Cytochrome P450 gene expression levels in peripheral blood mononuclear cells in comparison with the liver

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Cytochromes P450 (CYPs) compose a superfamily of similar proteins involved in detoxification and elimination, as well as activation of a wide variety of compounds. Most CYP family members are localized in the liver. In order to assess whether peripheral blood leukocytes (PBL) are available as a surrogate for the determination of *CYP* gene expression levels in the liver, we compared *CYP* gene expression levels in PBL with those in liver tissues from patients with hepatocellular carcinoma (HCC). We measured *CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 3A4, 3A5, 3A7, 4A11, 4B1* and *CYP27* gene expressions in PBL and in the liver by real-time reverse-transcription (RT)-PCR. We could detect expression of *CYP1A1, 1A2, P1B1, 2A6, 2B6* and *2E1* genes in PBL and all the genes except for *CYP2F1* in the liver. Although gene expression levels within each subfamily were closely correlated within PBL and within the liver, a clear correlation of gene expression levels between PBL and liver tissues was found only for *CYP4B1*. Although inter-individual variation of the expression level of each *CYP* gene was wide, the induced level was proportional to the basal expression level. Therefore, monitoring of *CYP* gene expression levels in PBL, especially those of *CYP4B1*, could be available as a biomarker for monitoring of exposure to environmental pollutants and assessing the associated risk. Compared with non-tumor tissue, HCC tissues tended to show overexpression of multiple *CYP* genes, indicating that individualized selection and more effective administration of chemotherapeutic agents could perhaps be based on the pattern of *CYP* overexpression. (Cancer Sci 2004; 95: 520–529)

Metabolism of foreign compounds in the body to polar, hydrophilic metabolites is an important prerequisite for detoxification and elimination of xenobiotics from the body. The cytochromes P450 (CYPs) are a superfamily of similar heme-containing proteins that are involved in the oxidative metabolism of xenobiotics. CYPs also catalyze the bioactivation and inactivation of a wide variety of endogenous compounds, including steroid hormones and eicosanoids. Most of the CYP family members are located in the liver.[1–3] Many environmental factors, stress, drugs and diseases influence the expression levels of CYP family members, which may lead to alterations of hepatic drug metabolism in man. Studies on such alterations generally require systemic administration of a drug or probe substance and the application of standard pharmacokinetic approaches.[4, 5] Although small amounts of needle biopsy material from the liver can be used for assessment of gene expression, the process of biopsy is accompanied by practical and ethical problems. Some CYPs are also expressed in extra-hepatic tissues such as the lung, the kidney and the small intestine. Although xenobiotics are transported via the blood and peripheral blood is obtained for routine medical examination, little is known about CYP expression in peripheral blood cells. So far, *CYP1A1, 1B1, 2D6, 2E1* and *3A* genes have been shown to be expressed in peripheral blood.[6, 7] In order to determine whether peripheral blood leukocytes (PBL) are applicable as a surrogate for assessment of *CYP* gene expression in the liver, we measured *CYP* gene expression levels in PBL and the liver, and studied the intra-individual correlation between them. Approximately 70% of human liver CYPs is accounted for by CYP1A2, 2A6, B6, 2C, 2D6, 2E1 and 3A.[8] Here, we measured the expression levels of 19 *CYP* genes, i.e., *CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 3A4, 3A5, 3A7, 4A11, 4B1* and *CYP27*, by real-time reverse-transcription (RT)-PCR. Furthermore, we compared the *CYP* gene expression levels in tumor and non-tumor tissues from liver cancers to assess whether carcinogenesis is associated with specific changes in *CYP* gene expression levels or not.

Materials and Methods

We investigated 18 patients with hepatocellular carcinoma (HCC), 2 patients with intrahepatic cholangiocarcinoma (ICC), 2 with bile duct cancer (BDK) and 1 with colon cancer metastasized to the liver (META) who underwent surgical resection at the Department of Gastroenterological Surgery, Kyoto University. In all cases, pathological diagnosis was confirmed independently by 2 different pathologists. The patients were 62.6±12.0 years old at diagnosis (mean±SD, range 48–76 years old) and consisted of 9 males and 1 female. The tumor part and non-tumor part were separately dissected from extirpated tissues. Among 24 HCC cases, liver tissues were available from 18 patients and peripheral blood from 20 patients. Peripheral blood was obtained with sodium citrate 2 or 3 days before surgical operation (pre-operation), and at 1 day, 7 days and 30 days after operation. As a control, peripheral blood was collected from healthy volunteers consisting of 20 males (mean: 35.7 years old, 31–46 years old). Those were composed of 10 smokers, 1 ex-smoker and 9 non-smokers, and 11 alcohol drinkers and 9 non-drinkers according to the definitions of the

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Abbreviations: CYP, cytochrome P450; PBL, peripheral blood leukocytes; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; BDK, bile duct cancer; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

National Nutrition Survey, Japan (http://www.nih.go.jp/eiken/nns/kokumin/). In addition, as a control for liver disease, peripheral blood from patients with gastric cancers (5 cases) without liver metastasis (non-liver cancers) was obtained before surgical operations. Written informed consent was obtained from each patient and volunteer according to the guidelines of the Ethical Committee of Kyoto University, Faculty of Medicine.

RNA preparation and real-time RT-PCR. Peripheral blood (10 ml) obtained with 3.8% sodium citrate was centrifuged at 3000 rpm for 15 min, and the buffy-coat was separated. Total RNA was extracted using a QIAGEN miniprep kit (QIAGEN, Germany) according to the manufacturer's protocol. Total RNA from tissues was extracted using a QIAGEN mediumprep kit after tissues had been homogenized in a homogenizer (Mini Beadbeater 8, Biospec Products, Inc., OK).

RT-PCR was performed as described by Nishimura *et al*.[9] Briefly, *CYP* and control *β-actin* and *GAPDH* gene expression levels were determined using *Taq*-Man One-Step RT-PCR Master Mix reagent. Design of the primers and *Taq*Man probes and the conditions of RT-PCR performed in an ABI PRISM 7700 sequence detector system (Applied Biosystems, CA) were the same as described previously.[9] The sequences of primers and probes are presented in Table 1. Gene expression levels are presented as the ratio of target mRNA to *β-actin* mRNA. The gene expression levels were rounded to three decimal places.

Specificity of RT-PCR. We performed cross-reactivity assay to determine whether our assay system could exclusively quantify the target molecule. Total RNA was extracted from *Escherichia coli* transfected with human *CYP1A1* or *1A2*.[10] *CYP1A1* and *1A2* gene expression levels were measured individually using *CYP1A1* and *1A2* specific primer and probe sets.

Immunohistochemistry. Human CYP molecules such as CYP1A2, 2A6, 2C8, 2D5, 2E1 and 3A4 were purified from *Saccharomyces cerevisiae* expressing transfected *CYP* genes. Immunohistochemistry was performed using rabbit polyclonal antibodies raised against each molecule as the primary antibody.[11] Anti-CYP1B1 polyclonal antibody was purchased from Daiichi Pure Chemical (Tokyo).

