Characterization of Genomic Alterations in Hepatoblastomas

A Role for Gains on Chromosomes 8q and 20 as Predictors of Poor Outcome

Ruthild G. Weber,^{*,†} Torsten Pietsch,[‡]
Dietrich von Schweinitz,[§] and Peter Lichter^{*}

From the Abteilung Organisation komplexer Genome, Deutsches Krebsforschungszentrum, Heidelberg, Germany; the Institut für Humangenetik,[†] Ruprecht-Karls-Universität, Heidelberg, Germany; the Institut für Neuropathologie,[‡] Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany; and the Abteilung für Kinderchirurgie,[§] Universitäts-Kinderspital Beider Basel, Switzerland*

As data on the genomic alterations in hepatoblastoma (HB) are limited, 34 HB tumors and three HB cell lines were screened for DNA copy number changes by comparative genomic hybridization. The average number of chromosomal imbalances per tumor was 2.3 ± 0.5 (mean $\pm$ SEM) with gains sevenfold more frequent than losses. The most frequent gains of chromosomal material in HB tumors were on 2q (44%), 1q (41%), 2p (29%), 20 (24%), 22q (18%), 8q (15%), 8p and 12q (9% each), as well as 7q, 12p, and 17 (6% each) and the only recurrent loss was on 4q in 12% of cases. Highly amplified sequences were identified in four tumors and mapped to 2q24 in two cases, to 8q in two cases (once to 8q11.2-q13 and once to 8q11.2-q21.3) as well as to 10q24-q26 in one case. In one cell line, highly amplified DNA sequences were mapped to 7p and 8q. Comparison to previously published data on this series of HB revealed that the number of chromosomal imbalances was significantly higher in HB tumors with loss of heterozygosity on 11p ($P = 0.03$), whereas in five of 10 HB biopsies without chromosomal imbalances, β -catenin gene mutations were found. HB patients were divided into a good (no evidence of disease) and a poor (died of disease) outcome group according to their clinical course after standard therapy. Two alterations were found to be significantly associated with poor outcome: gain on 8q ($P = 0.007$) and gain on 20 ($P = 0.009$). In summary, our analysis allowed the identification of gains on chromosomes 1q and 2 as hallmark DNA copy number changes in HB with 2q24 as a critical chromosomal band. Furthermore, this study provided evidence that gains on 8q and 20 play a role as markers

of prognostic significance in HB. (Am J Pathol 2000, 157:571–578)

Hepatoblastoma (HB) is a malignant embryonal liver tumor primarily affecting children 5 years of age or younger.¹ HB is the most common primary malignant hepatic tumor of childhood affecting $\sim 1/1,000,000$ population in the United States.² HBs are composed of immature epithelial cells and can contain admixtures of immature mesenchymal or teratomatous elements. Accordingly, they are histologically classified into epithelial or mixed, ie, epithelial and mesenchymal, types.³ A small-cell anaplastic variant was also incorporated into the classification.⁴

In contrast to hepatocellular carcinoma (HCC), in which the main etiological factor is chronic hepatitis virus infection,⁵ no exogenous causes are known in HB. Although most HBs are sporadic, the incidence of HB is highly elevated in certain hereditary diseases, such as Beckwith-Wiedemann syndrome (BWS)⁶ or familial adenomatous polyposis.⁷ These findings imply that alterations at 11p15 (the BWS critical region) and of the wingless signaling pathway involving the APC gene (which is constitutionally mutated in patients with familial adenomatous polyposis) could also play a role in the genesis of sporadic HBs. Indeed, loss of heterozygosity (LOH) at 11p was detected in sporadic HBs.^{8,9} Moreover, sporadic HBs were found to carry LOH at the APC locus as well as somatic mutations of the APC gene¹⁰ and of the β -catenin gene,¹¹ whose gene products interact in the wingless signaling pathway.

To date, cytogenetic analysis of chromosomal aberrations in HB was limited to an approximate total of 30 tumors.^{12–23} As most of the HB series examined were quite small, the significance of the chromosomal changes detected remains unclear. Double-minute chromosomes,

Supported by grants from the Deutsche Krebshilfe (10-1124-Li1), the Deutsche Forschungsgemeinschaft (Pi191/9-1), the BMBF (01GI 9964/5), and the Bennisgen-Foerder Foundation.

Accepted for publication May 9, 2000.

Address reprint requests to Dr. Ruthild G. Weber, Institute of Human Genetics, University of Heidelberg, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany. E-mail: Ruthild_Weber@med.uni-heidelberg.de.

a cytogenetic hallmark for gene amplification, were found in some HB tumors and a HB cell line.^{12-14,24} However, the DNA sequences contained in this extrachromosomal material have not been identified. Thus, the genes amplified in HB are as yet unknown. The prognostic relevance was not shown for any of the chromosomal changes previously found in HB.

