Table 2
Peptides

Peptide	Source	Start position	Amino acid sequence	HLA restriction
$MRP3_{503}$	MRP3	503	LYAWEPSFL	HLA-A24
$MRP3_{692}$	MRP3	692	AYVPQQAWI	HLA-A24
$MRP3_{765}$	MRP3	765	VYSDADIFL	HLA-A24
HIV env_{584}	HIV envelope	584	RYLRDQQLL	HLA-A24
CMV $pp65_{328}$	CMV pp65	328	QYDPVAALF	HLA-A24
AFP_{403}	AFP	403	KYIQESQAL	HLA-A24

was <15% of the maximum release for all experiments. For the assay using hepatoma cell lines, the cytotoxic activity was considered positive when it was higher than that of CTL against K562 which shows non-specific lysis. The assay was performed at least three times for each peptide.

2.7. ELISPOT assay

ELISPOT assays were performed as previously described with the following modifications [9,10]. Peptides $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$ were used for the detection of MRP3-specific T cells. Negative controls consisted of a HIV envelope-derived peptide ($HIVenv_{584}$) [24]. Positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$) [25]. The colored spots were counted with a KS ELISpot Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of antigen from the number of spots in its presence. Responses for peptides $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$ in HCC patients were considered positive if more than the mean + 3SD specific spots in healthy normal donors were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in its absence. Responses for peptides $HIVenv_{584}$ and $CMVpp65_{328}$ were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen.

2.8. Tetramer staining and flow cytometry

Peptide $MRP3_{765}$ specific tetramer was purchased from Medical Biological Laboratories Co., Ltd (Nagoya, Japan). Tetramer staining was performed according to a previously reported method with several modifications [10]. In brief, PBMCs were stained with CD8-PerCP (BD PharMingen, San Diego, CA, USA) and tetramer-PE (10 μl) for 30 min at room temperature. Cells were washed, fixed with 0.5% paraformaldehyde/PBS, and analyzed on a FACSCalibur™ flow cytometer. Data analysis was undertaken with CELLQuest™ software (Becton–Dickinson, San Jose, CA, USA).

2.9. Statistical analysis

Data are expressed as means ± SD. The χ^2 test with Yates' correction, Fisher's exact probability test, and the unpaired *t*-test were used for statistical analyses where appropriate. Linear regression lines for the relationship between expression of MRP3 mRNA and MRP3-specific immune responses were calculated using Pearson's correlation coefficient. A level of $P < 0.05$ was considered significant.

3. Results

3.1. Patient profile

The clinical profiles of the patients are shown in Table 1. In 52 patients, HCC was histologically classified as well-, moderately, and poorly differentiated HCC in 17, 31, and 4, respectively. In the other patients, HCC was diagnosed based on typical CT findings and AFP elevation. On tumor classification based on the size and number, the tumor was large (>2 cm) in 79, small (≤2 cm) in 24, multiple in 73, and solitary in 30. Vascular invasion was noted in 30 patients. On tumor classification using the TNM staging of the Union Internationale Contre Le Cancer (UICC) classification system (6th version), 24, 51, 16, 1, 3, and 8 patients were staged I, II, IIIA, IIIB, IIIC, and IV, respectively.

3.2. Expression of MRP3 in hepatoma cell lines and HCC tissues

To investigate the MRP3 expression level in HCC, we measured MRP3 mRNA in 8 hepatoma cell lines by real-time PCR. The expression ratio of MRP3 to β-actin, measured as an internal control, is shown in Fig. 1A. All hepatoma cell lines except HLF expressed MRP3, but the expression level varied among the cell lines. HepG2, Hep3B and Huh7 showed high expression levels, but Alex, HLE, SKHep1 and Huh6 showed low expression levels.

The MRP3 expression level in HCC tissues was compared with non-cancerous tissues in specimens obtained from 20 HCC patients by US-guided needle tumor biopsy or surgical resection. The MRP3 expression level was significantly higher in HCC tissue than in the non-cancerous tissue ($P < 0.05$) (Fig. 1B). In the analysis of the individual MRP3 expression levels, 11 of 20 (55%) HCC tissues showed higher expression level than that of Huh 7 whose average of expression level is 1.0 (Fig. 1C).

3.3. Cytotoxic activity of MRP3 peptide-specific CTL against hepatoma cell lines

Whether the MRP3-derived peptides used were capable of inducing peptide-specific CTL from PBMCs was investigated in at least 10 HCC patients. The CTLs specific for $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$ were induced in 3, 3 and 2 patients, respectively. As shown in Fig. 2A, all CTL induced with $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$ showed high-level cytotoxicity against C1RA24 cells pulsed with the corresponding peptides.

These CTLs exhibited cytotoxicity against hepatoma cell lines with the HLA-A24 molecule and high expression of MRP3, HepG2 and HLE, but not against MRP3-hypoexpressing Huh6 and MRP3-overexpressing Huh7 without HLA-A24 molecule (Fig. 2B).

3.4. T Cell responses to MRP3-derived peptides assessed by IFN-γ ELISPOT analysis

To determine a significant number of T cells that specifically reacted with $MRP3_{503}$, $MRP3_{692}$ and $MRP3_{765}$

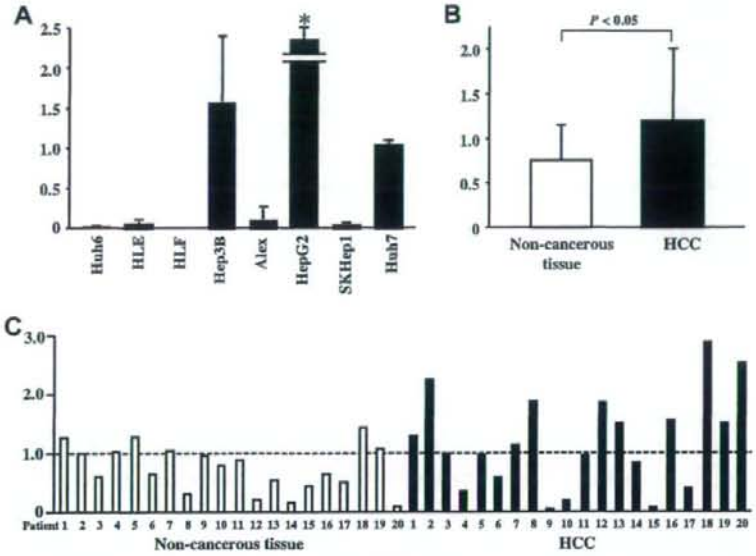

Fig. 1. Expression levels of MRP3 mRNA. (A) Expression of MRP3 mRNA was measured by real-time PCR in hepatoma cell lines. (B) Comparison of MRP3 mRNA expression levels between non-tumor (white bar) and tumor (solid bar) tissues. The data are expressed as means + SD. The unpaired *t*-test was used for a statistical analysis. (C) Comparison of MRP3 mRNA expression levels between non-tumor (white bar) and tumor (solid bar) tissues in individual HCC patients. * denotes 6.15 + 4.21.

peptides in HCC patients, ELISPOT assays were performed using PBMCs from 11 healthy donors. The number of specific spots was 0.2 ± 0.5, 1.5 ± 2.1, 0.9 ± 1.0, 1.3 ± 2.0, and 13.3 ± 15.7 cells/3×10^5 PBMCs, respectively (Fig. 3). Similarly, cells that specifically reacted with the peptides were counted in HCC patient-derived PBMCs. Regarding a number of T cells that specifically reacted with the peptide of larger than the mean + 3SD of that in healthy donor-derived PBMCs as a significant response, 20.0, 14.1, and 21.4% of the patients showed significant responses to $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$, respectively (Fig. 4A). A significant response specific for $CMVpp65_{328}$ was detected in 51.0% and 36.4% of the HCC patients and healthy donors, respectively, showing no significant difference between the 2 groups. On the other hand, no significant response for $HIVenv_{584}$ was observed in both groups.

On similar analysis of TIL, 75.0, 75.0, and 37.5% of the patients showed significant responses to $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$, respectively, revealing that the frequencies were higher than those in PBMCs (Fig. 4B).

3.5. Detection of $MRP3_{765}$ tetramer$^+$ and CD8$^+$ T lymphocytes in PBMCs

The frequency of MRP3-specific T cells was also investigated using $MRP3_{765}$ tetramer in 20 HCC patients. To confirm the specificity of $MRP3_{765}$ tetramer, we tried to detect the tetramer$^+$ cells in a CTL line induced by stimulation with $MRP3_{765}$ peptide. The frequency of $MRP3_{765}$ tetramer$^+$ cells in CD8$^+$ cells was increased from 0.03% before to 9.15% after stimulation (Fig. 5A). When PBMC was stimulated with irrelevant peptide (AFP_{403}), the frequency of $MRP3_{765}$ tetramer$^+$ cells was only 0.08%.

To count the frequency of tetramer$^+$ cells in peripheral blood, we used freshly isolated non-stimulated PBMCs for the assay. The tetramer$^+$ and CD8$^+$ T cells accounted for 0.00–0.23% in PBMCs of HCC patients (Fig. 5B). Next, the results were compared with those of ELISPOT assay. In patients 1 to 7, both $MRP3_{765}$ tetramer$^+$ and IFN-γ producing cells responding to the peptide in ELISPOT assay were detected. In contrast, in patients 8 to 13, the frequency of tetramer$^+$ cells was high, but no significant increase in the $MRP3_{765}$ peptide-specific T cell count was detected by the ELISPOT assay.

3.6. MRP3-specific T cell responses and clinical features of HCC patients

To clarify the clinical characteristics of MRP3-specific T cell responses in HCC patients, the clinical background was compared between patients who showed positive responses to MRP3-derived peptides on ELISPOT assay and those who did not. No significant differences were noted between the 2 groups (Table 3).

