Comparative analysis of ATP-binding cassette (ABC) transporter gene expression levels in peripheral blood leukocytes and in liver with hepatocellular carcinoma

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ATP-binding cassette (ABC) transporters comprise a superfamily of similar proteins involved in transmembrane transport of various substances. ABC transporter family members in the liver participate in bile formation and lipid metabolism. In order to assess whether peripheral blood leukocytes (PBL) are available as a surrogate for determination of the expression of ABC transporter genes in the liver, we compared ABC transporter gene expression levels in PBL with those in liver tissues from patients with hepatocellular carcinoma (HCC). We measured *ABCA1, A2, B1–B4, C1°C5, G1* and *G2* gene expression levels in PBL, and cancerous and non-cancerous portions of liver from patients with hepatocellular carcinoma by means of real time reverse-transcription (RT)-PCR. We could not detect *ABCC5* expression in any tissue of the liver. Close correlations between *ABCA2, C1* and *G1* in PBL and in non-tumor tissues of the liver were found. Compared with the non-tumor part, HCC tissue expressed lower levels of *ABCA1, B4* and *G2*. We think monitoring of *ABCA2, C1* and *G1* gene expression levels in PBL will be useful for selection of anti-cancer agents and monitoring of drug resistance of HCC. Administration of chemotherapeutic agents which are substrates of ABCA1, B4 and G2 should be effective for the treatment of HCC. (Cancer Sci 2004; 95: 530–536)

The ATP-binding cassette (ABC) transporter superfamily is one of the largest gene families of transmembrane proteins.[1–3] ABC transporters bind their substrates and move them through the membrane against a substrate concentration gradient by utilizing energy from hydrolysis of ATP. In humans, the number of known ABC transporters is about 50, which have been classified into 7 subfamilies (ABCA–G) (http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html) based on their homology; they are involved in translocation of a wide variety of substrates across cells and subcellular membranes. The genetic variation in ABC transporter genes is a cause or contributor to a wide variety of drug-resistance phenotype of cancers and metabolic diseases.[4,5] In the normal liver, several ABC transporters are expressed, and are involved in the secretion of components of the bile[6] and the regulation of plasma HDL levels.[3] In humans, at least 8 ABC transporters are known to confer resistance to cytotoxic compounds. Among these, the ABCB (MDR) and ABCC (MRP) subfamilies are well documented. ABCB1 and 3 are localized at the apical (canalicular) membrane of hepatocytes. Amphipathic organic cations and neutral compounds are transported via ABCB1 and phospholipids via ABCB3.[7] ABCC1 and 3 are located in the basolateral membrane, whereas ABCC2 is localized in the canalicular membrane. ABCC2-mediated transport leads to secretion of organic anions into bile and ABCC1 transports glutathione conjugates into blood. ABCC3 is also expressed in bile duct epithelial cells. Hepatic ABCC3 is induced by various conditions and transports substances into blood when ABCC2 is deficient.[8]

Human macrophages contain a variety of ABC transporters involved in cholesterol uptake and/or HDL-dependent cholesterol efflux.[9] Expression of ABCA1 is induced during monocyte differentiation into macrophages and is up-regulated by cholesterol influx.[10]

Since ABC transporters are involved in excretion of xenobiotics and drug resistance of cancer cells, characterization of ABC transporter gene expression in liver cancer in comparison with the non-tumor part of the liver might allow better selection of therapeutic modalities. Although anticancer agents are transported via the blood stream and peripheral blood is obtained for routine medical examination, little is known about ABC transporter expression in peripheral blood leukocytes (PBL). We studied whether ABC transporter gene expression is coordinately regulated and whether ABC transporter gene expression levels in PBL can be used as a surrogate for assessment of those in the liver. The intra-individual correlations of ABC transporter gene expression levels between PBL and liver were also determined. Furthermore, we compared ABC transporter gene expression in tumor and non-tumor tissues from liver cancers to assess whether or not carcinogenesis-associated changes in ABC transporter gene expression levels occur.