In the present study, it was our aim to identify chromosomal imbalances in a large series of HBs and to determine the chromosomal regions of origin of amplified DNA sequences. Thus, we applied comparative genomic hybridization (CGH), which allows the genome wide screening for DNA copy number gains and losses in a single experiment^{25,26} to a series of 34 HB tumors and three HB cell lines. To elucidate the prognostic value of the chromosomal imbalances found, the changes detected in HBs of patients with good *versus* poor outcome were compared. In addition, chromosomal imbalances were analyzed in the context of genetic alterations formerly identified to play a role in the pathogenesis of HB, LOH on chromosomes 1 and 11p as well as mutations of the *β-catenin* gene, which had been determined for the present series of HBs previously.^{9,11,27}

Materials and Methods

Tumors and Cell Lines

A total of 34 sporadic HB tumors and three HB cell lines from sporadic HBs were analyzed. The histology of the 34 HB tumors was classified as epithelial in 25 cases, as mixed in eight cases, and as small-cell anaplastic in one case according to Weinberg and Finegold.⁴ A part of each tumor was snap-frozen immediately after surgical removal and stored at -80°C . Histological evaluation of these tumor pieces revealed an estimated tumor cell content of at least 80% in all cases. The HB cell lines investigated were HepT1,²⁴ HUH-6,²⁸ and HepT3. The HepT3 cell line was derived from tumor D204, so that both the primary tumor and the respective cell line could be studied.

Patients

HB tumors from 18 female and 16 male patients were examined. The patients' ages ranged from 2 to 144 months with an average of 23.3 months. All but six patients were treated according to the HB-89 or HB-94 protocol of the German Society of Pediatric Hematology and Oncology (surgery followed by a standardized chemotherapy protocol with ifosfamid, cisplatin, and adriamycin.²⁹) Only four patients had received chemotherapy before the operation in which tumor samples were obtained. In all patients, clinical course after therapy was monitored. Patients were divided into two outcome groups: a good outcome group (patients had no evidence of disease, NED group) and a poor outcome group (patients died of disease, DOD group). Age and gender were not significantly different in the two outcome groups. Average follow-up was >5 years.

DNA Extraction and CGH

Fragments from frozen tumor samples were analyzed by staining a frozen section with hematoxylin and eosin (H&E) to exclude necrotic or contaminating normal tissue. For DNA extraction, one representative tissue area was selected per tumor. DNA from the same tumor area was used for all experiments performed including the LOH studies^{9,27} and the mutation analysis of the *β-catenin* gene.¹¹ The preparation of high molecular weight DNA from frozen tumor samples, cultured cells, and peripheral blood leukocytes was carried out by proteinase K digestion and phenol/chloroform extraction. Following standard procedures, metaphase spreads were prepared from stimulated peripheral blood lymphocytes obtained from a healthy male patient (46,XY). CGH was performed as previously described.³⁰

For CGH analysis, image acquisition and processing were performed using the Cytovision System version 3.1 (Applied Imaging, Richmond, UK). Average ratio profiles were determined from the analysis of 12 metaphase spreads. The diagnostic threshold values used to score losses and gains were 0.75 (lower threshold) and 1.25 (upper threshold), respectively, in accordance with previously reported CGH analysis protocols.³¹ High-level amplifications were defined as gains of chromosomal material which led to either a very bright and distinct fluorescent band in the hybridization pattern of the tumor DNA and/or a deviation of the average ratio profile beyond the 2.0 threshold. Banding assignment of losses, gains, and high-level amplifications was based on the comparison of CGH average ratio profiles and chromosome ideograms, except for the banding assignment of the high-level amplification on 2q which was achieved by electronically overlaying the 4,6-diamidino-2-phenylindole and the fluorescein isothiocyanate images. By the CGH analysis procedure applied here, low copy number changes can be detected with a resolution of ~ 10 Mbp,³² which corresponds to the size of an average chromosomal band. CGH can also detect and map amplified DNA sequences if the product of amplicon size and copy number exceeds 2 Mbp.^{33,34}

Negative control experiments, in which differently labeled normal DNAs were co-hybridized to normal metaphase chromosomes, were regularly performed in parallel with the tumor hybridizations. As artifactual results have been occasionally observed in our laboratories and other laboratories on chromosome 19 and chromosomal bands 1p34-p36^{30,35,36} these regions were excluded from the analysis.

Statistics

Comparisons between groups were performed by Fisher's exact test or Mann-Whitney *U* test, as appropriate. $P < 0.05$ was considered as statistically significant.

Results

A series of 34 sporadic HB tumors and three HB cell lines from sporadic HBs was investigated by CGH. Clinical

Table 1. Summary of Selected Clinical Data, Histopathological Characteristics, and Genomic Alterations Identified by CGH and Other Molecular Genetic Techniques in HB Tumors and HB Cell Lines Investigated

