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Frequent Loss of Heterozygosity on Chromosomes 16 and 4 in Human Hepatocellular Carcinoma

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By restriction fragment length polymorphism analysis, we examined loss of heterozygosity at 34 loci on 23 chromosomes in 35 surgically resected human hepatocellular carcinomas. Allele losses at the *HP* locus on chromosome 16q22 and at the *MT2P1* locus on chromosome 4p11-q21 were detected in 57% (8/14) and 50% (8/16) of cases, respectively. Loss of heterozygosity on chromosomes 16q and 4 occurred simultaneously in 4 of 7 informative cases for both loci, and seemed to be important in the development of human hepatocellular carcinoma irrespective of the presence of hepatitis B virus infection. In contrast, the incidence of allele loss was low at the other loci, e.g., chromosome 1p, 3p, 11p, 13q or 17p, where one allele is frequently lost in other cancers.

Key words: Human hepatocellular carcinoma — Restriction fragment length polymorphism

Hepatocellular carcinoma (HCC)[5] is a primary malignant tumor of the liver parenchyma, and is one of the most prevalent types of malignancy in Africa, Southeast Asia, China, Korea and Japan. In Japan, patients with HCC almost always have associated chronic active hepatitis and/or liver cirrhosis. Although the main cause of these chronic liver diseases is considered to be hepatitis B virus (HBV) infection, recent epidemiological studies and our study of HBV DNA in liver tissues have disclosed an increasing incidence of non-A, non-B hepatitis virus-associated HCCs in Japan.[1,2)]

Little is known about the genetic alteration responsible for the development or progression of human HCCs *in vivo,* but many reports have suggested that integration of the HBV genome is involved in human hepatocarcinogenesis.[3-5)] For example, there are reports of a deletion in chromosome 11p or a rearrangement on chromosome 4 associated with the HBV integration site.[4,5)] Findings of amplification and a single base mutation of some oncogenes have been reported in HCC, but their incidence was low.[6)] Using the restriction fragment length polymorphism (RFLP) in the human genome, two studies on human HCC have been published to date: one found loss of heterozygosity on chromosomes 11p and 13q[7)] and the other loss of heterozygosity on chromosome 4q.[8)] All cases examined in those studies carried HBV infection. There has been no study on non-A, non-B hepatitis virus-associated HCCs. This prompted us to study possible loss of heterozygosity in HCC using polymorphic DNA markers in order to find the genetic alteration responsible for development or progression of HCC, particularly non-A, non-B hepatitis virus-associated HCCs.

Thirty-five primary tumors and their respective non-neoplastic liver tissues were obtained from 35 patients at surgery. The tissue samples were stored at $-80°C$ until isolation of DNAs. Histopathological diagnosis of HCC was made for all the tumors examined. Non-neoplastic liver tissues in all 35 cases showed chronic hepatitis and/or cirrhosis. Serum HBV surface antigen (HBsAg) was positive in 10 cases, and a majority of the remaining 25 cases was regarded as carrying non-A, non-B type chronic hepatitis.

High-molecular-weight DNA was isolated from frozen tissue samples of both cancerous and non-cancerous portions for each case as described previously.[9)] Ten micrograms of DNA was digested to completion with appropriate restriction enzymes, *Msp*I (Takara, Kyoto), *Eco*RI, *Bam*HI, *Taq*I, *Hin*dIII or *Bcl*I (Toyobo, Tokyo), subjected to 0.8% agarose-gel electrophoresis, and transferred to Nitroplus 2000 nylon filters (MSI, Westboro, MA) by Southern blotting.[10)] The filters were prehybridized, hybridized to ^{32}P-labeled probe DNA, washed and exposed for autoradiography. The signal intensity of fragments was approximately quantified with a Bio-Rad Model 620 videodensitometer (Bio-Rad Japan, Tokyo). The filters were hybridized repeatedly to several kinds of probes localized on different chromosomal loci.

