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Original Paper

Chromosomal Abnormality in Hepatocellular Carcinoma by Comparative Genomic Hybridisation in Taiwan

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The elucidation of the genetic changes of hepatocellular carcinoma (HCC) is very important for understanding the molecular mechanism of liver carcinogenesis. In order to identify the gains or losses in DNA sequence copy number in HCC, we used comparative genomic hybridisation to study 40 cases (44 tumours) of HCC. Tumour DNA and DNA from non-neoplastic liver tissue were labelled with different fluorochromes and then simultaneously hybridised to normal metaphase spread chromosomes. An image acquisition system was used to quantitate signal intensities contributed by tumour and reference DNA along the entire length of each chromosome. Regions of amplification and deletion were demonstrated as quantitative alterations. Losses were prevalent on chromosome regions 16q (43%), 17p (20%), 13q (20%), 4q (15%) and 8p (15%). Gains frequently occurred on 8q (30%), 1q (20%), 6p (20%) and 17q (18%). Hepatitis B virus carriers had a significantly higher frequency of losses on chromosome 16q. Furthermore, the minimal region of losses was narrowed down to 16q11-q22. This study confirms the presence of previously known chromosomal aberrations in HCC and highlights a new significant correlation between losses on chromosome 16q and hepatitis B virus carriers.

Key words: DNA loss, DNA gain, comparative genomic hybridization
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INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the leading malignancies in the world [1]. In Taiwan, it ranks first in terms of cancer mortality [2, 3]. Different aetiological factors such as hepatitis viral infection, dietary aflatoxins, or chemical carcinogens are associated with the development of liver cancer [2–5]. Nevertheless, the molecular mechanism remains to be clarified.

The genesis of human cancers is a multistep process reflecting cumulative genetic alterations that include the activation of oncogenes or the inactivation of tumour suppressor genes [6, 7]. Traditionally, cytogenetics have been used to detect the genetic changes of cancer [8]. Chromosomal aberrations have been reported in leukaemia for many years [7, 8]. For solid tumours, the identification of chromosomal aberrations is in its infancy because of technical difficulties in obtaining sufficient numbers of dividing cells [9–11]. Molecular genetic studies of isolated tumour DNA have been more successful and have been used to detect common regions of allelic loss, mutation, or amplification [12, 13]. In HCC, by using restriction fragment length polymorphism (RFLP), loss of heterozygosity (LOH) on chromosome 1p, 4q, 5q, 8p, 11p, 13q, 16q and 17p has been described [14–18]. Microsatellite analysis provides another simple way to study systematically genetic changes in tumour tissue [19–22]. However, genome-wide surveys of LOH are tedious and time consuming [23].

Recently, a new molecular cytogenetic method has been developed [11]. This method, comparative genomic hybridisation (CGH), is capable of detecting and mapping relative DNA sequence copy number between genomes. Tumour

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DNA and DNA from non-neoplastic tissue are labelled with different fluorochromes and then simultaneously hybridised into normal metaphase spread chromosomes. An image acquisition system is used to quantitate signal intensities contributed by tumour and reference DNA along the entire length of each chromosome. Regions of amplification and deletion are demonstrated as quantitative alterations.

CGH adapts molecular biological techniques for simultaneous analysis of the entire genome [24–28]. As no specific probes or previous knowledge of aberrations are required, CGH is especially suitable for the identification and mapping of previously unknown DNA copy number changes that may highlight the locations of important genes in neoplasia. Furthermore, the possibility that more than one locus is involved in tumour initiation and progression can be assessed [26, 29, 30]. Genomic DNA from tumour specimens is used so that genetic alterations identified with CGH are not artifactually altered by propagation in cell culture [24, 27]. In this study, we used CGH to identify the gains or losses in DNA sequence copy number in 40 HCC patients.

