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Chromosomal aberrations in human hepatocellular carcinomas associated with hepatitis C virus infection detected by comparative genomic hybridization

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Summary Thirty-five hepatocellular carcinomas (HCCs) associated with hepatitis C virus (HCV) were analysed by comparative genomic hybridization (CGH), to screen for changes in copy-number of DNA sequences. Chromosomal losses were noted in 1p34–36 (37%), 4q12–21 (48%), 5q13–21 (35%), 6q13–16 (23%), 8p21–23 (28%), 13q (20%), 16q (33%) and 17p13 (37%). Gains were noted in 1q (46%), 6p (20%), 8q21–24 (31%) and 17q (43%). High level gains indicative of gene amplifications were found in 7q31 (3%), 11q13 (3%), 14q12 (6%) and 17q12 (3%); amplification at 14q12 may be characteristic for HCCs. No significant difference in chromosomal aberrations was noted between carcinomas associated with HCV-infection in our study and those reported earlier in HCCs infected with hepatitis B virus (HBV), indicating that both HBV- and HCV-related carcinomas may progress through a similar cascade of molecular events.

Keywords: hepatocellular carcinomas; CGH; chromosomal aberrations

Hepatocellular carcinoma (HCC) is one of the most frequent human cancers worldwide, and it carries a very poor prognosis (Whelan et al, 1993). Although genetic changes underlying the development and progression of HCC are poorly understood, there exist some well-known predisposing factors such as persistent hepatitis due to viral infection and exposure to mycotoxins (Rensburg et al, 1985; Nalpas et al, 1991; Tanaka et al, 1991). In fact, most of HCC are associated with a background of chronic liver disease (chronic viral hepatitis or cirrhosis). Both activation of cellular oncogenes and inactivation of tumour suppressor genes have been implicated in previous studies (Rogler et al, 1992; Tabor et al, 1994; Di Bisceglie et al, 1997; Nishida et al, 1997).

Cellular proto-oncogenes may be activated through insertion of a viral genome, a process similar to integration of retrovirus; in a few cases, mutagenesis resulting from integration of hepatitis B virus (HBV) into cellular genes has appeared to be linked to hepatocarcinogenesis (Wang et al, 1990). However, since most HCCs accompanied by infection with HBV contain viral DNA integrated in the genome, this mechanism, as well as oncogene activation, has been proposed as a general contributor to the development of hepatocellular carcinoma (Simon and Carr, 1995). By contrast, to our knowledge hepatitis C virus (HCV) is never integrated into the genomes of hepatocytes (Fong et al, 1991; Kasai et al, 1996; Di Bisceglie et al, 1997). The carcinogenic mechanisms of both viruses are still under intensive investigations.

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Among the tumour suppressors most frequently inactivated in HCCs is the p53 gene, mutated in 25–60% of these tumours (Oda et al, 1992; Fujimoto et al, 1994). Losses of heterozygosity (LOH) at chromosomes 1p, 4q, 5q, 8p, 11p, 13q, 16q, 17p and/or 22q are common in HCC (Tsuda et al, 1990; Zhang et al, 1990; Fujimori et al, 1991; Simon et al, 1991). Classical cytogenetic studies have detected losses of the same chromosome arms in HCCs (Fourel et al, 1994). Therefore, some putative tumour suppressor genes at these loci may be involved in the development and progression of HCC.

Comparative genomic hybridization (CGH) is a molecular cytogenetic method that makes it possible to survey the entire genome for gains and losses of DNA sequences (Kallioniemi et al, 1992, 1994). The utility of CGH is based on the concept that regions with increased copy number reveal chromosomal sites that may contain dominant oncogenes, whereas regions with decreased copy number may be loci of putative tumour suppressor genes (Kallioniemi et al, 1992, 1994). CGH reliably screens the entire human genome, and therefore allows detection of any chromosomal sites that are likely to contain genes with an important role in tumour development (Ariyama et al, 1998; Sakakura et al, 1999).

In the study reported here, CGH analysis was performed in 35 cases of HCC with HCV infection (hereafter, HCV-HCC) in order to identify those regions that contain potential oncogenes or tumour suppressor genes responsible for hepatocellular carcinogenesis.