Materials and Methods

Materials. We investigated 21 patients with hepatocellular carcinoma (HCC), 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 63.1±7.6 years old at diagnosis (mean±SD, range 46–75 years old) and consisted of 16 males and 5 females. Tumor and non-tumor regions were separately dissected from extirpated tissues. Peripheral blood was obtained with sodium citrate 2 or 3 days before surgical operation, and at 1 day, 7 days and 30 days after operation. As a control, peripheral blood was collected from 20 healthy male volunteers (mean=35.7 years old, 31–46 years old). In our preliminary study, we measured ABC transporter gene expression

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levels in PBL from 2 females and found no significant differences between males and females in any of the genes examined. 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 reverse-transcription (RT)-PCR. Peripheral blood (10 ml) obtained with 3.8% sodium citrate was centrifuged at 3000 rpm for 15 min, and the buffy-coat fraction 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 Bead-beater 8, Biospec Products, Inc., OK).

In our preliminary study, we tried to measure PBL expression levels of 23 ABC transporter genes, i.e., *ABCA1, 2* and *7*, *B1–4 and 7–9*, *C1, 2, 4, 5*, *D1–4*, *E1*, *F1*, *2* and *G1*, *2*, for which primer sets for real time RT-PCR could be designed. We could detect expression of all the ABC transporter genes examined. Because of the limited amount of RNA obtained from PBL, we arbitrarily chose 10 representative ABC transporter genes, i.e., *ABCA1, A2, B1, B4, C1, C2, C4, C5, G1* and *G2*, for expression analysis in PBL and liver tissues. In addition, *ABCB2, B3* and *C3* expression levels were determined in liver tissues. The method for measuring gene expression levels of ABC transporters and control *GAPDH* and *β-actin* genes, and the design of primer sequences and RT-PCR conditions were the same as previously described.[11] Briefly, a *Taq*Man One-Step RT-PCR