MATERIALS AND METHODS

Patients

Primary HCC tissues and non-neoplastic liver tissues were obtained from 40 HCC patients who underwent surgical resection in National Taiwan University Hospital. The diagnosis of HCC was confirmed by histology. A total of 40 HCC patients were included in this study (Table 1). One patient (case 29) had two operations for primary and recurrent HCCs and there were two tumours in each operation. Another patient

Table 1. *Clinical characteristics of the* 40 *hepatocellular carcinoma (HCC) patients studied by comparative genomic hybridisation*

Case	Sex	Age	HBsAg	Anti-HCV	Tumour-diameter (cm)	Liver state	Familial HCC tendency
1	F	55	N	P	3.6	Cirrhosis	N
2	M	41	P	N	7	Cirrhosis	N
3*	M	59	P	N	2.6	Cirrhosis	P
4	F	67	N	P	1.8	Cirrhosis	N
5	M	60	P	N	5	Cirrhosis	N
6	M	64	P	?	5	Cirrhosis	N
7†	M	46	P	N	2.2	Cirrhosis	P
8	M	67	P	N	3.6	CPH	N
9	M	39	P	?	8	Cirrhosis	N
10	M	29	P	?	4.3	Cirrhosis	N
11	F	43	P	?	4.5	Non-cirrhosis	N
12	F	68	N	P	4.5	Cirrhosis	N
13	M	65	P	P	2.2	Cirrhosis	N
14	F	57	N	N	7.5	Cirrhosis	N
15	M	50	P	N	2.5	Cirrhosis	N
16	M	44	P	?	5	Cirrhosis	N
17	F	47	P	N	5	CPH	N
18	F	64	N	P	3	Cirrhosis	N
19	M	31	P	N	4	Cirrhosis	N
20‡	M	56	P	P	17	CPH	P
21	F	60	N	P	4.8	Cirrhosis	N
22	M	60	N	P	2.5	Cirrhosis	N
23‡	M	60	P	N	20	CPH	P
24	M	63	N	P	2	Cirrhosis	N
25	M	63	N	P	3.2	Cirrhosis	N
26	F	65	P	N	7	Cirrhosis	N
27	M	52	P	N	4	Non-cirrhosis	N
28	M	54	P	N	13	CPH	N
29	M	57	P	N	$T_a = 3$	Cirrhosis	N
					$T_b = 2$		N
					$T_1 = 2$		N
					$T_2 = 1.7$		N
30	M	64	N	P	$T_a = 2.8$	CPH	N
					$T_b = 2.2$		N
31*	M	60	P	N	5	Cirrhosis	P
32§	M	58	P	N	4.5	Non-cirrhosis	P
33§	M	49	P	N	2.7	Cirrhosis	P
34§	M	46	P	N	3.5	Non-cirrhosis	P
35¶	F	33	P	N	2.5	Cirrhosis	P
36¶	M	40	P	N	2.5	Non-cirrhosis	P
37‖	F	65	N	P	2.2	Non-cirrhosis	P
38‖	F	57	N	P	1.2	Non-cirrhosis	P
39**	M	62	P	N	3	Cirrhosis	P
40**	M	36	P	N	1.5	Non-cirrhosis	P

T_a, T_b, primary tumours; T_1, T_2, recurrent tumours; ?, not determined; F, female; M, male; N, negative; P, positive; CPH, chronic persistent hepatitis; HBsAg, hepatitis B surfice antigen; anti-HCV, hepatitis C virus antibody. *Brothers. †The patient had one brother but tissue was not available in this study. ‡Brothers. §Brothers. ¶Brother and sister. ‖Sisters. **Father and son.

(case 30) had two small tumours. Therefore, a total of 44 tumours were studied. The tumour size was less than 3 cm in 21 tumours, between 3 and 5 cm in 16 tumours, and greater than 5 cm in the remaining seven tumours. 27 patients were male and 13 female. Ages ranged from 29 to 67 years. 28 were positive for hepatitis B surface antigen (HBsAg) and 12 were negative for HBsAg. Hepatitis C virus antibody (anti-HCV) was positive in 2 of the 28 patients who were positive for HBsAg. However, in the 12 patients negative for HBsAg, 11 patients were positive for anti-HCV and only 1 was negative for anti-HCV. There were 14 patients (seven families) who had family clustering of HCC in this study; case 3 and case 31 were brothers; case 20 and case 23 were brothers; case 32, case 33 and case 34 were brothers; case 35 and case 36 were brother and sister; case 37 and case 38 were sisters; case 39 and case 40 were father and son, and case 7 had a brother who died of HCC but tissue was not available for analysis.

