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Original Article

Gene expression profiling in hepatoblastoma cases of the Japanese Study Group for Pediatric Liver Tumors-2 (JPLT-2) trial

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ABSTRACT

Background: Over the past two decades, significant improvements in the outcomes of children diagnosed with hepatoblastoma (HBL) have resulted from developments in diagnostic methods and treatments. However, the outcomes of some cases remain unfavorable, and others suffer from late complications caused by treatment.

Procedure: We elucidated the genetic profile of HBLs to identify biological markers for diagnosis and to determine the grade of malignancy. RNA samples extracted from 53 specimens of fresh-frozen HBL and corresponding noncancerous liver tissues were used for gene expression profiling and pathway analysis.

Results: In the comparison between HBL and noncancerous liver tissues, genes involved in several transcription pathways including glypican 3 and Wnt signaling pathway members were upregulated, whereas cytochrome p450 family genes were downregulated. The analyses of high-risk (metastatic) HBL and HBL progression or recurrence cases revealed upregulated expression of histone cluster genes and upregulation of RXR activators molecular signaling. Clustering analysis of the tumor samples revealed three distinct groups among the HBL cases.

Conclusion: Aberrant expression of genes involved in tissue differentiation pathways may be related to the development of HBL, and such genes, including *AFP*, *PGC*, *SPINK1*, and *NQO1*, may be putative therapeutic targets for HBL progression

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Introduction

Hepatoblastoma (HBL) is the most common primary liver tumor in children and is usually diagnosed during the first 3 years of life. Before 1980, the only curative treatment for children with malignant hepatic tumors was complete surgical resection of the tumor. The introduction of effective chemotherapeutic regimens in the 1980s resulted in an increased number of patients ultimately eligible for tumor resection and a reduced postoperative recurrence rate [1-3]. However, poor outcomes are still seen in several HBL cases, especially those with distant metastasis. The identification and development of new prognostic

stratifications have led to novel treatments for high-risk patients and reduced treatment for low-risk patients to avoid the delayed effects and unnecessary toxicities associated with treatment [4]. Until now, few biological markers to stratify high-risk tumors have been reported, and no biological markers have been applied in the clinic [5, 6]. To identify new molecular biomarkers for risk stratification and malignant grade, we performed microarray analysis of gene expression in samples from HBL patients prior to treatment.

Materials and Methods

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I Patients

Among approximately 400 patients enrolled in Japanese Study Group for Pediatric Liver Tumors -2 (JPLT-2) trial, approximately 360 hepatoblastoma (HBL) patients underwent this protocol between December 1999 and November 2012 at the institutions of the JPLT. Tumor and noncancerous liver tissue (NCL) samples from more than 100 of these HB patients were obtained at diagnosis before chemotherapy and stored at -80°C . The JPLT-2 consisted of two different treatment protocols: cisplatin and pirarubicin as first-line treatment and ifosfamide, pirarubicin, etoposide, and carboplatin as second-line treatment [7]. The human ethics review committee of our university approved the study protocol, and signed informed consent was obtained from the parent of each patient (Ethics Committee Approval No. 20). The clinical disease stage was determined at the time of initial diagnosis according to the classification of the pre-treatment extent of disease (PRETEXT) staging system, which is based on the number of liver segments involved, extent of local invasion, extent of regional lymph node involvement, and presence of distant metastasis [8]. The Childhood Hepatic Tumors International Collaboration (CHIC) is a new international hepatoblastoma database created by four major study groups: the International Childhood Liver Tumors Strategy Group (SIOPEL), Children's Oncology Group, German Association of Pediatric Hematology and Oncology, and JPLT [9, 10]. Risk stratification was also performed according to the CHIC classification criteria based on clinical backbones. Briefly, standard-risk disease is defined as PRETEXT I/II tumors or PRETEXT III tumors with negative PRETEXT annotation factors, intermediate-risk disease as PRETEXT IV tumors or PRETEXT I–III tumors with positive PRETEXT annotation factors, and high-risk disease as metastatic disease. The pathological classification of HBLs by Haas *et al.* and the Japanese Society of Pathology grouped HBLs into two major subtypes: well-differentiated (fetal) and poorly differentiated (embryonal) types [8, 11]. In this study, we enrolled 53 patients whose tumor RNA quality was sufficient for microarray analysis. The clinical features of the patients are summarized in (Table 1).