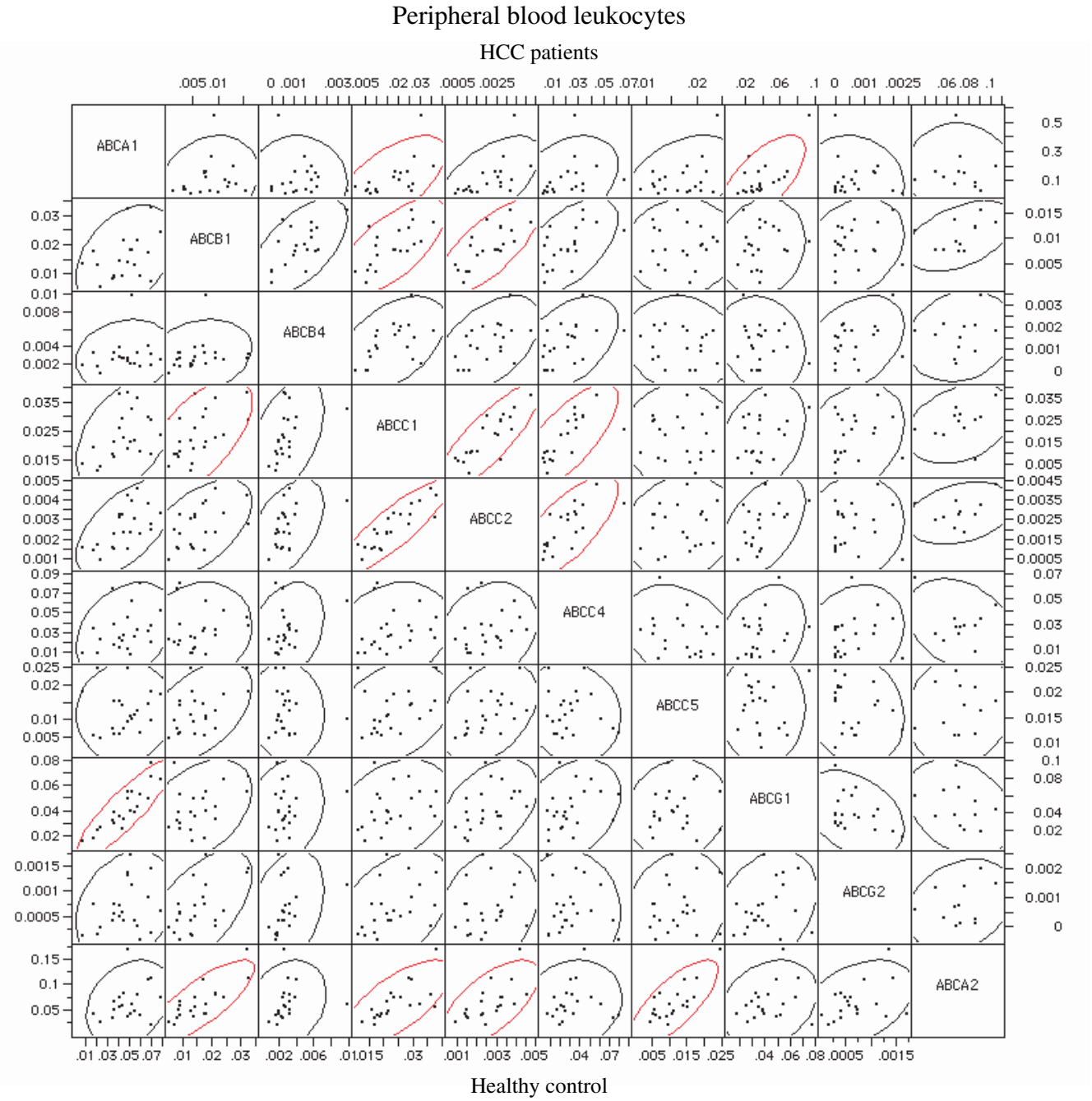

Fig. 1. Correlation plot matrix of ABC transporter gene expression levels in peripheral blood leukocytes. The lines show the range of 1 SD of correlation coefficient calculated by linear regression and are drawn in red in the case correlation coefficient >0.6.

Master Mix Reagents Kit (Applied Biosystems, CA) containing 300 n*M* forward primer, 900 n*M* reverse primer, and 200 n*M* *Taq*Man probe was used at 50 μl/tube. Amplification and detection were performed using the ABI PRISM 7700 Sequence Detector System (Applied Biosystems) with the following profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles each at 95°C for 15 s and 60°C for 1 min. Samples were deemed positive at any given cycle when the value of the emitted fluorescence was greater than the threshold value calculated by the instrument's software (Sequence Detector Ver. 1.6.3). The threshold cycle (C_t), which is defined as the cycle at which PCR amplification reaches a significant value, that is, 15 times greater than the standard deviation of the baseline is given as the mean value. Gene expression is presented as the ratio of target mRNA to *β-actin* mRNA. The expression levels were rounded to three decimal places.

Statistical analysis. Correlation coefficients among expression levels of ABC transporter genes were calculated by linear regression and are presented as a correlation plot matrix. In the present study, if the correlation coefficient of two genes was more than 0.6, we arbitrarily defined that the expression levels of those genes were closely correlated. The significance of the difference between ABC transporter gene expression levels in PBL from HCC patients and healthy controls was determined by using Student's *t* test and that in different preparations from the same patients was determined by use of the Tukey-Kramer test. Cluster analysis of ABC transporter gene expression levels was performed by the Wars method. Statistical analysis was performed using JMP 5.0J (SAS Institute Japan, Inc., Tokyo).

Results

ABC gene expressions in PBL. Among 13 ABC transporter genes, expression levels of *ABCB2, B3* and *C3* in PBL were not determined because of the shortage of total RNA from PBL. *ABCG2* gene expression in PBL was quite low and was not detectable in 4 out of 20 cases with HCC. Only *ABCA1, C1* and *C4* genes showed close correlations between expression levels preoperation and at 1 day after operation (data not shown). We regarded gene expression levels in PBL from preoperation as representative because the patients did not receive any medical treatment at this time.