MATERIALS AND METHODS

Primary tumour specimens

The material consisted of 35 primary human hepatocellular carcinomas with HCV infection. The clinical stage distribution of these

Table 1 DNA copy number changes in each of 35 hepatocellular carcinomas

Sample no.	Gain	Loss	Histology, stage
1	–	12q, X	Ed1, T2N0M0 Stage 2
2	1q, **7q31**, 12q, **14q12**	1p	Ed3, T3N0M0 Stage 3
3	**14q12**, 8q,9q,16p,19p,20	1p,4q,5q,8p,10,11q,13q	Ed3, T1N0M0 Stage 1
4	8q	1p31–36, 4q, 6q, 8p,	Ed2, T2NXM0 Stage 2
5	1q,3,6,17q	4,11,12p,13q,14q,15q,18p	Ed3, T3N0M0 Stage 3
6	1q,5p, 8q	4q,5,8p,14q,17p,19	Ed2, T2N0M0 Stage 2
7	–	–	Ed2, T3N0M0 Stage 3
8	1q, 7,15q,16p	1p,4q,5q,13q,16q	Ed2, T4N0M0 Stage 4a
9	1q,8q,17q, 20	2,3,4,5q,6q 14–26,11	Ed2, T2N0M0 Stage 2
10	1q,8q,10,14q	1p,7q,10q,16,17,18q,19,20,21q	Ed2, T1N0M0 Stage 1
11	3,10q,12,17q,20	1p34–36,3p12–13,4q,7,16,17p13	Ed2, T4N0M0 Stage 4a
12	1q,3, 4p,5q,6,16,17q	10,15q,21q,Xp,Xq12–21	Ed4, T3N0M0 Stage 3
13	3,17q,Xq	5p,5q11–32,15q,18	Ed2, T1N0M0 Stage 1
14	7,8q,17q,19q,20,22q	8p,12p	Ed3, T4NXM0 Stage 4a
15	1q,6p,7p14–22,14q,15q,17q,21q	3p22–26,4q,6q,7p,12q	Ed2, T2N0M0 Stage 2
16	1q,1q,8q,15q	9q,12q24,16,17,19,20,21q	Ed2, T2N0M0 Stage 2
17	15q	1p	Ed2, T3N0M0 Stage 3
18	1q,2,8q,11,12,14q,17q,18,19	1p,4q	Ed3, T3N0M0 Stage 3
19	1q,3,4p,11p,17,19q,21q	1p,4q,6q,9q22–24,16,17p13,18,19	Ed4, T3N0M0 Stage 3
20	–	17p	Ed2, T2N0M0 Stage 2
21	1q,2,3,**11q13**,17q,X,	4q12–31,9q31–34,17p,19,21q,22q	Ed1, T3N0M0 Stage 3
22	2,4,11p,13q,15q,19	5q14–23,6q	Ed2, T3N0M0 Stage 3
23	6p,17q,	6q,17p,8p21–23	Ed2, T3N0M0 Stage 3
24	1q,8q,10p,17q,20,21q	1p,2,4,5,8q,9p22–24,13q,16q,17p,X	Ed3, T2N0M0 Stage 2
25	2,6,16p,X	1p,4q,13q,16q,18q	Ed2, T3N0M0 Stage 3
26	1q,2p,6p,8q,17,20,X	1p,5q,6q,16,18,20	Ed2, T4N0M0 Stage 4a
27	**17q12**	17p,19,22q	Ed4, T4N0M0 Stage 4a
28	–	–	Ed2, T2N0M0 Stage 2
29	–	–	Ed2, T3N0M0 Stage 3
30	8q,18,19q,X	1q22–32,2q,4,6q,13q,17,19	Ed2, T4N0M0 Stage 4a
31	1q,4p	4q,5q,8p,16,19,22q	Ed3, T2N0M0 Stage 2
32	2p,17,8q21–24	5q,6,8p,17p,21q	Ed2, T3N0M0 Stage 3
33	11p,16p,17q,19p,20	4,8p,9p,16q,19,21q	Ed3, T4N0M0 Stage 4a
34	1q,11p,15q, 21q	5q, 8p, 9,16q,17p13,19	Ed2, T3N0M0 Stage 3
35	6p,9q,11, 12q14–22	1p, 4, 5q, 8p21–23,12p,13q,16q,17p	Ed3, T2N0M0 Stage 2

*The regions of high level gain are written in bold. TNM classification of UICC was used for staging system. Edmondson classification was used for histological classification.

cases was as follows: stage 1, three cases; stage 2, 11 cases; stage 3, 14 cases; stage 4, seven cases. High molecular weight tumour DNA was isolated from homogenized tumour specimens using standard protocols. Normal human male DNA was also isolated from the peripheral blood of a male volunteer as using a reference DNA for CGH.

