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Frequent Increase of DNA Copy Number in the 2q24 Chromosomal Region and Its Association with a Poor Clinical Outcome in Hepatoblastoma: Cytogenetic and Comparative Genomic Hybridization Analysis

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In a cytogenetic and comparative genomic hybridization (CGH) study of 38 hepatoblastomas, we found gain of 1q in 17 tumors (44.7%), that of 2/2q in 14 (36.8%), that of 20/20q in 9 (23.7%) and that of 8/8q in 8 (21.0%), loss of 4q in 4 (10.5%) and no DNA copy changes with normal karyotype or no mitotic cells in 11 (28.9%). Eleven tumors with 2/2q gain detected by CGH had a total chromosome 2 gain, a partial 2q gain, or a total chromosome 2 gain with an augmented partial 2q region; the common region for DNA copy gain was 2q24. Two-color fluorescence *in situ* hybridization (FISH) analyses using probes covering the centromere of chromosome 2 or *HOXD13* (2q31) confirmed the CGH findings, and showed that the common region for gain in 2q was centromeric to *HOXD13*. Event-free survival (EFS)±standard error (SE) at 5 years was lowest in patients with 2q gain [37±15%], highest in those with no DNA copy changes [82±12%], and intermediate in those with DNA copy changes other than 2q gain [74±13%] (P=0.0549). Multivariate analysis showed that 2q gain was an independent factor predicting a poor outcome. These findings suggest the presence of a growth-promoting gene or an oncogene in the 2q24 chromosome band, and a tumor suppressor gene in terminal 4q, which have important roles in the development and progression of hepatoblastoma.

Key words: Hepatoblastoma — CGH — FISH — Chromosome abnormalities — 2q gain

Hepatoblastoma is a malignant hepatic tumor in children, which occurs mostly in the first 3 years of life.[1)] Due to the rare incidence, cytogenetic and molecular-genetic studies in hepatoblastoma have been limited compared with those of other childhood tumors. Common cytogenetic abnormalities reported included trisomies of chromosome 2, 5, 8 and/or 20, and 1q trisomy, which is occasionally associated with 4q deletion; namely, der(4)t(1;4)(q12;q34).[2–5)] Recently, comparative genomic hybridization (CGH) analysis has been performed in 3 series of hepatoblastomas, which confirmed cytogenetic findings reported previously.[6–8)]

Although most hepatoblastomas are sporadic, they also occur in association with Beckwith-Wiedemann (B-W) syndrome and familial adenomatous polyposis (FAP).[9, 10)] *APC* mutation and/or loss of heterozygosity in the *APC* locus were reported in some children with both sporadic and the FAP-associated hepatoblastomas.[11, 12)] More recently, mutation of the *β-catenin* gene was reported in approximately half of the sporadic hepatoblastomas examined.[13)]

Although previous cytogenetic and CGH analyses reported common chromosomal regions of gain or loss, only one study attempted to determine the prognostic implication of these genetic changes,[8)] because of the small number of hepatoblastomas included in each series. We performed cytogenetic and CGH analysis in 38 children with hepatoblastoma, and identified frequent DNA copy changes in distinct chromosomal regions including 1q and 2q. Moreover, fluorescence *in situ* hybridization (FISH) and chromosome analyses narrowed down the region of 2q gain, and revealed a cytogenetic mechanism of DNA copy gain in 2q in one tumor. We also found that 2q gain was associated with poor outcome of hepatoblastoma patients.

MATERIALS AND METHODS

Patients and samples (Table I) Tumors were obtained from 38 Japanese children with hepatoblastoma who underwent biopsy or surgery between March 1989 and September 1999. Thirteen and 25 tumors were obtained

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Table I. Clinical and Pathological Characteristics of 38 Patients with Hepatoblastoma