DNA isolation

Genomic DNAs were extracted from the tumour and non-tumour liver tissues using standard methods as described previously [5].

CGH

The target metaphase slides were prepared from phyto-haemagglutinin-stimulated peripheral blood lymphocytes from a normal male or female. To assess the hybridisation characteristics, each batch of slides was extensively tested with labelled normal genomic DNA and with whole chromosome painting probes. If evidence of dim or non-uniform hybridisation was detected, the entire batch of slides was abandoned, and another batch was prepared.

CGH was performed essentially as described previously [11]. Briefly, DNA samples were labelled either with SpectrumGreen dUTP (test samples) or SpectrumRed dUTP (normal reference DNA) using the CGH nick translation kit (Vysis, Illinois, U.S.A.). The amounts of DNase and DNA polymerase I was adjusted so that the probe fragment size distribution after labelling was 300–3000 bp (a smear in an agarose gel). A probe fragment of this size was necessary to obtain uniform, intense hybridisation. Two hundred nanograms of the SpectrumGreen-labelled probe, 100 ng of the SpectrumRed-labelled probe, as well as 10 μg of unlabelled Cot-1 DNA were precipitated with ethanol. The DNAs were dissolved in 10 μl hybridisation buffer [50% formamide, 10% dextran sulphate, 2×SSC (0.15 M sodium chloride and 0.015 M sodium citrate, pH7)], denatured at 70°C for 5 min and hybridised to denatured normal metaphase spreads. After a 3 day hybridisation, the slides were washed. Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in an antifading solution.

Digital image analysis

The results of the hybridisation were evaluated using a digital image analysis system based on a fluorescence microscope and charge-coupled device camera (Photometrics, Tucson, Arizona, U.S.A.) interfaced to a Quips XL workstation [11] and a filter system consisting of a triple-band pass beam splitter and emission filters [11, 24]. Excitation of each fluorochrome was accomplished by using single band pass excitation filters in a computer-controlled filter wheel. Three-colour images, green for tumour hybridisation, red for normal reference DNA hybridisation, and blue for DNA counterstain, were acquired from five to 10 metaphase spreads after hybridisation. The quality of the hybridisation was first assessed by visual inspection of the three-colour images. The following criteria were used for acceptable hybridisation: uniform strong hybridisation over all metaphase spreads; and the consistency of colour ratio changes in chromosome homologues within one metaphase spread and between different spreads. Chromosomes were then identified based on the DAPI banding pattern. Calculation of the green/red fluorescence ratio was performed using the Xwoolz software as described previously [11, 24].

Interpretation of CGH results

The green/red ratio changes along chromosomes reflect alterations in the DNA sequence copy number in the tumour genome [9, 23–27]. Chromosomal regions where the mean

Table 2. Abnormality detected by comparative genomic hybridisation analysis in 40 hepatocellular carinoma patients