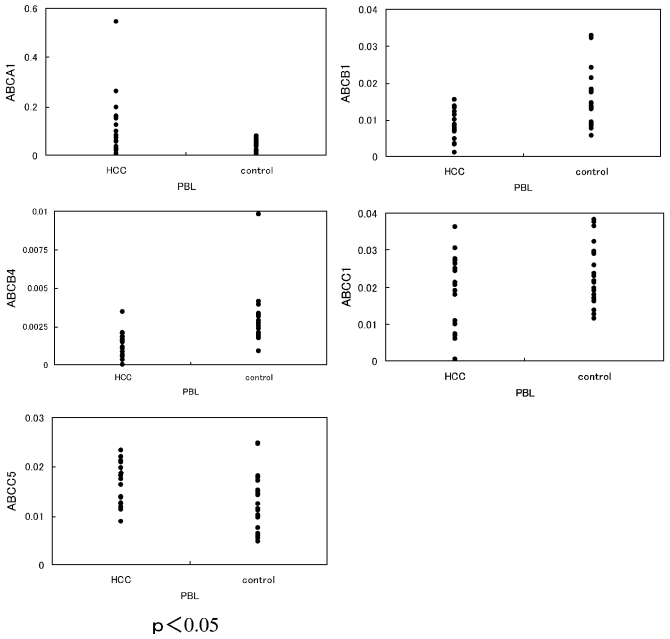

Fig. 2. Comparison of ABC transporter gene expressions between peripheral blood leukocytes from patients with hepatocellular carcinoma and from healthy controls. ABC transporter genes for which the expression levels were significantly different are depicted ($P<0.05$, Student's *t* test).

Expression levels of *ABCB1* and *C1*, *ABCC1* and *C2*, and *A1* and *G1* were closely correlated irrespective of whether PBL was obtained from patients with HCC or healthy volunteers (Fig. 1). In PBL, gene expressions of *ABCA1* and *C5* of HCC patients were significantly higher and those of *ABCB1, B4* and *C1* were significantly lower than those of healthy volunteers (Fig. 2).

ABC gene expression in liver tissues. *ABCC5* gene expression in liver tissues was not detectable. In liver tissues, gene expression levels of *ABCA1*, *A2* and *B1* were closely correlated with those of several other genes in the non-tumor part (Fig. 3). The association between *ABCB2* and *B3*, *B4* and *G2*, and *C2* and *G2* was significant, irrespective of tumor tissues or non-tumor tissues. In liver tissues, *ABCA1, B4* and *G2* gene expression levels were significantly lower, and *ABCC4* and *G1* gene expressions were significantly higher in tumor tissues than in non-tumor tissues (Fig. 4). A close correlation between tumor and non-tumor tissues was found for *ABCC4* gene expression (r=0.67).

Association of ABC gene expression levels in PBL and liver tissues. The expression level of ABC transporter genes tended to be the highest in the non-tumor part, followed by the tumor part of liver tissues and PBL in decreasing order. However, *ABCC4* gene expression in PBL was at the same level as that in the non-tumor part of the liver. Close correlations between the non-tumor part of liver tissues and PBL were observed for *ABCA2, C1* and *G1* (Fig. 5), but no close correlation was found between PBL and tumor tissues. We could not find any correlation of any ABC transporter gene expression and hepatitis B or C virus infection, or total cholesterol level of peripheral blood.

Cluster analysis. In order to identify associations among ABC transporter gene expression levels, we carried out cluster analysis. The expression profiles of ABC transporter genes could be divided into two groups, one containing *ABCG1* and the other not. *ABCA1* was in the *ABCG1* group, except for non-tumor tissue. *ABCC4* in PBL was in the *ABCG1* group in healthy controls, whereas it was in the other group in HCC patients. In liver tissues, *ABCC1* and *C4* were always in the *ABCG1* group and *ABCA1* was in the same group in tumor tissues, but in the other group in non-tumor tissues (Fig. 6).

Discussion

ABC transporters are involved in bile acid formation in the liver and hepatic clearance,[12, 13] and some ABC transporters in leukocytes regulate reverse cholesterol transport.[14] Aberrations of ABC transporters result in various diseases mainly associated with lipid metabolism and liver functions.[4, 5] Recently expression profiling of 47 ABC transporter genes in 20 different human tissues was performed.[15] However, RNA analyzed in that study was commercially purchased and each RNA preparation was a mixture from different individuals. Since the extent of inter-individual variation of ABC transporter gene expression is unknown, a comprehensive and quantitative study to correlate individual ABC transporters is necessary to elucidate their clinical relevance. Although the number of ABC transporter genes examined was limited, the present study correlated individual ABC transporters in PBL or in liver tissues. Furthermore, this study is the first to directly compare ABC transporter gene expression levels in human PBL and in liver tissues. In the present study, we analyzed expression of 10 ABC transporter genes both in PBL and in liver tissues, including HCC.

