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Allele loss on chromosome 16 associated with progression of human hepatocellular carcinoma

(tumor differentiation/hepatitis C virus infection/hepatitis B virus integration)

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ABSTRACT **Loss of heterozygosity on chromosome 16 is a common genetic alteration in human hepatocellular carcinoma (HCC). To clarify the pathogenetic significance of allele loss on chromosome 16, we performed restriction fragment length polymorphism analysis of 70 surgically resected tumors by using 15 polymorphic DNA markers for chromosome 16. Loss of heterozygosity on chromosome 16 was detected in 36 (52%) of 69 informative cases, and the common region of allele loss in these 36 tumors was located between the *HP* locus (16q22.1) and the *CTRB* locus (16q22.3–q23.2). These losses occurred more frequently in HCCs of poor differentiation, of larger size, and with metastasis, whereas they were not detected in HCC at the earliest stage. In addition, these losses were not associated with presence or absence of hepatitis B virus DNA integration or hepatitis C virus infection. These results show that loss of heterozygosity on chromosome 16 is a late event occurring after hepatocarcinogenesis and strongly suggest that this phenomenon is involved in enhancement of tumor aggressiveness during progression of HCC.**

Many studies have demonstrated deletions in specific chromosomal regions that are suggested to be responsible for development of human cancers (1–15). In such cases, mutations of specific genes located in specific chromosomal regions and elimination of their normal allele are considered to be involved in carcinogenesis and/or tumor progression (16). Such genes are considered to be recessive oncogenes, or tumor-suppressor genes, because their presence in normal cells seems to be required for prevention of tumor emergence (17). The first example of a recessive oncogene was the *RB* gene, which is localized on chromosome 13q14 and contained in the region commonly deleted in retinoblastoma (1, 2), osteosarcoma (3), and lung cancer (4, 18). Recently, the p53 gene, localized on chromosome 17p13, and the *DCC* gene, localized on chromosome 18q, regions commonly deleted in colorectal cancer (5–8), were suggested to be further examples of recessive oncogenes (19–22). Frequent genetic losses have also been reported on chromosome 1p (9, 10), 3p (4, 11, 12), 5p (5), and 11p (13–15) in human cancers, suggesting the presence of recessive oncogenes other than *RB*, p53, and *DCC*.

Our previous study on human hepatocellular carcinoma (HCC) demonstrated high incidence of loss of heterozygosity (LOH) on chromosome 16q (23). LOH in this region seemed to play an important role in hepatocarcinogenesis or progression of HCC, because there had been no report of frequent LOH on chromosome 16 in other tumors, although deletion or inversion of chromosome 16 has been detected cytogenetically in a special type of myelomonocytic leukemia (24, 25). In the present study, further analysis of restriction fragment length polymorphism was performed using DNA isolated from a larger number of primary HCCs (70 tumors) and 15 polymorphic DNA markers localized on chromosome 16 in order to clarify the commonly deleted region possibly containing the putative recessive oncogene and to understand the role of LOH on chromosome 16 in hepatocarcinogenesis or progression of HCC.

MATERIALS AND METHODS

Patients. Seventy lesions of primary HCC and corresponding noncancerous liver tissues were obtained from 57 patients who had undergone surgical therapy for HCC. Among the 70 lesions, 25 were resected from the patients with multiple primary lesions of HCC, and diagnosis of primary HCC in these cases was performed according to the criteria of Kanai *et al.* (26). All specimens were kept frozen at −80°C until DNA isolation. By macro- and microscopic observation of surgically resected specimens, 6 lesions were defined as early HCC, because the lesions had characteristic features of the earliest developmental stage and of low-grade malignancy (26)—i.e., small size (less than 2 cm), preservation of underlying liver structure, and very well differentiated cancer cells. The other 64 lesions were determined to be advanced HCC and classified into well, moderately, and poorly differentiated groups according to the criteria of Edmondson and Steiner with modification (26). Among these cases, 3 showed a "nodule-in-nodule" appearance in which an HCC lesion of poorer differentiation newly occurred within early HCC. This type of lesion is considered to be a transitional form from early to advanced HCC (27, 28).