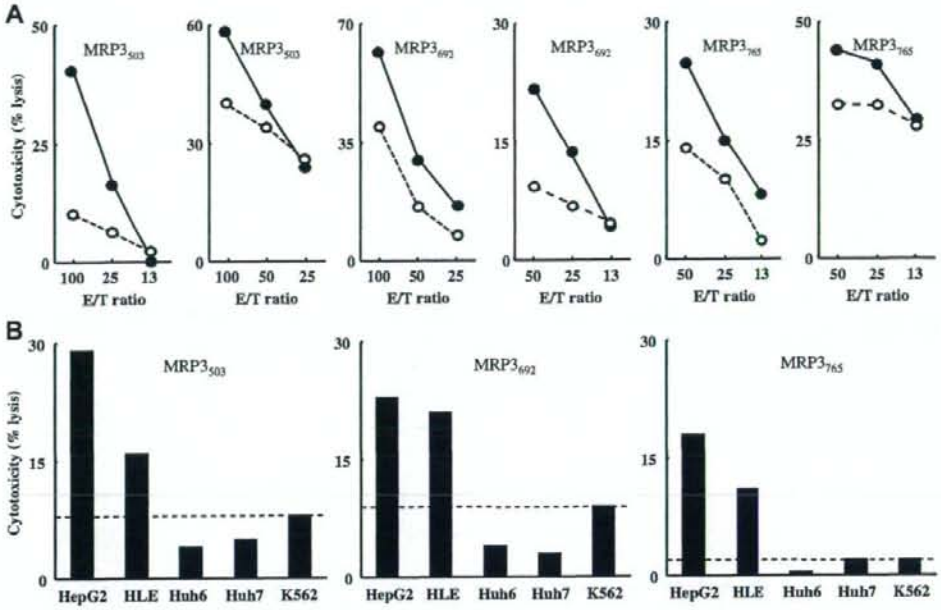

Fig. 2. Cytotoxicity of MRP3-specific T-cell lines derived with peptide in patients with HCC. (A) The cytotoxicity of T-cell lines was determined by a standard 6-h cytotoxicity assay at various effector to target (E/T) ratios against C1R-A*2402 cells pulsed with or without one of the MRP3-derived peptides listed in Table 2. The open circle shows the cytotoxicity against C1R-A*2402 cells pulsed without a peptide. The closed circle shows the cytotoxicity against C1R-A*2402 cells pulsed with a peptide. (B) Cytotoxicity of MRP3-specific T-cell lines derived with peptide was also measured against hepatoma cell lines. The cytotoxicity was considered positive when it was higher than that against K562 which shows non-specific lysis. HepG2 expresses MRP3 and has HLA-A*2402. Huh 6 and HLE show a low expression of MRP3 and have HLA-A*2402. Huh 7 shows MRP3 expression, but does not have HLA-A*2402. Cytotoxicity was determined by a standard 6-h cytotoxic assay (E/T ratio of 50:1).

In 20 HCC patients in whom the MRP3 expression level in HCC tissue could be measured, the relationship between the expression level and frequency of MRP3-specific T cells was investigated. A significant negative correlation was present between the MRP3 expression level in HCC tissue and MRP3-specific T cell frequency ($r = -0.54$, $P < 0.05$) (Fig. 6A). When the relationship between the MRP3 expression level in HCC tissue and CMVpp65-specific T-cell frequency was similarly analyzed, no significant correlation was present. Furthermore, when the patients were divided into groups with high and low HCC tissue MRP3 expression levels, setting the border to the mean MRP3 expression level in the normal liver tissues, 0.743, the peripheral blood MRP3-specific T cell frequency was significantly higher in the low- than in the high-level group ($p < 0.05$) (Fig. 6B). The CMVpp65-specific T cell frequency was not significantly different between the 2 groups.

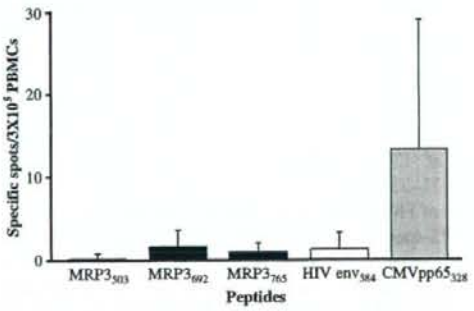

Fig. 3. Direct ex-vivo analysis (IFN-γ ELISPOT assay) of peripheral blood T cell responses to MRP3-derived peptides (peptides $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$: solid bars) or control peptides (peptides $HIVenv_{584}$ and $CMVpp65_{328}$: open and grey bars, respectively) in healthy normal donors. The data are expressed as means + SD.

3.7. Enhancement of MRP3-specific T cell responses after anti-cancer treatment

Several studies including our report have clarified that HCC treatment enhanced HCC-specific immune responses [9,26,27]. We investigated whether MRP3-specific T cell responses observed in HCC patients were enhanced by HCC treatment. In 12 patients who underwent TAE or radiofrequency ablation (RFA) or both

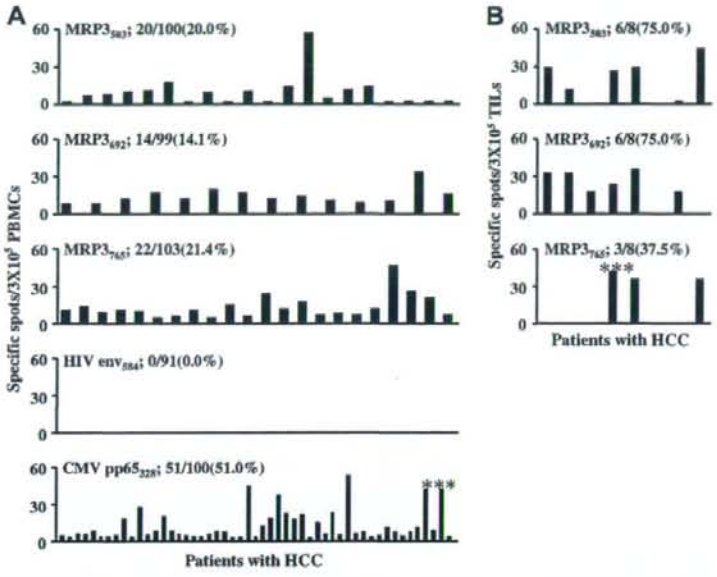

Fig. 4. Direct ex-vivo analysis (IFN-γ ELISPOT assay) of PBMCs (A) and TILs (B) response to MRP3-derived peptides (peptides $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$) or control peptides (peptides $HIVenv_{584}$ and $CMVpp65_{328}$) in HCC patients. Only significant IFN-γ responses are included. Responses to peptides $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$ were considered positive if more than the mean + 3SD specific spots in healthy normal donors were detected and if the number of spots in the presence of antigen was at least twofold greater than that in its absence. Responses to peptides $HIVenv_{584}$ and $CMVpp65_{328}$ were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than that in its absence. The peptide sequences are described in Table 2. * denotes 770 specific spots. ** denotes 210 specific spots. * denotes 72 specific spots.**

without MRP3-specific T-cell responses before treatment, changes in the MRP3-specific T cell frequency were investigated by measuring the frequency by ELISPOT assay before and after treatment. The $MRP3_{503}$, $MRP3_{692}$, or $MRP3_{765}$ peptide-specific T cell frequency was increased after treatment in 8 of the 12 patients (Table 4). In contrast, the immune response to $HIVenv_{584}$ peptide was not enhanced in any patient, and that to $CMVpp65_{328}$ peptide was enhanced in one patient.

4. Discussion

The expression of MRP3 has been reported in several normal tissues and cancer cells [14,15,28,29]. Although MRP3 expression in HCC tissue was confirmed by immunohistochemical staining [16], the expression level varied among patients, and a conclusion has not been reached as to whether the expression is increased compared to that in normal liver tissue [16,17]. In this study, MRP3 expression in HCC tissue was detected in all 20 HCC patients, and the expression level was significantly higher than that in non-cancerous tissue.

The presence of MRP3-recognizing CTL has been reported in lung, colon, bladder, and renal cancer patients [13,30]. However, to our knowledge, there is no report showing the presence of MRP3-specific CTL in HCC patients. In this study, we showed that MRP3-specific CTL could be induced by stimulating PBMCs with MRP3-derived peptides, and the induced CTL showed cytotoxicity against hepatoma cell lines overexpressing MRP3. Based on these findings, we confirmed that MRP3-specific CTLs exist in HCC patients and MRP3 serves as an immunogenic antigen in HCC.

The frequency of peripheral blood CTL specific to each MRP3 epitope was similar to the reported frequencies of CTL against other tumor antigen epitopes [9,10,31–33]. The CTLs were induced even in an early stage of HCC and regardless of HCV infection. In TILs, MRP3-specific CTL were more frequently detected, compared to that in peripheral blood, suggesting that MRP3-specific CTL are not only present in peripheral blood but also infiltrate into the tumor.

The presence and frequency of MRP3-specific CTL were also confirmed using $MRP3_{765}$ tetramer. However, MRP3-specific CTL could not be detected by ELISPOT assay in 6 patients despite a high frequency being

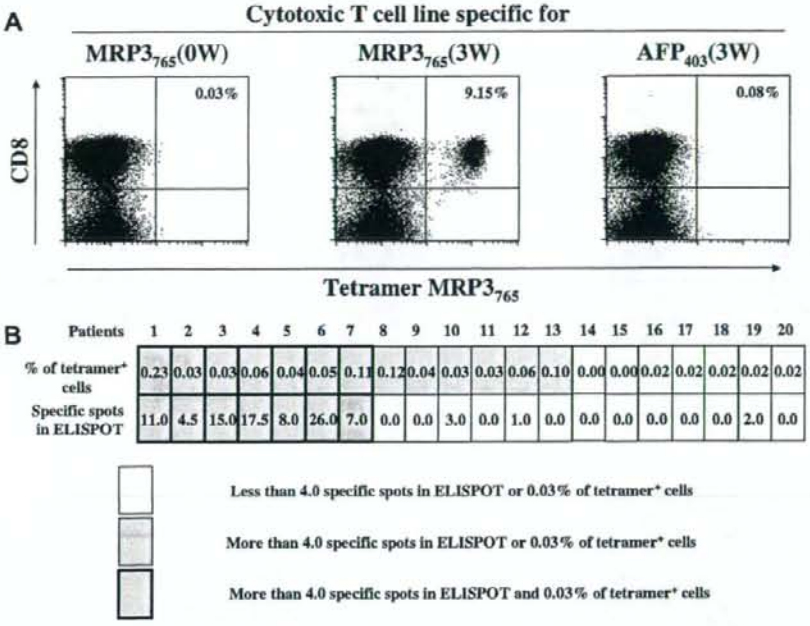

Fig. 5. Detection of MRP3-specific, HLA-A*2402-tetramer$^+$ and CD8$^+$ T lymphocytes in the peripheral blood. (A) Specificity of the $MRP3_{765}$ tetramer was confirmed by staining peptide-specific and non-specific in vitro-expanded T-cell lines. (B) Analysis of the association between the frequency of tetramer$^+$ cells and IFN-γ-producing cells detected on ELISPOT assay was performed in 20 patients.

detected by the tetramer. These findings were similar to those of hTERT-specific CTL in our previous study [10], suggesting the presence of MRP3-specific non-functional T cells in HCC patients.

In the analysis of association between the HCC tissue MRP3 expression level and MRP3-specific T-cell frequency in peripheral blood, a negative correlation was detected, suggesting that MRP3-specific immune responses exert an immune pressure on MRP3-expressing HCC cells. Recent studies have shown the involvement of MRP3 in the resistance to anti-tumor drugs and poor prognosis in several cancer patients [14,15,28,29,34,35]. Taken together with these reports, our results suggest the possibility that MRP3-targeting immunotherapy not only simply eliminates cancers but also improves drug resistance and the prognosis by inhibiting MRP3 expression in cancer cells.