II Tissue samples

Tumor tissue were obtained during surgery or biopsy from more than 100 patients prior to any chemotherapy and corresponding noncancerous liver (NCL) tissue specimens were also obtained to the extent possible. Among the 53 cases whose tumor RNA was high quality, 14 NCL samples were available. These samples were immediately stored at -80°C until use. The tissue adjacent to each frozen tissue specimen was examined by pathological review for diagnosis and confirmation.

III DNA and RNA extraction

Tissue DNA samples were extracted and purified using standard methods. Total cellular RNA was extracted from tumor tissues by the acid guanidinium thiocyanate–phenol–chloroform method [12]. The quantity and quality of the extracted RNA were assessed using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA, USA). We selected 53 RNA samples whose RNA integrity number (RIN) values were greater than 7.4.

IV Detection of *CTNNB1* (Catenin Beta 1) gene mutations and deletions

To detect mutations and deletions in *CTNNB1*, genomic DNA from each tumor specimen and corresponding noncancerous liver tissue was amplified by PCR using primers targeting exon 3 of *CTNNB1*, as described previously [13]. To detect amplicons harboring a large deletion event involving exon 3, the sizes of the PCR products were analyzed by 2% agarose gel electrophoresis. To detect point mutations in exon 3, the PCR products were reamplified using the following internal primers: 5'-AAAATCCAGCGTGGACAATGG-3' and 5'-TGTGGCAAGTCTGCATCATC-3'. The resulting PCR products were sequenced using the ABI 3100 DNA sequences. Mutations were confirmed by at least two amplification reactions from the original DNA.

V Microarray analysis

Microarray experiments were performed using the Affymetrix Gene Chip (Affymetrix, Inc., Santa Clara, CA, USA), according to the standard protocols, and the Ambion WT Expression kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). First-strand cDNA was generated from 250 ng total RNA obtained from HB tumor and normal liver tissues using reverse transcriptase and the T7 primer. Second-strand cDNA was generated using a DNA polymerase mix containing RNase H. Antisense complementary RNA (cRNA) was generated via *in vitro* transcription using the T7 RNA polymerase and was purified using nucleic-acid-binding beads. Second-cycle cDNA was generated from the purified antisense cRNA; 5.5 μg of the resulting cDNA were then subjected to fragmentation and terminal labeling and hybridized to the Human Gene 1.0 ST array (Affimatrix, Thermo Fisher Scientific, Santa Clara, CA), which contains 36,079 probes including 32,020 annotated RefSeq transcripts. After hybridization, the arrays were rinsed and labeled with streptavidin–Cy5, scanned using a scanner (GeneChip™ Scanner 3000 7G, Affimatrix, Thermo Fisher Scientific), and then analyzed using Gene Spring Software (Gene Spring GX ver. 14.9, Agilnet, Santa Clara, CA). The microarray data were registered in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>).

VI Microarray data analysis

For comparisons of gene expression fold changes determined from the microarray data, we considered the RefSeq probes on the array to be nonredundant and representative of the whole transcriptome. First, we compared gene expression changes between HBL and NCL tissues. In the 14 cases with available tumor and NCL tissues, gene expression levels between paired tumor and NCL tissues were performed. Otherwise, gene expression levels were also compared between whole expression data of 53 tumor samples and that of 14 noncancerous liver tissue. Second, among the HBL samples, we compared gene expression levels among the standard-, intermediate-, and high-risk HBL cases. Gene expression between the event-free surviving and recurrence or progression cases was also compared. The resulting gene lists were filtered and uploaded to the Ingenuity Pathway Analysis (TOMY Digital Biology, Inc., Tokyo, Japan) and Key Molnet ver. 2 (KM Data Co., Tokyo, Japan) software programs.

Table 1. Clinicopathological features of the hepatoblastoma samples used for microarray analysis.

