

Copy-number variations in hepatoblastoma associate with unique clinical features

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Abstract

Purpose Hepatoblastoma is a rare childhood liver malignancy with limited relevant cytogenetic data. This study aimed to discover common genomic copy-number variations (CNVs) in subjects with hepatoblastoma and its relevance to the clinical course.

Methods Gene copy-number was systemically rated by high-resolution comparative genomic hybridization (CGH) DNA oligonucleotide microarray. The study group consisted of 12 children (7 males and 5 females) with hepatoblastoma and another 20 healthy individuals (10 males and 10 females) as controls. The influence of recurrent CNVs on clinical outcomes was analyzed.

Results Four highly recurrent CNVs were identified in these 12 hepatoblastoma children after comparison with controls, including a gain on 1p13.3 ($n = 3$, 25%) and

losses on 5p15.33 ($n = 4$, 33.3%), 16q12.2 ($n = 4$, 33.3%), and 19q13.42 ($n = 3$, 25%). The most prevalent sites of genomic deletion were 5p15.33 and 16q12.2. Zinc finger, DHHC-type containing 11 (ZDHHC11) and DHHC-type containing 11B (ZDHHC11B) were mapped to 5p15.33, which was associated with a lower rate of survival with native liver ($p = 0.03$). The carboxylesterase 4-like (CES4) gene that mapped to 16q12.2 was associated with smaller tumor size at presentation.

Conclusions Deletions of 5p15.33 (33.3%) and 16q12.2 (33.3%) are the most frequent hepatoblastoma-related events in our patient group with 5p15.33 microdeletion as a potential biomarker for the fate of survival with native liver.

Keywords Comparative genomic hybridization · Hepatoblastoma

Abbreviations

array-CGH Array-based comparative genomic hybridization

CNVs Copy-number variations

J.-F. Wu and C.-H. Lee contributed equally to this work.

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Background and purpose

Hepatoblastoma is the most common primary liver malignancy in children in Western countries, and the second most common primary hepatic malignancy during infancy and early childhood in Taiwan [1]. With the existence of universal hepatitis B vaccination program since July 1984, the incidence of pediatric hepatocellular carcinoma has declined over the last decade [2, 3]. The exact genetic background of hepatoblastoma remains largely unknown, although most cases occur sporadically, others arise with

Beckwith–Wiedemann syndrome or familial adenomatous polyposis [4, 5].

Conventional cytogenetic methods, such as fluorescence in situ hybridization and comparative genomic hybridization (CGH) analysis, have been applied to hepatoblastoma and have defined chromosomal breakpoints at 1q, 2q, 4q, 7q, and 11q [6–8]. They also have identified copy-number variations (CNVs) in the form of losses on 4q, 9p, and 13q and identified gains on 1q, 2q, 8q, 12q, 20q, and 22q [9–11]. However, inconsistent results exist among studies, which may be due to different genomic backgrounds of patient populations and technical constraints. A rapidly developing molecular genetic tool, oligonucleotide array-based CGH (array-CGH), has greatly improved the resolution to a few kilobases and has provided the means for screening the human genome to discover novel CNVs associated with the disease. Using oligonucleotide microarray technologies, amplifications at 7q34, 14q11.2, and 11q22.2 and deletions at 11p15 have emerged in certain tumor samples of hepatoblastoma [12]. Another array-CGH study has shown that chromosomal loci on 1q, 2q, 4q, 6q, 8q, and 20q are prominent in the etiology of hepatoblastoma [13].

The prime objective of this study was to define high-resolution regions of CNVs in hepatoblastoma and noninvasively identify possible prognostic biomarkers using oligonucleotide array-CGH to survey CNVs throughout the genomes of peripheral lymphocytes.

Patients and methods

Subjects

Twelve children (7 males and 5 females) with hepatoblastoma were enrolled in this study from 2005 to 2008. They were diagnosed with hepatoblastoma and had regular follow-up visits at the Department of Pediatrics of National

Taiwan University Children's Hospital. Their clinical characteristics and outcomes are summarized in Table 1.