Case No.	Age/sex	Histologic type[a]	AFP[b] (ng/ml)	Stage	Chemotherapy before surgery	Survival[c] (months)	Present[d] status	Comment[e]
N patients (n=11): normal CGH pattern and normal karyotype or no mitotic cells								
356	6m/M	Unclassifiable	Negative	1	–	134+	NED	
669	1/F	Embryonal	330 000	4	–	107+	NED	
834	6m/F	Embryonal	2 600 000	4	+	94+	NED	
893	5m/F	Fetal	2 070 000	2	+	92+	NED	
1303	3/F	Fetal	398 106	1	–	52+	NED	
1384	8/M	Macrotrabecular	Negative	4	+	8	DOD	
1471	2/F	Embryonal	525 800	4	+	48+	NED	
1891	1/M	Fetal	21 845	1	–	24+	NED	
1918	4m/M	Fetal	378 500	2	+	27+	NED	
2116	11m/M	Fetal	905 160	2	+	13+	AWD	
2201	5/F	Embryonal	600 000	4	+	10	DOD	
A1 patients (n=14): 2q gain detected by CGH and/or cytogenetic analysis								
843	8/F	Embryonal	1 110 000	4	+	8	DOD	
972	1/F	Fetal	1 400 000	2	+	16	DOD	
991	7m/M	Embryonal	776	4	+	80+	NED	
1067	1/M	Mixed (E & M)	300 000	2	+	89+	NED	
1103	2/M	Embryonal	16 348	4	+	22	DOD	
1117	1/F	Fetal	414	1	–	83+	NED	CHD
1134	2/M	Embryonal	2 110 000	3	–	5	DOD	CHD, epilepsy, MR
1408	1/F	Embryonal	611 596	4	+	15	DOD	
1694	1/M	Embryonal	252 250	2	+	24+	NED	CHD, microcephalus
1975	2/F	Embryonal	64 000	2	+	21+	NED	
2093	1/M	Embryonal	87 411	3	+	14+	AWD	
2150	3/F	Unclassifiable	846 214	4	+	11+	AWD	
2198	1/F	Embryonal	177 190	1	–	10+	NED	
2230	1/F	Fetal	53 350	4	+	10+	AWD	
A2 patients (n=13): CGH and/or cytogenetic changes other than 2q gain								
692	3m/F	Fetal	329 287	1	–	105+	NED	B-W syndrome
769	1/F	Embryonal	390 000	1	–	105+	NED	
990	1/F	Fetal	920	1	–	82+	NED	
1057	8m/M	Unclassifiable	5 480	2	+	85+	NED	
1107	2/M	Unclassifiable	45 830	3	+	22	DOD	Prematurely born
1131	4/M	Unclassifiable	875	4	+	116+	NED	
1148	6m/M	Fetal	290 000	2	+	69+	NED	
1194	8m/M	Fetal	420 000	2	–	65+	NED	
1358	7/M	Small cell	Negative	2	–	54+	NED	Li-Fraumeni syndrome
1416	3/M	Fetal	41 987	3	–	17	DOD	
1495	4m/M	Fetal	1 405 020	2	+	39+	NED	
1748	4m/M	Embryonal	500 000	2	+	24+	NED	B-W syndrome
1905	1m/F	Fetal	5 723 300	2	+	15+	NED	

a) Mixed (E & M), mixed epithelial and mesenchymal; small cell, small cell undifferentiated.
b) AFP, α-fetoprotein.
c) + after survival indicates that the patient is alive.
d) NED, no evidence of disease; AWD, alive with disease; DOD, died of disease.
e) B-W, Beckwith-Wiedemann; CHD, congenital heart disease; MR, mental retardation.

before and after chemotherapy, respectively. There were 21 males and 17 females, ranging in age from 3 months to 8 years with a median age of 1 year. Patients were staged according to the system proposed by the Children's Cancer Study Group.[14] The majority of patients were treated according to the protocols of the Japan Hepatoblastoma Study Group.[15] The pathological diagnosis was made based on routine hematoxylin/eosin-stained slides by

pathologists at each institution according to the classification proposed by Haas *et al*.[16] Of the 38 hepatoblastomas, 15 were classified as fetal histologic type, 15 as embryonal histologic type, 1 as mixed epithelial and mesenchymal histologic type, 1 as macrotrabecular histologic type, 1 as small cell undifferentiated histologic type, and 5 as unclassifiable because of necrosis due to preoperative chemotherapy. Hepatoblastomas occurred in 2 patients with B-W syndrome (Nos. 692 and 1748), in 3 patients with congenital heart disease with or without other malformations (Nos. 1117, 1134 and 1694), in 1 patient with Li-Fraumeni syndrome (No. 1358), and in 1 patient (No. 1107) who was born prematurely.

Cytogenetic studies Chromosomes were studied as described previously.[17] Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN) 1995.[18]

CGH study Genomic DNA was isolated from 38 hepatoblastoma tumors and from lymphocytes of a normal male and a normal female. Tumor DNA was labeled by nick translation with biotin-11-dUTP, and control DNA was labeled with digoxigenin-11-dUTP. Four hundred to 800 ng of labeled tumor and normal DNA were combined with 15 μg of human *Cot*1 DNA and precipitated with ethanol. The precipitate was resuspended in 12 μl of the hybridization mixture, denatured, and hybridized to normal metaphase cells for 48 h. Hybridized DNA fragments were detected with avidin-fluorescein isothiocyanate (FITC) and antidigoxigenin rhodamine. Chromosomes were counterstained with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI). Digital images of FITC, rhodamine and DAPI fluorescence were acquired separately with a cooled charge-coupled device (CCD) camera. The ratios of the FITC-to-rhodamine signal intensity were calculated using the automated CGH analysis software (CytoVision, Applied Imaging, Newcastle, UK). The ratio profiles were averaged from at least 10 metaphase cells. A chromosomal region was considered to be overrepresented (gain) or underrepresented (loss), if the average ratio profile was above 1.25 or below 0.75, respectively.