CGH and digital image analysis

CGH was performed using directly fluorochrome-conjugated DNA as described elsewhere (Ariyama et al, 1998; Sakakura et al, 1999). Three single-colour images (DAPI, Spectrum green and Texas red fluorescence) were collected from each metaphase spread using an epifluorescence microscope (Nikon, Tokyo, Japan), a cooled charge-coupled device (CCD) camera (Hamamatsu Photonics, Hamamatsu, Japan), and analysed using a digital image analysis system, The Power Gene, Mac Probe (PSI, Perceptive Scientific Instrument). Chromosomal regions where the mean ratio fell below 0.8 were therefore considered to reflect losses of DNA (underrepresentation), whereas regions where the mean ratio exceeded 1.2 were considered gained (overrepresentation) in the tumour genome. Overrepresentations were considered to be high-level amplifications when the fluorescence ratio exceeded 1.5 (Sakakura et al, 1999). Heterochromatic regions near the centromeres and the entire Y chromosome were excluded from analysis.

RESULTS

An overview of genetic changes in 35 HCCs is shown in Figure 1 and listed in Table 1. Thirty-two tumours (92%) showed DNA sequence copy number changes, which was significantly higher than previous cytogenetic studies had indicated (Lowichik et al, 1996). This reflects the power of CGH in revealing aberrations across the genome in uncultured cells. Three tumours had no copy number changes. None of six normal liver tissues showed any gains or losses of DNA sequence copy number (data not shown). Losses in HCC predominated over gains with a ratio of 1:1.2. On average, there were 7.6 (range 0–12) aberrations per primary tumour: 3.4 gains (range 0–6) and 4.2 losses (range 0–7).

The most common copy number changes were losses at 1p34–36 (13/35; 37%), 4q12–21 (17/35; 48%), 5q13–21 (12/35; 35%), 6q13–16 (8/35; 23%), 8p21–23 (10/35; 28%), 13q (7/35; 20%), 16q (12/35; 33%) and 17p13 (13/35; 37%). Gains were seen in 1q (16/35; 46%), 6p (7/35; 20%), 8q21–24 (11/35; 31%) and 17q (15/35; 43%). Minimal overlapping regions of common DNA copy number changes in these HCCs, and their relationship to known locations of oncogenes, tumour suppressor genes, or