Data were also available regarding the presence of portal vein tumor thrombi or intrahepatic metastasis at the time of surgery and the development of extrahepatic metastasis after initial surgery.

Hepatitis B virus (HBV) DNA integration was examined by Southern blot hybridization analysis using a ^{32}P-labeled HBV DNA probe (29). Serum anti-hepatitis C virus (HCV) antibody was measured by an enzyme-linked immunosorbent assay (30, 31). Assay kits were provided by Ortho Diagnostics.

DNA Probes. The 15 polymorphic markers examined are localized on 13 loci of chromosome 16: *HBA1* (32), *D16S32*, *D16S34*, and *D16S35* (33), *D16S131* (34), *CETP* (35), *MT2* (36), *D16S4* (37), *HP* (38), three regions in *TAT* (HP0.4, BB0.4, and BS0.9) (39, 40), *CTRB* (39, 41), *D16S7* (42), and *APRT* (43) (Table 1).

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Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LOH, loss of heterozygosity.

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Table 1. Incidence of LOH detected by 15 polymorphic DNA markers localized on chromosome 16 in primary HCC

Marker locus	Localization	No. of tumors examined	Incidence of LOH (%), loss in tumor/heterozygotes
HBA1	p13.3	67	16/36 (44)
D16S32	pter–p13	70	8/26 (31)
D16S34	pter–p13	69	9/20 (45)
D16S35	pter–p13	69	9/24 (38)
D16S131	p13.1	70	6/13 (46)
CETP	q21	69	20/44 (45)
MT2	q13–q22.1	70	15/36 (42)
D16S4	q22.1	70	12/31 (39)
HP	q22.1	70	10/28 (36)
TAT			
HP0.4	q22.1	70	14/29 (48)
BB0.4	q22.1	70	13/25 (52)
BS0.9	q22.1	70	13/23 (57)
CTRB	q22.3–q23.2	68	17/38 (45)
D16S7	q24	70	24/53 (45)
APRT	q24	70	17/26 (65)

Southern Blot Analysis. High molecular weight DNA was isolated and completely digested with restriction enzymes. The enzymes used were *Sac* I for *HBA1*, *Pst* I for *D16S34*, *Msp* I for HP0.4, *Bam*HI for BB0.4, *Hae* III for BS0.9, *Eco*RI for *HP*, and *Taq* I for markers of other loci. DNA was electrophoresed in 0.8% agarose gel, transferred to nylon filter, hybridized to a radiolabeled DNA probe, and autoradiographed as described (23).

Association of allele loss on chromosome 16 with HBV DNA integration, HCV infection, and degree of tumor differentiation and spread was calculated by χ^2 test.

RESULTS

Commonly Deleted Region on Chromosome 16 in HCC. Among 57 patients with 70 primary HCC lesions, constitutional heterozygosity was detected at at least three loci on chromosome 16 in 56 patients with 69 lesions. In one patient, constitutional heterozygosity was not detected by any of the 15 DNA probes used. Among the 69 informative lesions, 36 tumors (52%) showed LOH at one or more of the 15 RFLP sites on chromosome 16 (Figs. 1 and 2). The remaining 33 tumors showed no LOH at any loci examined. The incidence of LOH was higher than 30% at all involved loci and was highest at the *APRT* locus (65%) (Table 1). All cases with LOH were determined densitometrically to be caused by simple allele deletion without duplication.