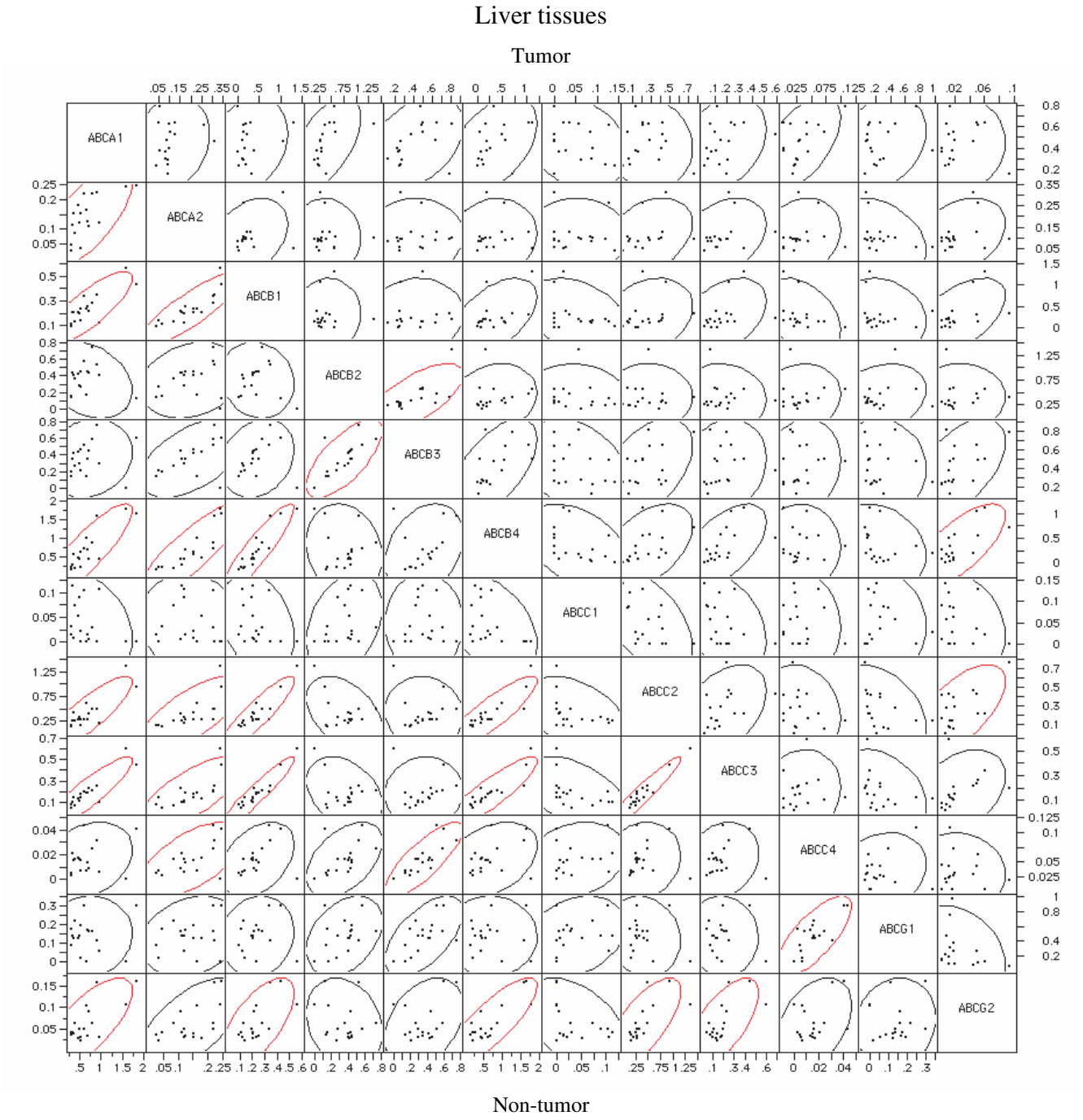

Fig. 3. Correlation plot matrix of ABC transporter gene expression levels in liver tissues. The right upper half shows the relationship in the tumor part and the left lower half is that in the non-tumor part. The lines show the range of 1 SD of correlation coefficient calculated by linear regression and are drawn in red in the case correlation coefficient >0.7.

Except for *ABCA1, C1* and *C4*, no close correlation was found among levels at different days after operation within each ABC transporter gene, indicating that the transcriptional level of ABC transporter genes is affected by medical treatment and the general condition of patients. Although their physiological functions are not known, both *ABCC3* and *G2* are associated with drug resistance. In the present study, *ABCG2* (also known as *BCRP*, *MXR* or *ABCP*) gene expression was low or not detectable in PBL. However, it is highly expressed in a subpopulation of hematopoietic stem cells.[16] Thus, *ABCG2* gene expression could be a marker of hematopoietic malignancy.