Case	Age*/ sex	Histology [†]	Stage [‡]	Pre-OP chemo	Outcome	Gains/amplifications	Losses	LOH 11p15.5**	LOH 1 ^{††}	β-Catenin mutation [#]
D269	9/f	Mixed (F/E)	III	No	NED	amp(2q24)	—	Yes	Yes (1p)	Yes
D272	12/m	Mixed (F > E)	I	No	NED	enh(2q24–q37)	—	No	No	Yes
D268	6/f	Mixed (F/E)	III	No	NED	enh(22q)	—	Yes	No	Yes
D266	8/f	Mixed (F/E)	I	No	NED	—	—	No	No	Yes
D314	7/f	Mixed (F [§])	I	No	NED	enh(7q33–q36,8,10p,12,13q,20,22q); amp(8q11.2–q13,10q24–q26)	dim(10q11.2–q23)	Yes	n.a.	No
D310	30/m	Mixed (F > E)	I	No	NED	enh(1q,2,5,17,20)	—	No	Yes (1q)	Yes
D401	9/f	Mixed (F)	I	No	NED	enh(1q42–q44)	dim(10p,16q)	No	n.a.	Yes
D263	12/m	Epithelial (F)	III	No	NED	—	—	No	No	No
DZ23II	22/m	Epithelial (F/E)	III	No	NED	—	—	No	No	No
DZ29II	13/m	Epithelial (F)	I	No	NED	enh(1q,2)	—	No	No	No
D315	12/m	Epithelial (F)	I	No	NED	enh(X)	—	No	No	No
DZ18	6/m	Epithelial (F/E)	I	No	NED	enh(20)	—	Yes	No	No
D104	27/f	Epithelial (E)	I	No	NED	enh(1q,2,11)	—	Yes	Yes (1p)	Yes
D6II	11/f	Epithelial (F)	I	No	NED	—	—	No	No	Yes
D161	11/f	Epithelial (F/E)	I	No	NED	enh(1q,2q23–q32)	—	No	No	No
D175	16/m	Epithelial (F/E)	I	No	NED	—	—	No	Yes (1p,1q)	Yes
D204	4/f	Epithelial (F/E)	I	No	NED	enh(1q);amp(2q24)	—	No	No	Yes
D264	57/m	Epithelial (F/E)	III	No	NED	enh(1q,2)	—	No	No	No
D319	4/m	Epithelial (F)	I	No	NED	—	—	No	n.a.	No
D399II	2/m	Epithelial (F)	III	No	NED	—	—	No	n.a.	Yes
D381	51/f	Epithelial (F/E)	III	Yes	NED	enh(1q32–q44,22q)	—	No	n.a.	No
D377	20/m	Epithelial (F)	III	No	NED	enh(22q13)	—	No	n.a.	No
D564	3/f	Epithelial (F)	I	No	NED	enh(1q,2)	—	n.a.	n.a.	No
D566	86/f	Epithelial (F)	I	No	NED	—	—	n.a.	n.a.	No
D675	144/f	Epithelial (F)	I	No	NED	enh(1q)	dim(1p,4q)	No	n.a.	n.a.
D169II	27/f	Small-cell anaplastic	I	No	NED	—	—	No	n.a.	Yes
D312	15/f	Mixed (F/E)	III	No	DOD	enh(2,20)	—	No	No	Yes
D497	6/f	Epithelial (E)	III	Yes	DOD	enh(2);amp(8q11.2–q21.3)	—	n.a.	n.a.	No
D306IV	12/m	Epithelial (F < E)	III	No	DOD	enh(20)	dim(4q13–q24)	Yes	No	Yes
D304	42/f	Epithelial (E)	III	No	DOD	enh(1q31–q44,2,8,20)	dim(4q34–q35)	Yes	Yes (1q)	No
D308	25/m	Epithelial (F/E)	III	No	DOD	enh(1q,2q,22q13)	—	No	Yes (1q)	No
D318	17/f	Epithelial (E [¶])	III	No	DOD	—	—	No	n.a.	No
D162	19/m	Epithelial (E), multifocal	III	Yes	DOD	enh(1q,2,8,12q23–q24,17,20)	dim(4p12–p15, 4q12–q35,18q)	Yes	No	Yes
D166	48/m	Epithelial (F < E), multifocal, rec.	III	Yes	DOD	enh(1q,2p23–p25,2q,6p21–p25,7q, 8q,12,14q,16,20,21q22,22q,Y)	—	Yes	Yes (1q)	No
HepT3 [†]	4/f	Epithelial	I	No	NED	enh(1q,2,8,17,20)	—	No	No	Yes
HUH6 [†]	12/m	Mixed (E)		No	DOD	enh(2,12,20,22q); amp(7p,8q)	dim(8p)	n.a.	n.a.	Yes
HepT1 [†]	34/f	Epithelial (E), multifocal	III	No	DOD	enh(1q,2p,7p,9p,12p,16p12–p13, 17q22–q25,20,22q)	dim(13q21–q34, 14q24–q32)	Yes	No	Yes

* Age is given in months.

† Cell line. f, female; m, male.

‡ The predominant epithelial component is given in brackets: F/E, equal amounts of fetal and embryonal tissue; F > E, more fetal than embryonal tissue; F < E, more embryonal than fetal tissue; F, fetal tissue only; E, embryonal tissue only.

§ Predominantly sarcoma-like tissue.

¶ HCC-like features (transitional HB); rec., recurrent tumor.

|| According to ref. 54: chemo, chemotherapy; NED, no evidence of disease; DOD, died of disease; LOH, loss of heterozygosity; n.a., not analyzed; enh, enhanced/gained; amp, amplified; dim, diminished/lost.

** Published previously.⁹

†† Published previously.²⁷

Published previously.¹¹

data, the histopathological diagnosis, and genomic imbalances identified by CGH are summarized in Table 1.

Chromosomal Imbalances in 34 HB Biopsies

Chromosomal imbalances were detected in 24 HB biopsies. The average number of aberrations per tumor was 2.3 ± 0.5 (mean $\pm$ SEM). Gains, including amplifications, were almost sevenfold more frequent than losses (two gains/amplifications per tumor *versus* 0.3 losses per tumor).

The gains/amplifications found in more than one case were on 2q (15 of 34, 44%), 1q (14 of 34, 41%), 2p (10 of 34, 29%), 20 (eight of 33, 24%), 22q (six of 34, 18%), 8q (five of 34, 15%), 8p and 12q (three of 34, 9% each), as well as 7q, 12p, and 17 (two of 34, 6% each). Losses were detected on 4q in four of 34 cases (12%) and once each on 1p, 4p, 10, 16q, and 18q (Table 1; Figure 1).

