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Genetic Changes and Histopathological Grades in Human Hepatocellular Carcinomas

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Loss of heterozygosity (LOH) on chromosomes 1p, 4q, 5q, 8p, 13q, 16q, 17p, and 22q, and mutation of the p53 gene were simultaneously analyzed in 63 hepatocellular carcinomas (HCCs) with distinct histopathological grades, 80% of the tumors being from patients who had been exposed to hepatitis B virus (HBV) or hepatitis C virus (HCV). The frequencies of LOH on 8 chromosomes were 0–25% in 10 well differentiated HCCs, LOH being observed on 4q, 5q and 17p, 21–53% in 26 moderately differentiated HCCs, LOH on 8p and 17p being high, and 29–75% in 27 poorly differentiated HCCs, LOH on 17p, 4q and 8p being the most frequent. p53 gene mutation was detected in moderately and poorly differentiated HCCs at 15% and 52%, respectively, but not at all in well differentiated HCCs. Of the mutations detected, 42% were transition mutation and only 5% were CpG transition, in contrast to the high frequencies of these types of mutations in colon tumors (78% and 54%, respectively). LOH on every chromosome and p53 mutation were more frequent in more advanced tumors, and accumulation of genetic changes increased with increase of the histopathological grade. Frequency of genetic changes in HCCs from HBV-positive patients was comparable to that from HCV-positive patients. The present results suggest that accumulation of genetic changes in multiple tumor suppressor genes, especially LOH on 17p, 4q and 8p, and mutation in p53 gene, are involved in the progression of liver cancer, and LOH on 17p and 4q precedes other genetic changes. Differences in the direction of p53 mutation between HCC and colon carcinoma suggest that liver carcinogens are distinct from colon carcinogens. Furthermore, mechanisms affecting the frequency of LOH in HCCs in HBV-infected patients may be similar to those in HCV-infected patients.

Key words: Hepatocellular carcinoma — LOH — p53 mutation — Histopathological diagnosis — Hepatitis virus infection

HCC[8] is one of the most common tumors in Asia and Africa,[1,2] where hepatitis virus infection and exposure to specific liver carcinogens are prevalent. However, the mechanisms by which these factors contribute to liver carcinogenesis are still unclear. Examination of genetic changes in tumor suppressor genes and oncogenes should be a valuable approach to elucidate the mechanisms of carcinogenesis. LOH has been frequently detected in HCC at the chromosomal regions 1p, 4q, 5q, 8p, 11p, 13q, 16q and 17p.[3–10] Mutation of the p53 gene, which occurs in a wide variety of human tumors, has also been detected in HCC.[11–14] However, the correlation between accumulation of these genetic changes and histopathological grade of liver tumors is not fully understood. In the present study, we analyzed LOH on chromosomes 1p, 4q, 5q, 8p, 13q, 16q, 17p, and 22q, and mutation in the p53 gene simultaneously in 65 liver tumors with distinct histopathological grade. We also examined the difference in frequency of LOH between tumors from HBV-positive patients and those from HCV-positive patients.

MATERIALS AND METHODS

Histopathological classification of tumors and preparation of DNA Sixty-five liver tumors obtained from 52 patients were classified according to histopathological diagnosis[15,16] into 10 well differentiated HCCs (corresponding to Edmondson grade I), 26 moderately differentiated HCCs (Edmonson I-II and II), and 27 poorly differentiated HCCs (Edmondson II-III, III, III-IV and

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[8] The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; LOH, loss of heterozygosity; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; RFLP, restriction fragment length polymorphism.

IV) and 2 atypical adenomatous hyperplasias. Fifty-two of these tumors were obtained from 41 patients positive for HB surface antigen and/or HCV antibody.

High-molecular-weight DNA was prepared from each tumor and corresponding non-tumorous tissue using SDS-pronase K and phenol-chloroform.