Statistical analysis. Correlation coefficients among *CYP* gene expression levels were calculated by linear regression and are presented as a correlation plot matrix. Correlation coefficients between fractions of neutrophils and lymphocytes were calculated by linear regression. Cluster analysis of *CYP* gene expres-

Table 1. Primers and probes used for RT-PCR analysis

CYP	Forward primer	Reverse primer	Probe	Amplicon size
1A1 (NM_000499)[1]	5′-GTCATCTGTGCCATTTGCTTTG-3′ (589–610)[2]	5′-CAACCACCTCCCCGAAATTATT-3′ (685–664)	5′-CGCTATGACCACAACCACCAAGAACT-3′ (616–641)	97[3]
1A2 (AF182274)	5′-TGTTCAAGCACAGCAAGAAGG-3′ (860–880)	5′-TGCTCCAAAGACGTCATTGAC-3′ (951–931)	5′-CTAGAGCCAGCGGCAACCTCATCCCA-3′ (884–909)	92
1B1 (NM_000104)	5′-ACCGTTTTCCGCGAATTC-3′ (766–783)	5′-GTACGTTCTCCAAATCCAGCC-3′ (961–941)	5′-AGCAGCTCAACCGCAACTTCAGCAACTT-3′ (785–812)	196
2A6 (AF182275)	5′-TTTTGGTGGCCTTGCTGGT-3′ (20–48)	5′-GGAGTTGTACATCTGCTCTGTGTTCA-3′ (171–146)	5′-TGCCTGACTGTGATGGTCTTGATGTCTGTT-3′ (40–69)	152
2B6 (AF182277)	5′-CCCCAAGGACACAGAAGTATTTC-3′ (1146–1168)	5′-GATTGAAGGCGTCTGGTTTTTC-3′ (1228–1207)	5′-TGAGCACTGCTCTCCATGACCCACACTA-3′ (1175–1202)	83
2C8 (NM_000770)	5′-GGACTTTATCGATTGCTTCCTG-3′ (783–804)	5′-CCATATCTCAGAGTGGTGCTTG-3′ (926–905)	5′-TTGGCACTGTAGCTGATCTATTTGTTGCTGGA-3′ (863–894)	144
2C9 (M61857)	5′-GACATGAACAACCCTCAGGACTTT-3′ (766–789)	5′-TGCTTGTCGTCTCTGTCCCA-3′ (910–891)	5′-AAAACACTGCAGTTGACTTGTTTGGAGC-3′ (863–890)	145
2C18 (M61856)	5′-AGGATATTGACATCACCCCCA-3′ (1394–1414)	5′-TCAGACAGGAATGAAGCAGAGCT-3′ (1473–1451)	5′-AATGCATTTGGTCGTGTGCCACCCT-3′ (1420–1444)	80
2C19 (NM_000769)	5′-GAACACCAAGAATCGATGGACA-3′ (748–769)	5′-TCAGCAGGAGAAGGAGAGCATA-3′ (943–922)	5′-TAATCACTGCAGCTGACTTACTTGGAGCTGGG-3′ (863–894)	196
2D6 (NM_000106)	5′-CCTACGCTTCCAAAAGGCTTT-3′ (720–740)	5′-AGAGAACAGGTCAGCCACCACT-3′ (912–891)	5′-CAGCTGGATGAGCTGCTAACTGAGCACA-3′ (748–775)	193
2E1 (AF182276)	5′-TTCAGCGGTTCATCACCCT-3′ (1070–1088)	5′-GAGGTATCCTCTGAAAATGGTGTC-3′ (1146–1123)	5′-TCCAACCTGCCCCATGAAGCAA-3′ (1096–1117)	77
2J2 (NM_000775)	5′-AGCTTAGAGGAACGCATTCAGGA-3′ (460–482)	5′-CGAAGGTGATGGAGCAAATGAT-3′ (592–571)	5′-AGGCCCAACACCTCACTGAAGCAA-3′ (485–508)	133
3A4 (AF182273)	5′-GATTGACTCTCAGAATTCAAAAGAAACTGA-3′ (825–854)	5′-GGTGAGTGGCCAGTTCATACATAATG-3′ (973–948)	5′-AGGAGAGAACACTGCTCGTGGTTTCACAG-3′ (946–918)	149
3A5 (NM_000777)	5′-CCTTACCCCAGTTTTTGAAGCA-3′ (684–705)	5′-TCCAGATCAGACAGAGCTTTGTG-3′ (881–859)	5′-TTTCTTTCGAATTCTGGGAGTCAATCATC-3′ (850–822)	198
3A7 (NM_000765)	5′-CCTTACCCCAATTCTTGAAGCA-3′ (684–705)	5′-TCCAGATCAGACAGAGCTTTGTG-3′ (881–859)	5′-AGTCTTTTGAATTCTGAGAGTCAATCATCAGC-3′ (850–819)	198
4A11 (NM_000778)	5′-AGGAGCTACAACGGATTCAGAA-3′ (209–230)	5′-ACGAACTTTGCCTCCCCATAG-3′ (288–268)	5′-ACATTCCCAAGTGCCTGTCCTCATTG-3′ (241–266)	80
4B1 (NM_000779)	5′-CCTGGTTTCTCTACTGCATGGC-3′ (974–995)	5′-CCAGATCATCCCACTGGAAGA-3′ (1081–1061)	5′-CTGTACCCTGAGCACCAGCATCGTTGTA-3′ (997–1024)	108
27 (M62401)	5′-AGAGGAGATTCCACGTCTAGGAC-3′ (171–193)	5′-ACATCCACATTGGACCGTACTT-3′ (292–271)	5′-TGCGCTTCTTCTTTCAGCTGTTCGTTCA-3′ (197–224)	122

1) GenBank accession number. *2*) Position. *3*) bp.

Table 2. *CYP* gene expressions in peripheral blood leukocytes

	CYP1A1	*CYP1A2*	*CYP1B1*	*CYP2A6*	*CYP2B6*	*CYP2E1*	*CYP4B1*
HCC[1] (Pre[2])	0.019±0.023	0.021±0.021	0.037±0.016	0.017±0.020	0.040±0.044	0.055±0.071	0.005±0.010
non-HCC[3] (Pre)	0.030±0.048	0.032±0.044	0.039±0.033	0.029±0.044	0.066±0.092	0.083±0.132	0.013±0.020
Gastric Ca[4] (Pre)	0.007±0.002	0.014±0.008	0.033±0.018	0.003±0.002	0.015±0.007	0.025±0.018	ND[6]
Control[5]	0.003±0.002	0.005±0.002	0.027±0.010	0.004±0.002	0.009±0.004	0.015±0.006	0.000±0.000

1) Hepatocellular carcinoma. *2*) Pre-operation. *3*) Metastatic cancer to the liver. *4*) Gastric cancer without liver metastasis. *5*) Healthy control. *6*) Not detectable. * $P<0.05$ (Tukey-Kramer's HSD test).