Among HCCs with LOH at one or more loci, 58% (21/36) of tumors were considered to have total loss of one chromosome: e.g., in cases 8 and 12, allele loss was detected at all loci examined (Fig. 1 *A* and *B* and Fig. 2). In the other 15 tumors (42%), allele loss on chromosome 16 was partial: e.g., in case 31, allele loss was detected at *D16S131* and *APRT* but was not detected at other informative loci (Fig. 1*C*). In case 33, allele loss was detected at *HBA1*, *CETP*, *MT2*, *D16S4*, and *TAT* but not at other informative loci (Fig. 1*D*). In case 34, the *TAT* locus alone showed allele loss (Fig. 1*E*). Among these 15 tumors, the commonly deleted region was shown to be located between the loci *HP* at 16q22.1 and *CTRB* at 16q22.3–q23.2, containing the *TAT* locus, except for 1 tumor (case 32) (Fig. 2).

Association of LOH on Chromosome 16 with Progression of HCC. Integration of HBV DNA into cellular DNA and serum anti-HCV antibody were detected in 11 (16%) and 41 (59%) cases, respectively. There was no significant difference in incidence of the LOH between the cases with HBV DNA integration (73%) and those without it (48%), nor between the cases with positive serum anti-HCV antibody (46%) and those without it (63%) (Table 2).

Although LOH on chromosome 16 was detected in none of the 6 early HCCs, it was detected in 57% (36/63) of HCCs at the more advanced stage. Incidence of LOH was markedly higher in HCCs showing poorer differentiation: allele loss on chromosome 16 was detected in 88% of poorly differentiated HCCs, whereas it was detected in 52% and 18% of moderately and well differentiated HCCs, respectively ($P < 0.001$, Table 2). Eighteen out of the 21 poorly differentiated HCCs with LOH showed total loss of one chromosome or total loss of the long arm. In the 3 "nodule-in-nodule" lesions, LOH on chromosome 16 was detected in 1 (case 33).

Incidence of allele loss on chromosome 16 was also significantly high in tumors with intrahepatic metastasis or portal vein tumor thrombi ($P < 0.005$, Table 2) and in tumors