Further to evaluate the usefulness of MRP3 in HCC immunotherapy, we also investigated the association between HCC treatment and the MRP3-specific CTL frequency. As we and other groups previously reported [9,26,27], the MRP3-specific CTL frequency was increased after treatment in 8 of the 12 patients in whom no immune response to MRP3 was detected before treatment, whereas the HIV env_{584}- and $CMVpp65_{328}$-specific CTL frequencies were not increased, excluding one patient, suggesting that this phenomenon represents the enhancement of MRP3-specific immune responses. These findings also confirmed that MRP3 is an antigen expressed in HCC tis-

Table 3
Univariate analysis of the effect of variables on the T cell response against MRP3

	Patients with positive T cell response	Patients without positive T cell response	*p*-value[a]
No. of patients	38	65	
Age (years)[b]	61.4 ± 10.0	64.3 ± 9.7	NS
Sex (M/F)	30/8	49/16	NS
AFP level (≦20/>20)	17/21	21/44	NS
Diff. degree of HCC (well/moderate or poor/ND)[c]	5/14/19	12/21/32	NS
Tumor multiplicity (multiple/solitary)	30/8	43/22	NS
Vascular invasion (+/−)	12/26	18/47	NS
TNM factor			
(T1/T2–4)	6/32	18/47	NS
(N0/N1)	36/2	64/1	NS
(M0/M1)	36/2	57/8	NS
TNM stage (I/II–IV)	6/32	18/47	NS
Histology of non-tumor liver (LC/chronic hepatitis)	30/8	55/10	NS
Liver function (Child A/B/C)	23/14/1	38/25/2	NS
Etiology (HCV/HBV/others)	26/7/5	49/12/4	NS

[a] NS, not significant.
[b] Data are expressed as means ± SD.
[c] ND, not determined.

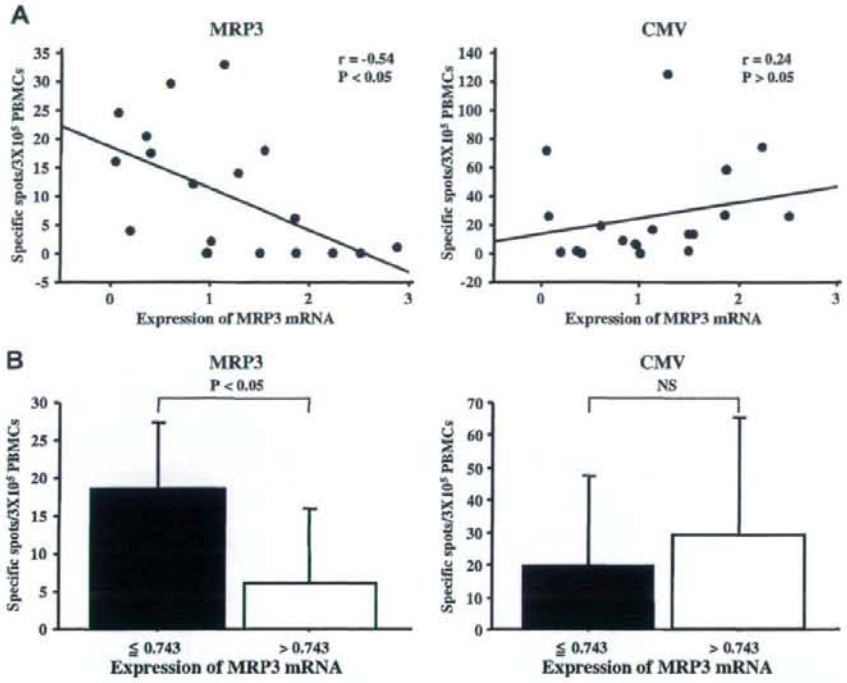

Fig. 6. Analysis of the association between the frequencies of MRP3- specific T cells detected on ELSPOT assay and the expression levels of MRP3 mRNA in HCC tissues. The frequency of MRP3-specific T cells was calculated by the sum of specific spots against $MRP3_{503}$, $MRP3_{692}$, and $MRP3_{765}$ peptides. (A) Linear regression lines for the relationship between the expression of MRP3 mRNA and the frequency of MRP3- or CMVpp65-specific T cells were calculated using Pearson's correlation coefficient. (B) Analysis of the frequency of MRP3- or CMVpp65-specific T cells in patients with low and high expression levels of MRP3 mRNA in HCC tissues.

sue, and has strong immunogenicity that readily induces CTL in vivo.

In conclusion, our study demonstrates that MRP3 is a potential candidate for tumor antigen with strong immunogenicity in HCC immunotherapy.

Acknowledgements

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Table 4
T cell response to MRP3-derived peptides by ELISPOT assay before and after treatment

	Treatment[a]	Before treatment					After treatment				
		$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$HIVenv_{584}$	$CMVpp65_{328}$	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$HIVenv_{584}$	$CMVpp65_{328}$
Patient 1	TAE + RF	1	0	0	0	9	**5**	5	3	2	6
Patient 2	TAE + RF	0	0	0	0	0	**2**	2	0	0	2
Patient 3	TAE + RF	0	2	0	4	5	**8**	1	**6**	2	**13**
Patient 4	TAE + RF	0	0	0	0	**16**	0	0	0	0	**46**
Patient 5	TAE + RF	0	4	0	1	3	**5**	0	**37**	0	ND[b]
Patient 6	TAE + RF	0	0	0	0	**61**	0	0	4	1	**59**
Patient 7	TAE	0	0	0	0	**92**	0	0	**6**	0	**129**
Patient 8	TAE	0	1	0	1	9	0	1	0	0	0
Patient 9	RF	0	0	0	0	**74**	**6**	7	2.5	2.5	**65**
Patient 10	RF	0	0	0	0.5	0	**3**	0	1	0.5	9.5
Patient 11	RF	1.5	0	1	1	9.5	0	3	**9.5**	1	5.5
Patient 12	RF	0	0	0	0	**13**	0	0	0	0	6

Bold and underlined letters indicate a significant increase as described in materials and methods.

[a] TAE, transcatheter arterial embolization; RF, radiofrequency ablation.

[b] ND: not determined.

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Common Transcriptional Signature of Tumor-Infiltrating Mononuclear Inflammatory Cells and Peripheral Blood Mononuclear Cells in Hepatocellular Carcinoma Patients

Yoshio Sakai, Masao Honda, Haruo Fujinaga, Isamu Tatsumi, Eishiro Mizukoshi, Yasunari Nakamoto, and Shuichi Kaneko

Department of Gastroenterology, Kanazawa University, School of Medicine, Kanazawa, Japan

Abstract

Hepatocellular carcinoma (HCC) is frequently associated with infiltrating mononuclear inflammatory cells. We performed laser capture microdissection of HCC-infiltrating and noncancerous liver-infiltrating mononuclear inflammatory cells in patients with chronic hepatitis C (CH-C) and examined gene expression profiles. HCC-infiltrating mononuclear inflammatory cells had an expression profile distinct from noncancerous liver-infiltrating mononuclear inflammatory cells; they differed with regard to genes involved in biological processes, such as antigen presentation, ubiquitin-proteasomal proteolysis, and responses to hypoxia and oxidative stress. Immunohistochemical analysis and gene expression databases suggested that the up-regulated genes involved macrophages and Th1 and Th2 CD4 cells. We next examined the gene expression profile of peripheral blood mononuclear cells (PBMC) obtained from CH-C patients with or without HCC. The expression profiles of PBMCs from patients with HCC differed significantly from those of patients without HCC ($P < 0.0005$). Many of the up-regulated genes in HCC-infiltrating mononuclear inflammatory cells were also differentially expressed by PBMCs of HCC patients. Analysis of the commonly up-regulated or down-regulated genes in HCC-infiltrating mononuclear inflammatory cells and PBMCs of HCC patients showed networks of nucleophosmin, SMAD3, and proliferating cell nuclear antigen that are involved with redox status, the cell cycle, and the proteasome system, along with immunologic genes, suggesting regulation of anticancer immunity. Thus, exploring the gene expression profile of PBMCs may be a surrogate approach for the assessment of local HCC-infiltrating mononuclear inflammatory cells. [Cancer Res 2008;68(24):10267–79]

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies worldwide (1). It commonly develops from chronic liver diseases, such as viral hepatitis (2) and chronic hepatitis, resulting from hepatitis C virus (HCV) infection, is a major risk factor. Indeed, 7% of patients with liver cirrhosis (LC) caused by persistent HCV (LC-C) infection develop HCC annually (3).

Cancer tissues are often associated with infiltrating inflammatory cells, such as tumor-associated macrophages (4), T lymphocytes (5), and antigen-presenting cells (6). These tumor-infiltrating mononuclear inflammatory cells are thought to be important modulators of HCC (7). However, their actual role remains controversial. Increased numbers in HCC have been correlated with a fair prognosis (8), but tumor-infiltrating mononuclear inflammatory cells in HCC tissues have also been found to involve more $FOXP3^+$ regulatory T cells (9) and provide a cancer-favorable environment that leads to resistance to therapy. Characterization of tumor-infiltrating mononuclear inflammatory cells may be valuable in understanding tumor immunology and, possibly, in predicting the prognosis of HCC patients (7).

Peripheral blood mononuclear cells (PBMCs) consist of immune cells, such as monocytes and lymphocytes, and are essential players in the host immune defense system, which responds to various abnormal conditions in the host (10). PBMCs and tumor-infiltrating mononuclear inflammatory cells contain CTLs, specifically cytocidal to cancer tissues (11) and regulatory T cells that can suppress the host immune response against cancer (9). Thus, PBMCs may potentially reflect host immune status. However, there are limited assays for assessing the immune status of PBMCs, such as a proliferation assay, measurements of cytokine production, and the assessment of cytocidal potential.

The advent of cDNA microarray technology for the analysis of gene expression profiles has been useful in comprehensively disclosing underlying molecular features and has provided considerable information for basic science and clinical medicine. We have analyzed gene expression in liver diseases (12, 13) and believe it may become a useful diagnostic tool using liver tissue biopsy samples (14). We have also reported that gene expression profiling of PBMCs predicted the effect of IFN for the eradication of HCV (15) and can provide biomarkers not only for the control of blood sugar but also possibly for predisposing diabetic factors (16). Gene expression profiling of PBMCs from patients with renal cell carcinoma can be used to predict their response to systemic chemotherapy (17). Thus, gene expression information from the cellular components of peripheral blood may be useful in interpreting the internal condition of the patient.

In this study, we used DNA microarray technology to examine differences in gene expression profiles between HCC-infiltrating and noncancerous liver-infiltrating mononuclear inflammatory cells, which were selectively microdissected (12), and the gene expression profiles of PBMCs from LC-C patients with or without HCC. We observed distinct transcriptional features of HCC-infiltrating mononuclear inflammatory cells, reflecting the immune status of the local environment. Intriguingly, the transcriptional features of the HCC-infiltrating mononuclear inflammatory cells were shared with PBMCs from HCC patients. Thus, we suggest the possibility that the gene expression profile of PBMCs may be useful as a clinical surrogate biomarker for the assessment of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Shuichi Kaneko, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan. Phone: 81-76-265-2233; Fax: 81-76-234-4250; E-mail: skaneko@m-kanazawa.jp.

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the internal environment of HCC patients with chronic hepatitis C (CH-C) infection.