Table 3. *CYP* gene expressions in liver tissues

		CYP1A1	*CYP1A2*	*CYP1B1*	*CYP2A6*	*CYP2B6*	*CYP2C8*
HCC[1)]	T[2)]	0.047±0.099	0.615±1.739	0.045±0.050	2.223±3.352	1.984±3.326	1.059±1.965
	N[3)]	0.096±0.083	5.700±4.490 *	0.039±0.032	3.855±3.846	11.338±11.771 *	6.196±4.306 *
		CYP2C9	*CYP2C18*	*CYP2C19*	*CYP2D6*	*CYP2E1*	*CYP2J2*
HCC	T	2.452±5.175	0.909±0.895	0.013±0.011	0.680±0.720	23.866±37.109	0.157±0.149
	N	10.477±7.518 *	2.178±1.922 *	0.040±0.032 *	0.943±0.678	61.312±42.265 *	0.502±0.299 *
		CYP3A4	*CYP3A5*	*CYP3A7*	*CYP4A11*	*CYP4B1*	*CYP27*
HCC	T	2.538±7.415	0.231±0.291	0.076±0.127	2.762±3.157	0.001±0.001	2.816±2.635
	N	6.199±3.849	0.488±0.560	0.233±0.212 *	14.772±8.268 *	0.005±0.011	3.977±2.396

1) Hepatocellular carcinoma. *2*) Tumor part. *3*) Non-tumor part. * $P<0.05$ (Tukey-Kramer's HSD test).

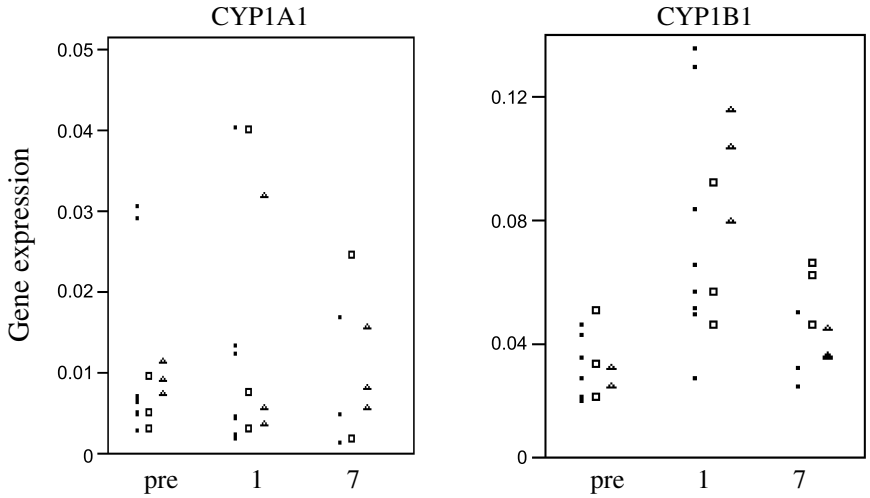

Fig. 1. Comparison of *CYP1A1* and *1B1* gene expression levels between the 3 patients with the highest neutrophil fraction (□), the 3 with the lowest neutrophil fraction (△) and the others (■). The differences in expression levels of both genes are not significant. Values represent the ratio of target (*CYP*) mRNA to *β-actin* mRNA.

sion was performed by the Wars method and the significance of differences between *CYP* gene expression levels was determined by means of the Tukey-Kramer test using JMP 5.0J (SAS Institute Japan, Inc., Tokyo), or Student's *t* test.

Results

We measured the expression levels of the *CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2E6, 2F1, 2J2, 3A4, 3A5, 3A7, 4B1* and *CYP27* genes in PBL and in liver tissues. Effective discrimination between *CYP1A1* and *1A2* was confirmed (data not shown). Only the *CYP1A1, 1A2, 1B1, 2A6, 2B6, 2E1, 2F1* and *4B1* genes were expressed in PBL (Table 2), whereas all *CYP* genes except for *CYP2F1* were expressed in liver tissues (Table 3). Expression levels of *CYP2F1* were less than 10^{-2} times those of other *CYP* genes, if detectable, and therefore, we excluded the *CYP2F1* gene from the present examination of PBL. In order to determine the effect of the period from blood sampling to RNA extraction, gene expression was compared among RNA preparations extracted immediately after drawing the blood from 3 healthy volunteers, and after storage for 3 h and 24 h in a refrigerator. We could not observe any effect of the period between blood gathering and RNA extraction on the results (data not shown).

Since leakage of tumor cells into the systemic blood circulation has been reported to occur, we calculated the ratio of tumor cells in the peripheral blood leukocytes by use of the following equation: Total gene expression in peripheral blood cells=gene expression of tumor cells×R+gene expression of blood cells×(1−R), R=the ratio of tumor cells in the peripheral blood. It was assumed that the level of gene expression is similar between tumor cells in the liver and in the peripheral blood, and between PBL in patients and those in healthy volunteers. For the calculation of R, we used *transferrin* and *albumin* gene expression levels because the expression levels of these genes in tumor cells were remarkably high compared with those in PBL.

Table 4. Fractionation of PBL form 3 each patients with the highest and the lowest neutrohil percentages

Day	HCC	Neutrophil (%)	Lymphocyte (%)	Others (%)
pre[1)]	308	78.0	15.6	6.4
	293	74.9	18.8	6.3
	297	71.7	18.5	9.8
	299	47.8	37.5	14.7
	300	47.6	44.6	7.8
	303	38.9	41.2	19.9
1[2)]	302	88.5	5.0	6.5
	308	87.4	6.8	5.8
	290	84.5	10.5	5.0
	288	74.8	15.4	9.8
	303	74.1	18.0	7.9
	299	69.8	21.9	8.3
7[3)]	293	79.1	9.5	11.4
	296	75.8	13.2	11.0
	294	70.3	20.9	8.8
	303	63.0	20.7	16.3
	290	58.7	25.9	15.4
	300	57.2	25.6	17.2

1) Before medical treatments. *2*) One day after surgical operation. *3*) Seven days after surgical operation.