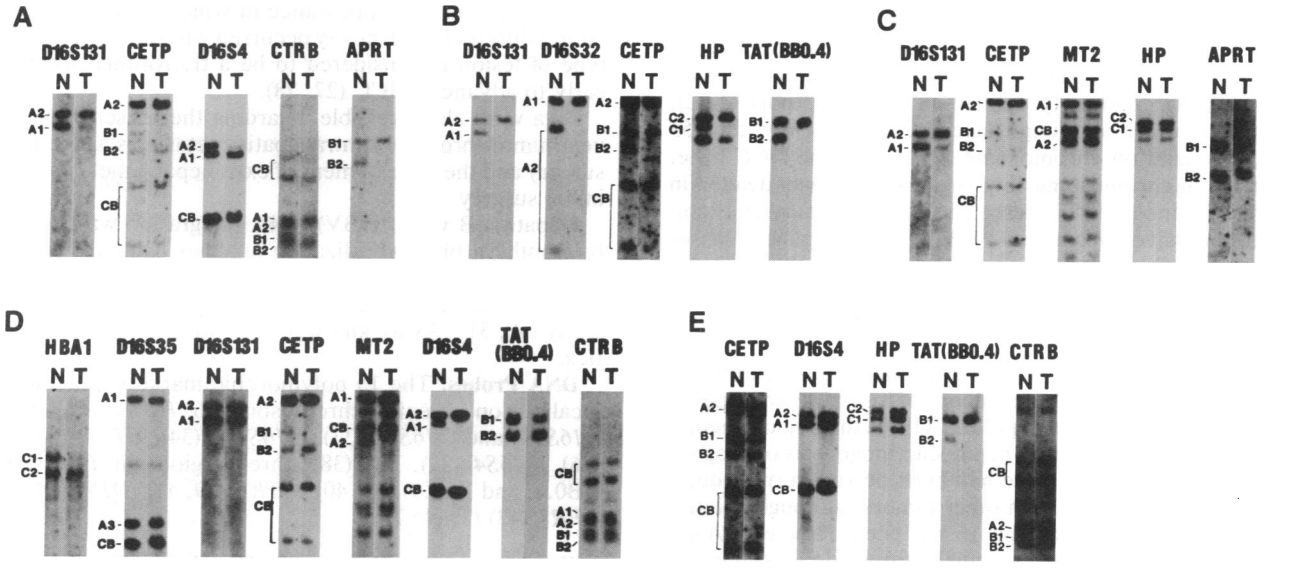

FIG. 1. Restriction fragment length polymorphism on chromosome 16 in HCC. DNA samples were isolated from tumor (T) and nontumorous liver (N) tissue surgically resected from patients. Cases 8 (*A*), 12 (*B*), 31 (*C*), 33 (*D*), and 34 (*E*) are presented. Ten micrograms of DNA was digested with appropriate restriction enzymes as described in the text, electrophoresed, and analyzed by Southern blot hybridization as described (23). Allele designation in autoradiograms was done according to refs. 34, 40, and 44. CB denotes constant bands.

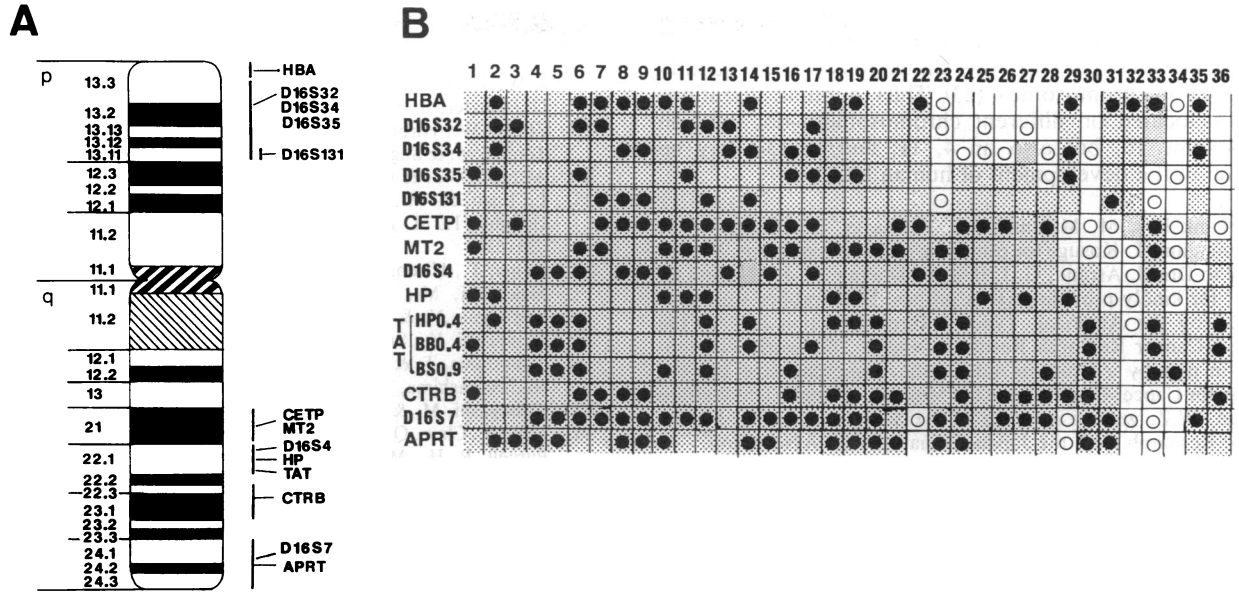

FIG. 2. Map of the common region showing allele loss on chromosome 16 in human HCC. (*A*) Localization of markers on chromosome 16 (45). (*B*) Thirty-six primary HCC lesions (nos. 1–36) in which LOH was detected at one or more of the 15 sites indicated on the left are presented. Informative sites are indicated by symbols (●, LOH; ○, no LOH). Areas with no symbol are noninformative regions. Maximum extent of putative deleted regions is shown by shading.

of larger size ($P < 0.05$, Table 2). Furthermore, allele loss on chromosome 16 was detected in all 4 patients (cases 10–12 and 35) that later developed metastasis to the lung, brain, or adrenal glands.