All the hepatoblastoma patients were diagnosed through clinical and laboratory examinations, including α -fetoprotein, radiographic, and histologic inspections. The staging system at diagnosis was based on the Strategy Group of the International Society of Pediatric Oncology (SIOPEL) pretreatment extent of disease (PRETEXT) grading system [14, 15]. All children received the treatment protocol (including neoadjuvant chemotherapy, surgical tumor excision/transplantation, and adjuvant chemotherapy) designed by the Childhood Liver Tumors Strategy Group [15]. The primary study endpoint was patient mortality with native liver or liver transplantation (failure of native liver survival).

Twenty healthy individuals (10 males and 10 females) were recruited as controls. Those with major systemic disorders, mental retardation, and clinical evidence of brain, trunk, or limb anomalies were excluded because these disorders might involve genetic defects. Because several hereditary diseases do not affect carriers until adulthood, the age criterion of the normal controls was set at 30 years to exclude subjects who appeared "healthy" but had genetic defects.

The study protocol was approved by the Institutional Review Board of the National Taiwan University Hospital. Informed consent was signed by either the parents or subjects included in the present study.

Genomic DNA extraction

Genomic DNA was prepared from peripheral blood lymphocytes using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA), according to the manufacturer's instructions [16]. The reference DNA pool for array-CGH analysis was obtained by pooling equal amounts of chromosomal DNA extracted from lymphocytes of ten

Table 1 General characteristics of hepatoblastoma patients in this study

Case	Gender	Age (month)	α -fetoprotein (ng/mL)	Histology	Survival status
HB1	Male	9	855220	Epithelial ^a	Survival/native liver/no residual tumor
HB2	Male	2	6652	Epithelial ^a	Mortality/native liver with residual tumor
HB3	Male	12	378860	Mixed ^b	Survival/liver transplantation/no residual tumor
HB4	Male	82	885600	Epithelial ^a	Survival/liver transplantation/no residual tumor
HB5	Male	16	946520	Epithelial ^a	Mortality/native liver with residual tumor
HB6	Female	31	1042808	Epithelial ^a	Mortality/native liver with residual tumor
HB7	Male	9	62795	Epithelial ^a	Survival/native liver/no residual tumor
HB8	Female	48	801000	Epithelial ^a	Survival/native liver/no residual tumor
HB9	Female	8	124196	Epithelial ^a	Survival/native liver/no residual tumor
HB10	Female	18	461000	Epithelial ^a	Survival/native liver/no residual tumor
HB11	Male	2	301401	Mixed ^b	Survival/native liver/no residual tumor
HB12	Female	12	1161536	Epithelial ^a	Survival/liver transplantation/no residual tumor

^a Epithelial, pure epithelial-type hepatoblastoma, including fetal and embryonal type

^b Mixed epithelial- and mesenchymal-type hepatoblastoma

healthy males and ten healthy females. The reference genome for array-CGH analysis was taken from a sex-matched normal DNA pool.