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[5] Abbreviations: HCC, hepatocellular carcinoma; RFLP, restriction fragment length polymorphism; HBV, hepatitis B virus; SDS, sodium dodecyl sulfate; HBsAg, hepatitis B surface antigen.

Table I. Loss of Chromosomal Heterozygosity in Human Hepatocellular Carcinoma

Marker locus	Chromosome location	Enzyme	No. of cases	Heterozygosity	
				Constitutional	Loss in tumor
LMYC	1p	*Eco*RI	32	18	2
REN	1q	*Hin*dIII	23	14	0
CRYG1	2q	*Taq*I	6	1	0
D3S2	3p	*Msp*I	35	14	1
RAF1P1	4p	*Bam*HI	35	13	2
MT2P1	4	*Eco*RI	35	16	8
D4S16	4	*Msp*I	35	5	2
ADH3	4q	*Msp*I	35	4	0
D5S2	5	*Msp*I	27	8	0
MYB	6q	*Eco*RI	26	13	0
COLIA2	7q	*Eco*RI	35	15	1
MOS	8q	*Eco*RI	16	0	0
		*Bcl*I	27	0	0
D9S1	9	*Taq*I	24	7	0
PLAU	10q	*Bam*HI	32	20	0
HRAS	11p	*Bam*HI	27	5	0
INS	11p	*Taq*I	25	9	0
D11S24	11q	*Bam*HI	27	2	0
D12S17	12	*Msp*I	35	15	1
D13S1	13q	*Msp*I	11	9	0
D13S2	13q	*Msp*I	27	13	0
D13S3	13q	*Hin*dIII	12	4	0
D13S4	13q	*Msp*I	15	4	0
D14S1	14q	*Hin*dIII	35	17	6
D15S1	15q	*Msp*I	35	10	1
D16S32	16p	*Taq*I	35	16	4
D16S37	16p	*Taq*I	35	2	0
HP	16q	*Bam*HI	35	13	7
		*Eco*RI	35	14	8
D17S1	17p	*Msp*I	14	7	1
D18S5	18q	*Taq*I	35	18	1
D19S7	19	*Msp*I	34	11	0
D20S4	20q	*Msp*I	35	15	0
D21S52	21q	*Hin*dIII	10	4	1
D22S1	22	*Taq*I	25	10	0
DXYS1	Yp	*Taq*I	18	14	1
	X	*Taq*I	6	1	0

The DNA probes used to detect the various polymorphic human chromosomal loci used in this study are listed in Table I. The length of each allelic fragment observed was identical to those published previously.[11]

As summarized in Table I, information on loss of heterozygosity occurring in HCCs at 33 loci on 22 different chromosomes was obtained except for the *MOS* locus on chromosome 8. Loss of heterozygosity was detected at 15 loci on 12 different chromosomes. The frequency of allele loss at an individual locus ranged between 57% (8/14) at *HP* on chromosome 16 and 5.6% (1/18) at *D18S5* on chromosome 18.