Case	Gain	Loss
1	–	6q22-qter
2	1q, 3q	8p, 13q, 16q, 17p
3	8q	5q, 6q, 8p, 16q
4	–	–
5	–	–
6	8q, 9p, 17q	4q, 11p, 13q, 16q
7	1q, 14q, 17q	4q, 8p21-p23, 11p, 16q
8	–	1p, 8p, 16q
9	–	–
10	3q, 4p, 8q	1p, 6q, 16q, 17p
11	1q, 6p, 8q	–
12	–	–
13	1q, 6p	11p, 13q, 16q, 17p12-pter
14	6p	–
15	–	–
16	1q41-qter, 12p13-pter	6q, 14q
17	6p, 17q	8p, 14q
18	1q, 8q	–
19	7q, 17q	–
20	6p22-pter	13q, 16q
21	–	–
22	–	–
23	8q, 9p	–
24	–	–
25	11q, 17q	4q21-qter
26	6p, 20q13-qter	5q, 9p21-pter, 16q, 17p
27	8q	–
28	8q24-qter	–
29 T_a	1q	4q21-q28, 5q32-qter, 11p, 13q, 17p
T_b	–	5q, 17p13-pter, 16q
T_1	8q	11p, 16q, 17p
T_2	–	5q, 16q
30 T_a	1q, 11p13-pter	–
T_b	–	4q, 13q, 16q
31	8q	1p36-pter, 6q, 8p, 16q
32	19q13	11q, 16q11-q24
33	3q28-qter	13q
34	8q	9p, 16q12-q24
35	14q24-qter, 17q	4q, 13q, 16q
36	6p, 11q	1p, 9q34-qter, 17p13-pter
37	–	–
38	–	–
39	17q, 19q13	11p, 16q11-q22
40	6p, 8q	16q11-q22

For abbreviations see Table 1

green/red ratio (five to 10 metaphase spreads) exceeded 1.25 were considered over-represented (gained), whereas regions where the mean ratio was below 0.75 were considered under-represented (lost). If the mean of the green/red ratio exceeded 1.5 in a region of a chromosome arm, these regions were considered to represent high level DNA amplification. Telomeric and heterochromatic regions were excluded from the analysis. It should be emphasised that the green/red ratio profiles do not provide information on absolute copy numbers, such as the level of DNA amplification.

Statistical analysis

We correlated the CGH results of 40 HCC patients with several clinical aspects, including sex, age, tumour size, liver state, tendency of familial clustering of HCC and status of hepatitis B or C virus infection. The statistical analysis was performed by the computer program SPSS (SPSS for Windows, SPSS Inc.). The *P* values were obtained by Chi-square analysis.

RESULTS

Gains and losses of DNA sequence in HCC detected by CGH

Of the 40 HCC patients analysed, 30 HCC patients (75%, 30/40) clearly showed a number of chromosomal alterations, whilst the remaining 10 HCCs had no detectable changes (Tables 1 and 2). Among the 30 HCC patients who had chromosomal aberrations, 21 HCCs had both gains and losses, seven HCCs had gains only, whilst two HCCs had losses only (Table 1). After analysing the 24 chromosomes, a total of 15 chromosomes had abnormalities (Figure 1). Among the 15 chromosomes which had aberrations, eight chromosomes had both gains and losses, four chromosomes had gains only, whilst three chromosomes had losses only. There were four chromosomes which had a frequency of gains of more than 15% (Figure 1, Table 3). The highest frequency of gains was on 8q (30%, 12/40), followed by 1q (20%, 8/40), 6p (20%, 8/40) and 17q (18%, 7/40) (Table 3). DNA gain at limited 8q24-qter was found in 1 of the 12 cases with DNA gain on the whole 8q arm. The minimal overlapping regions of DNA gain on 8q were narrowed down to 8q24-qter (Tables 2 and 3). The other minimal overlapping regions were narrowed down to 1q41-qter from 1 of the 8 cases with gain on the whole 1q arm and 6p22-pter from 1 of the 8 cases on the whole 6p arm (Tables 2 and 3). High level amplifications of several small regions were found in four tumours (10%, 4/40) including 1q (5%, 2/40), 7q (3%, 1/40) and 11q11-12 (3%, 1/40) (Table 3).

In this study, there were four chromosomes which had a frequency of losses of more than 15%. The highest frequency of DNA losses was on 16q (43%, 17/40), followed by 13q (20%, 8/40), 17p (20%, 8/40), 8p (15%, 6/40) and 4q (15%, 6/40) (Table 3). In the 17 cases who showed DNA losses on the 16q arm, 2 cases had limited loss at 16q11-22. Therefore, the minimal overlapping regions for the DNA losses were narrowed down to 16q11-22. The other minimal overlapping regions of loss were narrowed down to 17p13-pter from 2 of the 8 cases showing loss on the 17p arm, 8p21-23 from 1 of the 6 cases showing loss on the 8p arm and 4q21-28 from 1 of the 6 cases showing loss on the 4q arm (Tables 2 and 3). Figure 2 illustrates the mean green/red fluorescence ratio profiles of a tumour obtained from CGH analysis. Figure 2(a) shows the DNA gain of 1q and Figure 2(b) illustrates the DNA loss of 16q.