Southern hybridization Southern blot analysis of DNA from tumors was performed as described previously.[17] Pairs of probes and enzymes were D1S7(1p33-p35)/*Hin*fI,[18] MT2P1(4p11-q21)/*Eco*RI,[19] D5S81(5q21-q22)/*Msp*I,[20] D5S43(5q34-qter)/*Hin*fI,[21] MSR(8p22)/*Msp*I,[10] D13S1(13q13)/*Msp*I,[22] D16S7(16q24)/*Taq*I,[23] D17S30(17p13.3)/*Bam*HI,[24] hp53B(17p13.1)/*Bgl*II[25] and IGLC(q11.1-11.2)/*Eco*RI.[26]

PCR-SSCP analysis Exons 5, 6, 7, 8, 9 and 10 of the p53 gene were amplified separately by PCR, and mutation was analyzed by the SSCP method.[27] DNA samples (0.1 μg each) were amplified in the presence of [α-^{32}P]-dCTP using appropriate pairs of primers as described.[28] The reaction mixture was diluted with formamide-dye solution containing EDTA, then heated for 5 min at 80°C, and an aliquot was applied to 5% polyacrylamide gel containing 5% glycerol. Electrophoresis was performed for 3–4 h at 40 W with fan cooling. The gel was exposed to X-ray film at −80°C. Primers used for PCR were the same as those described[28] except for the primers for exons 9 and 10. Exon 9, sense: 5′TGATGAGAATTCGCCTCTTTCCTAGCACTG3′ and antisense: 5′TGATGAGAATTCCCAAGACTTAGTACCTGA3′. Exon 10, sense: 5′TGATGAGAATTCCTCTGTTGCTGCAGATCC3′ and antisense: 5′TGATGAGAATTCGCTGAGGTCACTCACCT3′.

Direct sequencing An aberrant single-strand DNA band was eluted from PCR-SSCP gel as described,[29] and amplified by asymmetrical PCR[30] (100:1 primer ratio) under the same conditions as used for the PCR-SSCP analysis. The amplified DNA was purified from the reaction mixture by using a QIAGEN Spin-20 column (QIAGEN, Inc., Chatsworth, CA) and subjected to dideoxy chain-termination reaction using Sequenase Version 2.0 (United States Biochemical Co., Cleveland, OH). The primers used for sequencing were the same as those in PCR-SSCP, and were prelabeled with [γ-^{32}P]ATP. The reaction mixture was applied to 6% polyacrylamide gel containing 7 *M* urea, which was electrophoresed, and then exposed to X-ray film.

RESULTS

LOH and histopathological grade in HCC LOH was analyzed on chromosomes 1p, 4q, 5q, 8p, 13q, 16q, 17p and 22q in HCC with distinct histopathological grades, well, moderately or poorly differentiated carcinoma. The RFLP probes used were those used previously to detect

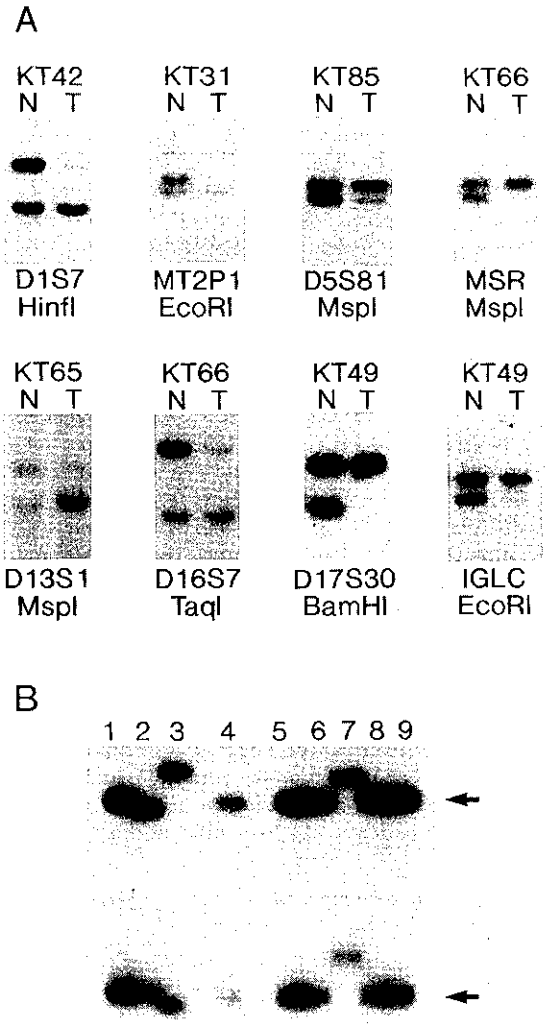

Fig. 1. A, LOH analysis of HCCs using RFLP probes on chromosome 1p(D1S7), 4q(MT2P1), 5q(D5S81), 8p(MSR), 13q(D13S1), 16q(D16S7), 17q(D17S30) and 22q(IGLC). N, DNA from normal tissue; T, DNA from HCC. B, PCR-SSCP analysis of HCCs for exon 7 in p53 gene. Arrows indicate the normal bands. Mutant bands are seen in lanes 2, 3 and 7.