Age (months)	
Median	22
Range	0-109
Sex	
Female	25 (47.2%)
Male	28 (52.8%)
Histologic type	
Well differentiated	30 (56.6%)
Poorly differentiated	21 (39.6%)
Other	2 (3.8%)
PRETEXT classification ^a	
I	9 (18.0%)
II	15 (28.3%)
III	18 (34.0%)
IV	11 (20.7%)
Distant metastasis	14 (26.4%)
CHIC risk stratification ^b	
Standard	31 (58.5%)
Intermediate	8 (15.1%)
High	14 (26.4%)
CTNNB1 gene alteration	
Deletion	23 (43.4%)
Mutation (exon 3)	16 (18.9%)
Event	
Free	32 (60.4%)
+	21 (39.6%)
Clinical course	
Alive	38 (71.7%)
Dead	15 (28.3%)

^aPRETEXT: PRETreatment of EXTent of disease ^{2,7}, ^bCHIC: Children Hepatic tumor International Collaboration ^{9,10}

VII Statistical analysis

The Mann–Whitney *U* test and Chi-square test were used to examine the relationships between clinical factors and gene expression profiles. All tests were two tailed, and differences with a *P*-value < 0.05 were regarded as statistically significant.

Results

I Gene expression profiles of HBL tissue compared with noncancerous liver tissue

The gene expression profiles of noncancerous liver (NCL) and HBL tissues were obviously different. Therefore, the differences in expression of specific genes between the tumor and NCL tissue samples were analyzed. The lists of the upregulated and downregulated genes are shown in Table 2. The upregulated genes were those with a greater than 10-fold increase in expression in more than half of HBL samples compared with the paired NCL tissues, and the downregulated genes were those with a less than 15-fold decrease in expression in more than half of HBL samples. Of these genes, *REG3A* (Regenerating Family Member 3 Alpha), glypican 3 (GPC3), and Wnt signaling-related genes were upregulated in HBL. The network analysis of differentially expressed genes encoding transcription factors, including *HNF1A* (Hepatocyte Nuclear Factor 1 Alpha), *HNF4A* (Hepatocyte Nuclear Factor 4 Alpha), *C/EBP*, *CTNNB1*, and *FOXA3*, indicated several pathways including FXR (farnesoid X receptor) /RXR (retinoid X receptor activation), LXR (liver X receptor) /RXR and PXR (pregnane X receptor)/RXR pathways potentially related to tumor development [14]. The downregulated genes (Table 2) included cytochrome P450 (CYP) family genes, solute carrier family genes, and genes encoding enzymes, such as tyrosine aminotransferase and tryptophan 2,3-dioxygenase. Of the *CYP* family genes, *CYP2B6*, *CYP2C8*, *CYP2B7P1*, and *CYP2B7P1* were markedly downregulated. The pathway analysis indicated upstream genes including *PXR* and *CAR* are suspected as regulatory factors in these genes. The network analysis of differentially expressed genes also indicated genes associated with complement activation and blood coagulation.

Table 2: Up- and downregulated genes in hepatoblastoma tissues compared with noncancerous tissues

Gene symbol	Gene	Location
Upregulated genes		
REG3A	regenerating islet-derived 3 alpha	chr2
DPEP1	dipeptidase 1 (renal)	chr16
SNORD114-26	small nucleolar RNA, C/D box 114-26	chr14
LGR5	leucine-rich repeat-containing G protein-coupled receptor 5	chr12
DKK1	dickkopf homolog 1 (Xenopus laevis)	chr10
EPCAM	epithelial cell adhesion molecule	chr4
TSPAN5	tetraspanin 5	chr4
APCDD1	adenomatosis polyposis coli down-regulated 1	chr18
SNORD113-4	small nucleolar RNA, C/D box 113-4	chr14
NOTUM	notum pectinacylesterase homolog (Drosophila)	chr17
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	chr12
NKD1	naked cuticle homolog 1 (Drosophila)	chr16