Materials and Methods

Study subjects. All patients participating in this study had advanced chronic liver disease, cirrhosis, or persistent HCV infection. Twelve patients who developed HCC as a consequence of advanced chronic liver disease related to hepatitis C and who underwent surgical treatment were enrolled (Supplementary Table S1). HCC and noncancerous liver tissues were obtained and frozen. For analysis of gene expression profiles in PBMCs, 32 LC patients without HCC and 30 LC patients with HCC (Supplementary Table S2) were included. Development of HCC was diagnosed by computed tomography (CT) or magnetic resonance imaging with contrast reagents and abdominal angiography with CT imaging in arterial and portal flow phases (18). The pathologic tumor node metastasis classification system of the Liver Cancer Study Group of Japan was used for the staging of HCC. LC was diagnosed by pathologic findings in biopsy specimens where available; otherwise, radiological imaging, platelet counts, serum hyaluronic acid levels, and indocyanine green retention rates were considered for the diagnosis of cirrhosis. The study has been approved by the institutional review board, and informed consent was obtained from all patients enrolled in the study.

Isolation of PBMCs. PBMCs were isolated from heparinized blood samples by Ficoll-Hipaque density gradient centrifugation, as reported previously (15).

Laser capture microdissection. HCC and noncancerous liver tissues obtained during surgery were frozen in optimum cutting temperature compound (Sakura Finetech; ref. 13). All HCC tissues were nodular and clearly separated by noncancerous tissues macroscopically. Cells infiltrating HCC tissues were visualized under a microscope and precisely excised by laser capture microdissection (LCM) using a CRI-337 (Cell Robotics, Inc.), as previously performed (Supplementary Fig. S1*A*; ref. 12). Cells infiltrating noncancerous tissues of CH-C patients were visualized and excised similarly.

RNA isolation and amplification. Total RNA was isolated from PBMCs or tissue samples using a microRNA isolation kit (Stratagene) in accordance with the supplied protocol with slight modifications. Isolated RNA was then amplified twice using antisense RNA and an Amino Allyl MessageAmp aRNA kit (Ambion), as described previously (13). The reference RNA sample was isolated from the PBMCs of a 29-yr-old healthy male volunteer and was amplified in the same manner. Amplified RNAs from the PBMCs of patients and the healthy volunteer were labeled with Cy5 and Cy3 (Amersham), respectively. Equal amounts of amplified RNAs were hybridized to an oligo-DNA chip (AceGene Human Oligo Chip 30K, Hitachi Software Engineering Co., Ltd.) overnight and were then washed for image scanning.

DNA microarray image analysis. The fluorescence intensity of each spot on the oligo-DNA chip was determined using a DNA Microarray Scan Array G (PerkinElmer). The images obtained were quantified using a DNASIS array (v2.6, Hitachi Software Engineering Co., Ltd). For normalization, the intensity of each spot without oligo-DNA was subtracted from that with oligo-DNA in the same block. A validated spot was determined when the intensity of the spot was within the intensity ± 2 SDs for each block. By calibrating the median to base quantity, the intensities of all spots were adjusted for normalization between Cy5 and Cy3.

Quantitative real-time detection PCR. Real-time detection PCR (RTD-PCR) was performed as previously described (15). Briefly, template cDNA was synthesized from 1 μg of total RNA using SuperScript II RT (Invitrogen). Primer pairs for chemokine (C-C motif) receptor 1 (*Ccr1*), histone acetyltransferase 1 (*Hat1*), mitogen-activated protein kinase kinase 1 interacting protein 1 (*Map2k1ip1*), phosphatidylinositol glycan anchor biosynthesis, class B (*PigB*), toll-like receptor 2 (*Tlr2*), superoxide dismutase 2 (*Sod2*), cytokeratin 8 (*Krt8*), *Krt18*, *Krt19*, and glyceraldehydes-3-phosphate dehydrogenase, as an internal control of expression, were purchased from the TaqMan assay reagents library (Applied Biosystems). Synthesized cDNA was mixed with the TaqMan Universal Master Mix (Applied Biosystems), as well as each primer pair and reaction was performed using ABI PRISM 7900HT. Relative expression level of each gene was calculated compared with that of internal control in each sample. Results are expressed as means $\pm$ SE.

Flow cytometry analysis. Flow cytometry analysis was performed as described previously (19). Briefly, isolated PBMCs were incubated in PBS supplemented with 2% bovine serum albumin (Sigma-Aldrich JAPAN K.K.) with antihuman CCR1 and CCR2 antibodies labeled with Alexa Fluor 647 (Becton Dickinson Pharmingen). The fluorescence intensity of the cells was measured using a FACSort (Becton Dickinson).

Immunohistochemistry. Surgically obtained HCC and noncancerous liver tissues were fixed with neutral buffered formalin, embedded in paraffin, cut into 4-μm sections, and mounted on microscope slides. The fixed slides were deparaffinized and subjected to heat-induced epitope retrieval 98°C for 40 min. After blocking endogenous peroxidase activity in the tissue specimen using 3% hydrogen peroxide, the slides were incubated with appropriately diluted primary antibodies, antihuman CD4 or antihuman CD14 mouse monoclonal antibodies (Visionbiosystems Novocastra). The reaction was visualized by the REAL EnVision Detection System (DAKO) followed by counterstaining with hematoxylin.

Statistical analysis. Hierarchical clustering and principal component analysis of gene expression was performed using BRB-ArrayTools.[1] Fisher's exact test was used to examine the significance of hierarchical clustering in the dendrogram. A class prediction was performed by three nearest neighbors, incorporating genes that were differentially expressed at the $P = 0.002$ significance level, as assessed by the random variance t test (BRB-ArrayTools). For genes to analyze in a pathway, we used a P value of <0.05 with 2,000 permutations to avoid underestimating the presence of meaningful signaling pathways that were coordinately up-regulated or down-regulated with subtle differences (13). The cross-validated misclassification rate was computed, and at least 2,000 permutations were performed for a valid permutation P value. The univariate t values for comparing the classes were used as weights. Student's t-test was performed for RTD-PCR data, and P values of <0.05 were deemed to be statistically significant. The population of CCR1-positive or CCR2-positive cells in PBMCs by flow cytometry analysis was tested for differences (with $P < 0.05$) by the Mann-Whitney U-test, using SPSS software (SPSS Japan, Inc.).

Analysis of expression data for biological processes and networks. As for genes significantly up-regulated or down-regulated in HCC-infiltrating mononuclear inflammatory cells compared with noncancerous liver-infiltrating mononuclear inflammatory cells or in PBMCs in LC without HCC compared with LC with HCC at $P < 0.05$, we have performed analysis of the biological processes using the MetaCore software suite (GeneGo), as described previously (13). Possible networks were created according to the list of the differentially expressed genes using the MetaCore database, a unique curated database of human protein-protein and protein-DNA interactions, transcription factors, and signaling, metabolic, and bioactive molecules. The P value was calculated as described previously (13).

Gene expression data of major leukocyte types and analysis of DNA microarray expression data. Gene expression data for leukocytes were retrieved through publicly accessible databases.[2] The gene set database GDS1775, which includes gene expression data for major leukocyte types, was obtained and subjected to one-way clustering analysis using BRB-Array Tools with genes that were up-regulated in HCC-infiltrating mononuclear inflammatory cells for the enrolled cases above.

Results

Gene expression in mononuclear inflammatory cells infiltrating into HCC tissue. HCC is frequently associated with infiltrating mononuclear inflammatory cells (20), and various attempts have been made to understand their biological significance

[1] http://linus.nci.nih.gov/BRB-ArrayTools.html
[2] http://www.ncbi.nlm.nih.gov/geo/

(8, 9, 21). We selectively obtained HCC-infiltrating mononuclear inflammatory cells by LCM and compared their gene expression profiles with those of noncancerous liver-infiltrating mononuclear inflammatory cells obtained in the same way (Supplementary Fig. S1A; Supplementary Table S1). The gene expression profiles of HCC-infiltrating mononuclear inflammatory cells showed that 115, 206, and 773 genes were up-regulated and 52, 114, and 750 genes were down-regulated compared with those of noncancerous liver-infiltrating mononuclear inflammatory cells at P levels of <0.005, <0.01, and <0.05, respectively (Geo accession no.[3] GSE 10461; Supplementary Fig. S1B).

Genes at the $P < 0.05$ level were analyzed with regard to their role in biological processes in HCC-infiltrating mononuclear inflammatory cells compared with noncancerous liver-infiltrating mononuclear inflammatory cells using the MetaCore pathway analysis software. The significant processes, in which the up-regulated genes in HCC-infiltrating mononuclear inflammatory cells were involved, included antigen presentation, an immunologically important process in antigen-presenting cells, such as monocyte/macrophages and dendritic cells (Table 1; ref. 22). The genes involved in this process were the genes for the CD1d molecule and C-type lectin domain family 4 for glycolipid antigen recognition (23, 24) and CD86, an accessory molecule indispensable for provoking an immune response (25), suggesting an activated immune reaction in these cells. The up-regulated genes in HCC-infiltrating mononuclear inflammatory cells were also involved in the ubiquitin-proteasomal proteolysis process, with significant genes, such as those encoding ubiquitin-conjugating enzymes and proteasome subunits. This process is required to eradicate unnecessary proteins, which are ubiquitinated, and then degraded in proteasomes (26). Processes related to the steps of gene expression, such as transcription by RNA polymerase II, mRNA processing, and the process of the cell cycle were also represented in the genes up-regulated in HCC-infiltrating mononuclear inflammatory cells, indicating enhanced cellular activity. Genes involved in the process of double-strand breaks, such as topoisomerase II α4 (27), and proliferating cell nuclear antigen (PCNA; ref. 28) genes involved in responses to hypoxia and oxidative stress, such as thioredoxin, peroxiredoxin, and antioxidant protein, were also up-regulated, suggesting that HCC-infiltrating mononuclear inflammatory cells were in an activated inflammatory status and under hypoxic or oxidative stress, presumably caused by the HCC. Thus, the profile of up-regulated genes in HCC-infiltrating mononuclear inflammatory cells suggested an inflammatory status, possibly triggered by antigenic stimulation of HCC tissues.

Fewer processes were identified for the down-regulated genes. One intriguing process identified was that of integrin-mediated cell matrix adhesion, suggesting that HCC-infiltrating mononuclear inflammatory cells may be less adhesive in the local tissues where they were found (Supplementary Table S3).

Subpopulation analysis of HCC-infiltrating mononuclear inflammatory cells using immunohistochemistry and transcriptional analysis. Tumor-infiltrating mononuclear inflammatory cells consist of a mixed cell population, including macrophages, effector T cells, and regulatory T cells, which have been considered to be both cancer-favorable or cancer-unfavorable (8, 21). HCC-infiltrating and noncancerous liver-infiltrating mononuclear inflammatory cells were immunohistochemically evaluated to examine the characteristics of the subpopulations. CD14-positive monocytes/macrophages were prominent in HCC-infiltrating mononuclear inflammatory cells, whereas they were rarely observed in noncancerous liver-infiltrating mononuclear inflammatory cells (Fig. 1A). CD4-positive helper T cells were observed in both HCC tissues and noncancerous liver tissues, although in noncancerous liver tissues, these cells tended to accumulate within the aggregates of mononuclear inflammatory cells, whereas they seemed to be scattered in HCC-infiltrating mononuclear inflammatory cells (Fig. 1A).