frequent LOH in HCC or colorectal tumor.[5–11, 17] Examples of LOH patterns are demonstrated in Fig. 1A. Frequencies of LOH with all 63 HCCs were 30% (15/50) at D1S7 on 1p, 43% (9/21) at MT2P1 on 4q, 31% (14/45) at D5S81 and D5S43 on 5q, 42% (14/33) at MSR on 8p, 20% (5/25) at D13S1 on 13q, 28% (14/50) at D16S7 on 16q, 57% (32/56) at D17S30 and the p53 locus on 17p, and 32% (9/28) at IGLC on 22q. The LOH in every chromosome was higher in the more advanced grades of HCC, as shown in Fig. 2. In well

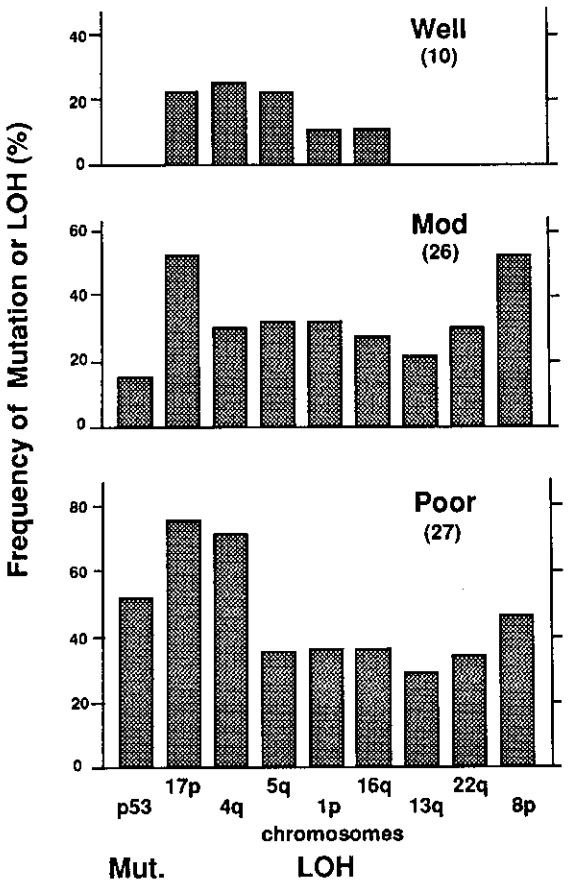

Fig. 2. Frequency of loss of heterozygosity, p53 mutation and histopathological grade in hepatocellular carcinomas. The frequency of LOH on an individual chromosome was defined as the number of tumors that showed allele loss divided by the number of informative tumors. The frequency of p53 mutation was defined as the number of tumors with mutation divided by the number of tumors analyzed. Well, well differentiated HCC; Mod, moderately differentiated HCC; Poor, poorly differentiated HCC. The number of tumors analyzed is shown in parentheses.