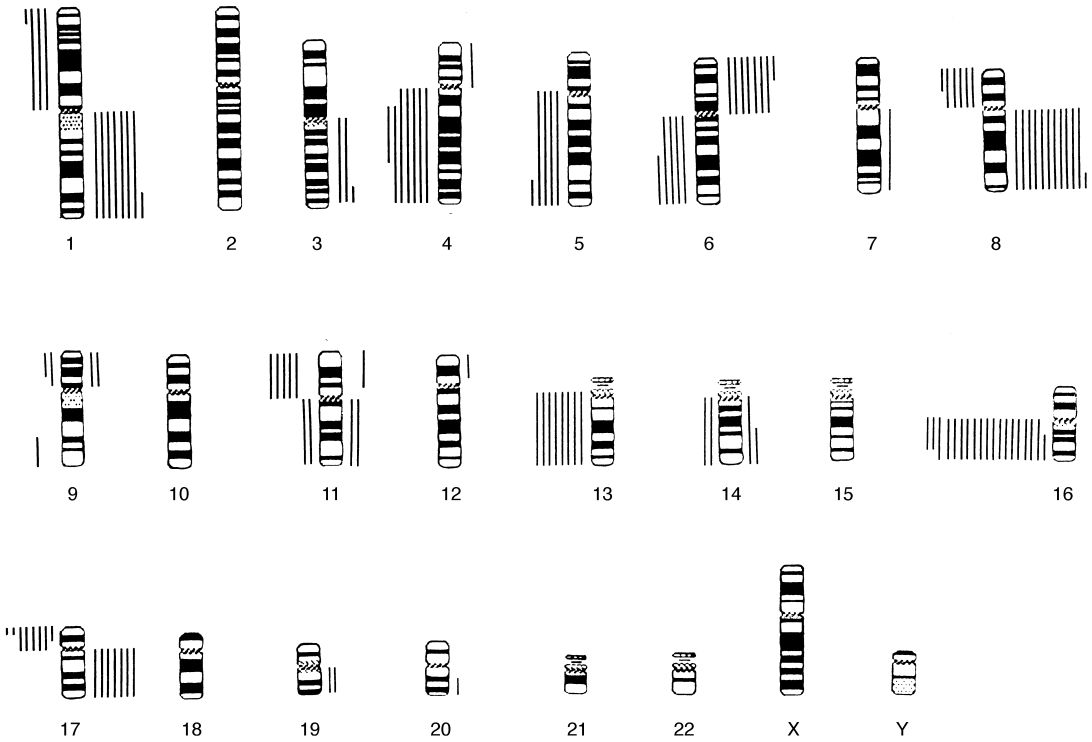

Figure 1. Summary of genetic imbalances detected by comparative genomic hybridisation in 40 hepatocellular carcinoma (HCC) patients (44 tumours). Vertical lines on the left of each chromosome ideogram represent losses of genetic material in a given tumour and those on the right correspond to a gain.