FISH studies To detect the copy number of chromosome 2 and gain of the 2q31 chromosomal region, we used an α satellite DNA probe covering the centromeric region of chromosome 2 (CEP 2, Spectrum Orange) (VYSIS, Downers Grove, IL), and a BAC probe (RBP1) covering *HOXD13*, which is located in 2q31.[19] The BAC probe was labeled with biotin (bio)-16-dUTP (Boehringer, Mannheim, Germany) by nick translation. Interphase and metaphase cells were stained with DAPI. Two-color FISH was performed as described.[20]

Statistical analysis Patients were grouped according to various biological and clinical aspects of the disease. Event-free and overall survivals for each group of patients were estimated on October 30, 1999 using the Kaplan-Meier method, and compared using log-rank tests. Time to failure was defined as the interval between surgery or preoperative chemotherapy and relapse or death from any cause. Failure to achieve complete remission by chemotherapy or gross residual disease after the initial surgical procedure was considered an event at day 1.

Influence of various biological and clinical factors on event-free and overall survival was estimated using the Cox proportional-hazards model. The analysis was performed using the SPSS software for Windows, version 7.5.1 (SPSS, Chicago, IL).

RESULTS

Cytogenetic findings Of 38 hepatoblastomas in which cytogenetic analysis was performed, 9 (23.7%) had no mitotic cells, 13 (34.2%) had only normal mitotic cells, and 16 (42.1%) had clonal abnormal cells (Table II). Twelve tumors had trisomy 2 or trisomy or pentasomy 2q (Fig. 1), 12 had trisomy, tetrasomy, or hexasomy 1q, 6 had trisomy 8, and 4 had trisomy 20. Two tumors had an unbalanced 1;4 translocation resulting in a deletion of 4q34−4qter and 1q trisomy.

CGH findings Findings from CGH analysis of 38 hepatoblastomas are presented in Table II and Fig. 2. Of 38 tumors, 24 (65.8%) had DNA copy number changes in at least one chromosomal region. Fourteen tumors had gain of 1q, 11 had gain of 2/2q, 5 had gain of 20/20q and 3 had gain of 8/8q. Three tumors had loss of chromosome arm 4q. Of the 11 tumors with 2/2q gain, 4 (Nos. 1003, 1117, 1134 and 1975) had a total chromosome 2 gain, 3 (Nos. 1694, 2198 and 2230) had a partial 2q gain, 3 (Nos. 843, 972 and 991) had a total chromosome 2 gain with an augmented partial 2q region (Fig. 1), and one (No. 1408) had gains of 2pter−2q24 and 2q32−qter. The most common region for increased DNA copy number was 2q24.

Combined findings of cytogenetic and CGH analyses Of 29 tumors from which mitotic cells were obtained, 6 tumors had normal karyotypes and normal CGH patterns, 7 had normal karyotypes and abnormal CGH patterns, 3 had abnormal karyotypes and normal CGH patterns, and 13 had abnormal karyotypes and abnormal CGH patterns. Of the other 9 tumors from which no mitotic cells were obtained, 5 and 4 had normal and abnormal CGH patterns, respectively.

Combined findings of cytogenetic and CGH analyses showed gain of 1q in 17 tumors (44.7%), that of 2/2q in 14 (36.8%), that of 20/20q in 9 (23.7%) and that of 8/8q in 8 (21.0%), and loss of 4q in 4 (10.5%). The most common regions for gain were 1q32−1qter and 2q24, and the most common region for loss was 4q34−4qter.

Interphase and metaphase FISH findings Two-color FISH analysis using probes covering the centromere of chromosome 2 or *HOXD13* (2q31) was performed in 9 of