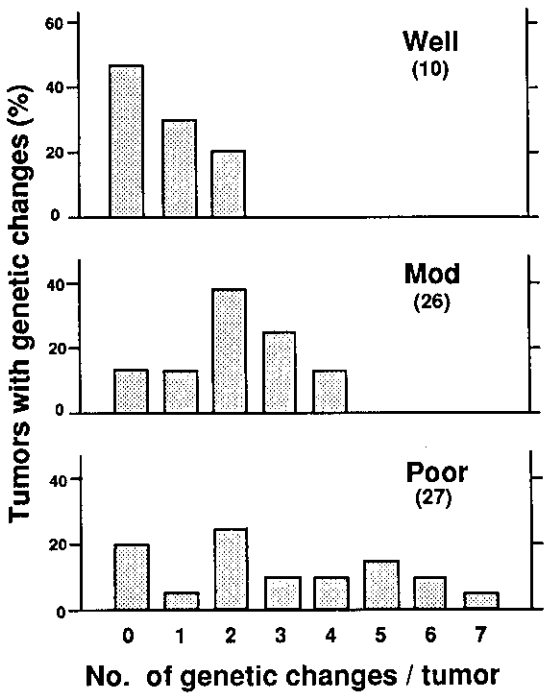

Fig. 3. Cumulative number of genetic changes and histopathological grade of hepatocellular carcinoma. Genetic changes include LOH on chromosome 1p, 4q, 5q, 8p, 13q, 16q, 17p and 22q, and p53 mutation. Well, well differentiated HCC; Mod, moderately differentiated HCC; Poor, poorly differentiated HCC. The number of tumors analyzed is shown in parentheses.

differentiated carcinomas, LOH was observed on chromosomes 4q, 5q and 17p at a frequency of 22–25% but LOH on other chromosomes was less than 11%. In moderately differentiated carcinoma, LOH on 17p (52%) and 8p (53%) was the highest, and LOH on every chromosome (21–32%) was more frequent than that in the well differentiated HCC. In poorly differentiated carcinomas, high LOH was observed on chromosomes 17p (75%), 8p (46%) and 4q (71%), and LOH on other chromosomes was 29–37%. In addition, genetic change was hardly detected in 2 atypical adenomatous hyperplasias except for 5q LOH in one tumor (data not shown). LOH on chromosome 18q, which has been detected frequently in advanced colorectal carcinomas,[17] was not detected even in poorly differentiated HCC (data not shown).

A correlation was also observed between accumulation of genetic changes and histopathological grade. Fig. 3 indicates the cumulative number of genetic changes per tumor. In well differentiated carcinoma, tumors having multiple genetic changes numbered only 2 out of 10 tumors (average number of genetic changes was 0.7/tumor). In moderately differentiated carcinoma 75% of tumors had multiple changes (average: 2.1/tumor), and 75% of poorly differentiated carcinomas had multiple changes (average: 3.0/tumor).

Mutation of the p53 gene and histopathological grade in HCC p53 gene mutation was analyzed in exons 5 to 10 by PCR-SSCP (Fig. 1B) and the direct sequencing method. Nineteen mutations were detected in 18 tumors as summarized in Table I. Mutations in the p53 gene consisted of missense (17 of 19) and nonsense (2 of 19) mutations, and one carcinoma had two mutations in one allele.

Table I. p53 Mutation and Loss of Heterozygosity on Chromosome 17p in HCCs

Tumor (HCC)	Histopathological grade	Exon	Codon	Base change	Amino acid change	17p LOH	HBV or HCV infection
KT778	Mod	5	151	CCC→TCC	Pro→Ser	loss	B+
KT49	Mod	5	171	GAG→GAC	Glu→Asp	loss	B−C+
KT740	Mod	7	239	AAC→AAA	Asn→Lys	loss	B+
KT74	Mod	7	244	GGC→GAC	Gly→Asp	loss	B+C−
KT495	Poor	5	163	TAC→CAC	Tyr→His	loss	B+
KT13	Poor	5	176	TGC→TCC	Cys→Ser	loss	B−
KT800	Poor	6	192	CAG→TAG	Gln→Stop	loss	B−
KT50	Poor	6	197	GTG→GAG	Val→Glu	loss	B+
KT12	Poor	6	204	GAG→TAG	Glu→Stop	loss	B+
KT874	Poor	7	236	TAC→GAC	Tyr→Asp	loss	B−
KT79	Poor	8	273	CGT→AGT	Arg→Ser	loss	B−C+
KT44-C1	Poor	8	273	CGT→TGT	Arg→Cys	loss	B+
KT303	Poor	8	275	TGT→TAT	Cys→Tyr	1,2	B−C+
KT66	Poor	8	281	GAC→AAC	Asp→Asn	loss	B+C−
KT69	Poor	8	282	CGG→GGG	Arg→Gly	loss	B−C+
KT589	Poor	8	285	GAG→GTG	Glu→Val	loss	B+
KT43	Poor	8	287 288	GAG→AAG AAT→TAT	Glu→Lys Asn→Tyr	loss	B−
KT44-C3	Poor	10	339	GAG→TAG	Glu→Stop	loss	B+

Mod, moderately differentiated HCC; Poor, poorly differentiated HCC.