Amplified sequences were identified in four tumors and mapped to 2q24 in two cases, to 8q in two cases

Figure 1. Location of chromosomal imbalances detected by CGH in 34 HB tumors (solid lines) and three HB cell lines (dashed lines). Vertical lines on the right side of a chromosome ideogram represent gains of chromosomal material whereas vertical lines on the left side correspond to losses. Chromosomal region 1p34-p36 and chromosome 19 were excluded from the evaluation (see Methods).

Figure 2. Amplified DNA sequences were identified in four HB tumors and mapped to three different chromosomes by CGH. The images revealing the hybridization pattern of the tumor DNA are shown next to the average ratio profiles. The value for the balanced threshold (1.0), the diagnostic threshold for losses (0.75), the diagnostic threshold for gains (1.25), and the highest threshold depicted are given for each profile. High-level amplifications were defined as gains of chromosomal material which led to either a very bright and distinct fluorescent band in the hybridization pattern of the tumor DNA and/or a deviation of the average ratio profile to the right beyond the 2.0 threshold.

(once to 8q11.2-q13 and once to 8q11.2-q21.3) as well as to 10q24-q26 in one case (Table 1; Figure 2). Amplified DNA from 8q11.2-q13 and 10q24-q26 was detected in the same tumor (D314).

Smallest regions of common gain/amplification were 1q42-q44, 2q24, 8q11.2-q13, and 22q13. Smallest commonly deleted regions on 4q were detected at 4q13-q24 and 4q34-q35.

Genetic Alterations in HB Biopsies without Chromosomal Imbalances

In 10 HB biopsies, representing approximately one-third of the cases, no imbalances were found by CGH. By microsatellite analysis, LOH on chromosome 1 (analysis of 17 microsatellite markers from 1p and 1q²⁷) had only been found in one of these tumors and LOH on 11p (analysis of 10 microsatellite markers from 11p⁹) in none of these cases. However, mutations of the β -catenin gene had been found in half of the tumors without CGH imbalances.¹¹

Table 2. Comparison of Chromosomal Imbalances in the Two Outcome Groups

	NED	DOD	P value
Gain on 1q	10/26 (38%)	4/8 (50%)	0.7
Gain on 2q	9/26 (35%)	6/8 (75%)	0.1
Loss on proximal 4q	1/26 (4%)	2/8 (25%)	0.13
Loss on distal 4q	1/26 (4%)	2/8 (25%)	0.13
Gain on 8q	1/26 (4%)	4/8 (50%)	0.007*
Gain of 20	3/26 (12%)	5/8 (63%)	0.009*
Gain on 22q	4/26 (15%)	2/8 (25%)	0.6

* Statistically significant ($P < 0.05$); NED, no evidence of disease; DOD, died of disease.

Chromosomal Imbalances in Three HB Cell Lines

In all HB cell lines, chromosomal imbalances were found. The average number of alterations per cell line was 7.7 ± 1.8 (mean $\pm$ SEM), which is more than three times higher than in tumors. Comparable to HB tumors, gains/amplifications were almost sevenfold more frequent in cell lines than losses (6.7 gains/amplifications per cell line *versus* one loss per cell line). The chromosomal imbalances identified are indicated in Table 1 and Figure 1.

Cell line HepT3 was derived from tumor D204 which was also analyzed. In the cell line, gain of chromosomes 2, 8, 17, and 20 were found in addition to the gain on 1q present in both tumor and cell line. Amplification of DNA sequences from 2q24 detected in the tumor was not found in the cell line.

Predictors of Outcome in HB Biopsies

Two alterations were found to be significantly more frequent in the poor outcome group (DOD group): gain on 8q (one in 26 NED group, four in eight DOD group; $P = 0.007$) as well as gain on 20 (three in 26 NED group, five in eight DOD group; $P = 0.009$) (Table 2). When taken together, both alterations would correctly predict outcome in six of eight DOD cases, thus their sensitivity is 75%. The specificity of the two alterations combined is 88%, ie, 23 of 26 tumors would be correctly determined not to have poor outcome (NED group).

The other common imbalances, gain on 1q, 2q, and 22q as well as loss on proximal or distal 4q were not differently distributed in the two outcome groups (Table 2). Likewise, there was no statistically significant effect on outcome for the three genomic alterations analyzed previously in this series of HB biopsies: mutation of the β -catenin gene,¹¹ LOH on chromosome 1,²⁷ and LOH on chromosome arm 11p,⁹ nor for patient age, patient gender, or tumor histology. There was also no significant correlation between the number of imbalances detected per tumor and outcome or between tumors with and without imbalances and outcome.

Comparative Analyses

There was a significant difference between the number of chromosomal imbalances in HBs with and without LOH

on 11p ($P = 0.03$). In contrast, no significant difference was found in the number of chromosomal imbalances in HBs with and without β -catenin gene mutations and in HBs with or without LOH on 1. There was no significant correlation between the number of chromosomal imbalances and the patient age. The imbalances identified by CGH, the numbers of chromosomal imbalances detected per tumor, and the age of patients were not significantly different in HB biopsies of the two main histological subtypes (epithelial versus mixed).

Discussion

We report on chromosomal imbalances in a large series of 34 HB biopsies and three HB cell lines from patients predominantly not subjected to pre-operative chemotherapy so that no chemotherapy-induced alterations were present in the DNA used for CGH analysis. The CGH data indicate that gains of chromosomal material are characteristic imbalances in HBs, as they were almost seven-fold more frequent than losses. Because gains can be identified by CGH in addition to losses but are underestimated by techniques like microsatellite analysis, CGH proved to be a particularly useful method in the study of HBs.