The present study showed that *ABCB1, C1* and *C2* gene expression levels in PBL are closely correlated. Since all these 3 transporters are well known to be involved in multidrug resistance, it would be interesting to know if the expression levels of these transporter genes are also correlated in stem cells. If they are, then combination chemotherapy with a broader spectrum of anti-cancer agents than is generally used could be useful for cancer treatment.

Both ABCA1 and G1 are involved in cholesterol efflux and these genes show striking similarities in expression pattern in monocytic cells *in vitro*.[17] The present study revealed a close

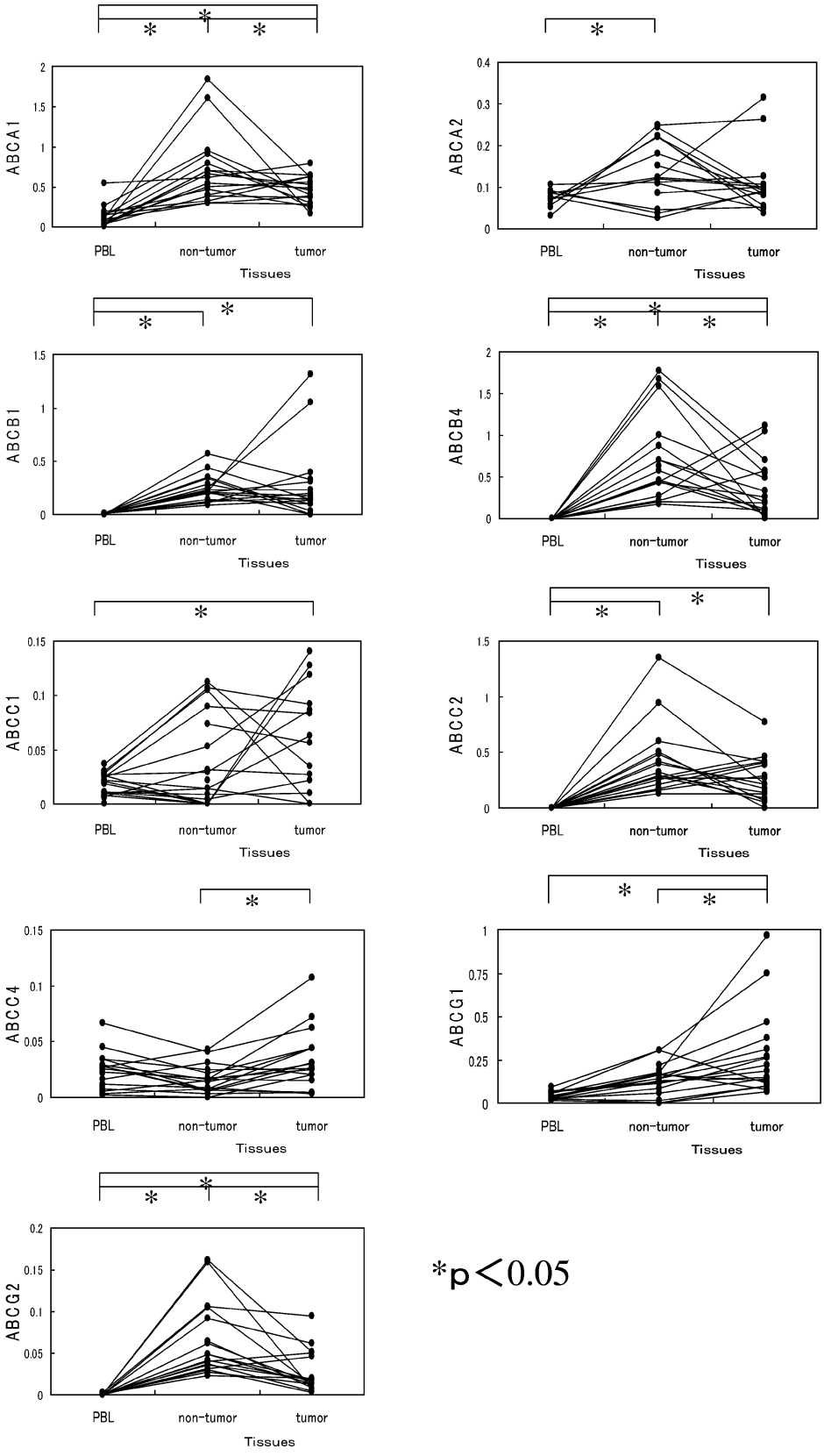