DKK4	dickkopf homolog 4 (<i>Xenopus laevis</i>)	chr8
MEP1A	meprin A, alpha (PABA peptidase hydrolase)	chr6
SLC7A11	solute carrier family 7, member 11	chr4
PEG10	paternally expressed 10	chr7
DLK1	delta-like 1 homolog (<i>Drosophila</i>)	chr14
C9orf4	chromosome 9 open reading frame 4	chr9
GPC3	glypican 3	chrX
Downregulated genes		
CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	chr19
CYP2C8 CYP2C19	cytochrome P450, family 2, subfamily C, polypeptide 8 19	chr10
TAT	tyrosine aminotransferase	chr16
CYP2B7P1	cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1	chr19
SLC10A1	solute carrier family 10, member 1	chr14
TDO2	tryptophan 2,3-dioxygenase	chr4
CYP2A6 CYP2A7 CYP2A13	cytochrome P450, family 2, subfamily A, polypeptide 6 7 13	chr19
HSD17B6	hydroxysteroid 17-Beta Dehydrogenase 6	chr12
APOF	apolipoprotein F	chr12
HAL	histidine ammonia-lyase	chr12
C9	complement component 9	chr5
FCN3	ficolin 3 (Hakata antigen)	chr1
SLC22A1	solute carrier family 22, member 1	chr6
HSD17B13	hydroxysteroid (17-beta) dehydrogenase 13	chr4
F9	coagulation factor IX	chrX
HAO2	hydroxyacid oxidase 2 (long chain)	chr1
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	chr1
CRP	C-reactive protein, pentraxin-related	chr1
GLYAT	glycine-N-acyltransferase	chr11
ABCB11	ATP-binding cassette, sub-family B, member 11	chr2
NNMT	nicotinamide N-methyltransferase	chr11
SLC1A1	solute carrier family 1, member 1	chr9

II Analysis of gene expression according to risk stratification

Clinically, among the several risk stratification systems proposed for HBL, we used the CHIC classification criteria to classify HBL into three risk categories: standard, intermediate, and high risk [9, 10]. The high-risk group consisted of HBL cases with distant metastasis, and the primary tumors showed significantly upregulated expression of *TERC* (*Telomerase RNA Component*), *GFRA3* (GDNF Family Receptor Alpha 3), *SPINK1* (Serine Peptidase Inhibitor, Kazal Type 1), *DUSP9* (Dual Specificity Phosphatase 9), and histone cluster genes such as *HIST1H3B*. On the other hand, *AFM* (*Aflandin*, a member of albumin gene family), *HAO1* (Hydroxyacid Oxidase 1), *BAATI* (Bile Acid-CoA:

Amino Acid N-Acyltransferase) and cytochrome P450 family genes (*CYP3A43*, *CYP2C9* etc.) were downregulated. In the comparison the standard- and intermediate-risk groups, *SPINK1* and *KRT23* (Keratin 23) were upregulated more than twofold, and *HSD17B2* (Hydroxysteroid 17-Beta Dehydrogenase 6), *PIPOX* (Pipecolic Acid And Sarcosine Oxidase), *ABCAB* (ATP-binding cassette, sub-family B, member 11), *CYP8B1*, *BDH1* (3-Hydroxybutyrate Dehydrogenase 1), and *XPNPEP2* (X-Prolyl Aminopeptidase 2) were downregulated more than twofold. These down regulate genes were correlated with hepatocyte differentiation. The pathway analysis of these genes indicated downregulation of bile acid biosynthesis signaling and correlate with liver hyperplasia and hyperproliferation.