Table 3. Summary of the high frequent losses, gains and amplifications of DNA sequences detected with comparative genomic hybridisation in hepatocellular carcinoma

Chromosome	Frequency % (number/total)				
	Minimal overlapping regions			HBsAg	
	Locus	Case no.		Positive	Negative
Gains					
8q	8q24-qter	1	30% (12/40)	39% (11/28)	8.3% (1/12)*
1q	1q41-qter	1	20% (8/40)	21% (6/28)	17% (2/12)*
6p	6p22-pter	1	20% (8/40)	25% (7/28)	8.3% (1/12)*
17q			18% (7/40)	21% (6/28)	8.3% (1/12)*
Losses					
16q	16q11-22	2	43% (17/40)	57% (16/28)	8.3% (1/12)†
13q			20% (8/40)	25% (7/28)	8.3% (1/12)*
17p	17p13-pter	2	20% (8/40)	21% (6/28)	0% (0/12)*
8p	8p21-23	1	15% (6/40)	21% (6/28)	0% (0/12)*
4q	4q21-28	1	15% (6/40)	14% (4/28)	17% (2/12)*
High level amplification					
1q			5% (2/40)	3.6% (1/28)	8.3% (1/12)*
7q			3% (1/40)	3.6% (1/28)	0% (0/12)*
11q (11q11-12)			3% (1/40)	3.6% (1/28)	8.3% (1/12)*

*$P > 0.05$. †$P < 0.05$. HBsAg, hepatitis B surface antigen.

Correlation between chromosomal alterations and clinical parameters

We correlated the chromosomal regions which had gains or losses in HCC samples of more than 15% with clinical parameters. There was no significant correlation with sex, age, tumour size, status of HCV antibody and liver state. Interestingly, we found a significant correlation between losses on chromosome 16q and positivity of HBsAg. For the 40 patients (44 HCCs), losses on chromosome 16q were found in 16 of 28 (57%) HCC patients who were positive for HBsAg but only 1 in 12 (8.3%) HCC patients who was HBsAg negative. A significant difference between these two groups was observed ($P = 0.042$). However, other regions of chromosomal gains or losses were not significantly correlated with the positivity of HBsAg ($P > 0.05$) (Table 3).

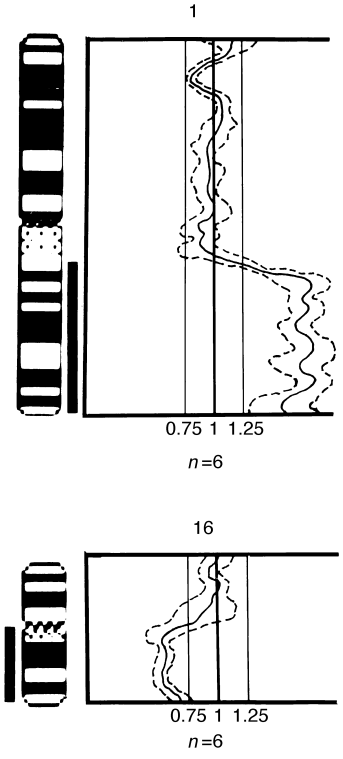

Figure 2. Examples of mean green to red ratio profiles for a tumour reflecting DNA sequence copy number changes. The mean green to red fluorescence ratio profile (±standard deviation) are shown for chromosome 1 and 16 from pter to qter. The number of observations (*n*) are shown on the bottom of each profile. Profiles on the top and bottom of the figure illustrate the DNA gain of chromosome 1q (top) and the DNA loss of chromosome 16q (bottom).

DISCUSSION

In the 44 HCCs studied by CGH, 77% of the tumours showed losses and gains of DNA sequences in at least one chromosome arm. Furthermore, most of the changes involved many different chromosomal regions. Losses were prevalent on chromosome regions 16q (43%), 17p (20%), 13q (20%), 4q (15%) and 8p (15%). Gains frequently occurred on 8q (30%), 1q (20%), 6p (20%) and 17q (18%). These results suggest that the genetic changes of HCC are highly complex. Similar complexity has been reported in a variety of human cancers such as bladder cancer, breast cancer and small cell lung cancer studied by CGH [27–31].