Fig. 4. Comparison of ABC transporter gene expression levels among peripheral blood (PBL), and non-tumor and tumor parts of the liver with hepatocellular carcinoma. Each line shows different preparations from the same individual. * $P<0.05$ (Tukey-Kramer test).

correlation between the expression levels of these genes in PBL, indicating that the genes may be transcriptionally regulated in concert to control cholesterol metabolism in PBL under physiological conditions. Cluster analysis revealed that *ABCA1* and *G1* gene expression levels in tumor tissue are regulated similarly, whereas this is not the case in non-tumor tissue.

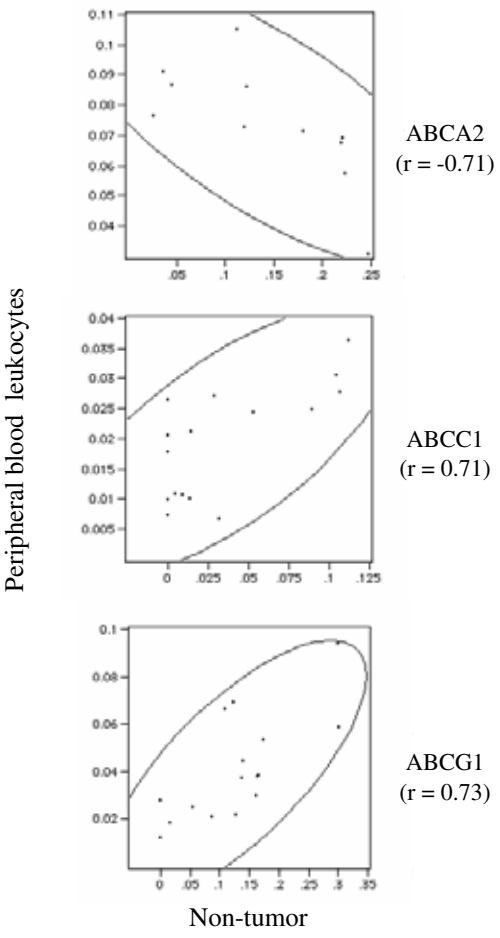

Fig. 5. Association of *ABCA2, C1* and *G1* gene expression levels between peripheral blood leukocytes and liver tissues.