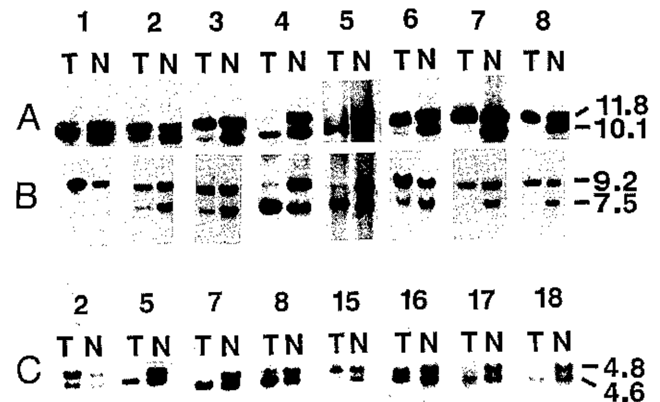

Fig. 1. Southern blot hybridization analysis on loss of heterozygosity at the *HP* locus on chromosome 16q (A and B) and at the *MT2P1* locus on chromosome 4 (C) in hepatocellular carcinoma. DNA samples from tumor (lanes T) and non-cancerous liver (lanes N) tissues obtained from cases 1 to 18 were digested with *Eco*RI (A and C) or with *Bam*HI (B), electrophoresed in 0.8% agarose gel and transferred to nylon filters. The filters were prehybridized in 50% formamide/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/0.1 *M* piperazine-N,N′-bis (2-ethanesulfonic acid) (pH 6.8)/0.65 *M* NaCl/5 m*M* EDTA/0.1% sodium dodecyl sulfate (SDS)/100 μg/ml denatured salmon testis DNA (Sigma) for at least 2 h and then hybridized to ^{32}P-labeled DNA probes at 42°C for 16–48 h in a solution identical to that used for prehybridization except for the presence of 10% dextran sulfate. Unbound probe was removed from the filters by washing twice at room temperature with 2×SSC (1×SSC is 0.15 *M* NaCl/0.015 *M* sodium citrate, pH 7.0)/0.1% SDS and twice (30 min each) at 65°C with 0.1×SSC/0.1% SDS. Probes used for hybridization were hp2-alpha[12] in A and B to examine the *HP* locus on chromosome 16q22.1, and pHM6[15] in C to examine the *MT2P1* locus on chromosome 4p11-q21. Washed filters were exposed for autoradiography using Kodak XAR-5 film for 12 h to 1 week at −80°C. Numbers to the right of autoradiographs indicate the molecular size of the polymorphic alleles in kilobases. The larger and smaller restriction fragments correspond to the alleles 1 and 2 in Table II, respectively.

Loss of heterozygosity for chromosome 16 was assessed with three polymorphic DNA markers: *HP* (hp2-alpha), a DNA fragment from the haptoglobin gene localized at 16q22.1[12]; *D16S32* (16/118) and *D16S37* (16/02), DNA fragments clustering on 16pter-p13.[13] Using the hp2-alpha probe, loss of heterozygosity in HCC tissue was detected in 8 of 14 informative cases with *Eco*RI digestion (cases 1 to 8 in Fig. 1A), and in 7 of 13 informative cases with *Bam*HI digestion (cases 2 to 8 in Fig. 1B). The cases showing the loss of heterozygosity were the same for both *Eco*RI and *Bam*HI diges-

Table II. Genotypes in HCC DNA at the *HP* Locus on Chromosome 16 and the *MT2P1* Locus on Chromosome 4, and Serum HBsAg Status

Patient	Locus[a]		Serum HBsAg[b]	Other loci with loss of heterozygosity
	HP (*Eco*RI)	*MT2P1* (*Eco*RI)		
1	2	–	–	
2	1	1	–	
3	1	1,2	–	*D16S32, RAF1P1, D21S52*
4	2	–	–	
5	2	2	–	*D16S32*
6	1	–	+	
7	1	2	–	*D16S32, D14S1, LMYC*
8	1	2	+	*D14S1, COL1A2*
9	1,2	1,2	–	*D3S2*
10	1,2	–	–	
11	1,2	–	+	
12	1,2	1,2	–	
13	1,2	–	+	
14	1,2	–	–	*D14S1*
15	–	1	–	*RAF1P1, D4S16, D14S1, D12S17, D18S5*
16	–	2	+	
17	–	2	–	*D4S16, LMYC*
18	–	2	+	
19	–	1,2	–	
20	–	1,2	–	
21	–	1,2	–	
22	–	1,2	–	
23	–	1,2	+	
24	–	–	–	*D14S1*
25	–	–	–	*D14S1*
26	–	–	–	
27	–	–	+	
28	–	–	–	
29	–	–	–	*D17S1*
30	–	–	–	*DXYS1*
31	–	–	–	*D16S32*
32	–	–	+	
33	–	–	+	*D15S1*
34	–	–	+	
35	–	–	–	

a) In the column for the locus, 1 and 2 refer to larger- and smaller-sized allele fragments, respectively. 1,2 indicates that heterozygosity remained in the tumors; a minus sign indicates constitutional homozygosity. 1 indicates loss of the smaller constitutional allele; 2 indicates loss of the larger allele.
b) Serum HBsAg was measured by reversed passive hemagglutination test.

tion except for case 1, which was informative only after digestion with the former.