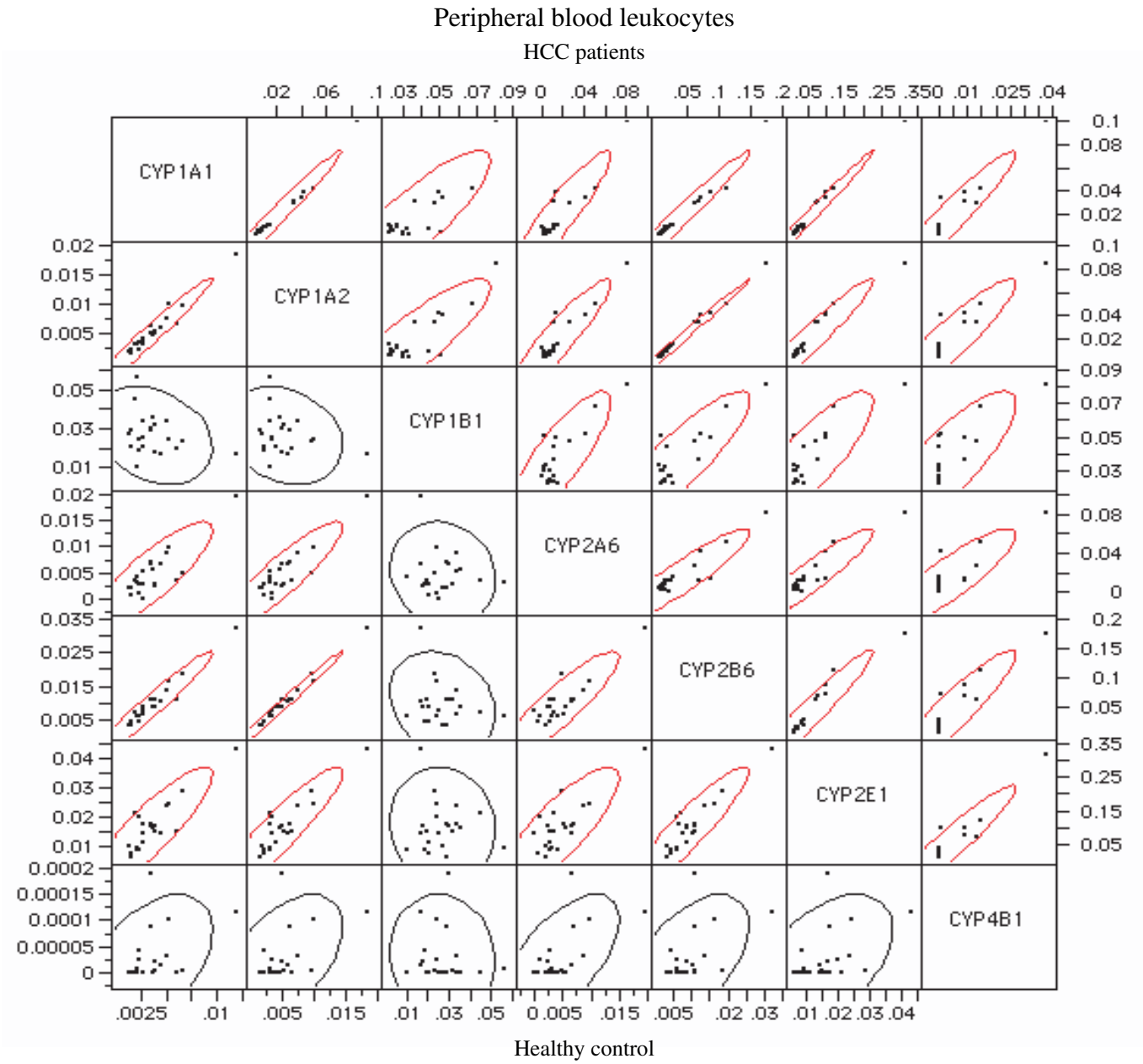

Fig. 2. Correlation plot matrix of *CYP* gene expression levels in peripheral blood leukocytes from patients before medical treatment (right upper part) and in healthy controls (left lower part). The line shows the range of 1 SD of correlation coefficient calculated by linear regression and is drawn in red in the case of correlation coefficient >0.7. Values represent the ratio of target (*CYP*) mRNA to *β-actin* mRNA.

Therefore, net gene expression was obtained by subtracting the mean value of gene expression in healthy volunteers. Average calculated *R* values were $6.4/10^6$ and $1.1/10^5$ cells using the *albumin* and *transferrin* gene expression data, respectively (data not shown).

***CYP* gene expressions in PBL.** Healthy volunteers were divided into 3 groups according to smoking and drinking habits: non-smokers, ex-smokers who had ceased to smoke after smoking for various periods, smokers, non-drinkers, ex-drinkers who had ceased to drink after drinking for various periods and drinkers, respectively. There was no significant difference of any *CYP* gene expression in PBL among volunteers with various habits of smoking or drinking. Therefore, all volunteers were combined in one group (Table 2).

In order to minimize cell loss from the limited amount of blood available, we did not fractionate leukocytes into each subgroup before RNA extraction. We examined whether *CYP* gene expression levels are different depending on the cell lineage or not. The levels of *CYP* gene expression in PBL showed no significant difference between the 3 patients with the highest neutrophil counts and the other patients, or between the 3 patients with the lowest neutrophil counts and the others. Representative results are shown in Fig. 1. Furthermore, a good inverse correlation was observed between percentages of neutrophils and lymphocytes (Table 4).

The preoperative stage is considered to provide basal expression levels of *CYP* genes in patients, because the patients were not under medical treatment. Therefore, *CYP* gene expression levels before surgical operation were taken as representative, unless otherwise specified. *CYP* gene expression in PBL from

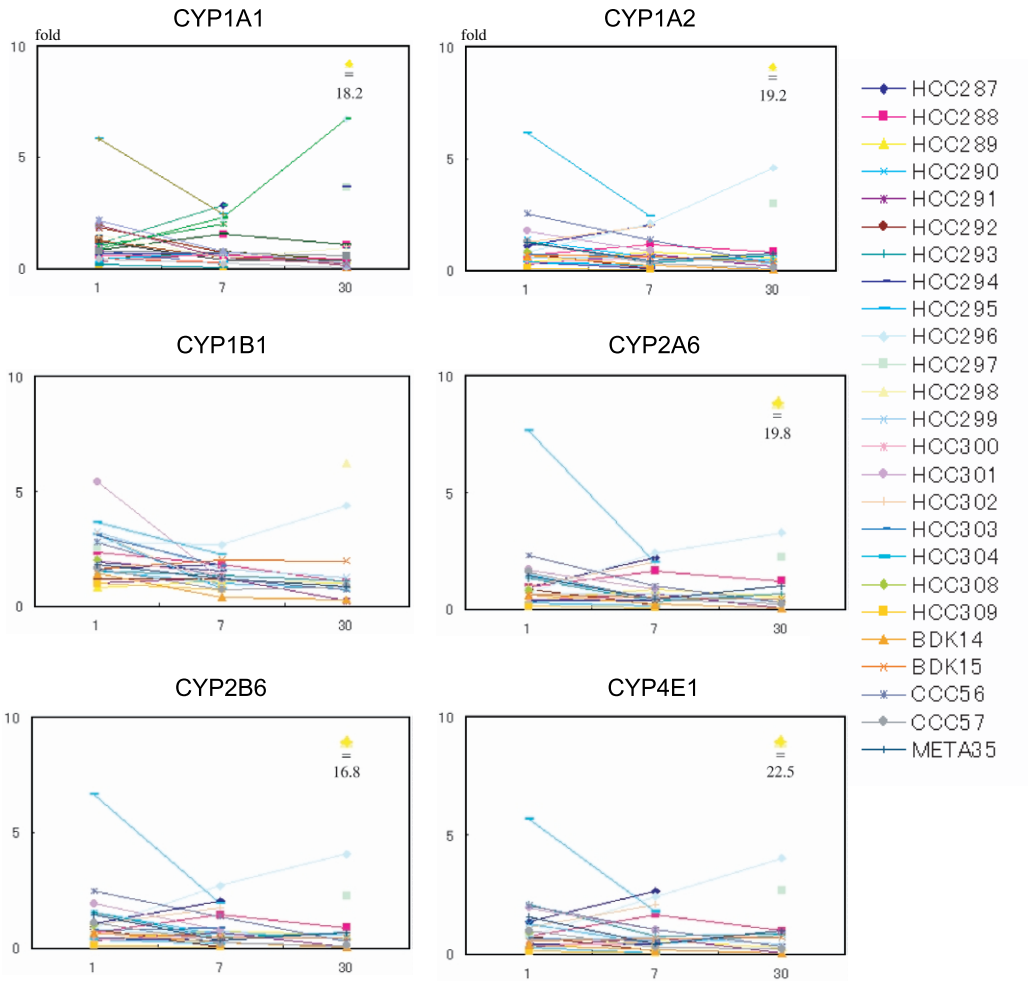

Fig. 3. Fluctuation pattern of *CYP* gene expression levels in peripheral blood leukocytes from patients with hepatocellular carcinoma (HCC), bile duct cancer (BDK), cholangiocellular carcinoma (CCC) and metastatic liver cancer (META). *CYP* gene expression levels at 1, 7 and 30 days after surgical operation divided by those at the pre-operative stage are presented.