Next, we examined the genes that were significantly up-regulated in HCC-infiltrating mononuclear inflammatory cells compared with noncancerous liver-infiltrating mononuclear inflammatory cells, relative to subpopulations of leukocytes, and explored how they may be relevant to leukocyte subpopulations, using the database of the human immune cell transcriptome in the Gene Expression Omnibus[3] (Geo accession no. GDS1775), which covers 26 immune regulatory cells, such as T cells, B cells, natural killer cells, macrophages, dendritic cells, basophils, and eosinophils. Among the 206 extracted, up-regulated genes in HCC-infiltrating mononuclear inflammatory cells (at the $P < 0.01$ level), 97 annotated genes were used for one-way hierarchical clusters (Fig. 1B). Most genes among 97 annotated up-regulated genes in HCC-infiltrating mononuclear inflammatory cells were shown to be expressed with higher magnitude in lipopolysaccharide-stimulated or lipopolysaccharide-unstimulated macrophages than in other types of major leukocytes. The next subpopulations, including the second most number of genes for relatively high magnitude of expression, were Th1 and Th2 CD4 cells under conditions supplemented with interleukin-12 (IL-12) and IL-4, respectively (Geo accession no.[3] GSM90858), secreting Th1 and Th2 cytokine profiles, respectively, suggesting that featured genes expressed in HCC-infiltrating mononuclear inflammatory cells were indicative of CD4 helper T cells, secreting a variety of cytokines.

Thus, this expression analysis showed that, in HCC lesions with tumor antigens, there was an accumulation of antigen-presenting cells, monocyte/macrophages, and CD4 helper T cells, which were in a cytokine-secreting condition, with enhanced cellular biological activities, including ubiquitin-proteasomal proteolysis, presumably under a hypoxic and oxidative stress environment caused by the HCC. The overall inflammatory status represented by HCC-infiltrating mononuclear inflammatory cells was not determined in terms of an anticancer effect, because no obvious shift of CD4 helper T cells to the Th1 or Th2 condition was indicated.

Distinct gene expression profile of PBMCs obtained from patients with cirrhotic liver disease complicated with HCC. The HCC-infiltrating mononuclear inflammatory cells were distinct in terms of expressed genes. The putative biological processes involving these up-regulated genes in tumor-infiltrating mononuclear inflammatory cells suggested a general influence of the HCC on the local environment of the host, represented by stress-response genes. We, thus, examined whether PBMCs in the systemic circulation of the patient might also be influenced by the development of HCC. PBMCs were obtained from 30 patients with LC associated with HCC and from 32 patients with LC not associated with HCC, and the gene expression profiles were compared (Geo accession no.[3] GSE10459).

Unsupervised hierarchical clustering analysis using 17,903 filtered genes, the expression values of which were not missing in >50% of the cases, identified two major clusters of patients, with and without HCC (data not shown). To examine the reproducibility and the reliability of the clustering, we excluded

Table 1. Biological processes for genes up-regulated in HCC-infiltrating mononuclear inflammatory cells

Biological process	−log(*P*)	Gene	ID	*t* (*T/[1]NT)	*P*	Cellular components [1]
Antigen presentation	8.526	CD163	NM_004244	3.96	0.001	M
		CD86 antigen	NM_006889	3.28	0.006	M
		IFN, α-inducible protein 6	NM_022872	2.99	0.031	M
		IFN, γ-inducible protein 30	NM_006332	2.89	0.011	M
		Fc fragment of IgG, high affinity Ia, receptor (CD64)	NM_000566	2.85	0.013	M
		C-type lectin domain family 4, member M	NM_014257	2.73	0.020	
		CD63	NM_001780	2.51	0.024	M
		CD1D antigen	NM_001766	2.19	0.049	
Ubiqutin-proteasomeal proteolysis	6.555	Nucleoporin 107 kDa	NM_020401	4.32	0.001	
		Proteasome subunit, β type, 5	NM_002797	3.80	0.002	T, M
		Ubiquitin-conjugating enzyme E2R 2	NM_017811	3.67	0.004	
		Proteasome subunit, α type, 5	NM_002790	3.64	0.003	
		Prostaglandin E synthase 3	NM_006601	3.53	0.003	
		Ubiquitin-conjugating enzyme E2 binding protein, 1	NM_005744	2.94	0.011	
		Ubiquitin-conjugating enzyme E2E 3	NM_006357	2.75	0.017	
		DnaJ (Hsp40) homologue, subfamily A, member 1	NM_001539	2.47	0.028	
		Syntaxin 5	BC012137	2.19	0.046	
ER and cytoplasm	5.704	Chaperonin containing TCP1, subunit 8 (θ)	NM_006585	3.71	0.002	T, M
		Peptidylprolyl isomerase A	NM_021130	3.69	0.002	
		ERO1-like	NM_014584	3.03	0.009	T, M
		Peptidylprolyl isomerase C	BC002678	2.68	0.017	M
		SEC63 homologue	AF119883	2.59	0.020	
		Peptidylprolyl isomerase B	NM_000942	2.54	0.023	
		Chaperonin containing TCP1, subunit 4 (δ)	NM_006430	2.53	0.023	
		FK506 binding protein 3, 25 kDa	NM_002013	2.46	0.026	T, M
		Heat shock 70 kDa protein 5	AF188611	2.45	0.027	
mRNA processing	5.143	Small nuclear ribonucleoprotein polypeptide B	NM_003092	4.65	0.000	
		Small nuclear ribonucleoprotein polypeptide F	BC002505	3.28	0.005	T
		DEAD (Asp-Glu-Ala-Asp) box polypeptide 20	NM_007204	3.22	0.006	
		Cleavage and polyadenylation specific factor 6	NM_007007	3.16	0.010	
		Cleavage stimulation factor subunit 2	NM_001325	3.10	0.008	T
		Heterogeneous nuclear ribonucleoprotein A2/B1	NM_031243	2.94	0.010	
		PRP4 pre-mRNA processing factor 4 homologue B	NM_003913	2.90	0.020	
		Gem-associated protein 4	NM_015721	2.64	0.019	T
		LSM6 homologue	NM_007080	2.63	0.019	
		Exportin 1	NM_003400	2.42	0.029	
		RNA-binding motif protein 8A	AF127761	2.41	0.030	
		Splicing factor, arginine/serine-rich 1	M72709	2.39	0.036	
Transcription by RNA polymerase II	4.298	TAF9 RNA polymerase II	NM_016283	5.01	0.001	
		General transcription factor IIH, polypeptide 3, 34 kDa	NM_001516	4.74	0.001	
		TAF6-like RNA polymerase II	NM_006473	3.91	0.002	
		Nuclear receptor corepressor 1	AF044209	3.64	0.007	
		TATA box binding protein	NM_003194	2.89	0.018	

(*Continued on the following page*)

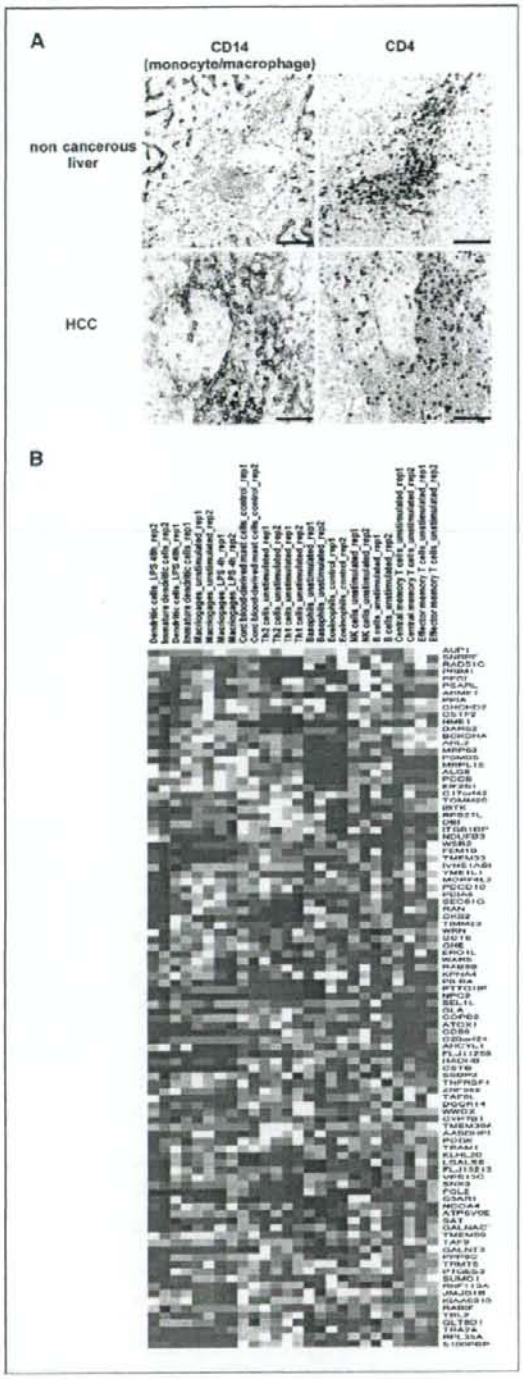
Table 1. Biological processes for genes up-regulated in HCC-infiltrating mononuclear inflammatory cells (Cont'd)

Biological process	−log(*P*)	Gene	ID	*t* (*T/†NT)	*P*	Cellular components‡
		Cofactor required for Sp1 transcriptional activation	NM_004270	2.82	0.014	T, M
		SUB1 homologue	NM_006713	2.59	0.021	
		General transcription factor II, I	NM_033001	2.55	0.023	T, M
		GCN5-like 2	NM_021078	2.34	0.048	
		TBP-like 1	NM_004865	2.24	0.043	
Double-strand breaks repair	3.289	RAD51 homologue C	NM_058216	5.24	0.000	T
		Werner syndrome	AF091214	4.99	0.000	T
		NIMA-related kinase 1	AK027580	3.27	0.007	
		Protein phosphatase 2	AF086924	3.24	0.023	
		Protein phosphatase 6	NM_002721	3.13	0.007	
		Proliferating cell nuclear antigen	NM_002592	2.80	0.014	T
		Topoisomerase II α-4	AF285159	2.57	0.033	T
ESR1-nuclear pathway	2.886	Nuclear receptor corepressor 1	AF044209	3.64	0.007	
		Nuclear receptor coactivator 4	X77548	3.19	0.007	
		Dopachrome tautomerase	NM_001922	3.04	0.019	
		COP9, subunit 5	NM_006837	2.77	0.014	
		Tissue specific extinguisher 1	NM_002734	2.70	0.018	M
		SCAN domain containing 1	NM_033630	2.50	0.026	
		Kinase insert domain receptor	NM_002253	2.35	0.047	
Cell cycle	2.241	Cyclin-dependent kinase inhibitor 3	NM_005192	4.60	0.000	
		Erythrocyte membrane protein band 4.1	NM_004437	3.47	0.014	
		RAN, member RAS oncogene family	NM_006325	3.38	0.004	T
		Cyclin C	NM_005190	3.14	0.008	
		Cell division cycle 42	NM_044472	3.14	0.007	
		Cyclin-dependent kinase-like 1	NM_004196	2.77	0.033	
		Cell division cycle 73	NM_024529	2.72	0.043	M
		Cell division cycle 27	NM_001256	2.57	0.043	
		Microtubule-actin cross-linking factor 1	AK023285	2.57	0.025	
		Histone cluster 1	NM_005323	2.30	0.047	
		Cyclin-dependent kinase 7	NM_001799	2.13	0.050	
		Cyclin G_2	NM_004354	2.48	0.038	
Response to hypoxia and oxidative stress	1.401	Thioredoxin	NM_003329	2.64	0.019	T, M
		Glutaredoxin 2	NM_016066	2.63	0.024	T, M
		Peroxiredoxin 3	NM_006793	2.81	0.016	T, M
		Peroxiredoxin 2	NM_005809	2.27	0.039	
		Antioxidant protein 2	NM_004905	2.22	0.042	
		Peroxiredoxin 1	NM_002574	2.21	0.043	T, M
		Microsomal glutathione *S*-transferase 2	NM_002413	2.41	0.031	M