From our data, gains on chromosome arm 2q and on chromosome arm 1q have emerged as the hallmark alterations in HB, as they are present in 75% of HB tumors with chromosomal imbalances. Cytogenetic analyses on a total of ~30 HB tumors reported on in the literature to date also reveal alterations of chromosomes 1 and/or 2,^{13,15-19,21,22} and so do recent CGH studies on smaller series of HBs.^{37,38}

Gain or amplification on chromosome 2 was the most common alteration in HB biopsies reported on here. Previously, the shortest region of overlap was shown to lie between 2q23 and 2q35.¹³ CGH allowed to pin-point the critical region to a single chromosomal band, 2q24, because amplified sequences were mapped to this band in two HBs each in our report and another CGH study.³⁸ As no obvious candidate proto-oncogenes have been found on 2q24 to date, experiments are underway to identify the amplified DNA sequences from these two HBs. Interestingly, frequent gain at 2q24-q32 was detected in *BRCA1* or *BRCA2* mutation-positive ovarian carcinomas but not in sporadic ovarian carcinomas.³⁹ Thus, gain on 2q might also define distinct subsets of HBs.

As gain on chromosome 2 is also commonly found in embryonal rhabdomyosarcoma,^{13,40-42} another pediatric neoplasm associated with BWS, this aberration might appear as a critical step in the development of embryonal tumors. However, in two other malignancies arising from immature cells, Wilms' tumor (which is also associated with BWS), and ovarian germ cell tumor (OGCT), gain on chromosome 2 was not or only rarely detected.⁴³⁻⁴⁵ Other chromosomal imbalances, like gains on 1q and 8, are shared by Wilms' tumor, OGCT and HB.⁴³⁻⁴⁵

When comparing chromosomal imbalances in HCC and HB, the frequency of gain on chromosome 2 seems to be the distinguishing alteration which was identified in

only 9% and 16% of HCC^{46,47} versus 47% of HB in our report. Loss on 4, gain on 8q and 20, however, are genomic alterations common to HB and HCC.⁴⁶ Interestingly, Wong et al⁴⁶ found these three imbalances significantly more frequently in HCCs without underlying liver cirrhosis. These particular HCCs have in common with HBs that they originate in the noncirrhotic liver. These data suggest that gains on 8q and 20 as well as loss on 4 are distinctive chromosomal changes leading to transformation in liver cells without previous cirrhosis irrespective of the fact whether an immature or adult hepatocyte is involved. In all types of HCC, gain on 1q is frequently detected,^{46,47} which makes this another imbalance common to HB and HCC. As gains on 1q, 8q, and 20 as well as loss on 4q are detected both in HCC from patients chronically infected with HBV^{46,47} and in HB tumors, which are thought not to be virus-associated, these particular chromosomal imbalances seem to be virus-independent.

The genomic basis of BWS are various alterations at 11p15.5 which cause an imbalance in the expression of imprinted growth stimulating (ie, *IGF2*) and growth repressing (ie, *CDKN1C/p57*) genes of this chromosomal region.⁴⁸ As the rate of HB is higher in BWS patients than in the general population, microsatellite analysis was performed to assess whether LOH on 11p also plays a role in sporadic cases of HB. Studies from other groups⁹ and an analysis of HBs from the present series⁹ showed LOH on 11p in a substantial fraction of sporadic HBs. A striking finding from our CGH data is that the number of chromosomal imbalances per tumor was significantly higher in the HBs with LOH on 11p than in those tumors without this allelic loss. It can therefore be speculated that in sporadic HBs, loss of genes at 11p is associated with an increase in genomic instability. As exclusively maternal alleles, preferentially expressing *CDKN1C/p57*, are lost in HB,⁹ it was presumed that a decrease of *CDKN1C/p57* expression would contribute to HB tumorigenesis. However, we could now show that this is not the case. In contrast, a significant up-regulation of *CDKN1C/p57* expression compared to mRNA levels in corresponding liver samples was found in nine of 12 HBs (W Hartmann, A Waha, A Koch, CG Goodyer, S Albrecht, D von Schweinitz, and T Pietsch. *p57/KIP2* is not mutated in hepatoblastoma but shows increased transcriptional activity in a comparative analysis of the three imprinted genes *p57/KIP2*, *IGF2* and *H19*; Am J Pathol (in press)). Thus, the putative tumor suppressor gene at 11p15.5, the inactivation of which causes genomic instability in HB, remains to be determined. It is another interesting point that by CGH no loss on 11p was found in any of the HBs analyzed. This could be because of the limited resolution of CGH (see Material and Methods) or to the presence of uniparental disomy at 11p15 as previously shown for HB by Koufos et al.⁸

Despite the fact that by cytogenetic analysis double-minute chromosomes, representing amplified DNA sequences, were found in a fraction of HB tumors and cell lines, no oncogenes representing the targets of these amplifications have been identified to date. The oncogenes studied for amplification in HB included *NMYC*,

ERBA, *ERBB*, *NRAS*, and *Shb*.^{13,49,50} Our study provides information about chromosomal sites harboring amplified DNA sequences implicated in the genesis of HB. Amplifications were detected in four HBs and mapped to three different sites: 2q24, 8q (8q11.2-q13 and 8q11.2-q21.3), and 10q24-q26. A number of interesting genes were mapped to distal 10q including the oncogenes *FGF8* and *FGFR2* as well as genes containing homeobox motifs known to be involved in development, ie, *EVX2*, *EMX2*, *HMX2*, and *HOX11*. Because mutations in homeotic genes are frequently found to be associated with tumorigenesis, these genes are good candidates for HBs.