pre-operative patients with non-liver cancers were also measured to assess whether there were alterations in *CYP* gene expression specific to liver tumor and stresses from surgical operation. Expression levels of all the *CYP* genes examined were highest in patients with HCC, followed by patients with non-liver cancers and healthy volunteers, in decreasing order. The differences of *CYP1A1, 1A2, 2A6, 2B6, 2E1* gene expression were significant between patients with HCC and healthy volunteers. The variation of *CYP* gene expression in PBL was the smallest in healthy volunteers. Correlations among expression levels of different *CYP* genes in PBL from pre-operative patients were examined (Fig. 2). If the correlation coefficient between expression levels of two *CYP* genes was higher than 0.7, we defined the expression levels of those *CYP* genes as closely associated. Although inter-individual variations were high among different *CYP*s, close associations were observed among expression levels of *CYP1A1, 1A2, 2B6* and *2E1* in healthy controls. In HCC patients, the expression levels of all the *CYP* genes were closely associated and a closer correlation was found between *CYP* genes for which expression was associated in healthy controls (Fig. 2).

Fluctuation of *CYP* gene expression levels in PBL after surgical operation. Since inter-individual variation was wide and preoperative PBL may reflect the basal status of individual patients, *CYP* gene expression levels divided by those at the pre-operative stage were plotted at 1, 7 and 30 days after surgical operation. Fig. 3 shows the pattern of *CYP* gene expression after surgical operation. Almost all the *CYP* genes examined showed similar fluctuation patterns. *CYP* gene expression was maximum on day 1 after surgical operations and decreased thereafter in most cases. Although the variation of *CYP* gene expression tended to become smaller with increasing period after surgical operation, *CYP* gene expression levels in PBL of patients with HCC tended to be higher than those of healthy volunteers even at 30 days after operation. Close correlations were observed between *CYP1A1* and *1A2* gene expression levels, and among *CYP2A6*, *2B6* and *2E1* gene expression levels in PBL from the same day after operation and between *CYP1A1* and *1B1* gene expression levels 30 days after operation (data not shown).

CYP gene expression in liver tissue. The *CYP* gene expression levels in the non-tumor part were generally higher than those in the tumor part, except for *CYP1B1* (Table 3). The correlation plot matrix is shown in Fig. 4. The values of the correlation coefficient between any two *CYP* gene expression levels in liver tissues were smaller than those of PBL. Although the number of pairs with close association was higher in non-tumor tissues than in tumor tissues, the number of close associations with *CYP1B1, 2A6, 2C8, 2C19* and *4A11* gene expression levels

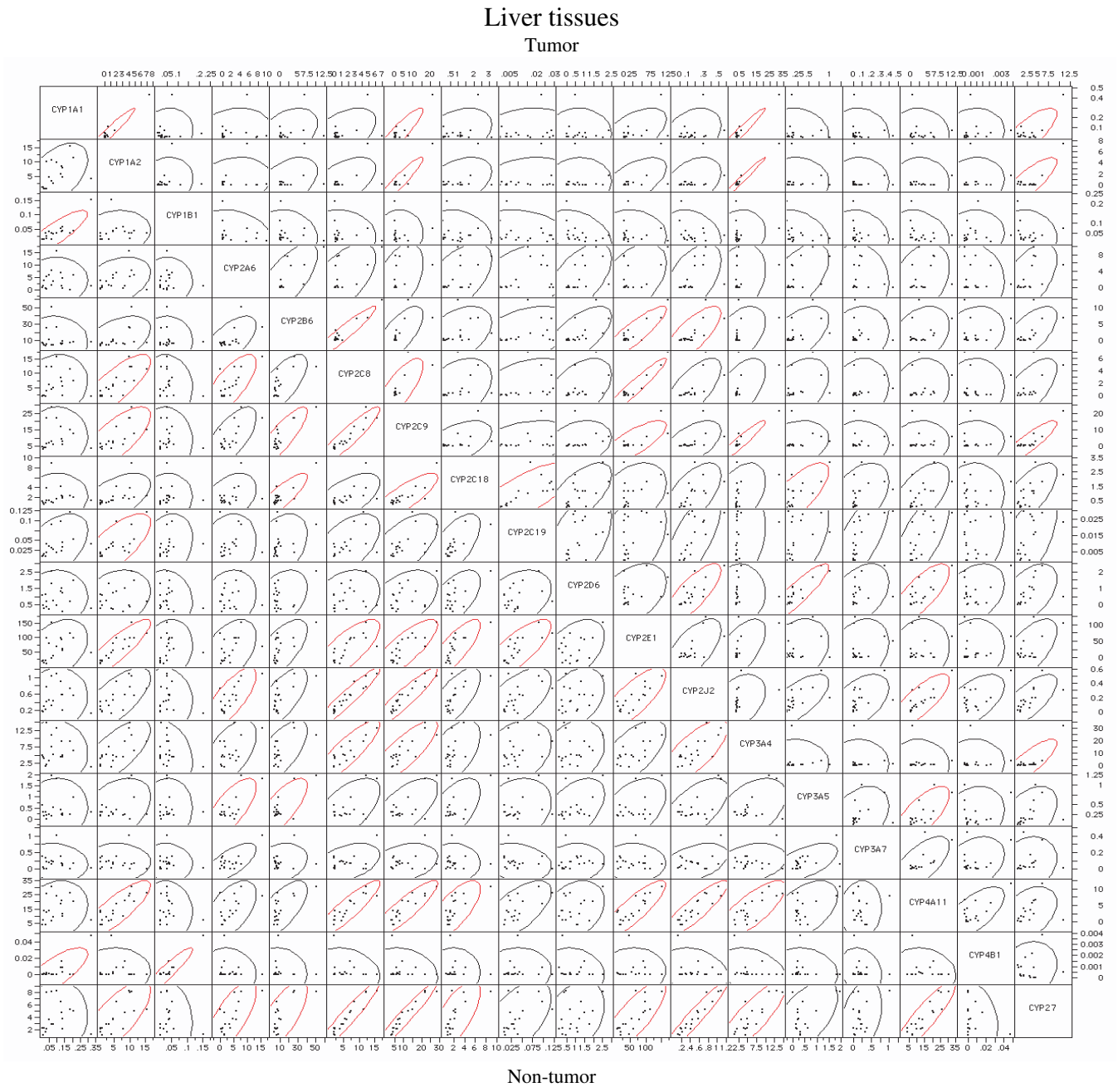

Fig. 4. Correlation plot matrix of *CYP* gene expression levels in liver tissues. The right upper half shows the relationship in the tumor part and left lower half shows that in the non-tumor part. The line shows the range of 1 SD of correlation coefficient calculated by linear regression, and is drawn in red in the case of correlation coefficient >0.7. Values represent the ratio of target mRNA to *β-actin* mRNA.