Array-CGH analysis

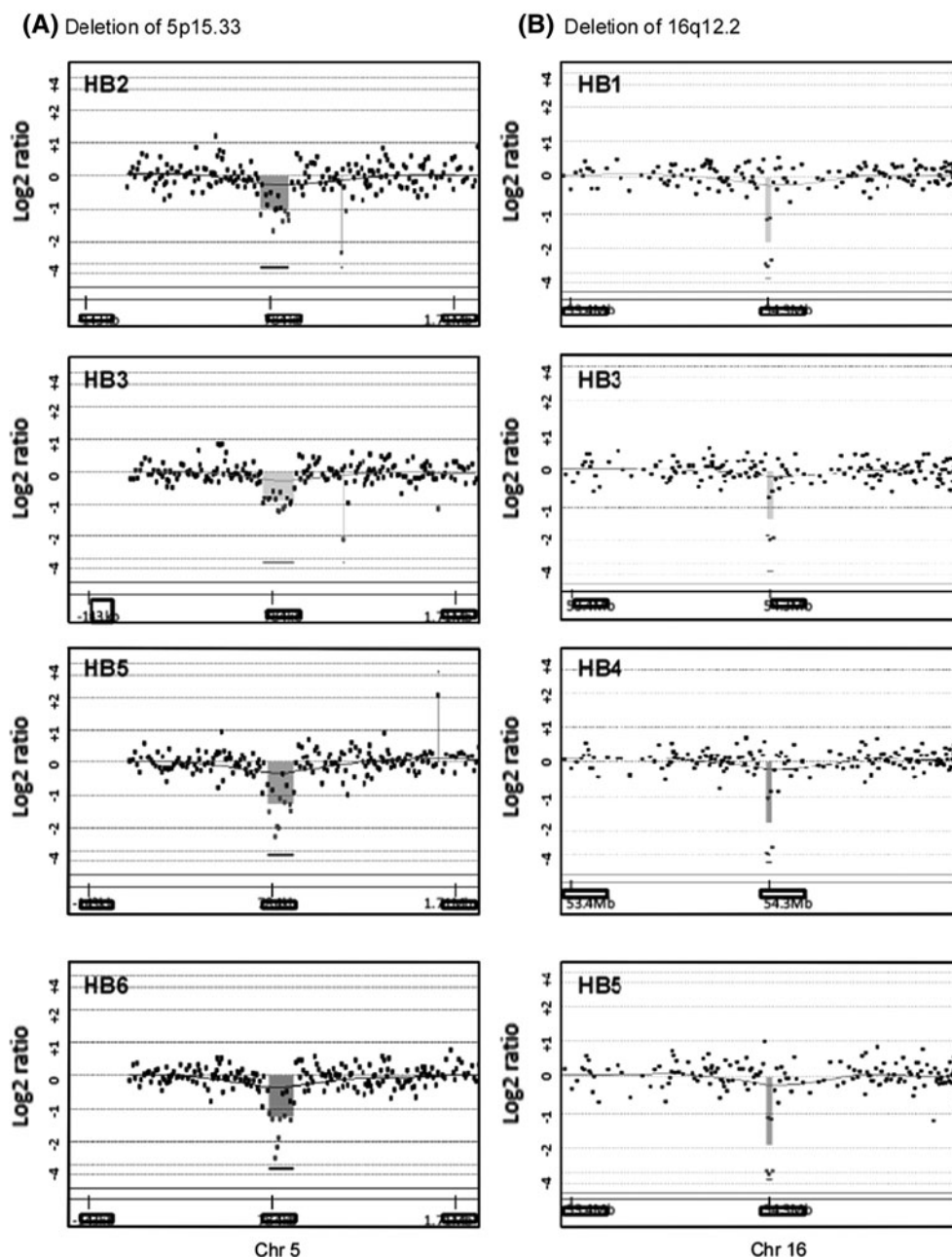
A microarray of over 244,000 oligonucleotide probes designed to cover coding, non-coding, and intergenic sequences in the human genome (Human Genome CGH microarray kit 244A, Agilent Technologies, Palo Alto, CA, USA) was used for array-CGH analysis. Genomic DNA fragmentation, labeling, and array hybridization were performed according to the standard array-CGH protocol

(Version 4) provided by Agilent Technologies, as previously described [16–18].

A G2565B DNA microarray scanner (Agilent Technologies) scanned the hybridized arrays and the Feature Extraction software (Version 8.1.1, Agilent Technologies) analyzed the microarray images. Another customized analytical software package, Agilent CGH Analytics, Version 3.4, analyzed subsequent data [16, 18]. The CNV locations were calculated using the ADM-2 statistical algorithm and the threshold for aberration was set at 9.0 for an amplification or deletion call (Fig. 1).