Table II. Cytogenetic, CGH and FISH Findings in 38 Hepatoblastomas

Case No.	Karyotype	CGH findings		HOXD /CEP2	Gains of	
		Gain	Loss		1q	2/2q
N patients (n=11): normal CGH pattern and normal karyotype or no mitotic cells						
356	46,XY	—	—			
669	NM	—	—			
834	46,XX	—	—			
893	46,XX	—	—			
1303	NM	—	—			
1384	46,XY	—	—			
1471	NM	—	—			
1891	NM	—	—			
1918	46,XY	—	—			
2116	46,XY	—	—			
2201	NM	—	—			
A1 patients (n=14): 2q gain detected by CGH and/or cytogenetic analysis						
843	53,X,–X,dup(2)(q23q37),+dup(2)(q23q37),+5,+12, +der(16)t(1;16)(q12;q24),+20,+3mar	1q21–qter,* 2pter–qter(2q13–q36),* 6,10q24–q26, 20*	Xp Xq26–qter	4/3	+	+
972	47,X,–X,+2,der(4)t(1;4)(q21;q34),+mar,ace	1q21–qter, 2pter–qter(2q23–q31)*	4q33–qter	3/3	+	+
991	48,XY,+i(1)(q10),dup(2)(q24q34),+dup(2)(q24q34)	1q12–qter, 2pter–qter(2q24–q34),* 9, 16, 17, 22	—	5/3	+	+
1067	51,XY,+2,der(4)t(1;4)(q12;q34),+5,+8,+8,+20	—	—		+	+
1103	NM	1q12–qter, 2, 8q*	—		+	+
1117	52,XX,+2,+3,+7,+8,+15,+20	2, 3, 7, 8, 15	—			+
1134	47,XY,add(2)(q37),+add(2)(q37), other changes	1q31–qter, 2, 17q22–qter	—	3/3	+	+
1408	48,XX,+20,+der(?)t(1;?)(q31;?),dmin	1q31–qter, 2pter–2q22, 2q23–qter, 20	4q34–qter	2/3	+	+
1694	46,XY,dup(2)(q22q31),der(21)t(1;21)(q21;p11) /48,idem,+dup(2)(q22q31),+mar	1q12–qter, 2q21–q31	—	3/2	+	+
1975	48,XX,+2,+7,der(22)t(1;22)(q12;p11)	1q12–qter, 2, 7	—	3/3	+	+
2093	47,XY,+2	—	—			+
2150	50,XX,+2,+5,+8,der(20)t(1;20)(q12;q11), +der(20)t(1;20)(q12;q11)	1q23–qter, 19	—		+	+
2198	51,XX,+2,+5,+8,+der(?)t(1;?)(q21;?),+mar	1q24–qter, 2q24-qter	—	4/3	+	+
2230	46,XX,dup(2)(q23q37),der(17)t(1;17)(q21;q12)	2q23–q37	—	3/2	+	+
A2 patients (n=13): CGH and/or cytogenetic changes other than 2q gain						
692	46,XX	1q31–qter, 5q31,* 18q21–qter, 19	—		+	
769	46,XX	20q	—			
990	46,XX	1q24–q44	4q21–qter		+	
1057	51,XY,+i(1)(q10),+i(1)(q10),+8,+20,+21	—	—		+	
1107	NM	1q24–qter, 8q, 17,* 20q	11p,18		+	
1131	61–91,i(1)(q10), other complex changes	1q23–qter, 6q13–q23	1p34–pter		+	
1148	46,XY	X*	—			
1194	46,XY	21q22–qter	—			
1358	46,XY	3p24–pter, 5q33–q35,* 12q13–qter, 17q22–ter*	—			
1416	NM	6p12–pter	—			
1495	NM	Xq21–q28	—			
1748	48,XY,+8,+20	6p12–p21	—			
1905	46,XY	1p34–pter, 19, 20	—			

NM, no mitotic cells; * high-level DNA copy gain; HOXD/CEP2, see the legend to Fig. 2.

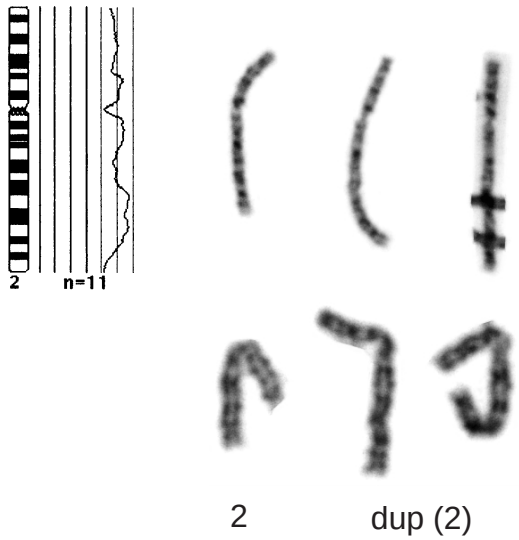

Fig. 1. CGH and cytogenetic examination of tumor 991. CGH analysis showed a total chromosome 2 gain with an augmented 2q24–q34 region, and 2 partial karyotypes show 1 normal chromosome 2 and 2 copies of dup(2)(q24q34), resulting in pentasomy of the 2q24–q34 region.