The frequency of p53 mutation and the histopathological grade in HCC are shown in Fig. 2. p53 mutation was detected in 15% (4 of 26) of moderately differentiated HCC and the frequency was significantly increased to 52% (14 of 27) with increase in the histopathological stage to poorly differentiated carcinomas. No mutation was detected in well differentiated carcinomas, although a considerable LOH on 17p was detected in these tumors. In all 3 grades of HCC, 17p LOH was more frequent than p53 mutation.

Directions of individual base changes are summarized in Table II. No hot spot of mutation was observed. Mutations involving transition, which was frequently detected (78%) in colorectal tumors,[28] amounted to 42% (8 of 19) in HCC. The CpG transition was 5% (1 of 19) in HCC, in contrast to a high frequency (54%) in colorectal carcinomas.[28]

Genetic changes in HCC from HBV-positive and HCV-positive patients Frequencies of LOH on chromosomes 1p, 4q, 5q, 8p, 13q, 16q, 17p and 22q in HCCs from HBV- and HCV-positive patients are shown in Table III. Of 63 HCCs, 16 were from HBV-positive but HCV-negative patients, and 15 were from HCV-positive but HBV-negative patients. Other cases were excluded from Table III because although they had been exposed to one type of virus, HBV or HCV, exposure to other types of viruses was unclear. There was no significant difference in the frequency of LOH between tumors from HB+HC− patients and those from HC+HB− patients. Also, no difference in the cumulative number of genetic changes in HCCs was observed between HB+HC− and HB−HC+ patients.

Table II. Direction of Base Change Mutations in Liver and Colon Tumors

Mutation	No. of mutations (%)	
	Liver	Colon[a]
GC→AT	7 (37)	39 (72)
GC→TA	4 (21)	6 (11)
AT→GC	1 (5)	3 (6)
AT→CG	1 (5)	1 (2)
GC→CG	3 (16)	1 (2)
AT→TA	3 (16)	4 (7)
Transition	8 (42)	42 (78)
Transversion	11 (58)	12 (22)
CpG→CpA or TpG	1 (5)	29 (54)

a) Data from the previous paper.[29]

DISCUSSION

The relationship between genetic changes and histopathological grade found in the present study suggests that inactivation of tumor suppressor genes on chromosomes 1p, 4q, 5q, 8p, 13q, 16q, 17p (or p53 genes) and 22q is involved in the development and/or progression of

Table III. Genetic Changes in HCCs from Patients Infected with Hepatitis Virus B or C

A. Frequency of genetic changes

	No. of tumors[a] with LOH or p53 mutation/informative tumors (%)	
	HB+HC−	HB−HC+
LOH on 1p	7/15 (47)	3/13 (23)
4q	3/6 (50)	3/7 (43)
5q	5/14 (36)	6/15 (40)
8p	3/9 (33)	3/7 (43)
13q	2/7 (29)	1/4 (25)
16q	6/16 (38)	3/14 (21)
17p	8/13 (62)	6/14 (43)
22q	1/9 (11)	5/9 (56)[b]
Mut. in p53 gene	2/16 (13)	3/15 (20)

B. Number of genetic changes in individual tumor

Histopathological grade	No. of genetic changes[c]/tumor	
	HB+HC−	HB−HC+
Well differentiated	0.5	0.8
Moderately differentiated	2.1	2.7
Poorly differentiated	3.4	3.3

a) Tumors included well, moderately and poorly differentiated HCCs.
b) Samples included 2 well, 6 moderately and 1 poorly differentiated HCCs from HB+HC− patients; and 2 well, 3 moderately and 4 poorly differentiated HCCs from HB−HC+ patients.
c) Numbers were mean values for 2 well, 9 moderately and 5 poorly differentiated HCCs from HB+HC− patients; and 5 well, 6 moderately and 4 poorly differentiated HCCs from HB−HC+ patients.

liver cancer. It is also shown that accumulation of genetic changes is associated with progression of HCC, and the Edmondson's classification is consistent with the accumulation of genetic changes.