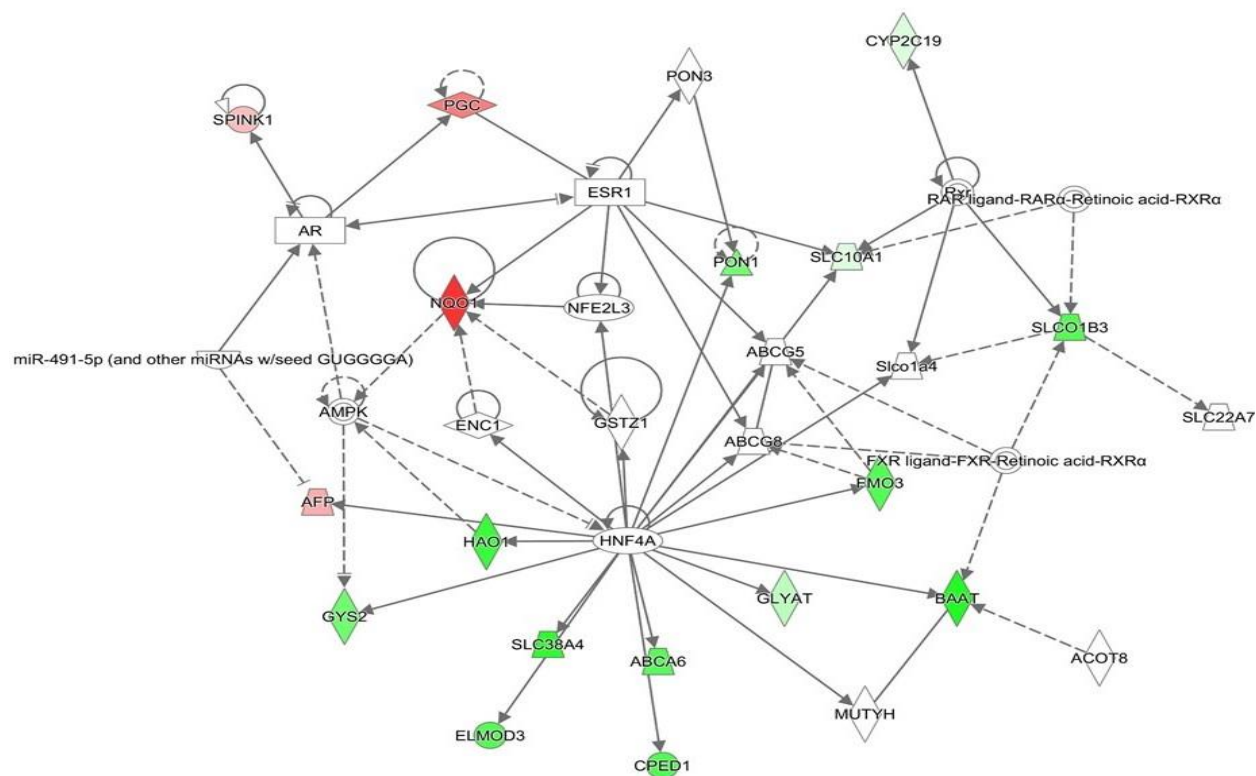


Figure 1: Network analysis using the upregulated or downregulated genes in progression or relapse cases.

The upregulated or downregulated genes more than twofold with the comparison of the relapse or progression cases to the other HBL cases. The genes upregulated or downregulated genes more than twofold are listed in Table 3. Red marks are up-regulated and blue marks are down-regulated genes. In this network, the core upstream genes of this network are *HNF4A* (Hepatocyte Nuclear Factor 4 Alpha), *GSTZ1* (Glutathione S-Transferase Zeta 1), *NFE2L3* (Nuclear Factor, Erythroid 2 Like 3) and *ESR1* (Estrogen Receptor 1).

III Gene expression analysis in the primary tumors of HBL progression or relapse cases

Among the HBL cases evaluated, 19 were patients with relapse or progression, and 13 were patients who subsequently died of HBL. The gene expression profiles of the relapse or progression cases were compared with those of the other HBL cases. The genes upregulated or downregulated genes more than twofold are listed in (Table 3). Among the upregulated genes, *AFP* is located on Chr. 4, *SPINK1*, and *PGC* (Progastricsin) were on chromosome 5, and *NQO1* (NAD(P)H Quinone Dehydrogenase 1) is on chromosome 16. The downregulated genes were *GLYAT* (Glycine-N-Acyltransferase), located on chromosome 11, *FMO3* (flavin containing monooxygenase 3), *GYS2* (glycogen synthase 2), *BAAT* (bile acid Coenzyme A), *SLCO1B3* (solute carrier organic anion transporter family, member 1B3), *HAO1* (hydroxyacid oxidase (glycolate oxidase) 1), *CYP2C19* (cytochrome P450, family 2, subfamily C), polypeptide 19), *SLC38A4* (solute carrier family 38, member 4), *SLC10A1* (solute carrier family 10 (sodium/bile acid cotransporter family), member 1), *ABCA6* (ATP-binding cassette, member 6), and *PON1* (paraoxonase 1). *GLYAT* located on chromosome 11 and *CYP2C19*, *SLCO1B3*, *GYS2*, and *SLC38A4*, located on chromosome 12.