*T represents tumor-infiltrating mononuclear inflammatory cells.
†NT represents non–tumor-infiltrating mononuclear inflammatory cells.
‡Cellular components predominantly expressed cellular components among 26 immune regulatory cells (T, Th cells; M, macrophage).

unchanged genes in all samples (genes with less than a 1.8-fold difference in >85% of samples) to remove noise. This hierarchical clustering analysis using 1,917 filtered genes confirmed two clear clusters in patients with or without HCC (Fig. 2*A*). In one major cluster, including the most LC cases, there was a subcluster, LC/HCC, which included more of the HCC patients located next to the cluster of patients with HCC (LC/HCC; Fig. 2*A*). The reproducibility of the clustering (proportion, averaged over replications and over all pairs of samples in the same cluster, BRB-ArrayTools) was 93%. Sensitivity and specificity to HCC in this cluster analysis is 88% and 76%, respectively. These cirrhotic patients without HCC were followed for at least a further 12 months to detect HCC; none of those in the LC group developed HCC over this time. The principal component analysis was performed with the filtered 1,917 genes and the two major groups; classifying LC and HCC were similarly observed (Fig. 2*B*).

To further confirm that gene expression in the PBMCs of patients with HCC was distinct from that in patients without HCC, analysis of PBMC gene expression was performed by a



supervised learning method using categories of LC-C or HCC, age, gender, serum alanine aminotransferase (ALT), and α-fetoprotein (AFP). It showed that patients with or without HCC were significant classifiers ($P < 0.0005$), assigned with 1,430 predictor genes ($P < 0.002$; Table 2). Of 32 patients with LC, eight (25%) were misclassified as having HCC, and 2 of 30 patients with HCC (6.7%) were misclassified as not having HCC, indicating that the overall accuracy of the prediction of a patient with or without HCC was 84% (Table 2). Other clinical variables supposed to be related to HCC occurrence, such as age (ref. 29; >68 or ≤ 68 years old), gender (30), and ALT(ref. 31; >50 or ≤50 IU/L), could not differentiate gene expression in PBMCs. AFP (>20 or ≤20 ng/mL) was actually significant but was a much less powerful classifier ($P < 0.02$, assigned with 301 classifier genes). The prediction accuracy for categories of LC-C versus HCC and the AFP value >20 versus ≤20 ng/mL is not significantly affected whenever the number of predictor genes is reduced to below 62 (Supplementary Fig. S2). Taken together, these results by unsupervised and supervised analysis methods indicate that HCC development in LC-C patients significantly affects the gene expression profile in PBMCs.

Features of biological processes for which gene expression was significantly altered in PBMCs in HCC patients. We next examined the biological processes possibly affected by HCC development, given the expression profiles in PBMCs from patients with HCC. Statistical analysis showed that 867 genes were up-regulated and 989 genes were down-regulated in PBMCs from patients with HCC, compared with those without HCC ($P < 0.005$). Six representative genes, *Ccr1, Hat, Map2k1ip1, PigB, Tlr2,* and *Sod2,* were randomly selected from genes which were biologically important and differentially expressed between LC and HCC groups, and their expression was confirmed by RTD-PCR (Supplementary Fig. S3*A*). To exclude the possibility of circulating cancer cells, we have also examined the expression of *Afp, Krt8, Krt18,* and *Krt19*. No expression was detected for *Afp* (data not shown), and no statistically significant difference was found for expression of *Krt8, Krt18,* and *Krt19* between patients with HCC and without HCC (Supplementary Fig. S3*A*). The expression data were also confirmed by flow cytometric analysis. We evaluated how many cells in blood expressed CCR1 and CCR2 and confirmed that populations expressing CCR1 and CCR2 were significantly higher in PBMCs from patients with HCC than those without (Supplementary Fig. S3*B*). To understand the biological processes in PBMCs for which up-regulated or

Figure 1. HCC-infiltrating mononuclear inflammatory cells involve monocyte/macrophage and helper T cell. *A*, immunohistochemical staining. Many of the HCC-infiltrating mononuclear inflammatory cells expressed monocyte/macrophage marker, CD14. In contrast, few CD14-positive cells were seen in noncancerous liver-infiltrating mononuclear inflammatory cells. *Bars*, 100 μm. *B*, one-way hierarchical clustering analysis of gene expression of immune-mediating cells with genes whose expression was up-regulated in HCC-infiltrating mononuclear inflammatory cells. Data for gene expression in immune-mediating cells were retrieved from Gene Expression Omnibus[2] (Geo accession no. GDS 1775). By excluding genes missing from over half of the immune-mediating cells, 206 genes up-regulated in HCC-infiltrating mononuclear inflammatory cells were filtered, and the remaining 97 genes were used for clustering. Transverse and longitudinal titles show the type of immune-mediating cell and gene symbols, respectively. Color indicates relative expression magnitude of 97 up-regulated genes HCC-infiltrating mononuclear inflammatory cells among retrieved expression data of major leukocyte types deposited in the public database. The red and blue color means relatively high or low magnitude of expression among 26 retrieved expression data of leukocytes. The heat-map shows that helper T cells and unstimulated or stimulated macrophages included more blocks with the red color.

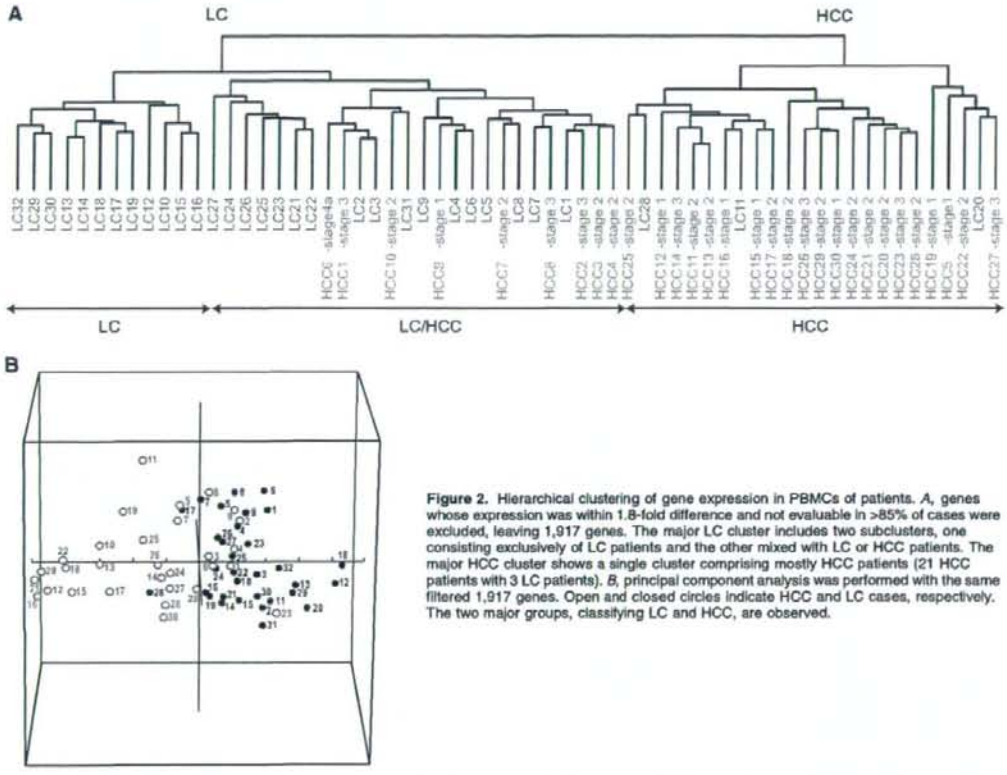


Figure 2. Hierarchical clustering of gene expression in PBMCs of patients. *A*, genes whose expression was within 1.8-fold difference and not evaluable in >85% of cases were excluded, leaving 1,917 genes. The major LC cluster includes two subclusters, one consisting exclusively of LC patients and the other mixed with LC or HCC patients. The major HCC cluster shows a single cluster comprising mostly HCC patients (21 HCC patients with 3 LC patients). *B*, principal component analysis was performed with the same filtered 1,917 genes. Open and closed circles indicate HCC and LC cases, respectively. The two major groups, classifying LC and HCC, are observed.

down-regulated genes were observed, we used MetaCore. The up-regulated genes in PBMCs from patients with HCC were involved in processes such as ubiquitin-proteasomal proteolysis (e.g., heat shock 70 kDa protein 4, ubiquitin conjugating enzymes), mRNA processing (e.g., heterogeneous nuclear ribonucleoproteins, RNA methyltransferase), antigen presentation (e.g., MHC class I polypeptide-related sequence A, B), cell cycle (e.g., HAT1, PCNA), and the response to hypoxia and oxidative stress (e.g., glutaredoxin 2, SOD2, thioredoxin; Table 3). These differentially up-regulated biological processes were also up-regulated processes in HCC-infiltrating inflammatory cells (Table 1). Thus, PBMCs from HCC patients present antigens in conditions of hypoxia and oxidative stress. Additionally, genes involved in other processes, such as apoptosis (e.g., apoptotic peptidase activating factor 1,

Table 2. Supervised learning methods for gene expression of PBMCs

Classifier category	Clinical groups	Total no. cases	No. cases misclassified	Classifier *P* values	No. genes in the classifiers ($P < 0.002$)
LC-C versus HCC	LC-C	32	8	<0.0005	1,430
	HCC	30	2		
Age (y)	>68	31	12	0.317	32
	≦68	31	16		
Gender	Male	25	15	0.178	20
	Female	37	9		
ALT (IU/L)	>50	26	20	0.82	28
	≦50	36	14		
AFP (ng/mL)	>20	29	10	0.02	301
	≦20	33	10		