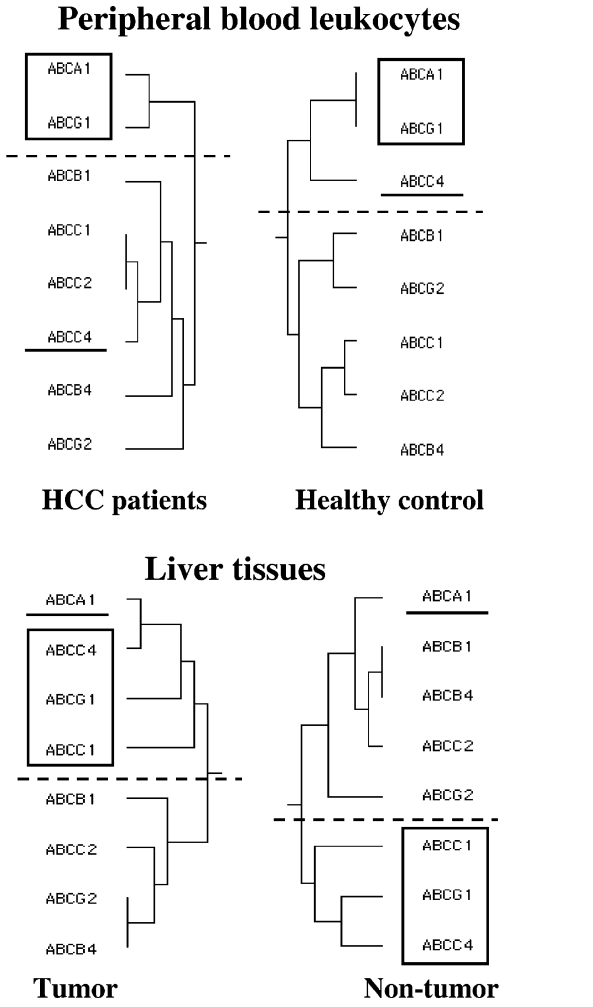

Fig. 6. Cluster analysis of ABC transporter gene expression levels in peripheral blood leukocytes and in liver tissues. The expression pattern of the ABC transporter genes can be roughly divided into two groups; that with ABCG1 (enclosed by the rectangle) and that without ABCG1. ABCA1 was in the ABCG1 group except in the non-tumor part of the liver. ABCC4 in PBL was in a different group between healthy controls and HCC patients.

Compared with the tumor region, the non-tumor part revealed higher expression of *ABCA1* and lower expression of *ABCG1*. These results suggest liver-specific suppressive regulation at the transcriptional level in normal liver. *ABCG2* is a placenta-specific ABC and confers a multidrug resistance phenotype if overexpressed in human cancer cell lines.[18] It is widely present in untreated human solid tumors, including HCC.[19] In the current study, *ABCG2* expression was closely correlated to *ABCC2* expression in the liver, suggesting that we need be more vigilant about natural cross resistance to chemotherapeutic agents than had been thought.

ABCB2 and *B3* genes are located in the same chromosomal locus at 6p21.3, the human leukocyte antigen (HLA) gene complex, and the gene products function by forming a heterodimer. These molecules are necessary for the expression of class I HLA molecules.[20] Although downregulation of HLA class I molecules in human tumor tissues has been suggested, HLA class I molecules form a set of collaborating gene products even in tumors.[21] This is consistent with the observations in the present study that *ABCB2* and *B3* gene expressions were closely correlated irrespective of tumor or non-tumor tissues of the liver, and that their expression levels were not different. These findings suggest that HCC does not necessarily evade cytotoxic effectors by downregulation of HLA class I molecules.

In the current study *ABCB4* and *C2* gene expression levels were closely correlated to the *ABCG2* gene expression level in liver tissues. Both ABCB4 and C2 are expressed in the bile canalicular membrane[6, 22] and secrete fatty acids and organic anions into bile, respectively. It is reasonable that these molecules are coordinately regulated to accomplish bile formation. ABCG2 is known to confer resistance to anthracycline anticancer drugs and C2 to a variety of natural products, as well as anthracyclines and cisplatinum.[23] Like ABCA1, which is involved in cholesterol transport, *ABCB4* and *G2* showed lower gene expression levels in tumor tissues than in non-tumor tissues. In HCC cells, especially in the early stages, fatty degeneration is not an uncommon finding in histological specimens. Underexpression of these genes in tumor tissues could be responsible for this histological finding. Furthermore, HCC with fatty degeneration could be more sensitive to anthracyclin anticancer drugs than HCC without fatty degeneration.

Liver tissues infected with hepatitis C virus (HCV) infection reveal significantly decreased *ABCC2* gene expression compared with tissues without infection. This reduction is attributed to pro-inflammatory cytokines secreted during hepatitis.[24] We could not observe significant differences of ABC transporter gene expression in association with HCV infection because, in the current study, only 1 case was negative for HCV and HBV infections, and this case was histologically accompanied with fibrosis and chronic inflammation. We need to analyze further

cases without hepatitis to assess the influence of hepatitis on ABC transporter gene expression.

The present study has shown that PBL can be used as a surrogate for *ABCA2, C1* and *G1* expression levels in the liver. Although ABCA2 is mainly localized in oligodendroglia[25] and its main function is thought to be neural transmembrane lipid export in the brain,[17] an ovarian cancer cell line with ABCA2 overexpresssion demonstrated estramustine resistance.[26] Since ABCC1 is involved in a multidrug resistance phenotype, *ABCA2* and *C1* expression levels in PBL should be taken into account in the selection of anti-cancer drugs for HCC treatment. ABCA2 and G1 are involved in cholesterol efflux and these genes are known to share target sites for a transcription factor, SP1 which can modulate the promoter activity of cholesterol-responsive transporters.[27] However, we could not find any association of these gene expression levels with blood levels of total cholesterol. We need to study blood levels of other lipids to look for possible associations.

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