The pathway analysis of these genes indicated upregulation of FXR/RXR, which is associated with regulation of *HNF4A* (Figure 1). In this network, the core upstream genes of this network are *HNF4A*, *GSTZ1*

(*Glutathione S-Transferase Zeta 1*), *NFE2L3* (*Nuclear Factor, Erythroid 2 Like 3*) and *ESR1* (*Estrogen Receptor 1*).

IV Clustering analysis

The results of unsupervised clustering of 16 genes up- and downregulated more than twofold is shown in (Figure 2). We found three clusters. In the upper cluster, none of the 20 patients died of HBL, whereas in the other two clusters, 13 of 33 patients died of HBL ($P < 0.01$). Interestingly, the two oldest patients (7 and 8 years old) with low-risk tumors were included in the upper cluster, whereas most of those represented in the bottom cluster were less than 1 year old. *CTNNB1* gene alterations and histological classification were not associated with these clusters, suggesting that these gene expression profiles may be independent prognostic factors for HBL. Since these patients were treated in the same clinical trial, the gene expression profiles provide information on the biological characteristics of HBL.

Discussion

There are some previous reports of gene expression data in HBL samples, but no data from untreated HBL patients who were enrolled in the clinical trial and treated under the same regimen have been reported [15-18]. This study consisted of tumor samples from patients who participated in the JPLT-2, and all tumor samples were collected prior to

treatment. Therefore, the gene expression data of our series were not influenced by treatments such as preoperative chemotherapy and vascular intervention.

The up- and down-regulated genes in the comparison between paired NLT and HBL tumor RNA showed activation of Wnt signaling genes and down-regulation of liver function correlating enzyme such as tyrosine aminotransferase and apolipoprotein F. The network analysis of the genes differentially expressed between HBL and NLT samples suggested *CTNNB1*, *HNFA1A*, and *HNFA4A* as upstream transcription factors. The *CTNNB1* gene is mutated at a high frequency in HBL, supporting our identification of these genes as distinguishing expressed in HBL. In addition, CYP family members were among the genes downregulated in HBL. These expression profiles suggest that activation of hepatocyte proliferation and inhibition of differentiation are associated with HBL. Among the upregulated genes, *DKK1* is an inhibitor of Wnt signaling, and *GPC3* overexpression has been reported previously in HBL as well as nephroblastoma [19-22]. Therefore, these gene expression profiles seemed to be reliable, and the oncogenesis of HBL may depend on aberrant Wnt signaling and ontogenesis.

To clarify the biological differences in HBL, we analyzed differences in the gene expression profiles according to risk stratification. Almost all high-risk HBL cases had distant metastasis and high expression of histone cluster genes and small nucleolar RNA, suggesting that distant

metastasis of HBL may be correlated with epigenetic regulation. Among these upregulated genes, *TERC* is the essential component of human telomerase, which is activated in human stem cells and immortalized cells. Our previous study already indicated the high telomerase activity of HBL is correlated with poor outcome of the patients [5, 23]. Therefore, telomerase activation and *TERC* expression might be the key factor for HBL progression. In the comparison between standard- and intermediate-risk tumors, the downregulation of genes involved in the *SIBLING* signaling pathway indicated that inhibition of tissue differentiation may be associated with local tumor infiltration. As tissue differentiation is regulated by epigenetic modifications, HBL development and progression may also be related to epigenetic regulation [24, 25]. The gene expression profiles of the HBL progression or relapse cases showed upregulation of four genes: *AFP*, *SPINK1*, *PGC*, and *NQO1*. An association between *AFP* overexpression and HBL progression has been reported previously [18]. These data also suggested that epigenetic regulation plays a role in HBL progression or relapse, because the differentially expressed genes are restricted to specific chromosomes. Chromosomal aberrations, including loss of chromosome 11, have been correlated with tumor progression [6, 26]. Some differentially expressed genes may result from chromosomal aberrations, including those in chromosomes 1 and 2, where had already reported as frequently aberrant regions in HBL [27]. Further analyses including genomic aberrations should be performed for clarification of the genomic characteristics of HBL.