11 tumors with gain of 2/2q detected by CGH (Fig. 2, Table II). The 2 probes were chosen because *HOXD13* was located close to the common region for 2q gain, and the centromere probe was needed as a control to show the number of chromosome 2. Two tumors (Nos. 1134 and 1975) with a total chromosome 2 gain had 3 centromeric and 3 *HOXD13* signals as expected. The signal number of *HOXD13* was greater than that of the centromere in 5 of the 6 tumors with a partial 2q gain (Nos. 1694, 2198 and 2230) or a total chromosome 2 gain with an augmented partial 2q region (Nos. 843 and 991), and the same signal number of *HOXD13* and the centromere was seen in the other (No. 972). One tumor (No. 1408) with gains of 2pter–2q24 and 2q32–qter had 3 centromeric and 2 *HOXD13* signals, and the finding confirmed the normal DNA copy of the 2q31 region. Thus, the interphase FISH findings were consistent with the CGH findings, and indicated that *HOXD13* was included in the common region for DNA copy gain of 5 out of 6 tumors, and that the common region was centromeric to the *HOXD13* (2q31) region.

FISH revealed metaphase cells in one tumor (No. 991) (Fig. 3). Normal chromosome 2 had 1 centromeric and 1

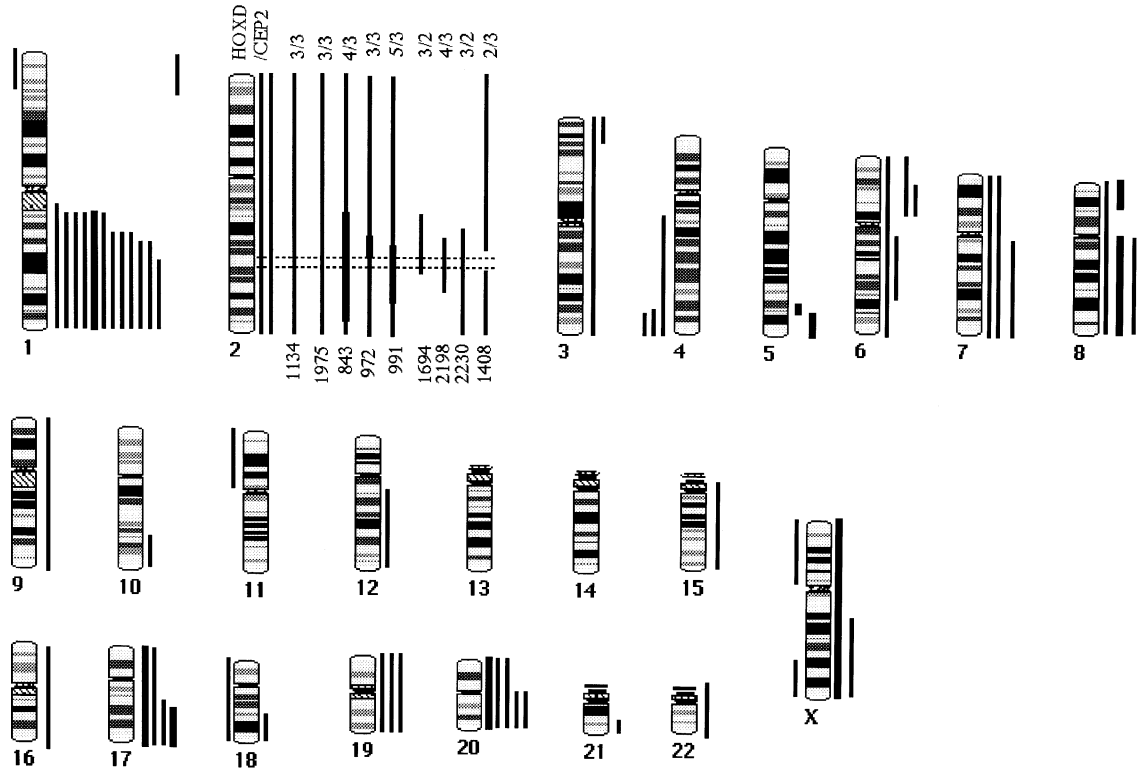

Fig. 2. Summary of CGH study of 38 hepatoblastomas. Regions of gain are shown by lines on the right, and regions of loss are shown by lines on the left. High-level DNA copy gain is marked by a bold line. Numbers below lines on the right side of chromosome 2 indicate the tumor number, and numbers above the lines indicate numbers of *HOXD13* and CEP2 signals; i.e. 3/3 shows that 3 *HOXD13* and 3 CEP2 signals were found on interphase cells. *HOXD13* is located in the area between the 2 horizontal dotted lines.