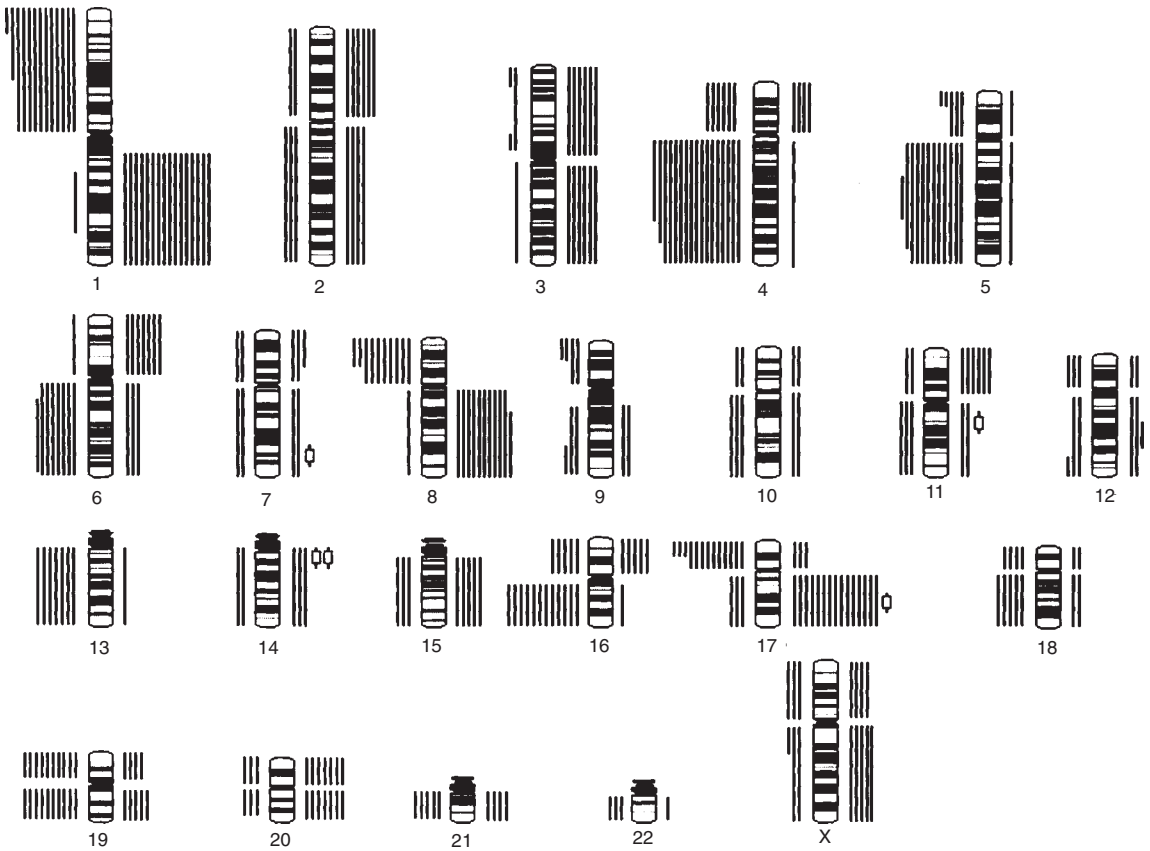

Figure 1 Schematic view of DNA copy number changes in 35 human hepatocellular carcinomas, with CGH data plotted in ideogram form. The 22 autosomal chromosomes and the X chromosome are represented by ideograms showing G-banding patterns, oriented with p-arms at the top. Each region of decrease in DNA is represented as a thin solid line to the left of and parallel to the chromosomal region where it occurs, as judged by computerized green-to-red profiles (see Materials and Methods). Each copy number increase is likewise represented as a thin solid line, to the right of the region where it occurs. Regions of frequent copy number change have multiple parallel lines beside them, and gene amplifications are indicated by open rectangles. The data are summarized in Tables 1 and 2

adhesion molecule genes are listed in Table 2. High level gains indicative of amplified genes were found in 7q31 (1/35; 3%), 11q13 (1/35; 3%), 14q12 (2/35; 6%) and 17q12 (1/35; 3%).

Figure 2A shows the typical two-colour image among our panel of CGH. The DAPI-stained image of the same metaphase from which the chromosomes were identified is shown in Figure 2B. Its copy number profile is shown in Figure 2C. DNA gains are evident on chromosomes 1q, 5p and 8q. Losses on chromosomes 4, 5q, 8p, 14q 17p and 19 are also readily apparent.

Table 2 Minimal overlapping regions of common DNA copy number changes in hepatocellular carcinoma

DNA copy number	Locus	Frequency	Putative target genes
Increase	1q	46% (16/35)	*PTPRC, ARG*
	6p	20% (7/35)	*PIM1*
	8q21–24	31% (11/35)	*MYC*
	17q	43% (15/35)	*ERBB2*
High level gain (amplification)			
	7q31	3% (1/35)	*MET*
	11q13	3% (1/35)	*HST1/INT2*
	14q12	6% (2/35)	
	17q12	3% (1/35)	*ERBB2*
Decrease	1p34–36	37% (13/35)	*TP73*
	4q12–21	48% (17/35)	
	5q13–21	35% (12/35)	*APC*
	6q13–16	23% (8/35)	
	8p21–23	28% (10/35)	
	13q	20% (7/35)	*RB*
	16q	33% (12/35)	*E-Cadherin (CDH1)*
	17q13	37% (13/35)	*TP53*