Allele loss was also detected in 4 of 16 heterozygotes (25%) identified by the DNA probe for the *D16S32* locus. Three of the cases with allele loss at *D16S32* also showed loss of heterozygosity at the *HP* locus, but in another case, information on the *HP* locus was not available. No loss of allele was detected in 2 heterozygotes identified by the DNA probe for *D16S37*. These results suggested that allele loss on chromosome 16 occurred more frequently in the region of 16q22 than between 16pter and 16p13 in HCC.

Four different polymorphic DNA markers were used to analyze the abnormality on chromosome 4: *RAF1P1* (c-raf-2 P52) on 4p16.1[14]; *MT2P1* (pHM6), a human metallothionein pseudogene on 4p11-q21[15]; *D4S16* (3E5) on 4p15.1-q11[16]; *ADH3* (pADH73) on 4q21-q23.[17] The incidence of loss of heterozygosity was high at the 2 loci; 50% (8 of 16) at *MT2P1* (cases 2, 5, 7, 8, 15–18 in Fig. 1C) and 40% (2 of 5) at *D4S16*. However, the incidence of loss of heterozygosity was low at the other loci; 15% (2 of 13) at *RAF1P1* and 0% (0 of 4) at *ADH3*. Cases 15 and 17 showed simultaneous allele loss at loci *MT2P1*, *RAF1P1* and *D4S16*, and at loci *MT2P1* and *D4S16*, respectively (Table II). These results suggested that allele loss on chromosome 4 occurred frequently in the region between 4p15.1 and 4q21.

As shown in Table II, 7 cases were informative for analysis of both loci *HP* and *MT2P1*. Four (cases 2, 5, 7 and 8) of them showed simultaneous loss of heterozygosity at these two loci. Another case with allele loss at *HP* (case 3) showed allele loss not at *MT2P1* but at *RAF1P1* on 4p16.1. The other two informative cases (cases 9 and 12) did not show any loss of heterozygosity at either locus. Accordingly, allele losses on chromosomes 16 and 4 together seemed to be strongly associated with human HCC.

Losses of heterozygosity on chromosomes 16 and 4 were detected not only in HBsAg-positive cases but also in HBsAg-negative cases: among 14 cases informative at the *HP* locus on chromosome 16, 10 were serum HBsAg-negative and 4 were positive. The incidence of loss of heterozygosity at the *HP* locus was 60% (6/10) for the serum HBsAg-negative group, and 50% (2/4) for the serum HBsAg-positive group. Among 16 cases informative at the *MT2P1* locus on chromosome 4, 13 were serum HBsAg-negative and the other 3 were positive. The incidence of loss of heterozygosity at the *MT2P1* locus was 54% (7/13) for the serum HBsAg-negative group, and 33% (1/3) for the HBsAg-positive group. This result suggested that these genetic abnormalities are common in human HCCs associated with HBV infection as well as those possibly associated with non-A non-B viral hepatitis.

Several common sites which are frequently deleted in many kinds of tumor have been detected by RFLP analysis, e.g., chromosomes 1p, 3p, 11p, 13q and 17p.[18] In this study, the incidences of allele losses at those loci were very low in human HCCs (Table I). On the other hand, there has been no report showing frequent loss of heterozygosity at chromosome 16 or chromosome 4 in cancers other than HCC. Alteration of these regions seems to be specifically associated with development and/or progression of human HCC.

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