The array-CGH data discussed in the present study were deposited in the Gene Expression Omnibus of NCBI [19],

Fig. 1 Array-CGH plots for recurrent deletions of 5p15.33 (a) and 16q12.2 (b) in hepatoblastoma. Hybridization results are shown for three representative cases. The x axis marks the chromosome coordinate and the y axis marks the normalized hybridization \log_2 ratio. A \log_2 signal ratio of 0 represents no aberrant copy number in an HB case. Where \log_2 is greater than 0, an amplified DNA copy-number for the hepatoblastoma genome relative to the reference genome is suggested. Where \log_2 less than 0, the reverse is inferred. The significant regions are highlighted in gray. Arrows indicate 5p15.33 (a) and 16q12.2 (b). Images were produced using CGH Analytics Version 3.5.14 software (Agilent Technologies)



which can be accessed through the GEO series accession number GSE18408 (<http://www.ncbi.nlm.nih.gov/geo/>).

Screening strategy for hepatoblastoma candidate CNVs

To build a list of hepatoblastoma candidate CNVs, a two-phased strategy was designed. In phase one, the copy-number polymorphisms found in healthy controls were excluded. Two array hybridization experiments were performed with sex-matched DNA pools from healthy Taiwanese and Caucasians (Promega, Madison, WI, USA). In addition, the CNVs listed in the Database of Genomic Variants (<http://projects.tcag.ca/variation/>) with relatively high incidences in the control subjects were excluded. In phase two, CNVs with inconsistent aberration patterns (i.e., showing a gain in some cases and loss in other cases) were extracted from the candidate list. To assess candidate CNVs regarding possible involvement in hepatoblastoma pathogenesis, the expression profiles of the genes contained in candidate CNVs were examined by an online search of UniGene (<http://www.ncbi.nlm.nih.gov/UniGene>). The CNVs involving nonhepatic expressed genes were excluded.

Statistical analysis

Statistical analyses were performed using the STATA package software (StataCorp LP, Texas, TX, USA). A Mann–Whitney *U* test was used to analyze continuous data, and Fisher's exact test was used for categorical variables. Native liver survival was defined as patient survival with the native liver, while patient survival was defined as survival of the study subject regardless of liver transplantation. Survival analyses for the native liver survival and patient survival durations were analyzed by the Cox's proportional hazards method and Kaplan–Meier plots. The assumption of the proportional hazards method was examined by checking the non-zero slope. The diagnostic age, PRETEX stage, initial AFP levels, and sex of patients were included into the multivariate Cox's proportional hazards method. A *p* value less than 0.05 was deemed to indicate statistical significance.

Results

General characteristics of the study population

The clinical data of the study subjects are listed in Table 1. These subjects ($n = 12$) were followed for 4.56 ± 4.09 years (95% confidence interval [CI], 2.00–7.13 years) after the diagnosis of hepatoblastoma.

Ten children were graded as PRETEX IV and two were PRETEX III at the time of diagnosis of hepatoblastoma.

All the children received chemotherapy using cisplatin and doxorubicin, according to the protocol designed by the Childhood Liver Tumors Strategy Group. These patients were treated according to the SIOPEL II ($n = 8$) and SIOPEL IV ($n = 4$) chemotherapy protocols.

Ten patients (83.4%) received primary tumor excision after neoadjuvant chemotherapy, followed by two courses of adjuvant chemotherapy. Among these 10 patients [HB1 (hepatoblastoma case 1) and HB3 to HB11], tumors recurred in four patients [two (HB3 and HB4) survived after salvage liver transplantation and two (HB5 and HB6) died while waiting for liver transplantation]. No recurrent tumor was found in the other six subjects (HB1 and HB7 to HB11) after primary tumor excision. One patient (HB12) received primary liver transplantation due to an unresectable tumor after neoadjuvant chemotherapy and survived without tumor recurrence. Another patient (HB2) had brain and lung metastasis at the time of diagnosis of hepatoblastoma and died of malignancy during the course of chemotherapy.