Table 3. Biological processes for genes up-regulated in PBMCs of HCC patients

Biological process	−log(*P*)	Gene	ID	*t* (T/NT)	*P*	Cellular components
Ubiquitin-proteasomal proteolysis and ER	22.237	Ubiquitin specific peptidase 8	D29956	5.54	0.0000	
		Protein phosphatase 3 (formerly 2B),	NM_000945	4.90	0.0000	
		Heat shock transcription factor 2	NM_004506	4.52	0.0000	
		Heat shock 90 kDa protein 1	NM_005348	4.45	0.0000	T, M
		Ubiquitin protein ligase E3A	NM_000462	4.27	0.0001	
		Ubiquitin-conjugating enzyme E2D1	NM_003338	3.62	0.0006	M
		Phosphatidylinositol glycan, class B	NM_004855	3.57	0.0007	
		Ubiquitin-conjugating enzyme E2D2	NM_003339	3.49	0.0009	
		Ubiquitin-conjugating enzyme E2D3	NM_003340	3.18	0.0023	
		RAN binding protein 2	NM_006267	3.11	0.0029	
		Ubiquitin-conjugating enzyme E2A	NM_003336	3.09	0.0030	
		Activating transcription factor 6	NM_007348	3.03	0.0037	T, M
		Ubiquitin specific protease 7	NM_003470	2.92	0.0050	
		Heat shock 70 kDa protein 9B	NM_001746	2.91	0.0050	
		T-complex 1	NM_030752	2.76	0.0077	
		Glutaredoxin 2	NM_016066	2.70	0.0093	
		Ubiquitin-conjugating enzyme E2N	NM_003348	2.68	0.0096	
		Ubiquitin-conjugating enzyme E2 variant 2	AF049140	2.66	0.0110	
		Ubiquitin specific protease 14	NM_005151	2.20	0.0322	
		Progesterone receptor-associated p48 protein	NM_003932	2.16	0.0353	
		Heat shock 70 kDa protein 4	AB023420	2.16	0.0346	
		Ubiquitin-conjugating enzyme E2L 3	NM_003347	2.14	0.0363	
		Tenascin XB	NM_004381	2.13	0.0377	
		Ubiquitin specific peptidase 33	AB029020	2.12	0.0385	M
mRNA processing	20.087	Heterogeneous nuclear ribonucleoprotein R	NM_005826	3.90	0.0003	T
		RNA (guanine-7-) methyltransferase	NM_003799	3.29	0.0024	
		Heterogeneous nuclear ribonucleoprotein D-like	NM_031372	3.23	0.0020	
		Survival motor neuron domain containing 1	NM_005871	3.12	0.0031	
		Ribonuclease, rnase a family, 4	NM_002937	2.93	0.0052	
		Heterogeneous nuclear ribonucleoprotein A1	NM_002136	2.68	0.0094	
		Heterogeneous nuclear ribonucleoprotein K	NM_002140	2.46	0.0170	
		Heterogeneous nuclear ribonucleoprotein U	NM_031844	2.36	0.0216	
		UPF3, yeast, homologue of, A	NM_023011	2.35	0.0228	
		Alternative splicing factor	M72709	2.03	0.0471	
Antigen presentation	10.124	Janus kinase 1	NM_002227	3.38	0.0013	
		MHC, class II, DO α	NM_002119	3.09	0.0031	
		MHC, class II, DR α	NM_019111	2.67	0.0098	
		MHC class I polypeptide-related sequence B	NM_005931	2.60	0.0122	
		MHC class I polypeptide-related sequence A	NM_000247	2.26	0.0276	
		Tumor necrosis factor receptor–associated factor 6	NM_004620	2.05	0.0456	
Cell Cycle	6.185	Karyopherin (importin) β 2	NM_002270	4.32	0.0001	
		Histone acetyltransferase 1	NM_003642	4.15	0.0001	T, M
		V-myc myelocytomatosis viral oncogene homologue	NM_002467	3.57	0.0008	
		Transforming, acidic coiled-coil containing protein 1	NM_006283	3.38	0.0014	

(*Continued on the following page*)

Table 3. Biological processes for genes up-regulated in PBMCs of HCC patients (Cont'd)

Biological process	−log(*P*)	Gene	ID	*t* (T/NT)	*P*	Cellular components
		Centromere protein B, 80 kDa	X05299	3.37	0.0014	
		Conductin	AF078165	3.07	0.0032	
		Amyloid β precursor protein-binding protein 1	NM_003905	2.99	0.0040	T
		Centromere protein C 1	NM_001812	2.90	0.0054	
		Heterochromatin-like protein 1	BC000954	2.72	0.0085	
		Mature T-cell proliferation 1	BC002600	2.49	0.0154	
		Proliferating cell nuclear antigen	NM_002592	2.46	0.0166	
		CSE1 chromosome segregation 1-like	NM_001316	2.42	0.0186	M
		Karyopherin α4 (importin α3)	NM_002268	2.37	0.0209	
		Signal transducers and activators of transcription-like protein	BC010854	2.36	0.0214	
		M-phase phosphoprotein 6	NM_005792	2.34	0.0228	
		Extra spindle pole bodies homologue 1	NM_012291	2.20	0.0316	
Apoptosis	4.811	Cathepsin S	NM_004079	5.59	0.0000	M
		YME1-like 1	NM_014263	5.49	0.0000	T, M
		Cullin 5	NM_003478	4.65	0.0000	M
		Apoptotic peptidase activating factor 1	NM_001160	3.53	0.0008	
		Cullin 2	NM_003591	3.43	0.0012	M
		Amyloid β precursor protein-binding protein 1	NM_003905	2.99	0.0040	T
		Caspase 9	NM_032996	2.96	0.0044	
		F-box only protein 5	NM_012177	2.88	0.0055	
		Cullin 1	NM_003592	2.52	0.0146	
		Caspase 4	NM_001225	2.23	0.0293	
		Caspase 1	NM_033293	2.02	0.0475	
TCR signaling and immune related	5.462	Protein tyrosine phosphatase, receptor type, C	NM_002838	5.72	0.0000	
		Phosphoinositide-3-kinase, catalytic, α polypeptide	NM_006218	5.38	0.0000	
		Activating transcription factor 2	NM_001880	3.98	0.0002	
		Chemokine (c-c motif) receptor 1	NM_001295	3.90	0.0003	
		NCK adaptor protein 1	NM_006153	3.18	0.0024	
		Chemokine (c-c motif) receptor 2	NM_000647	2.78	0.0075	
		Toll-like receptor2	NM_003264	2.75	0.0078	
		Inositol 1,4,5-triphosphate receptor, type 1	NM_002222	2.24	0.0290	
		T-cell receptor α-chain	X01403	2.05	0.0452	
Response to hypoxia and oxidative stress	2.655	MAP2K1IP1	NM_021970	6.51	0.0000	
		Glutathione s-transferase θ 2	NM_000854	3.43	0.0011	
		Hypoxia-inducible factor 1, α subunit	NM_001530	2.99	0.0040	
		MAP/ERK kinase kinase 5	NM_005923	2.73	0.0086	
		Glutaredoxin 2	NM_016066	2.70	0.0093	
		Peroxiredoxin 3	NM_006793	2.68	0.0157	
		Catalase	NM_001752	2.50	0.0151	
		Plasma glutathione peroxidase 3 precursor	NM_002084	2.19	0.0329	
		Superoxide dismutase 2	NM_000636	2.10	0.0400	
		Thioredoxin	NM_003329	2.05	0.0186	

caspase 9) and T-cell receptor (TCR) signaling (e.g., CCR1, CCR2, TCR α-chain), were also up-regulated in PBMCs from patients with HCC, suggesting vulnerabilities of PBMCs and activated T-cell signaling, respectively, in HCC development.

Biological processes involving the down-regulated genes in PBMCs from patients with HCC included skeletal muscle development, the estrogen receptor 1 (ESR1) nuclear pathway, NOTCH signaling, feeding, and neurohormones signaling, neurogenesis, leptin signaling, and IL-12, IL-15, and IL-18 signaling (Supplementary Table S4), showing no obvious connection compared with the down-regulated genes in HCC-infiltrating mononuclear inflammatory cells (Supplementary Table S3). These results indicate that HCC development in cirrhotic liver can influence PBMCs, providing distinct transcriptional features of up-regulated genes even during the operable stage of HCCs.

Networks of genes commonly up-regulated or down-regulated in both PBMCs and HCC-infiltrating mononuclear inflammatory cells. Analysis of the gene expression profiles of HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients showed that the development of HCC altered the gene expression of local infiltrating mononuclear inflammatory cells and systemically circulating PBMCs; interestingly, the affected biological processes were largely the same. To further explore these presumed local and systemic influences resulting from HCC development, we examined how individual genes were affected by constructing a network.

We found 773 up-regulated and 750 down-regulated significant genes in HCC-infiltrating mononuclear inflammatory cells compared with noncancerous liver-infiltrating mononuclear inflammatory cells at the $P < 0.05$ level. In PBMC gene expression, we observed 2,111 up-regulated and 2,027 down-regulated genes in the PBMCs of HCC patients, compared with LC patients at the $P < 0.05$ level. Among these genes, 378 were significant in both HCC-infiltrating mononuclear inflammatory cells and PBMCs from patients with HCC (Fig. 3*A*). For these 378 genes commonly altered genes, 70% of them were up-regulated or down-regulated in both HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients, whereas expression of the remaining 30% of them was discordant.

We used MetaCore software to perform network construction for 172 up-regulated and 93 down-regulated genes in both HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients. The signal pathway network revealed three central genes, PCNA (32), SMAD3 (33), and nucleophosmin (34), which were all up-regulated in HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients (Fig. 3*B*). PCNA had interactions with proteasome subunit genes, PSMC2, PSMC6, PSMD12, and thioredoxin and DNA polymerase iota genes. SMAD3 was linked with cyclin-dependent kinase 7 and cyclin G_2 with various genes related to the cell cycle. Nucleophosmin was connected to ubiquitin-conjugating enzyme e2e3 and glutaredoxins. Notably, FOXP3, a marker of regulatory T cells, and Janus-activated kinase 3 (JAK3), related to interleukin signaling (35), were up-regulated and down-regulated, respectively, in HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients in the constructed gene network.

The network constructed for individual genes whose expression was commonly altered in HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients also supported a condition of HCC-related stress. The network also indicated that immune reactions in patients with HCC are complex, because down-regulated JAK3, an interleukin signaling molecule, and up-regulated FOXP3 and SMAD3, known molecules of anticancer immunity, are involved in this network. Biological processes in HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients also included the antigen-presentation process.

Discussion

In this study, we explored gene expression in local infiltrating mononuclear inflammatory cells in HCC and noncancerous liver tissues and in PBMCs obtained from patients with hepatitis C–related LC, with or without HCC. Gene expression profiles of HCC-infiltrating mononuclear inflammatory cells were quite distinct from those of noncancerous liver-infiltrating mononuclear inflammatory cells, showing their differing roles in anticancer immunity. We also investigated gene expression in systemically circulating PBMCs from LC-C patients with or without HCC and found that PBMC gene expression profiles from patients with or without HCC were significantly different. Intriguingly, many biological processes involving the up-regulated genes were shared between HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients, suggesting that the local inflammatory effect evoked by HCC development is systemically projected in the host.