HBV infection has been regarded as an important factor in the development of HCCs [2, 3]. However, the molecular mechanism is unclear. Wang and colleagues demonstrated that allelic loss in 11p and 13q occurred in a higher percentage of HCCs arising from HBV carriers by RFLPs [14]. Recently, Becker and Zhou showed frequent loss of chromosome 8p in HBV positive HCC cases from China by microsatellite analysis [32]. In this study, we found that the frequency of chromosomal loss in HBsAg positive HCC patients was significantly higher on chromosome 16q than in those who were negative for HBsAg. There was no correlation between the other chromosomes which had gains or losses of more than 15% and positivity of HBsAg. Although further study is needed, we suggest that DNA loss at 16q may be important in the carcinogenesis of HCC and that the

changes on the other chromosome arms might not be important in HBsAg positive HCC patients.

In our study, the most frequent gains occurred at 8q (12/40, 30%). Recently, Marchio and colleagues showed a similar result, although at a higher incidence (60%) of gains at 8q by CGH [33]. This observation could correlate with overexpression of the *c-myc* gene (8q24) found in most HCCs [33]. Other common regions of gains including 1q, 6p and 17q have also been reported in HCC by CGH [33]. The significance of this finding needs further investigation. At the same time, we found three regions with high level copy number increases of chromosomal regions including 1q, 7q and 11q11-21. High level gains of small chromosomal regions are most informative in the identification of putative amplified oncogenes [34]. Amplification at 11q11-21 might be a loci for the presence of putative oncogenes. In agreement with our data, Marchio and colleagues also found 11q12 in a region of high level amplification [33]. Losses of DNA sequences by CGH analysis most often took place at 16q, 13q, 17p, 8p and 4q. Many previous studies of HCC by microsatellite analysis or RFLP have detected common losses at 16q, 13q, 17p, 5q, 11p, 9p, 4q, 8q and 11q [12–20]. Most of these chromosomal regions were also found to be lost by CGH but at a lower frequency than a previous study [33]. Chromosomal arms 13q, 17p and 8p which were lost in HCCs by CGH, have frequently been found to be lost in other tumour types such as myeloma, prostate cancer, small cell lung cancer and bladder cancer [31, 35–37]. It is possible that losses of these regions reflect inactivation of the same, currently unknown tumour suppressor genes in the different tumour types. Our data showed a lower frequency of DNA gains and losses by CGH than a previous report [33], the difference probably reflects the differences in tumour size between the two papers. However, another more likely possibility is the use of stricter criteria for gains and losses in our study than in the previous report.

DNA gain occurred more frequently on chromosomes 8q (30%), 1q (20%) and 6p (20%), with minimal overlapping regions at 8q24-qter, 1q41-qter and 6p22-pter. DNA loss occurred more frequently on chromosomes 16q (43%), 17p (20%), 8p (15%) and 4q (15%), with minimal overlapping regions at 16q11-22, 17p13-pter, 8p21-23 and 4q21-28. However, only 1/12 tumours showing 8q gain, 1/8 tumours showing 1q gain and 1/8 tumours showing 6p gain, showed less than a whole arm gain. Similar concern holds true for the minimal region of loss on 8p21-23 and 4q21-28. Because of limited cases, data are insufficient to demonstrate clearly the significance of these minimal overlapping regions of gain or loss in HCCs.

DNA losses on chromosome 16q(16q11-22) in this study confirm previous observations of DNA loss at the region 16q21-24 containing the E-cadherin gene [33, 38]. Decreases of DNA copy number of the 17p(17p13-pter) region is generally explained by the loss of the *p53* gene [33, 39]. At a lower but significant rate, loss of 8p was found in 15% of the tumours analysed in this study. This loss region containing the *PRLS* gene (PDGF-receptor betalike tumour suppressor) was reported as a consensus region in previous RFLP and LOH studies [33, 40].

CGH is an ideal technique for the analysis of HCC, since all chromosomal regions undergoing losses or gains can be unambiguously identified in a single hybridisation. Thus, despite the complexity of genetic changes, the most consistently altered chromosomal regions can be recognised and the minimal overlapping regions can be identified. This provides the basis for the mapping of chromosomal sites that are likely to harbour genes that are most important for the development or progression of these tumours. This study confirms the presence of previously known chromosomal aberrations in HCC and highlights new significant correlation between HBV positive HCC and loss of chromosome 16q.

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