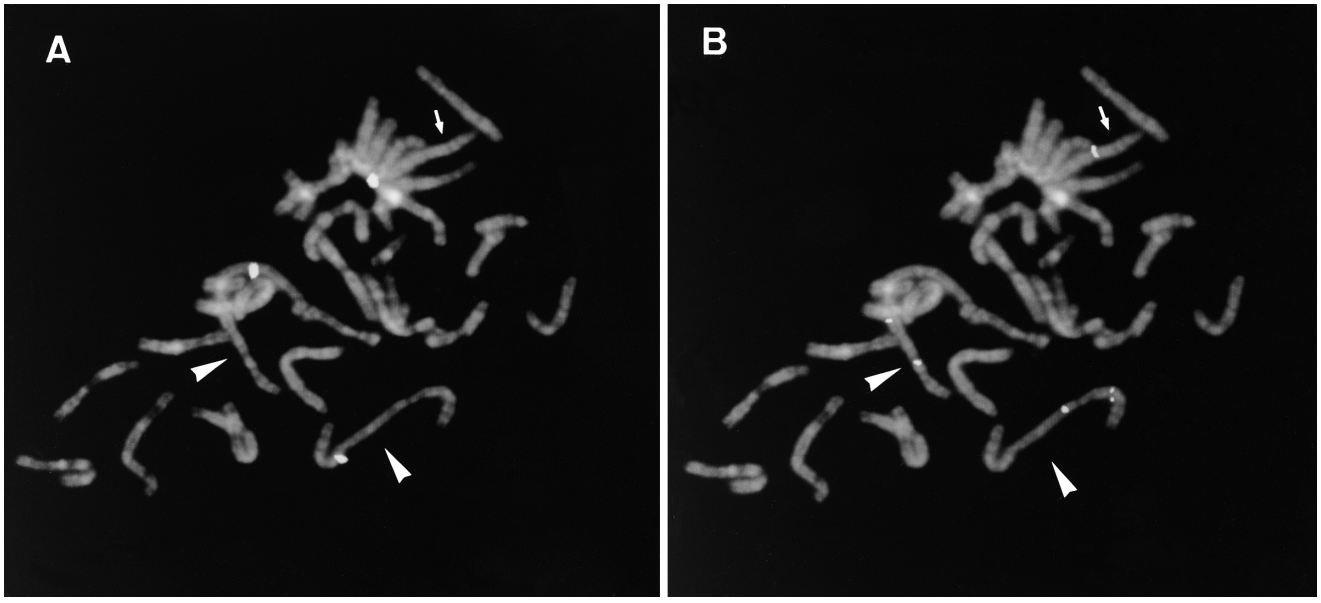

Fig. 3. A. Hybridization of Spectrum Orange-labeled CEP2 to a cell from tumor 991. An arrow and arrowheads show normal chromosome 2 and dup(2) chromosomes, respectively. B. Hybridization of a biotin-labeled BAC clone covering *HOXD13* to the same cell as in A. Normal chromosome 2 had 1 *HOXD13* signal, and dup(2) chromosomes had 2 *HOXD13* signals.

HOXD13 signals as expected, and 2 dup(2) chromosomes had 1 centromeric and 2 *HOXD13* signals, which were apart. Cytogenetic, CGH and FISH findings indicated that the tumor cells had 5 copies of the 2q24–q34 region as a result of duplication of the 2q24–q34 region, and the subsequent duplication of the dup(2) chromosome.

Statistical findings Patients were classified into 3 groups on the basis of combined cytogenetic and CGH findings; namely, 11 N patients with normal CGH and normal or no cytogenetic findings, and 27 A patients with abnormal CGH and/or abnormal cytogenetic findings. The 27 A patients were further classified as 14 A1 patients with 2q gain and 13 A2 patients with abnormalities other than 2q gain (cytogenetic and CGH classification 1), or 17 A3 patients with 1q gain and 10 A4 patients with abnormalities other than 1q gain (cytogenetic and CGH classification 2). Event-free survival (EFS)±standard error (SE) at 5 years was lowest in A1 patients [37±15%], highest in N patients [82±12%] and intermediate in A2 patients [74±13%] (P=0.0549) (Fig. 4). EFS±SE at 5 years was lower in A3 patients [40±14%] than in N patients [82±12%] or in A4 patients [80±13%] (P=0.1062). Overall survival (OS)±SE at 5 years was lower in A1 patients [48±17%] than in N patients [81±12%] or in A2 patients [82±12%] (P=0.1397) (Fig. 5). OS±SE at 5 years was lower in A3 patients [54±14%] than in N patients [82±12%] or in A4 patients [88±12%] (P=0.2286).

Seven of the 11 N patients were treated with chemotherapy before surgery. To avoid the possible influence of chemotherapy, the 7 patients were excluded from the next analysis. The P values for EFS and OS were 0.0378 and 0.0503, respectively, among 4 N, 14 A1 and 13 A2 patients, and 0.0758 and 0.1126, respectively, among 4 N, 17 A3 and 10 A4 patients. The findings indicated that the differences in EFS and OS became more significant in the analysis excluding the 7 N patients than in the analysis including them.

