Mesko, S. Poliska, A. Szegedi, Z. Szekanecz, K. Palatka, M. Papp, L. Nagy, Peripheral blood gene expression patterns discriminate among chronic inflammatory diseases and healthy controls and identify novel targets, BMC Med. Genomics 3 (2010) 15.
[12] M.X. Pham, J.J. Teuteberg, A.G. Kfoury, R.C. Starling, M.C. Deng, T.P. Cappola, A. Kao, A.S. Anderson, W.G. Cotts, G.A. Ewald, D.A. Baran, R.C. Bogaev, B. Elashoff, H. Baron, J. Yee, H.A. Valantine, Gene-expression profiling for rejection surveillance after cardiac transplantation, N. Engl. J. Med. 362 (2010) 1890–1900.
[13] K. Satake, T. Takeuchi, T. Homma, H. Ozaki, CA19-9 as a screening and diagnostic tool in symptomatic patients: the Japanese experience, Pancreas 9 (1994) 703–706.
[14] J.E. Kim, K.T. Lee, J.K. Lee, S.W. Paik, J.C. Rhee, K.W. Choi, Clinical usefulness of carbohydrate antigen 19-9 as a screening test for pancreatic cancer in an asymptomatic population, J. Gastroenterol. Hepatol. 19 (2004) 182–186.