The genes which have been implicated in HB tumorigenesis are those coding for members of the wingless signaling pathway, ie, the *APC* and the *β -catenin* gene. APC functions as a tumor suppressor by regulating cytoplasmic levels of *β -catenin*, a proto-oncoprotein. Patients with familial adenomatous polyposis, who are known to carry germline *APC* mutations, have a higher incidence of HB tumors.^{7,51,52} APC mutations were reported in sporadic HBs,¹⁰ whereas none were detected in the HBs of the present series.¹¹ However, activating mutations of the *β -catenin* gene were identified in 48% of HBs studied here.¹¹ Interestingly, *β -catenin* mutations were present in half of the HB tumors which contained no chromosomal imbalances. This finding suggests that mutation of the *β -catenin* gene is not associated with chromosomal instability in a substantial fraction of HBs.

It is of particular interest that this study has provided evidence for two genetic predictors of poor outcome in HB: gain on 8q and gain of 20. The notion that gain on chromosomes 8 and 20 is correlated with poor outcome in HB is further supported by a cytogenetic study reporting that two HB cases containing trisomy of chromosomes 8 and 20 presented with aggressive disease.²² Evidence for the growth advantage induced by the presence of gains on chromosome 8 and 20 in HB cells *in vitro* comes from the fact that these imbalances were found in cell line HepT3 but not in the primary tumor D204 that it was derived from. Thus, a subclone of cells with gains on 8 and 20, that was not detectable by CGH, was most probably present in the tumor and expanded to form a major clone, detectable by CGH, in the cell line. The region of interest on the long arm of chromosome 8 is defined by tumor D314, which was shown to contain amplified DNA sequences mapping to 8q11.2-q13. Candidate oncogenes located in this chromosomal region include *MOS* and *LYN*. On chromosome 20, our study could not define a critical area because all tumors with imbalances on 20 showed a gain of the entire chromosome.

In HCC cells, Wong et al⁴⁶ found evidence for the growth advantage of loss on 4q. Loss on 4q was significantly more common in HCCs with a diameter larger than 3 cm. 4q21-q22 and 4q35 were identified as commonly deleted regions in HCC and allelic losses on 4q35 were associated with larger tumor size and aggressive histological type.⁵³ Likewise, our study indicates that two critical regions on chromosome 4 are associated with HB. One of these is proximal at 4q13-q24, the other is distal at 4q34-q35. However, there was no significant correlation

between HBs with loss on proximal or distal 4q and poor prognosis in our study. Interestingly, der(4)t(1;4)(q12;q34) was described as a recurrent chromosome translocation in HB.²⁰ Thus, the underlying oncogenic event might be the loss of a gene on distal 4q or the gain of genes on 1q, as also suggested by our data.

A number of clinicopathological parameters have previously been shown to significantly correlate with disease-free survival in HB: tumor involvement of one *versus* both liver lobes, multifocal disseminated *versus* unifocal growth pattern in the liver, distant metastases, vascular invasion, fetal *versus* embryonal differentiation, and serum α -fetoprotein levels.⁵⁴ Our study provides evidence for novel genomic parameters with prognostic value for HB. Taken together, gains on 8q and 20 have a high sensitivity (75%) and an even higher specificity (88%). To validate the importance of these genomic alterations as prognostic factors, further studies are required in which the clinical course of patients is monitored in addition to the screening of HB tumors for the presence of these two chromosomal changes.

Acknowledgments

We thank Axel Benner (Heidelberg, Germany) and Dieter Haffner (Berlin, Germany) for statistical advice and calculations and Claus R. Bartram (Heidelberg, Germany) for generous support.

References

1. Stocker JT, Ishak KG: Neoplasms of the Liver. Edited by K Okuda, KG Ishak. Tokyo, Berlin, Heidelberg, Springer, 1987, pp 127-136
2. Young JLJ, Ries LG, Silverberg E, Horm JW, Miller RW: Cancer incidence, survival, and mortality for children younger than age 15 years. *Cancer* 1986, 58:598-602
3. Ishak KG, Glunz PR: Hepatoblastoma and hepatocarcinoma in infancy and childhood. Report of 47 cases. *Cancer* 1967, 20:396-422
4. Weinberg AG, Finegold MJ: Primary hepatic tumors of childhood. *Hum Pathol* 1983, 14:512-537
5. Beasley RP: Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer* 1988, 61:1942-1956
6. DeBaun MR, Tucker MA: Risk of cancer during the first four years of life in children from the Beckwith-Wiedemann syndrome registry. *J Pediatr* 1998, 132:398-400
7. Hughes LJ, Michels VV: Risk of hepatoblastoma in familial adenomatous polyposis. *Am J Med Genet* 1992, 43:1023-1025
8. Koufos A, Hansen MF, Copeland NG, Jenkins NA, Lampkin BC, Cavenee WK: Loss of heterozygosity in three embryonal tumors suggests a common pathogenetic mechanism. *Nature* 1985, 316:330-334
9. Albrecht S, von Schweinitz D, Waha A, Kraus JA, von Deimling A, Pietsch T: Loss of maternal alleles on chromosome arm 11p in hepatoblastoma. *Cancer Res* 1994, 54:5041-5044
10. Oda H, Imai Y, Nakatsuru Y, Hata J-I, Ishikawa T: Somatic mutations of the *APC* gene in sporadic hepatoblastomas. *Cancer Res* 1996, 56:3320-3323
11. Koch A, Denkhäus D, Albrecht S, Leuschner I, von Schweinitz D, Pietsch T: Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the β -catenin gene. *Cancer Res* 1999, 59:269-273
12. Mascarello JT, Jones MC, Kadota RP, Krous HF: Hepatoblastoma characterized by trisomy 20 and double minutes. *Cancer Genet Cytogenet* 1990, 47:243-247
13. Fletcher JA, Kozakewich HP, Pavelka K, Grier HE, Shamberger RC,