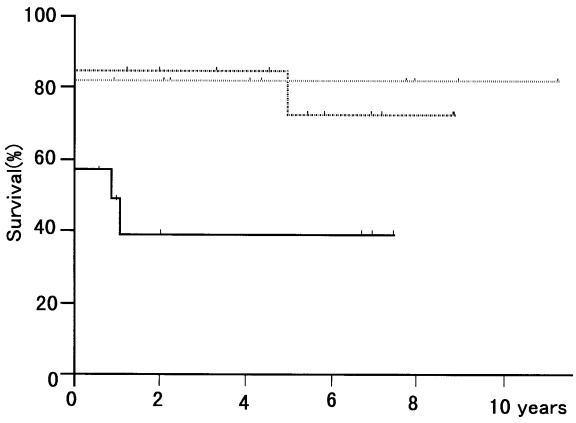

Fig. 4. Event-free survival curves for 3 groups of patients classified by CGH and cytogenetic findings (log-rank, P=0.0549). N patients had no DNA copy changes and normal karyotypes or no mitotic cells. A1 patients had 2q gain, and A2 patients had DNA copy and/or cytogenetic changes other than 2q. ---- N (11), – – – A2 (13), —— A1 (14).

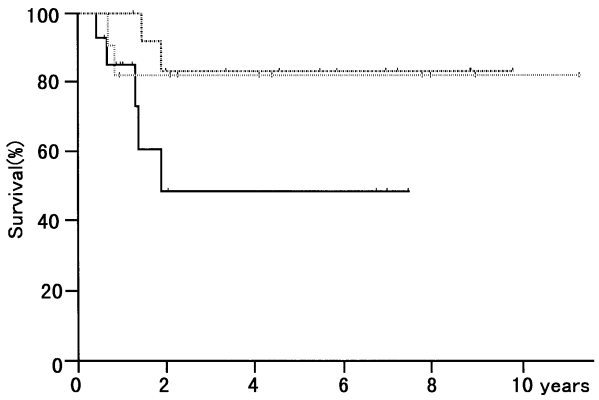

Fig. 5. Overall survival curves for the 3 (N, A1 and A2) groups of patients (P=0.1397). ---- N (11), – – – A2 (13), —— A1 (14).

Survival analyses on 3 groups of patients classified only by chromosome findings showed no differences among 13 N, 12 A1 and 4 A2 patients; 9 patients, whose tumors had no mitotic cells, were excluded from the analysis. The analysis on 3 groups of patients classified only by CGH findings showed a difference in OS (P=0.0356), but no difference in EFS (P=0.1724) among 14 N, 11 A1 and 13 A2 patients. Thus, the CGH analysis is more useful than the chromosome analysis to detect prognostic subgroups among hepatoblastoma patients.

Patients were grouped on the basis of various factors, including age (<5 years vs. ≥5 years), stage of the disease (I+II vs. III+IV), histological type of tumor (fetal histologic type vs. other types) and presence or absence of 2q gain (A1 patients vs. A2 patients; A1 patients vs. N patients). Cytogenetic and CGH classification 1 (N, A1, and A2), which showed a more significant P value on survival analysis than cytogenetic and CGH classification 2 (N, A3, and A4), was chosen for multivariate analysis on prognosis. The findings are summarized in Table III. Since no differences in EFS and OS were found between patients with and without fetal histologic type (data not shown), the histologic type was excluded from multivariate analysis. The patient's age and the groups classified by the cytogenetic and CGH classification had a similar contribution to the EFS. The patient's age had the largest contribution to the OS, followed by the cytogenetic and CGH classification and the stage of the disease. The findings show that the cytogenetic and CGH classification is an independent prognostic factor.

DISCUSSION

We examined 38 hepatoblastomas by both cytogenetic and CGH methods. CGH analysis detected the presence or absence of DNA copy changes in all 38 tumors including 9 that showed no mitotic cells by cytogenetic analysis. Moreover, CGH analysis detected DNA copy changes in 7 of 13 tumors that showed only normal mitotic cells; malignant cells may not have been in the mitotic phase, or were overlooked in the 7 tumors by cytogenetic analysis. However, cytogenetic analysis detected cells with chromosome abnormalities in 3 tumors that showed normal CGH pattern; the coexistence of tumor cells and an overwhelming number of non-tumor cells, which may have obscured the DNA copy changes, may explain the discrepancy. Thus, although CGH analysis gives more information on DNA copy changes than cytogenetic analysis, cytogenetic analysis sometimes complements CGH analysis.