The most remarkable change was LOH on chromosome 17p, which was detected from the stage of well differentiated HCC, and increased to 75% in the most advanced poorly differentiated HCC (Fig. 2). Chromosome 17p includes the p53 gene, which is frequently inactivated in various types of malignant human tumors by mutation and LOH. Both p53 mutation and 17p LOH are involved in the conversion of benign to malignant tumors in colorectal carcinogenesis.[17, 28] However, in liver carcinogenesis, p53 mutation does not seem to contribute to carcinoma formation, since no mutation was detected in well differentiated carcinomas which were already malignant. The appearance of p53 mutations at a later stage suggests that p53 mutation contributes to the progression of HCC, as previously proposed.[13, 14]

With respect to the direction of p53 mutation, G to T transversion at codon 249 has been frequently detected in HCC from patients in China and southern Africa,[11, 12] but no mutation was detected at this codon in the present study (Table I). On the whole, transversion mutation was rather high in the present samples of HCC (58%) compared to that of colorectal tumors (22%) analyzed previously by the same method.[28] High transversion was reported in mutation induced by aflatoxin B1 in bacteria,[31] and in animal hepatomas induced by carcinogens including aflatoxin B1 and aromatic hydrocarbons.[32] Transition at CpG, which frequently occurred in colorectal tumors (54%, 29 of 54), was infrequent in HCC (5%, 1 of 9). Similar results were obtained in other cases.[14] The results indicate that carcinogens causing p53 mutation of HCC in Japan are likely different from those in China and Africa, and that liver carcinogens are different from the causes of colorectal tumors. Recently, deletions or insertions have been observed in about 10% of 740 p53 mutations from a wide variety of human cancers.[33] Since these changes were not observed within exons 5 to 10 in our samples, genetic changes in other regions should be analyzed further. A rather low p53 mutation as compared to 17p LOH suggests that p53 mutation may possibly be present in regions other than exons 5 to 10 in low-grade HCC, or there is one more suppressor gene on chromosome 17p which contributes to liver carcinogenesis.

The second most frequent LOH was observed on chromosomes 4q, 5q and 8p. LOH on 5q was also detected in atypical hyperplasia. Chromosome 5q includes tha APC gene, which is frequently inactivated by LOH and mutation in colorectal carcinomas and adenomas with severe dysplasia. It should be examined whether or not the APC gene is mutated in HCC. Since LOH on 8p, which was not detected in well differentiated HCCs, was detected at a high frequency (53%) in moderately differentiated HCCs, a significant contribution of LOH on 8p to progression from well to moderately differentiated stage was suggested.

LOH on chromosomes 16q and 13q, the latter of which includes the RB gene, seems to contribute to the late progression of HCC, as previously described.[6, 34] These changes were obvious in more malignant (moderately and poorly differentiated) HCCs in our analysis. Furthermore, the present data show that the LOH on chromosome 1p and 22q is also involved in the late stage of progression. This region of 1p has been frequently lost in neuroblastomas, colorectal carcinomas and pheochromocytomas from patients with multiple endocrine neoplasia type 2, suggesting that the same tumor suppressor gene is involved in the progression of different types of carcinomas. *ras* gene mutation was detected in various human tumors,[35] but mutation at the K-*ras* gene at codon 12/13

was detected in only 1 of 63 HCCs, and no mutation was observed in N-*ras* codon 61 (data not shown). This and the previous reports[36] indicate minor importance of *ras* mutation in HCC formation.

The relation of genetic changes to infection with hepatitis viruses is still unclear, although a close correlation has been found epidemiologically between incidence of HCC and infection with HBV or HCV.[37,38] Integration of HBV DNA, accompanied by translocation and deletion of chromosomal DNA, has been observed in many HCCs from HBV-exposed patients,[39,40] though integration of HCV DNA has not been detected. The frequency of LOH in HCCs from HBV-infected patients was comparable to that in HCCs from HCV-infected patients in the present investigation (Table III). This may suggest that a similar mechanism, such as regeneration of liver cells through chronic hepatitis after virus infection, affects the incidence of genetic change in hepatic cells and/or HCCs in both HBV- and HCV-infected patients. It is not excluded that different mechanisms may underlie the initial step of carcinogenesis in HBV-infected patients and HCV-infected patients.

The present investigation, in conjunction with several previous reports, has revealed that inactivation of at least 8 tumor suppressor genes is involved in the development and/or progression of liver cancer. However, it is still unclear what gene(s) are involved in the early stage of liver tumor development. Genetic changes in atypical adenomatous hyperplasia and other benign liver tumors still need to be analyzed.

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