Candidate CNVs for hepatoblastoma

Excluding copy-number polymorphisms of the control genomes, Table 2 shows CNVs found in the 12 hepatoblastoma children. Patient HB10 had no CNV in this screening. Because CNVs affected by both gain and loss were not likely pathogenesis-relevant, recurrent CNVs on 1q21.3, 14q11.2, 14q24.3, 15q11.2, 16p11.2, and 20p13 were excluded from further study.

Among CNVs identified in the present study, four showed an incidence of at least 25%, including a gain on 1p13.3 ($n = 3$, 25%) and losses on 5p15.33 ($n = 4$, 33.3%), 16q12.2 ($n = 4$, 33.3%), and 19q13.42 ($n = 3$, 25%). The microdeletions of 5p15.33 and 16q12.2 detected in four patients were the most prevalent and noteworthy CNVs. The microdeletion of 5p15.33 ranged from 123.691 to 155.709 kb in length, with a minimum overlapping region of 107 kb containing coding sequences for tubulin polymerization-promoting protein (TPPP), AF251188, AK096220, and zinc finger DHHC-domain containing proteins 11B and 11 (ZDHHC11B and ZDHHC11) genes. The deleted regions in 16q12.2 were exactly matched among the four affected patients with 25.25 kb length, covering the complete coding sequence for carboxylesterase 4 (CES4). Both deletions were not detected in control genomes obtained from healthy females and males (Supplementary Fig. 1). In addition, ZDHHC11, ZFHHC11B, and CES4 are hepatic expressed genes mapped to 5p15.33 and 16q12.2, respectively. No hepatic expressed genes were mapped to the CNVs of 1p13.3 and 19q13.42.

Taken together, results from array-CGH identified 5p15.33 and 16q12.2 as the two CNVs with the highest

Table 2 Summary of genomic CNVs found in peripheral lymphocytes of hepatoblastoma patients

Subjects	DNA copy gains and losses
HB1	-1q21.3(2), -12p13.2(3), -14q11.2(36), -14q24.3(2), -16q12.2(5), +17q25.3(3), -20p13(3)
HB2	+1p13.3(3), -1q24.2(2), -1q31.1(10), -5p15.33(14), +16p11.2(41)
HB3	+1p36.21(8), +1p13.3(3), -5p15.33(15), -14q11.2(30), +14q24.3(4), -16q12.2(5), -19q13.42(2), +20p13(3)
HB4	-1p31.1(2), +1p13.3(2), +4q12(2), -6q27(2), +14q11.2 (30), -16q12.2(5)
HB5	+4q22.3(12), -5p15.33(13), -6q27(2), -16q12.2(5), -16q22.1(7), -19q13.12(2), -19q13.42(2)
HB6	-5p15.33(12), -20p13(2)
HB7	+17p12(75)
HB8	-5q33.1(2), -7q36.1(3), -12q24.33(98), -13q32.1(8), -16p11.2(32), -16q22.1(2), -19q13.42(2), -20q13.33(3), -22q13.2(3), -Xq26.3(3)
HB9	+20p13(3)
HB10	None
HB11	+1q21.3(6), -3q26.1(10), -7p14.1(11), -8p23.1(8), +9q22.2(3), -10p11.21(68), -12p13.2(4), -14q11.2(58), -14q32.33(4), -15q11.2(28), -15q14(10)
HB12	-3p12.1(10), -3q26.1(10), -4q13.2(11), -4q34.1(3), -6p21.32(2), -7q34(4), +8p11.23 - p11.22(20), -12p13.31(2), +14q11.1 - q11.2(36), +15q11.2(73), -22q11.23(6)

deletion rate among the hepatoblastoma patients. In addition, these two recurrent CNVs encompassed hepatic expressed genes.