By combined cytogenetic and CGH analysis we confirmed the findings previously reported that the most frequent chromosomal regions for DNA copy gain were 1q and 2q.[2–8] Frequent gain of 1q has been reported in various embryonal tumors and adult carcinomas by both cytogenetic and CGH analyses.[21, 22] In contrast, frequent gain of 2q has been reported only in ovarian carcinoma by CGH analysis.[23] Recently, three groups of investigators reported frequent gain of 2/2q in hepatoblastomas by

Table III. Results of Multivariate Analysis of 38 Patients with Hepatoblastoma Evaluated for Event-free and Overall Survival

Prognostic factors	Event-free survival		Overall survival	
	Relative risk (95% CI)	P	Relative risk (95% CI)	P
Patient's age				
≥5 years vs. ≤4 years	3.90 (1.05–14.51)	0.0423	27.28 (3.39–219.41)	0.0018
Stage of the disease				
III, IV vs. I, II	2.28 (0.64–8.11)	0.2047	2.98 (0.55–15.99)	0.2036
Cytogenetic and CGH classification				
A1 patients vs. A2 patients	3.80 (0.96–14.96)	0.0563	20.88 (1.99–218.84)	0.0112
A1 patients vs. N patients	4.94 (1.02–23.84)	0.0465	6.62 (1.05–41.68)	0.0441

CGH analysis.[6–8] Two of them reported high-level gain in a restricted region at 2q24.[7, 8] The present study also showed a partial 2q gain in 6 tumors by CGH analysis, and identified 2q24 as the most common region for DNA copy gain (Fig. 2). Furthermore, FISH and cytogenetic analysis revealed that the clone covering *HOXD13* (2q31) was located outside the common region for the gain, and that partial 2q gain detected by CGH was produced by a cytogenetic mechanism of duplication of the 2q24–q34 region followed by duplication of dup(2) chromosome in one tumor (No. 991). These findings suggest that a gene located at 2q24 may have multiplied and increased its product, and given the hepatoblastoma cells the growth advantage needed for proliferation. Candidate genes at 2q24 included the activin receptor-like kinases (*ALK*) gene, the tumor necrosis factor receptor-associated factor (*I-TRAF*) gene and the *FRZB-1* gene.[24–26] The *HOXD* genes, which are located close to the common region,[19] were excluded from candidacy by the present study.

In the present series of hepatoblastoma patients, EFS was lowest in A1 patients, highest in N patients and intermediate in A2 patients (Fig. 4). Seven (64%) of 11 N and 19 (70%) of 27 A patients received preoperative chemotherapy, and the ratios of treated to untreated patients with chemotherapy were similar between N and A patients. The absence of DNA copy changes found in the 7 N patients who were given preoperative chemotherapy may be interpreted as indicating that their tumor cells had no DNA copy changes detectable by CGH before chemotherapy, or that most tumor cells with DNA copy changes were killed by chemotherapy and non-tumor cells predominated in the tissue samples. We showed that the differences in EFS and OS among the 3 groups of patients became more significant in the survival analysis excluding the 7 N patients than in the analysis including them. Multivariate analysis showed 2q gain as an independent factor predicting a poor outcome. These findings further suggest that a growth-promoting gene or an oncogene may be present at 2q24.

Recently, Weber *et al.* compared an incidence of gains of 1q, 2q, 8q, 20, or 22q, or loss of 4q between 26 patients with no evidence of disease and 8 patients who died of disease, and found gains of 8q and 20 as predictors of poor outcome.[8] In contrast to their findings, we found gain of 2q, but not gains of 8q and 20, as a prognostic factor for poor outcome using log-rank tests of survival curves depicted by the Kaplan-Meier method. The different patient populations or different statistical methods used to analyze the prognostic significance of chromosome gains between the 2 studies may have contributed to the discrepancy. Because 2q gain showed a borderline significance in the survival analysis of the present study, further studies including more patients than those in the present series are required to prove the prognostic implication of gain of 2q, or other chromosomal regions.

Loss of a chromosome region detected by CGH was rather rare in the present series of hepatoblastomas. Four patients had either unbalanced 1;4 translocations resulting in the loss of terminal 4q detected by cytogenetic analysis, or loss of 4q21–qter or 4q33 or 34–qter detected by CGH analysis. Previous cytogenetic and CGH studies reported similar unbalanced 1;4 translocations in 5 hepatoblastomas, and loss of chromosome 4 in 6 hepatoblastomas, respectively.[2–4, 7, 8] Taken together, the terminal 4q is the most frequent region for chromosome loss, and is the target site for the molecular cloning of a tumor suppressor gene responsible for the development of hepatoblastoma.

Patients with familial adenomatous polyposis coli, which is caused by germline mutation of the *APC* gene, have an increased incidence of hepatoblastoma,[10] and somatic mutation of the *APC* gene was reported in some sporadic hepatoblastomas.[11, 12] The APC protein targets for β-catenin degradation.[27] Recently, a high frequency of *β-catenin* mutation has been reported in sporadic hepatoblastomas.[13] However, the present cytogenetic and CGH study showed no loss of 5q and no gain of 3p, where *APC* and *β-catenin* genes are located, respectively.

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