from tumor tissues was higher than from non-tumor tissues. In the tumor part, the expression levels of *CYP1B1* and *2A6* genes did not show any correlation with those of other *CYP* genes. The expression levels of *CYP2C8* and *4A11* genes in non-tumor tissues and those of *CYP1A1, 2B6* and *2C18* genes in tumor tissues were closely associated with those of more than half of the other *CYP*s examined.

Except for *CYP1B1* and *4B1*, cases in which the tumor part showed higher expression of one *CYP* gene compared with the non-tumor part also tended to show higher expression of multiple *CYP* genes versus the non-tumor part. All these cases (HCC288, 289, 290, 298, 300 and 302) were infected with hepatitis C virus, but there was no association of expression levels with histological grading of inflammatory status in the liver (data not shown), serum bilirubin levels or other clinico-pathological characteristics (Table 5).

The staining pattern of positive cells for CYP proteins was variable depending on the individual CYP species and case. Immuno-staining for CYP2A6 and 2D6 tended to be present uniformly in hepatocytes of the non-tumor part. In the tumor part, the staining pattern was generally speckled within the cancer nest. Hepatocytes positive for CYP3A4 were generally distributed to the centri-acinar region and showed fatty changes (data not shown). The intensity of staining for CYP1B1 was heterogeneous in the cancer part whereas non-tumor hepatocytes were weakly and homogeneously stained. However, overall intensity

Table 5. ***CYP*** **gene overexpressions in tumor part and clinicopathological characteristics**

	1A1	*1A2*	*1B1*	*2A6*	*2B6*	*2C8*	*2C9*	*2C18*	*2C19*	*2D6*	*2E1*	*2F1*	*2J2*	*3A4*	*3A5*	*3A7*	*4A11*	*4B1*	*27*	B[1]	C[1]	lesion[2]	a inf[8]	Plt[9]	T-Bil[10]	Alb[11]	AFP[12]
HCC288	●	●			●	●	●	●	●	●			●	●	●		●	●	●		+	LC[3]	–	110	0.8	3.6	6.6
HCC289				●				●		●					●				●		+	LC	?	55	1.2	3.2	220
HCC290	●		●					●		●	●				●			●	●	+	+	?[4]	?	160	1.1	4.2	87
HCC291														●								CH[5]		142	0.6	3.7	7.4
HCC292			●															●		+		LC		125	0.9	4.2	8011
HCC293			●													●		●		+		–	–[6]	241	0.9	5.3	16,368
HCC294			●															●		+	+	LC	–	86	1.7	3.9	45
HCC295																					+	LF[7]	+	109	0.8	3.6	703
HCC296			●																		+	LC	+	53	0.9	3.7	1864
HCC297			●															●				LF	–	211	0.3	4.3	10.4
HCC298	●			●	●	●	●		●				●						●	+	+	LC	–	129	0.3	4.3	69
HCC299				●																	+	CH	–	179	0.8	4.2	4636
HCC300			●															●	●		+	LC	–	88	1	3.5	587
HCC302	●		●	●						●	●			●	●	●		●	●		+	LC	+	20	0.5	4.8	5.8
HCC303																●				+		LC	–	162	0.7	4.6	215
HCC304																		●				LF	+	6.7	1	4	385.6
HCC305																				+		LC	+	109	0.9	3.8	105
HCC306																						CH	–	121	1.1	3.6	765
CCC56			●															●				–		212	0.9	4.2	<3.0
CCC57																				+	+	–		24	0.5	4.6	61
BDK15																				–	–	–				4.9	
META35																		●		–	–	–					

● *CYP* gene expressioion in tumor part>non-tumor part. *1*) Hepatitis virus infection. *2*) Lesion of non-tumor part. *3*) Liver cirrhosis. *4*) Unknown. *5*) Chronic hepatitis. *6*) No lesion. *7*) Liver fibrosis. *8*) Arterial invasion. *9*) Platelet count ($\times 10^4$/ml). *10*) Total bilirubin (mg/dl). *11*) Serum albumin (g/dl). *12*) α-Fetoprotein (ng/ml).

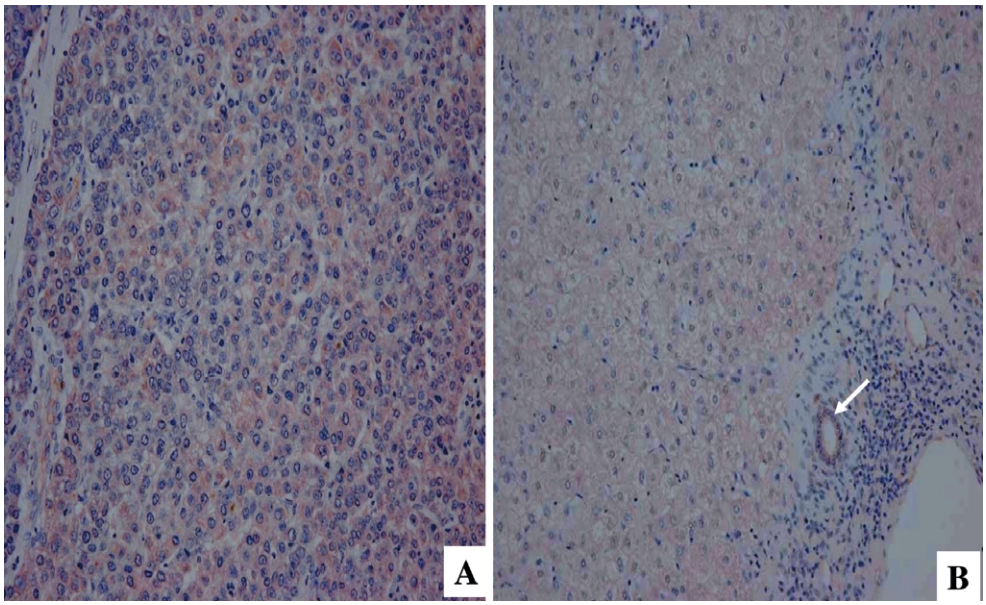

Fig. 5. Immunohistochemical staining of CYP1B1 in HCC300. Intensity of staining is heterogeneous in the cancer part (A). Non-tumor parenchymal cells are weakly and homogeneously stained, and epithelial cells of bile ducts (white arrow) are strongly positive for CYP1B1 (B).

tended to be higher in cancer cells than in hepatocytes and epithelial cells of bile ducts were strongly positive for CYP1B1 (Fig. 5).