Clinical significance of 5p15.33 and 16q12.2 deletion on hepatoblastoma

The effects of 5p15.33 and 16q12.2 deletion on clinical parameters and outcomes were further investigated. Four subjects (HB2, HB3, HB5, and HB6) had 5p15.33 deletion, demonstrating PRETEX 4 hepatoblastoma at presentation. HB2, HB5, and HB6 subjects died with native liver, and HB3 survived after liver transplantation. Subjects with 5p15.33 deletion were associated with a lower chance of survival with native liver [Hazard ratio (HR), 0.08; 95% CI 0.009–0.802; $p = 0.03$] (Fig. 2). No significant role of

5p15.33 deletion was identified regarding age at hepatoblastoma diagnosis, sex, maximum tumor diameter at initial presentation, tumor histology, and initial AFP levels. These clinical data are summarized in Table 3. In the multivariate Cox's proportional hazards method analysis stratified by initial diagnostic age, initial AFP, PRETEX stage, and sex, 5p15.33 deletion was still significantly associated with poor native liver survival (HR 0.12; $p = 0.02$).

The maximal tumor diameter on diagnosis was significantly smaller in subjects with 16q12.2 deletion (HB1, HB3, HB4, and HB5) as compared with others ($p = 0.03$; Mann–Whitney U test). The regression coefficient of the presence of the 16q12.2 deletion and tumor maximal diameter was -3.97 (95% CI -1.16 to -6.79 ; $p = 0.01$). No significant difference was found between hepatoblastoma with and without 16q12.2 deletion regarding all other clinical parameters, including the onset age, sex, initial serum AFP levels, and histology (Table 3).

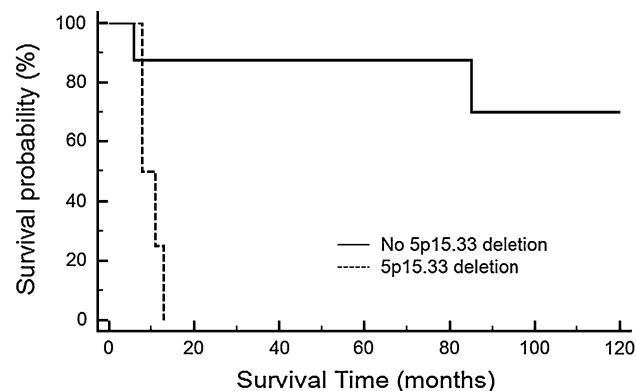


Fig. 2 The overall survival for patients was compared based on the results of array-CGH experiments, demonstrating the difference in the survival with native liver ($p = 0.03$, log-rank test) between hepatoblastoma patients with ($n = 4$) and those without ($n = 8$) 5p15.33 deletion

Discussion

Most hepatoblastoma arise sporadically, although some occur in association with a specific syndrome [4, 5, 20, 21]. However, no single chromosomal change in these syndromes is observed in all hepatoblastoma patients. Liver histology of hepatoblastoma can be classified as epithelial type (i.e., fetal, embryonic, and mixed fetal and embryonic), mixed mesenchymal-epithelial type, and small cell unclassified hepatoblastoma. Thus, hepatoblastoma pathogenesis may be a multifactorial and multihit process involving the development of various stages of hepatic stem cells in the embryo-fetus and even in the early infancy stage.

Table 3 Comparison of clinical features between hepatoblastoma subgroups with and without 5p15.33 or 16q12.2 deletion

Clinical features	5p15.33 deletion			16q12.2 deletion		
	Affected (<i>n</i> = 4)	Unaffected (<i>n</i> = 8)	<i>p</i>	Affected (<i>n</i> = 4)	Unaffected (<i>n</i> = 8)	<i>p</i>
Age ^a (month)	14 (2–31)	10.5 (2–82)	1	14 (9–82)	10.5 (2–48)	0.39
Log 10 AFP (ng/mL)	5.24 (3.86–5.45)	5.55 (4.65–6.41)	0.87	5.94 (5.58–5.98)	5.57 (3.82–6.01)	0.61
Tumor diameter (cm)	8.9 (5.8–12)	9.1 (5–12)	0.73	5.9 (5–7.2)	10.3 (5–12)	0.03
Female	0 (0%)	4 (50%)	0.21	0 (0%)	4 (50%)	0.21
Liver transplantation	1 (25%)	2 (25%)	1	2 (50%)	1 (12.5%)	0.24
Histology (mixed ^b)	1 (25%)	2 (12.5%)	1	1 (25%)	2 (12.5%)	1