DISCUSSION

Screening for HBV prior to blood transfusion has decreased the incidence of HCC with HBV infection in Japan, and more than 80% of HCC in Japan is now associated with HCV infection (Tanaka et al, 1991; The Liver Cancer Study Group in Japan, 1994; Takano et al, 1995). The role of HCV-infection in the aetiology of HCC and cirrhosis now seems to be more important than chronic hepatitis B infection. Although HBV is randomly integrated into the genome of hepatocytes in more than 90% of HCC with HBV infection (HBV-HCC) (Tokino et al, 1987; Tabor et al, 1994), HCV does not integrate into the genome as far as we know (Fong et al, 1991; Di Biscegli et al, 1997). The mechanism by which HCV contributes to development of HCC is unclear. However, as patients with HCV infection tend to be older and show more severe liver cirrhosis than patients with HBV-HCC, not only the background but also the mechanism involved in initiation of carcinogenesis may be different for the two viruses.

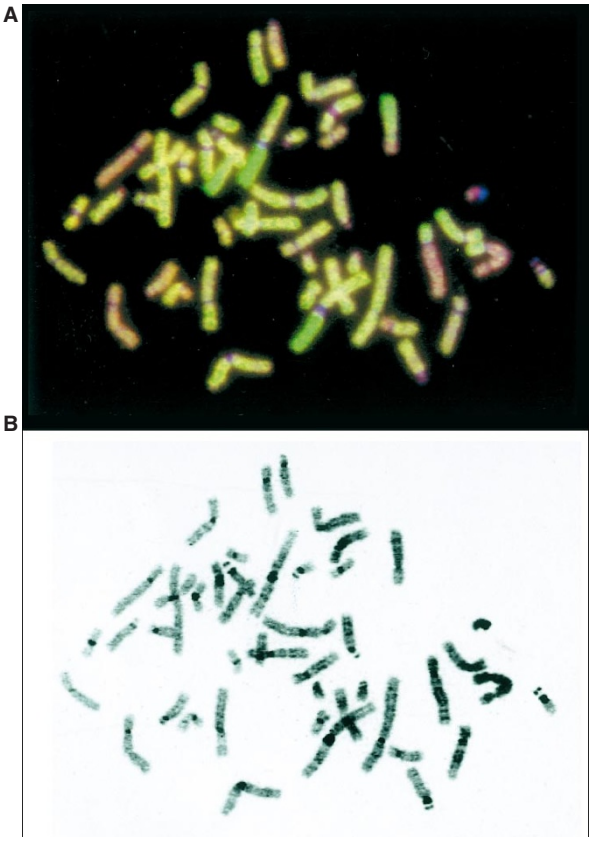

Figure 2 (A) Typical two-colour image of CGH in hepatocellular carcinoma. Differentially labelled tumour (green) and normal (red) DNAs were hybridized onto a normal human metaphase spread (46, XY). Chromosomal regions that are overrepresented in the tumour appear green, regions that are underrepresented appear red and unrelated regions appear yellow. Chromosomes showing copy number changes are numbered at their q ends; DNA gains are evident on chromosomes 1q, 5p and 8q. Losses on chromosomes 4, 5q, 8p, 14q and 17p are also readily apparent. **(B)** DAPI-stained image of the same metaphase cell shown in panel **A**. DAPI banding patterns were used for chromosome identification.

Our CGH analysis of 35 HCV-HCCs showed frequent imbalances on chromosomes 1, 4q, 5q, 6, 8, 16q and 17. These data are concordant with the results recently reported for HBV-HCCs (Marchio et al, 1997); a similar cohort of oncogenes and suppressor genes at these loci may be involved in the development and progression of both HBV- and HCV-related HCC. Our CGH results also agree with those of investigators who found no correlation between LOH in specific chromosomal regions and chronic infections with hepatitis B or C (Konishi et al, 1993; Nagai et al, 1997).