Correlation between *CYP* gene expression levels in liver tissues and PBL. *CYP4B1* gene expression was detectable in tumor tissues from 14 out of 17 cases and in non-tumor tissues from 10 of 18 cases, and in PBL from 18 out of 20 cases, and we found a correlation between *CYP4B1* gene expression in liver tissues from the non-tumor part and PBL before surgical operation (r=0.64, P<0.05) (Fig. 6). No association could be observed for expression of other *CYP* genes in liver tissue and PBL. We next performed cluster analysis, but although the combination of *CYP* members was different in each group, *CYP*s were clearly separated into 2 groups in terms of expression profile, that is, with and without *CYP1B1*, irrespective of PBL or liver tissue. In PBL *CYP2A6* was always in the *CYP1B1* group. *CYP4B1* was in the *CYP1B1* group in the healthy control, but in the other group in HCC patients. In liver tissues, *CYP4B1* was always in the *CYP1B1* group. In non-tumor tissues, *CYP1A1* was in the *CYP1B1* group, but was in the other group in non-tumor tissues (Fig. 7).

Discussion

Since *CYP* members play a crucial role in the metabolism of various substances, many studies on *CYP* gene expressions

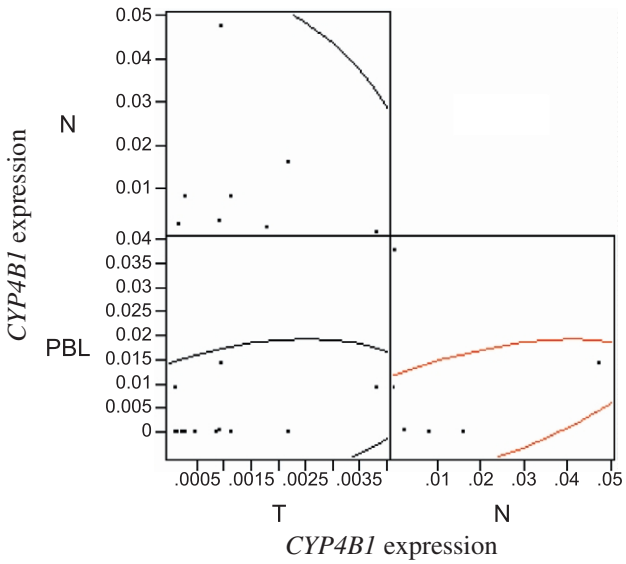

Fig. 6. Association of *CYP4B1* gene expression levels among peripheral blood leukocytes (PBL) before operation and in the tumor part (T) and non-tumor part (N) of liver tissues.

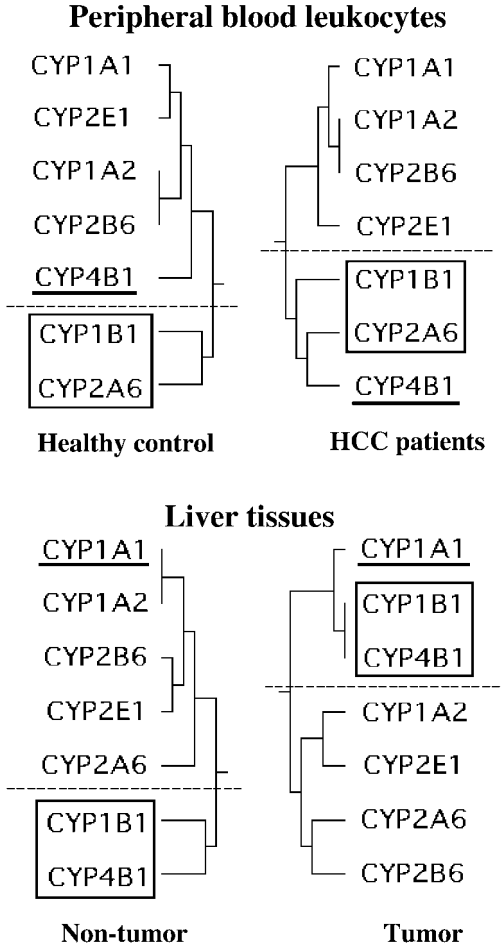

Fig. 7. Cluster analysis of *CYP* gene expression levels in peripheral blood leukocytes and in liver tissues. The expression pattern of the *CYP* genes is roughly divided into 2 groups (separated by a dotted line), i.e., the extrahepatic *CYP1B1* group (enclosed by the rectangle) and the hepatic group. *CYP4B1* in PBL and *CYP2A6* in PBL are in different groups between healthy control and HCC patients, and *CYP4B1* in liver tissues is in a different group between tumor tissues and non-tumor tissues.

have been reported. However, comprehensive and quantitative studies to correlate individual *CYP* molecules in human have not been performed either in PBL or liver tissue. Furthermore, this study is the first to directly compare *CYP* gene expression levels in human PBL and liver tissue. It is well established that biotransformation activity in cultured hepatic cells is regulated at the *CYP* transcription level.[12] In the present study, we analyzed the expression levels of 18 *CYP* genes both in PBL and in liver tissue, including liver cancers. *CYP1A1, 1A2, 1B1, 2A6, 2B6, 2E1* and *4B1* were expressed in PBL as determined by RT-PCR. Although we chose suitable primers and probes for specific RT-PCR detection of each *CYP* family member, we checked the specificity of the assay because homology of nucleotide sequences is quite high among *CYP* family members. We confirmed that we could accurately discriminate individual *CYP* molecules in the present study. From the calculations in the current study, the influence of tumor cells in the blood circulation might be the highest on *CYP2E1* expression because the difference between its expression levels in HCC tissues and PBL is the highest among the expressed *CYP* genes. Considering that the number of tumor cells was of the order of 10^{-6} at most, that the expression level of the *CYP2E1* gene in PBL was similar to those of other *CYP* genes and that *CYP2E1* gene expression was closely correlated with other *CYP* gene expressions, the influence of tumor cells in the circulation is considered to be negligible. In the present study it is indicated that the *CYP* gene expression levels in blood cells reflect those of PBL, excluding tumor cells, circulating in the peripheral blood. Our results also indicate that we do not need to fractionate leukocytes into subgroups for the analysis of *CYP* gene expression levels in PBL. *CYP* gene expression levels were not affected by differences in the cellular composition of blood cells. Thus, it is valid to use whole blood cells without fractionation. Alcohol consumption and cigarette smoking induce *CYP2E1* and *1A2*, respectively.[13] Theophyline, widely used in asthma therapy, is preferentially metabolized by *CYP1A2* and its systemic clearance is predicted to be about 50% greater in smokers than in non-smokers. In the present study, however, *CYP* gene expression levels in PBL were not affected by smoking or drinking habits. possibly because these individuals' drinking and smoking habits were not extreme, and induction levels, if any, might be within the level of inter-individual variations.