- Korf B, Morton CC: Consistent cytogenetic aberrations in hepatoblastoma: a common pathway of genetic alterations in embryonal liver and skeletal muscle malignancies? *Genes Chromosom Cancer* 1991, 3:37-43
14. Soukup SW, Lampkin BL: Trisomy 2 and 20 in two hepatoblastomas. *Genes Chromosom Cancer* 1991, 3:231-234
15. Annerén G, Nordlinder H, Hedborg F: Chromosome aberrations in an alpha-fetoprotein-producing hepatoblastoma. *Genes Chromosom Cancer* 1992, 4:99-100
16. Bardi G, Johansson B, Pandis N, Heim S, Mandahl N, Békassy A, Hägerstrand I, Mitelman F: Trisomy 2 as the sole chromosomal abnormality in a hepatoblastoma. *Genes Chromosom Cancer* 1992, 4:78-80
17. Tonk VS, Wilson KS, Timmons CF, Schneider NR: Trisomy 2, trisomy 20, and del(17p) as sole chromosomal abnormalities in three cases of hepatoblastoma. *Genes Chromosom Cancer* 1994, 11:199-202
18. Swarts S, Wisecarver J, Bridge JA: Significance of extra copies of chromosome 20 and the long arm of chromosome 2 in hepatoblastoma. *Cancer Genet Cytogenet* 1996, 91:65-67
19. Parada LA, Bardi G, Hallén M, Hägerstrand I, Tranberg K-G, Mitelman F, Johansson B: Cytogenetic abnormalities and clonal evolution in an adult hepatoblastoma. *Am J Surg Pathol* 1997, 21:1381-1386
20. Schneider NR, Cooley LD, Finegold MJ, Douglass EC, Tomlinson GE: The first recurring chromosome translocation in hepatoblastoma: der(4)t(1;4)(q12;q34). *Genes Chromosom Cancer* 1997, 19:291-294
21. Balogh E, Swanton S, Kiss C, Jakab ZS, Secker-Walker LM, Oláh É: Fluorescence *in situ* hybridization reveals trisomy 2q by insertion into 9p in hepatoblastoma. *Cancer Genet Cytogenet* 1998, 102:148-150
22. Sainati L, Leszl A, Stella M, Montaldi A, Perilongo G, Rugge M, Bolcato S, Iolascon A, Basso G: Cytogenetic analysis of hepatoblastoma: hypothesis of cytogenetic evolution in such tumors and results of a multicentric study. *Cancer Genet Cytogenet* 1998, 104:39-44
23. Nagata T, Mugishima H, Shichino H, Suzuki T, Chin M, Koshinaga S, Inoue M, Harada K: Karyotypic analyses of hepatoblastoma. Report of two cases and review of the literature suggesting chromosomal loci responsible for the pathogenesis of this disease. *Cancer Genet Cytogenet* 1999, 114:42-50
24. Pietsch T, Fonatsch C, Albrecht S, Maschek H, Wolf HK, von Schweinitz D: Characterization of the continuous cell line HepT1 derived from a human hepatoblastoma. *Lab Invest* 1996, 74:809-818
25. Kallioniemi A, Kallioniemi O-P, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 1992, 258:818-821
26. Du Manoir S, Speicher MR, Joos S, Schröck E, Popp S, Döhner H, Kovacs G, Robert-Nicoud M, Lichter P, Cremer T: Detection of complete and partial chromosome gains and losses by comparative genomic *in situ* hybridization. *Hum Genet* 1993, 90:590-610
27. Kraus JA, Albrecht S, Wiestler OD, von Schweinitz D, Pietsch T: Loss of heterozygosity on chromosome 1 in human hepatoblastoma. *Int J Cancer* 1996, 67:467-471
28. Doi I: Establishment of a cell line and its clonal sublines from a patient with hepatoblastoma. *Jpn J Cancer Res* 1976, 67:1-10
29. von Schweinitz D, Byrd DJ, Hecker H, Weinel P, Bode U, Bürger D, Erttmann R, Harms D, Mildenberger H: Efficiency and toxicity of ifosfamide, cisplatin and doxorubicin in the treatment of childhood hepatoblastoma. *Eur J Cancer* 1997, 33:1243-1249
30. Lichter P, Bentz M, Joos S: Detection of chromosomal aberrations by means of molecular cytogenetics: painting of chromosomes and chromosomal subregions and comparative genomic hybridization. *Methods Enzymol* 1995, 254:334-359
31. Du Manoir S, Schröck E, Bentz M, Speicher MR, Joos S, Ried T, Lichter P, Cremer T: Quantitative analysis of comparative genomic hybridization. *Cytometry* 1995, 19:27-41
32. Bentz M, Plesch A, Stiglenbauer S, Döhner H, Lichter P: Minimal size of deletions detected by comparative genomic hybridization. *Genes Chromosom Cancer* 1998, 21:172-175
33. Joos S, Scherthan H, Speicher MR, Schlegel J, Cremer T, Lichter P: Detection of amplified genomic sequences by reverse chromosome painting using genomic tumor DNA as probe. *Hum Genet* 1993, 90:584-589