DISCUSSION

A model of progression of human HCC is shown in Fig. 3. Integration of HBV DNA and persistent HCV infection, which are strongly suggested to be involved in hepatocarcinogenesis (30, 47, 48), were not associated with allele loss on chromosome 16. Allele loss on chromosome 16 did not occur in HCC at the earliest stage but occurred selectively in HCCs at the advanced stage, and the incidence of allele loss increased in accordance with spread and poorer differentiation of the tumor. These results show that allele loss on chromosome 16 occurs at a late stage of human HCC progression and that it is associated with enhancement of tumor aggressiveness rather than hepatocarcinogenesis.

Table 2. Association of allele loss on chromosome 16 with clinical and pathological parameters of primary HCC

Parameter	No. of tumors: Analyzed	Allele loss detected (%)	*P*
Integration of HBV DNA			
Present	11	8 (73)	NS
Absent	58	28 (48)	
Serum anti-HCV antibody			
Present	41	19 (46)	NS
Absent	27	17 (63)	
Undetermined	1	0	
Histological differentiation			
Early HCC	6	0 (0)	
Advanced HCC			
Well differentiated	17	3 (18)	<0.001
Moderately differentiated	21	11 (52)	
Poorly differentiated	25	22 (88)	
Intrahepatic metastasis or portal vein tumor thrombi			
Positive	31	22 (71)	<0.005
Negative	38	14 (37)	
Tumor size (cm)			
≤2.0	15	4 (27)	
2.1–5.0	29	14 (48)	<0.05
5.1–10.0	18	12 (67)	
≥10.1	7	6 (86)	
Total	69	36 (52)	

NS, not significant.

The region between the *HP* and *CTRB* loci containing the *TAT* locus is suggested to contain an unknown recessive oncogene. The physical distances between *TAT* and *HP* and between *TAT* and *CTRB* are estimated to be about 700 kilobase pairs and more than 800 kilobase pairs, respectively (49, 50). Although the distance is still long, the finding of a commonly deleted region in this study seems to be a first step for identification of a specific gene associated with progression of HCC.

In human HCCs, frequent LOH has been also reported on chromosomes 4 (23, 51, 52), 11p (53, 54), and 13q (54).

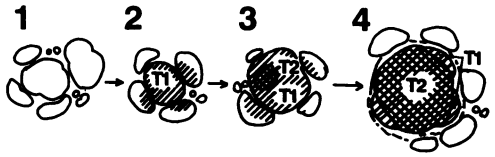

FIG. 3. A model of progression of human HCC (26–28). (Stage 1) Regenerative nodules in liver cirrhosis. At this stage, integration of HBV DNA and/or infection with HCV has already occurred. The constituent liver cells are not neoplastic and usually do not show clonal expansion. (Stage 2) Early HCC (T1) arises in the liver affected by chronic hepatitis or liver cirrhosis. The early HCC is already a clonal neoplastic lesion (46), although it is a very well differentiated, small-sized lesion with no metastatic potential at this stage (26). (Stage 3) HCC lesion(s) with less marked differentiation (T2) newly occurs within early HCC, grossly forming a "nodule-in-nodule" lesion. After this stage, allele loss on chromosome 16 is frequently detected, especially when the less differentiated HCC is of a highly aggressive nature. (Stage 4) Thereafter, the less differentiated lesion (T2) rapidly increases in size and finally compresses or replaces the preceding early HCC because of its more aggressive proliferative potential.

However, their association with tumor spread or grade of malignancy remains unknown. In the previous study, LOH on chromosome 16 in HCC was shown to coexist frequently with LOH on chromosome 4 (23). Thus, multiple alterations in DNA located on different chromosomes—not only on chromosome 16, but also on 4, 11, and/or 13—may be involved in the development of human HCC.

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