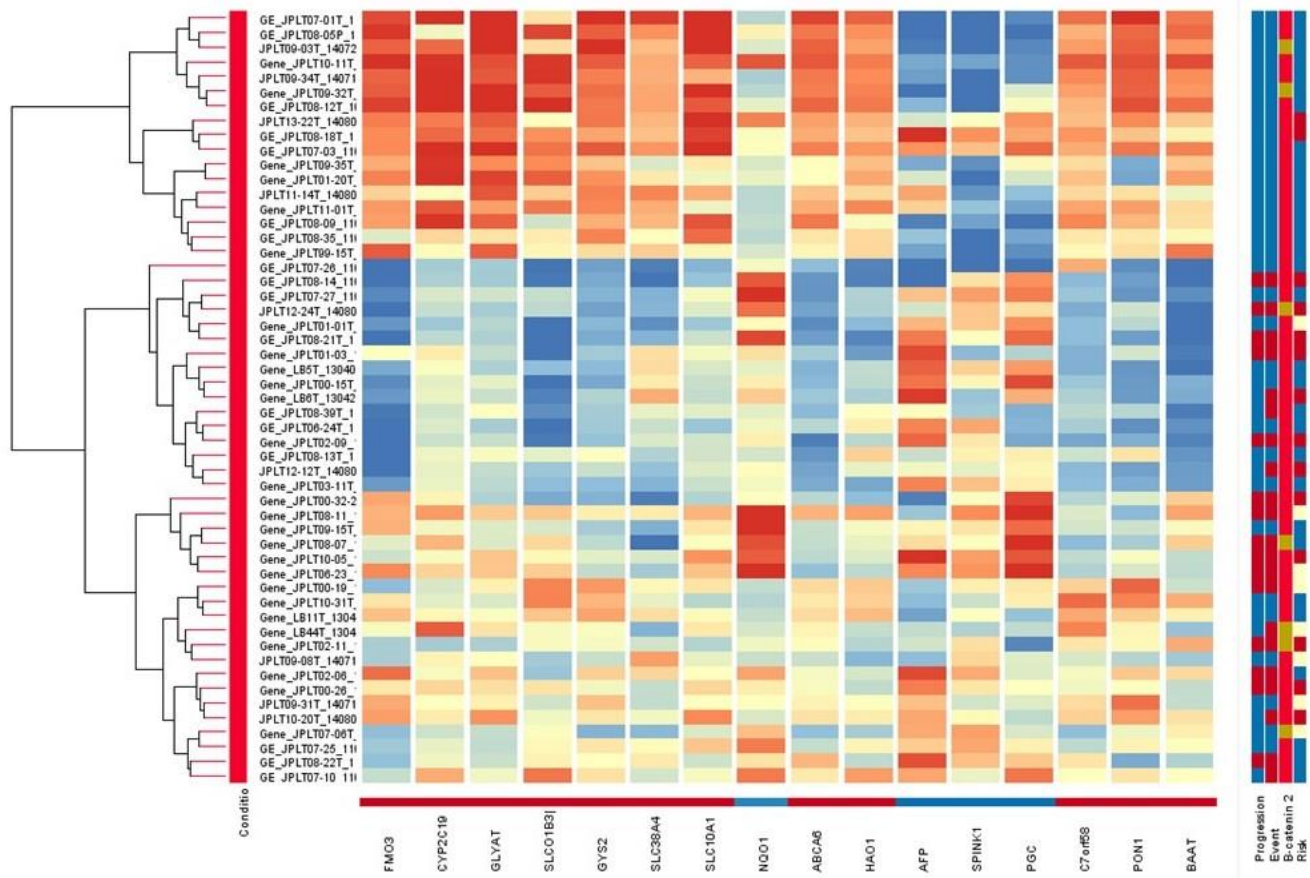


Figure 2: Unsupervised clustering of gene expression profiles of hepatoblastoma (HBL) tissue samples.