34. Piper J, Rutovitz D, Sudar D, Kallioniemi A, Kallioniemi O-P, Waldman FM, Gray JW, Pinkel D: Computer image analysis of comparative genomic hybridization. *Cytometry* 1995, 19:10-26
35. Kallioniemi O-P, Kallioniemi A, Piper J, Isola J, Waldman FM, Gray JW, Pinkel D: Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. *Genes Chromosom Cancer* 1994, 10:231-243
36. Weber RG, Boström J, Wolter M, Baudis M, Collins VP, Reifenberger G, Lichter P: Analysis of genomic alterations in benign, atypical, and anaplastic meningiomas: toward a genetic model of meningioma progression. *Proc Natl Acad Sci USA* 1997, 94:14719-14724
37. Steenman M, Tomlinson G, Westerveld A, Mannens M: Comparative genomic hybridization analysis of hepatoblastomas: additional evidence for a genetic link with Wilms tumor and rhabdomyosarcoma. *Cytogenet Cell Genet* 1999, 86:157-161
38. Hu J, Wills M, Baker BA, Perlman EJ: Comparative genomic hybridization analysis of hepatoblastomas. *Genes Chromosom Cancer* 2000, 27:196-201
39. Tapper J, Sarantaus L, Vahteristo P, Nevanlinna H, Hemmer S, Sepala M, Knuutila S, Butzow R: Genetic changes in inherited and sporadic ovarian carcinomas by comparative genomic hybridization: extensive similarity except for a difference at chromosome 2q24-q32. *Cancer Res* 1998, 58:2715-2719
40. Wang-Wuu S, Soukup S, Ballard E, Gotwals B, Lampkin B: Chromosomal analysis of sixteen human rhabdomyosarcomas. *Cancer Res* 1988, 48:983-987
41. Vagner-Capodano AM, Gentet JC, Gouzien M, Scheiner C, Raybaud C: Chromosome 2 abnormalities (+Der 2) in two cases of childhood rhabdomyosarcoma. *Pediatr Hematol Oncol* 1989, 6:187-192
42. Weber-Hall S, Anderson J, McManus A, Abe S, Nojima T, Pinkerton R, Pritchard-Jones K, Shipley J: Gains, losses, and amplification of genomic material in rhabdomyosarcoma analyzed by comparative genomic hybridization. *Cancer Res* 1996, 56:3220-3224
43. Steenman M, Redeker B, de Meulemeester M, Wiesmeijer K, Voûte PA, Westerveld A, Slater R, Mannens M: Comparative genomic hybridization analysis of Wilms tumors. *Cytogenet Cell Genet* 1997, 77:296-303
44. Getman ME, Houseal TW, Miller GA, Grundy PE, Cowell JK, Landes GM: Comparative genomic hybridization and its application to Wilms' tumorigenesis. *Cytogenet Cell Genet* 1998, 82:284-290
45. Riopel MA, Spellerberg A, Griffin CA, Perlman EJ: Genetic analysis of ovarian germ cell tumors by comparative genomic hybridization. *Cancer Res* 1998, 58:3105-3110
46. Wong N, Lai P, Lee S-W, Fan S, Pang E, Liew C-T, Sheng Z, Lau JW-Y, Johnson PJ: Assessment of genetic changes in hepatocellular carcinoma by comparative genomic hybridization analysis. *Am J Pathol* 1999, 154:37-43
47. Marchio A, Meddeb M, Pineau P, Danglot G, Tiollais P, Bernheim A, Dejean A: Recurrent chromosomal abnormalities in hepatocellular carcinoma detected by comparative genomic hybridization. *Genes Chromosom Cancer* 1997, 18:59-65
48. Li M, Squire JA, Weksberg R: Molecular genetics of Wiedemann-Beckwith syndrome. *Am J Med Genet* 1998, 79:253-259
49. Tsuda H, Shimamoto Y, Upton MP, Yokota J, Terada M, Ohira M, Sugimura T, Hirohashi S: Retrospective study on amplification of N-myc and c-myc genes in pediatric solid tumors and its association with prognosis and tumor differentiation. *Lab Invest* 1988, 59:321-327
50. Mares J, Polanska V, Gorgens H, Sedlacek Z, Marikova T, Bocek P, Kodet R, Schackert J, Goetz P: Oncogene amplification and expression in pediatric solid tumors. *Neoplasia* 1998, 45:123-127
51. Li FP, Thurber WA, Seddon J, Holmes GE: Hepatoblastoma in families with polyposis coli. *JAMA* 1987, 257:2475-2477
52. Giardiello FM, Petersen GM, Brensinger JD, Luce MC, Cayouette MC, Bacon J, Brooker SV, Hamilton SR: Hepatoblastoma and APC gene mutation in familial adenomatous polyposis. *Gut* 1996, 39:867-869
53. Bando K, Nagai H, Matsumoto S, Koyama M, Kawamura N, Onda M, Emi M: Identification of a 1-cM region of common deletion on 4q35 associated with progression of hepatocellular carcinoma. *Genes Chromosom Cancer* 1999, 25:284-289
54. von Schweinitz D, Hecker H, Schmidt-Von-Arndt G, Harms D: Prognostic factors and staging systems in childhood hepatoblastoma. *Int J Cancer* 1997, 74:593-599