LOH analyses have revealed that allelic loss of 4q is a frequent change in early-stage HCCs (Fujimoto et al, 1991; Nagai et al, 1996), and this chromosomal region is thought to harbour one or more tumour suppressor genes (Buetow et al, 1989; Yeh et al, 1996). To our knowledge, underrepresentation of this locus, as revealed by CGH, has never been reported in other types of cancers, so HCC-specific tumour suppressor genes may exist there. Another group of investigators reports that loss of 4q21–22 region occurred preferentially in well-differentiated HCC and they considered this a secondary event that increased the aggressiveness of established cancer (Kuroki et al, 1995). It has also been reported that loss of 4q is related to increased serum AFP (alpha-fetoprotein) levels (Yeh et al, 1996). In our CGH analysis of HCCs, the most commonly recurrent loss (4q) was observed in 45% of tumour specimens, the smallest region of overlap being 4q12–21. This result supported the view that chromosome 4q, particularly 4q12–21, harbours tumour suppressor activity that is lost during tumorigenesis in HCV-HCC.

We have explored possible correlations between clinicopathological characteristics and copy number changes in the tumours of our panel. The first objective of the statistical analysis was to examine relationship of total copy number changes with cancer stage. Information obtained from the univariate analysis (log-rank test) was applied to total copy number changes with cancer stage using the Cox model of proportional hazards, but we have found no relation between two factors. In the same way, we tried to analyse the relationship between copy number changes on specific chromosomes and tumour stage, but it could not be evaluated statistically because too few early-stage tumours (three cases) were among the total. Changes on 1p, 1q and 4q were detected in the HCCs classified as stage T1 (< 2 cm in diameter) with almost same frequency as more advanced HCCs.

The recurrently underrepresented sites observed in this CGH study, i.e. 1p, 5p, 16q and 17p, have also been reported to be common regions of allelic loss in HCC. Each of these segments is a known or suspected site of tumour suppressor genes, e.g. p53 at 17p13, *APC* at 5q21, *RB* at 13q14, *CDH1* at 16q21–24. Frequent genetic alterations in the distal region of chromosome 1p in HCCs suggest loss of this region is critical for initial hepatocarcinogenesis (Yeh et al, 1994). Recently p73, whose function is related to p53, was isolated from 1p36 (Kaghad et al, 1997). Unidentified suppressor genes associated with HCC appear to present on chromosome 8p (Emi et al, 1993; Fujiwara et al, 1994). The underrepresentation of 17p (45%) in our study is explained by loss of the p53 gene (17p13.1), a frequent feature of HCC (Fujimoto et al, 1994).

Over-representation of chromosome arm 1q was the most frequent feature in our series of tumours that shows copy-number increases; 16 of them exhibited gain involving 1q. Moreover, increased copy number at 1q has also been reported in a variety of other tumours including breast, gastric and neuroblastoma, all related to poor prognosis and sometimes to metastases (Borg et al, 1992; Tahara, 1995). Chromosome 1q contains the Abelson-related oncogene (*ARG*; Kruh et al, 1990) and the protein tyrosine phosphatase receptor type c polypeptide gene (*PTPRC*; Schaapveld et al, 1995), both of which are associated with cell growth and proliferation.

Overrepresentations of 17q and 8q, the second and third most common gains of DNA observed in this series, included amplification of 17q12 in one tumour. A potentially relevant gene on 17q12, *ERBB2*, encodes an EGF-like growth factor receptor (Kameda et al, 1990); another, whose product may contribute to control of cell proliferation and malignant transformation, is *MYC*; a gene that is over-expressed in most HCCs (Moroy et al, 1986). Gains at 6p (20% in our study) have not been previously described in HCC, although tumour-specific copy number increases at 6p have been reported in retinoblastomas and osteosarcomas (Cano et al, 1994; Forus et al, 1995), where *PIM1* may be one of the target genes.

CGH is particularly powerful in revealing discrete amplified loci. In our analysis, we detected four distinct amplification sites; 7q31, 11q13, 14q12 and 17q12. Putative target genes on 7q31, 11q13 and 17q12 are *MET*, *HST/INT2* and *ERBB2*, respectively, but amplification on 14q12 has never been reported in any other type of cancer. Therefore a novel gene whose overexpression is specific for HCC may exist there; amplification of this locus has