Tumor-infiltrating mononuclear inflammatory cells have been investigated to examine their roles in local cancer tissues. We have selectively obtained aggregates of infiltrating mononuclear inflammatory cells in HCC and noncancerous liver tissues by LCM without contamination of carcinoma or parenchymal cells. We have shown that the process of antigen-presentation (36) is a distinguishing feature for up-regulated genes in HCC-infiltrating mononuclear inflammatory cells compared with noncancerous liver-infiltrating mononuclear inflammatory cells. Consistently, immunohistochemical staining of HCC and noncancerous liver tissues revealed that the HCC-infiltrating mononuclear inflammatory cells are primarily monocytes/macrophages, a lineage of phagocytes and antigen-presenting cells (37). Helper CD4 T cells were also found but seemed to be scattered in the HCC-infiltrating mononuclear inflammatory cells, compared with their intensive accumulation in infiltrating mononuclear inflammatory cells in noncancerous liver tissues. Correspondingly, analysis using a publicly available gene expression database of major leukocytes showed that up-regulated genes in HCC-infiltrating mononuclear inflammatory cells were primarily featured for macrophages and Th1 and Th2 CD4 cells, preconditioned with IL-12 and IL-4, respectively. These findings could be interpreted in that HCC expresses tumor-antigens (38) different from the surrounding noncancerous liver tissues; consequently, phagocytes gather in HCC tissues, take up antigens expressed by HCC tissues, and interact with CD4 cells (39). The scattered distribution and transcriptional features of both the Th1 and Th2 predisposed status of CD4 helper T cells in HCC-infiltrating mononuclear inflammatory cells suggests their versatile inflammatory status in cancer immunity, although there was no obvious shift of the Th1/Th2 balance, which is considered to be important in cancer immunity (40).

Other characteristic biological processes involving the up-regulated genes in HCC-infiltrating mononuclear inflammatory cells included the response to hypoxia and oxidative stress (41), the ubiquitin-proteasome system, cell cycle, mRNA processing, ER, and cytoplasm. The ubiquitin-proteasome system is unique to eukaryotic cells and important in maintaining the normal biological activity of cells, with pleiotropic effects in higher animals (42). The cell cycle requires precise regulation of cyclin-dependent kinase under strict control by ubiquitination and subsequent protein degradation (32). Taken together, these processes involving the up-regulated genes may reflect a protective local response of the host, corresponding to the stress environment of HCC. In this sense, the double-strand break repair gene up-regulation may be interpreted as the cells responding to maintain normal cellular activities although they are exposed to a harmful environment by the HCC (43).

The biological processes involving the up-regulated genes in PBMCs from HCC patients, compared with those from LC-C patients without HCC, were, to a substantial degree, the same, involving the up-regulated genes in HCC-infiltrating mononuclear

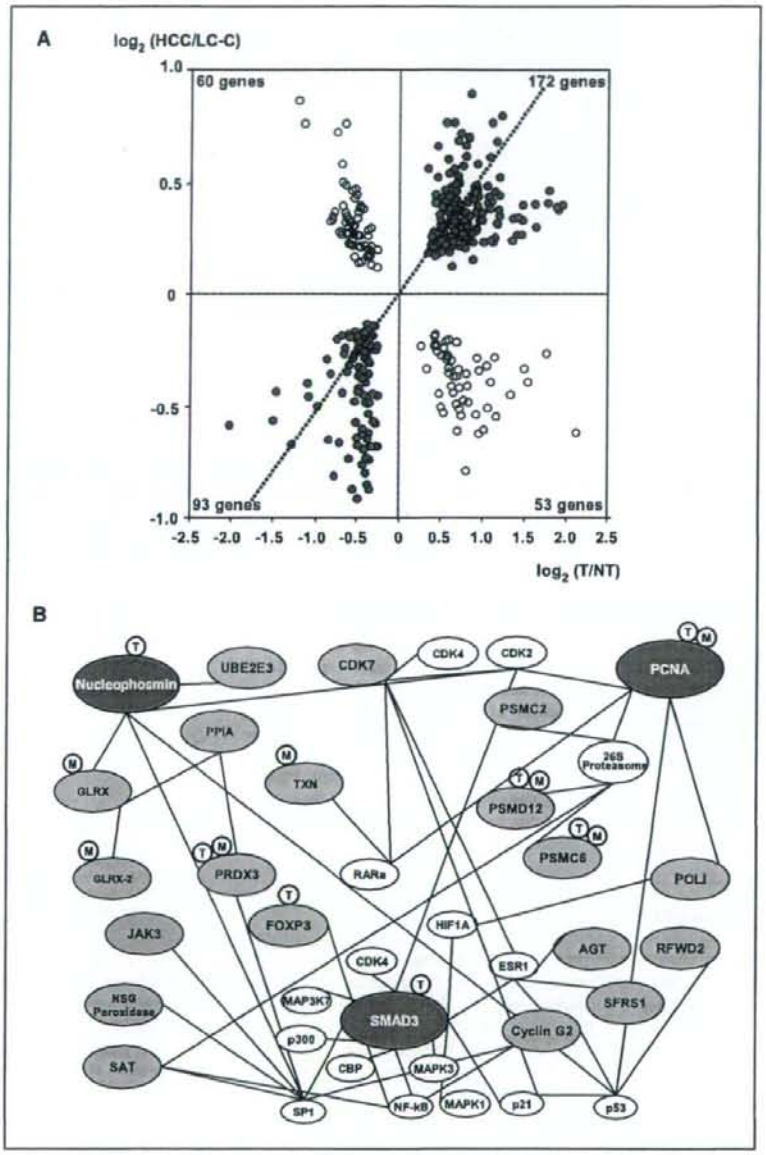


Figure 3. Features of commonly affected genes in PBMCs of HCC patients and HCC-infiltrating mononuclear inflammatory cells. *A*, scatter plots of gene expression ratios between local infiltrating mononuclear inflammatory cells and PBMCs. The axes show the binary logarithm value of the gene expression ratio of HCC-infiltrating mononuclear inflammatory cells over noncancerous liver-infiltrating mononuclear inflammatory cells on the *x* axis and the ratio of PBMCs from HCC patients over LC-C patients on the *y* axis. The right top quadrant includes 172 genes whose expression was up-regulated in HCC-infiltrating mononuclear inflammatory cells and in PBMCs from HCC patients, whereas the left bottom quadrant includes 93 genes down-regulated in both. *B*, interactive network for differentially expressed genes between PBMCs of HCC and LC-C patients and between infiltrating cells adjacent to HCC and noncancerous liver tissues. The three highlighted genes are PCNA, SMAD3, and nucleophosmin, which are related to the redox system, ubiquitin-proteasome system, and cell cycle, in addition to some immunologic gene connections. T or M at each node represent T lymphocytes or monocytes, respectively, and indicate the cell population in which each gene was expressed. The red-filled and blue-filled circles indicate up-regulation or down-regulation, respectively, in HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients.

inflammatory cells, such as ubiquitin-proteasomal proteolysis, ER, and cytoplasm, mRNA processing, antigen presentation, the cell cycle, and the response to hypoxia and oxidative stress. The reflection of these transcriptional features of HCC-infiltrating mononuclear inflammatory cells by PBMCs from HCC patients suggests a systemically projected influence of local HCC development, which is presumably the result of the stress environment caused by HCC and the host's reaction even when the size of the tumor is relatively small. In addition to exploring these biological processes, we also constructed networks of individual genes, the expression of which was similarly up-regulated or down-regulated, to depict commonly affected biological processes in tumor-infiltrating mononuclear inflammatory cells and PBMCs under HCC development in more detail. The networks highlighted three central genes, nucelophosmin, PCNA, and SMAD3, as up-regulated genes. They are connected to individual genes involved in ubiquitin,

proteasomes, the cell cycle, and oxidative stress (Fig. 3*B*). Interestingly, the immunologically important molecules, FOXP3 and JAK3, are in the network as up-regulated and down-regulated genes, respectively. FOXP3 is a transcriptional marker for regulatory T cells (44), and SMAD3 is also believed to be important in maintaining regulatory T cells (45). JAK3, which is associated with the interleukin receptor common γ chain (35) and is important in lymphoid development (46), was also involved in the network, suggesting that HCC influences the host immune system, which can be observed not only in HCC-infiltrating mononuclear inflammatory cells but also in the PBMCs of HCC patients. Thus, the network features of individual genes, commonly affected in HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients, further imply that the anticancer immunity of the host in response to HCC development involves the antigen presentation process to initiate the immune reaction.

The mechanism by which PBMCs from HCC patients reflect the transcriptional features of HCC-infiltrating mononuclear inflammatory cells requires further study. We observed that the population of CCR1-expressing and CCR2-expressing cells in PBMCs from HCC patients was higher than in those from LC-C patients. However, HCC-infiltrating mononuclear inflammatory cells did not show up-regulation of these genes. The meaning of the up-regulated CCR1 and CCR2 should be further investigated because chemokines are key molecules for the recruitment of inflammatory cells, regulating cellular adhesion and transendothelial migration, and the activation of inflammatory cells (47). The biological process of integrin-mediated cell matrix adhesion, genes involved in which were down-regulated in HCC-infiltrating mononuclear inflammatory cells, may suggest that these cells were able to remigrate into the microcirculation with the enriched blood flow in HCC tissues. The process of integrin-mediated cell matrix adhesion in HCC-infiltrating inflammatory cells may imply weaker adhesion of infiltrating mononuclear inflammatory cells to cancer tissues compared with noncancerous liver tissues (48). PBMCs are also presumed to be affected by humoral factors from HCC tissues (49). Another possibility is the presence of hematogenous spreading and circulating HCC cells because mRNA for AFP was detected in circulation (50). Because two-thirds of HCC patients enrolled for gene expression analysis of PBMCs showed serum AFP value <100, the presence of circulating HCC cells would not be evaluated by the detection of *Afp* gene expression alone. Therefore, we have examined expression of *Krt8*, *Krt18*, and *Krt19*, as well as *Afp*. Despite of the possibility of circulating cancer cells, we neither detected expression of *Afp* nor found significantly different expression of *Krt8*, *Krt18*, and *Krt19* between HCC and LC-C patients without HCC. Furthermore, genes up-regulated in HCC tissues compared with noncancerous liver tissues[3] did not correlate to up-regulated genes in PBMCs of HCC patients, indicating that different signature of gene expression in PBMCs between HCC and LC-C patients is not the reflection of the possible migrating cells from HCC tissues. In addition, all HCC cases, except for a case in gene expression analysis of PBMCs, were radiologically free of tumor thrombus in the vessel, which was indicative of microscopic invasion free or concomitant with invasion in the periphery of third or lower branch of vessels, suggesting that contribution of circulating cancer cells were presumed to be sufficiently small for the distinct difference of gene expression signature of PBMCs.

Although the number of enrolled HCC patients for analysis with local inflammatory cells was relatively small compared with the number of patients for analysis of PBMCs, our study has shown shared features of gene expression profiles of HCC-infiltrating mononuclear inflammatory cells and PBMCs from HCC patients, showing a complex immune status of the host in anticancer immunity. This finding suggests the possibility that readily accessible PBMCs can be used as a surrogate tissue to assess the local inflammatory environment surrounding cancers through examination of gene expression profiles.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[3] Unpublished data.

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