CYP gene expression levels in PBL from HCC patients were significantly higher than in the healthy control. Furthermore, those from patients with non-HCC were intermediate. *CYP* biomarker response may be influenced by stress, including increase in plasma corticosteroids[14] before surgical operation, and further induced by liver cell damage by cancer cells. Although inter-individual variations of *CYP* gene expression levels were large, a close association was observed between expression levels of any two *CYP* genes detectable in PBL from HCC patients. In addition, expression levels relative to pre-operation stage between *CYP1A1* and *1A2*, and among *CYP2A6, 2B6* and *2E1* were closely correlated irrespective of the day after surgical operation. These results suggest that basal levels of *CYP* gene expressions mainly reflect the constitutive expression. Furthermore, the major regulatory mechanism of *CYP* gene induction by exogenous stresses is common among *CYP*s expressed in PBL and the induced levels of these *CYP*s are proportional to the basal levels of expression.

The human CYP1 family is highly inducible by a wide range of toxicants, including polycyclic aromatic hydrocarbons. Cytokines generally have an inhibitory effect on *CYP* gene expression, whereas TNF-α enhances *CYP1B1* expression, indicating that *CYP1A* and *1B* expression levels are specifically regulated in relation to stimulants.[15] In the present study, higher expres-

sion levels of the *CYP1B1* gene were observed in 44.4% of HCC patients. Increased expression of *CYP1B1* is observed in a wide range of human tumors.[16] Elucidation of underlying common mechanisms leading to aberrant expression of the *CYP1B1* gene in cancer tissues is thus important for diagnosis and treatment of human cancers. Hepatic contents of *CYP1A2* and *CYP2C8/10* are reduced in individuals with elevated bilirubin.[13] In the present study, cases with hepatitis C viral infection tended to express lower levels of multiple *CYP* genes in non-tumor parts compared with tumor parts. The bilirubin levels of these cases were not always low. The number of cases examined in the current study was limited, and we need to determine which clinico-pathological characteristics are closely associated with expression of each *CYP* gene in the liver.

The CYP2 family is the largest and the most diverse of the CYP families and is divided into 5 subfamilies. In the present study, close associations among different subfamily members were found in PBL and in liver tissues. These findings indicate that a major common regulatory mechanism exists. Inflammation and cirrhosis are suggested to regulate the *CYP2A* expression pattern both in mouse models and human HCC. Liver tumors chemically induced in mice invariably overexpress *CYP2A* forms.[17] The prognosis is more unfavorable in patients with CYP2A6-negative tumors than in those with positive tumors.[18] In the current study, *CYP*2 gene expression levels in the tumor part were generally lower than in the non-tumor part in the same individuals. Although the expression level of *CYP2A6* in the non-tumor part was closely associated with those of other *CYP* genes, this was not the case in the tumor part of any HCC. These results suggest that decrease of *CYP2* gene expression, especially that of *CYP2A6*, is a crucial step toward hepatocarcinogenesis in humans. Although Raucy *et al.* reported that the *CYP2E1* mRNA level in PBL reflects *CYP2E1* activity in the liver, we could not find such a correlation. Their result could be indirectly explained by the fact that chlorzoxazone, a well-known substrate of liver CYP2E1, is metabolized in association with *CYP2E1* gene expression in PBL.[6] Furthermore, the patients in the present study had HCC, so the function of the non-tumor part would not be completely normal.

CYP3A enzymes are the most abundant CYPs in human liver and small intestine. CYP3A4 accounts for 28% of the total amount of CYP within the human liver, and is involved in the metabolism of 60% of medications.[4] Gene expression in PBL is controversial; *CYP3A5* but not *CYP3A4* is selectively expressed in human PBL.[19] *CYP3A4* gene expression in human PBL was measured by RT-PCR from 2 mg of total RNA.[20] *CYP3A5* and *3A7* but not *3A4* are detectable in a minority of samples from non-stimulated Caucasians.[21] In the present study, none of the *CYP3A4, 3A5* and *3A7* genes was apparently expressed, presumably because we used single-step RT-PCR from a small amount of total RNA (<100 ng) from only Japanese patients.

Approximately 70% of human liver CYPs are accounted for by *CYP1A2, 2A6, 2B6, 2C, 2D6, 2E1* and *3A4* and most of those are localized in the liver. CYP1A1, 1B1, 2F1 and 4B1 are mainly localized in extra-hepatic organs.[5, 8] Cluster analysis in the non-tumor part revealed that *CYP*s can be classified into two groups, that is, *CYP1B1* from the extra-hepatic group and members of the hepatic group. In PBL and HCC, *CYP1A1*, a member of the extra-hepatic group was always found in the hepatic group, and the structure of the cluster was quite similar between PBL from healthy control and HCC tissues. This indicates that the constitutive expression profile of the *CYP* genes is common among different somatic cells, but *CYP* gene expression levels in hepatocytes are regulated by hepatocyte-specific regulatory factors. Since various liver-specific key transcription factors for *CYP* genes are known,[12] it would be interesting to elucidate which factor is the most relevant to the function and phenotype of hepatocytes.

CYP4 activity is present in rat spleen lymphocytes and activities are high enough for selective studies of oxygenation reactions.[22] In the present study, *CYP4B1* expression was not detectable in the majority of both liver tissues and PBL; if it was detectable, its expression was closely associated between liver tissues and PBL. The *CYP4B1* expression pattern in PBL switched from the extra-hepatic *CYP* group in healthy controls to the hepatic group in HCC patients, indicating that induction of the *CYP4B1* gene by extrinsic factors is closely associated with induction of expression of other *CYP* genes. Furthermore, induced *CYP4B1* gene expression levels in PBL were closely correlated to those in the non-tumor part of the liver. *CYP4B1* is induced by hypoxia,[23] and hypoxia also stimulates the synthesis of CYP-derived inflammatory eicosanoids in a rabbit corneal epithelial model.[24] These results suggest that induced *CYP4B1* gene expression levels are a good index of the systemic status of metabolism and inflammation. In the current study, half of HCC cases revealed higher expressions of the *CYP4B1* gene in the tumor part than in the non-tumor part. Considering that human CYP4B1 is suggested to metabolize carcinogens,[11] the contribution of CYP4B1 to hepatocarcinogenesis needs to be elucidated. Also cytotoxic agents activated by *CYP4B1* such as 4-ipomeanol[25] should be potent chemotherapeutic drugs against HCC. Since the number of cases with a detectable level of *CYP4B1* expression in PBL was limited in the present study, we need to accumulate further cases to assess the clinico-pathological significance of CYP4B1 in liver diseases.

CYP gene expression levels in the tumor part were generally lower than in the non-tumor part of HCC. However, HCC tissues with overexpression of one *CYP* gene tended to show higher expression levels of multiple *CYP* genes, suggesting that chemotherapeutic drugs activated by *CYP* could be more effective than previously expected.

In view of the wide inter-individual and even intra-individual[26] variations of the *CYP* gene expression levels in PBL, changes of *CYP* gene expression divided by basal expression may be preferable to assess the alteration of individual metabolic status. Although the expresion levels of *CYP* genes except for *CYP4B1* in PBL did not reflect those in the liver, further study is needed to assess whether we can apply *CYP* gene expression in PBL as a biomarker for monitoring of the exposure to and risk associated with environmental pollutants.

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