The ranges for these clinical parameters in analyzed patients are indicated in the parenthesis. Mann–Whitney *U* test was used to test the difference in continuous data, and Fischer's exact test for the difference in categorical variables between different groups

^a Age, median values for age of diagnosed

^b Mixed, mixed mesenchymal-epithelial type hepatoblastoma

Because of the rarity of this disease, obtaining a sufficiently large sample size to achieve adequate statistical power in a single institute is difficult. The present study enrolled 12 hepatoblastoma patients to scan for CNVs using genome-wide array-CGH. The results suggest that CNVs, including gain on 1p13.3 and losses on 5p15.33, 16q12.2, and 19q13.42, may be pathogenic for this malignancy.

Alterations in the chromosomal region 5p15.33 have been reported for various human malignancies, including bladder cancer, non-small-cell lung cancer, breast cancer, and sporadic gastric cancer [22–25]. In the present study, the frequency of 5p15.33 microdeletion is 33.3% (*n* = 4) in the hepatoblastoma patients, three (75%) died with native liver, and only one (25%) survived after liver transplantation. Thus, the presence of genomic 5p15.33 microdeletion in hepatoblastoma patients was associated with higher mortality and native liver failure, which may serve as a prognostic marker.

Gene CNVs located at 5p15.33 included TPPP, AF251188, AK096220, DHHC11B, and ZDHHC11. Only ZDHHC11B and ZDHHC11 were expressed in hepatocytes. The Zinc finger DHHC-domain containing proteins are located at the cell membrane and show palmitoyl-transferase activity [26]. Although the exact function of these gene alterations, including those involving ZDHHC11B and ZDHHC11, in the pathogenesis of hepatoblastoma and other reported malignancies remains unknown [22–25], linkages among 5p15.33 microdeletion, hepatoblastoma occurrence, and poor outcome are evident in the present study. Further studies are needed to clarify the roles of these genes in hepatoblastoma pathogenesis.

The CES4, hepatic expressed gene, maps to the 16q12.2 region. The CES4 belongs to the carboxylesterase enzyme family, which is known to be involved in detoxification and metabolic activation of various drugs, environmental toxins, and carcinogens [27, 28]. The CES4 expression has been reported in various human tissues, including liver and placenta, and CES4 has both pharmacologic and

toxicologic significance in fetal development [27]. Thus, CES4 may be associated with hepatoblastoma development in the fetus and during early infancy.

Interestingly, subjects with 16q12.2 microdeletion had smaller tumor sizes on initial diagnosis in the present series, implying a possible role for the CES4 gene in tumor growth during the early stage of hepatoblastoma. Thus, the role of CES4 in hepatoblastoma tumorigenesis warrants further investigation.

Theoretically, CNVs with incidence less than 25% still may have a prognostic impact in subjects with hepatoblastoma. Because of the relatively small study population and lack of statistical power, further analyses on the clinical outcome in subjects with CNVs less than 25% were not done. Because hepatoblastoma is a rare malignant disease, performing large-scale studies at a single center is difficult. Although the sample size is relatively small, a relationship between the CNVs and clinical outcomes was clearly demonstrated. Further large-scale multicenter studies might be needed to confirm these observations.

In conclusion, the present study has explored CNVs in genomic DNA isolated from peripheral lymphocytes of hepatoblastoma patients and has shown that 5p15.33 and 16q12.2 microdeletions are frequent in hepatoblastoma. The microdeletion of 5p15.33 may be a valuable biomarker for hepatoblastoma patients at a high risk of native liver failure and may warrant earlier liver transplantation.

Conflict of interest This work was supported by the National Health Research Institute of Taiwan (NHRI-EX96-9418B1, NHRI-EX97-9418B1, NHRI-EX98-9418B1, and DOH99-TD-C-111-004).

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