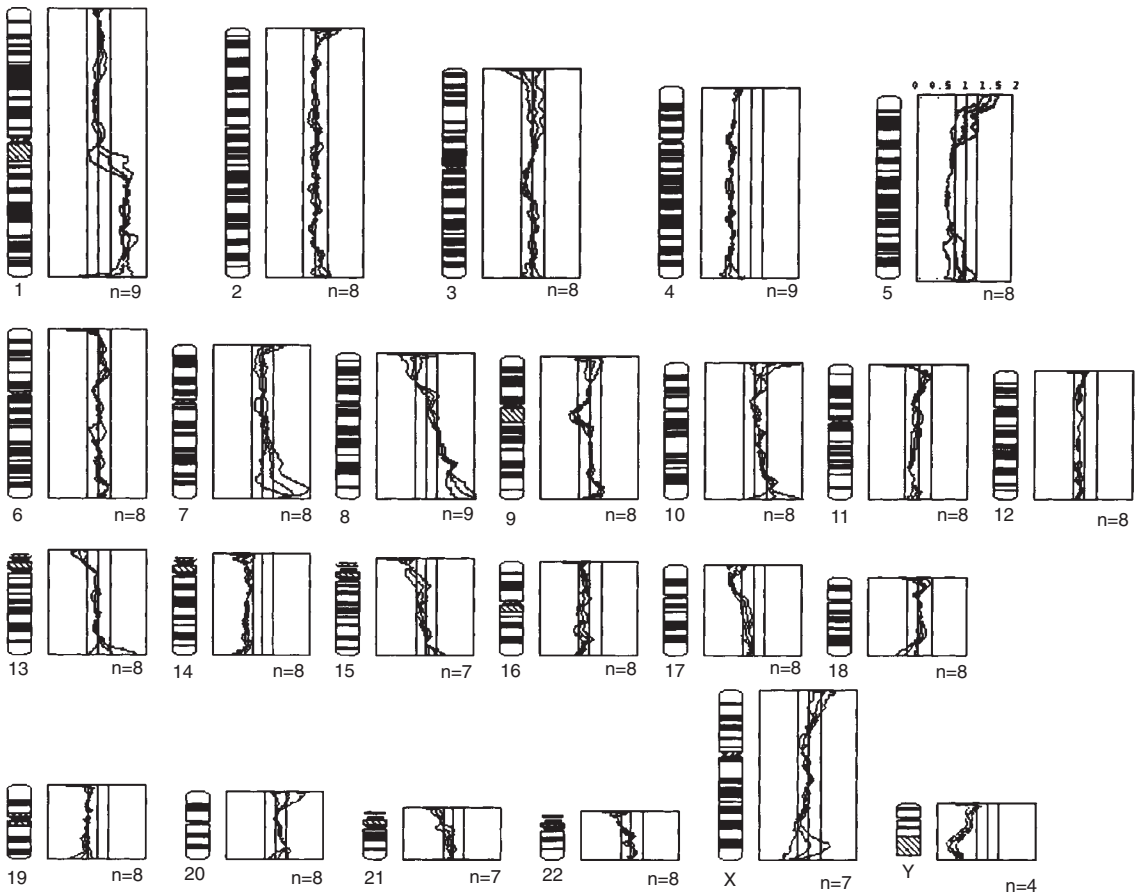

Figure 2C Computer-generated mean and standard deviation fluorescence ratio profiles for several metaphases from the hepatocellular carcinoma (Sample No 6). The central horizontal dashed line represents a green-to-red fluorescence ratio of 1 (no copy number change)

also been detected in HBV-HCC, along with amplifications of 11q13, 12p11 and 19q13 (Marchio et al, 1997). Amplifications we observed at 7q31, 11q13 and 17q12 have also been reported in other types of cancers (Tahara et al, 1994; Knuutila et al, 1998).

In summary, the recurrent copy number decreases we identified by CGH analysis support previous data on allelic loss in HCCs, and further implicate those sites as locations of tumour suppressor genes. Our study reveals that the pattern of chromosomal aberrations in HCC with HCV infection, although highly complex and involving virtually every chromosome, is clearly non-random and similar to CGH pattern of HCCs with HBV infection. In addition, many of the overrepresented chromosome segments observed in HCC are sites of known oncogene/growth-regulatory genes already implicated in tumorigenesis. Other overrepresented regions could be sites of novel amplified DNA sequences which may provide growth advantages in these aggressive neoplasms.

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