Cluster heatmap of the expression (median centered by row) of 16 genes that were more than two-fold up- or downregulated in all 53 HBL tissue samples. Clustering analysis revealed three distinct clusters: (A) upper, (B) middle, and (C) bottom. Of the 33 cases represented in the upper and middle clusters, 13 subsequently died of HBL, whereas none in the upper cluster ($n = 20$) died of HBL ($P < 0.01$).

The clustering analysis of the genes differentially expressed in the relapse or progression cases revealed three major tumor clusters (Figure 2). There were several differences among these HBL cases in terms of histology and patient age at onset. All deceased cases ($n = 13$) were included in the top and middle clusters, suggesting that this clustering analysis may be useful for risk stratification of HBL tumors. Sixteen genes discriminated in invasive and metastatic HBL samples by Cairo et al. were not included among our genes differentially expressed more than twofold, except for AFP [18]. Since most of the samples in their

study were isolated after chemotherapy, the difference in gene expression profiles compared with our study may have been due to treatment effects. Our pathway analysis of the gene set in (Table 3) revealed significant activation of RXR pathway and inhibition of LPS/IL-1 mediated pro-inflammatory cytokine signaling which leads to impaired metabolism, transport and/or biosynthesis of lipid, cholesterol, bile acid and xenobiotics. These genes may provide novel therapeutic targets.

Table 3: List of up- and downregulated genes in tissues from hepatoblastoma recurrence or progression cases

		Fold change
Upregulated (> 2-fold)		
SPINK1	serine peptidase inhibitor, Kazal type 1	2.92124
AFP	alpha-fetoprotein	2.49094
PGC	progastricsin (pepsinogen C)	2.45864
NQO1	NAD(P)H dehydrogenase, quinone 1	2.15604
Downregulated (> 2-fold)		
GLYAT		-2.57142
FMO3	glycine-N-acyltransferase	-2.51047
GYS2	flavin containing monooxygenase 3	-2.44638
BAAT	glycogen synthase 2	-2.21552
SLCO1B3	bile acid coenzyme A: amino acid N-acyltransferase	-2.16548
ELMOD3	solute carrier organic anion transporter family, member 1B3 ELMO/CED-12 domain containing 3	
HAO1	hydroxyacid oxidase (glycolate oxidase) 1	-2.15545
CYP2C19	cytochrome P450 family 2 subfamily C member 9	-2.12131
SLC38A4	solute carrier family 38 member 4	-2.11507
C7orf58	chromosome 7 open reading frame 58	-2.11388
SLC10A1	solute carrier family 10 (sodium/bile acid cotransporter family), member 1	-2.07694
	ATP-binding cassette, sub-family A (ABC1), member 6	
ABCA6	paraoxonase 1	-2.03363
PON1		-2.01788

Recent developments in whole-genome or exome analyses in tumor biology have revealed genomic alterations in childhood tumors including HBL. Further analysis of genomic alterations in HBL will be necessary to elucidate the driver mutations for diagnosis and targeted therapy in HBL.

Conflicts of Interest

The authors have no conflicts of interest.

Acknowledgments

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of Life Science, Natural Science Center of Basic Research and Development, and the Research Center for Molecular Center, Graduate School of Biomedical Science, Hiroshima University, for the use of their facilities.

Abbreviations

AFP	α -fetoprotein
CHIC	Childhood Hepatic tumors International Collaboration
COG	Children's Oncology Group
HBL	hepatoblastoma
JPLT	Japanese Study Group for Pediatric Liver Tumors
NCL	noncancerous liver tissue
PRETEXT	PRE-Treatment EXTent of tumor
SIOPEL	International Childhood Liver Tumor Strategy Group

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