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ヒト肝細胞癌の癌抑制遺伝子異常に関する研究

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ACCUMULATION OF ALLELIC LOSS ON ARMS OF CHROMOSOMES 13q, 16q AND 17p IN THE ADVANCED STAGES OF HUMAN HEPATOCELLULAR CARCINOMA

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We examined loss of heterozygosity at 13 loci on 5 chromosomes in hepatocellular carcinomas (HCCs) from 56 patients. In 42 of these cases, regenerative nodules of liver cirrhosis were also analyzed. High frequencies of allelic losses were detected on chromosomes 13q (47%), 16q (40%) and 17p (64%), whereas losses on chromosome 4p and 11p were observed in less than 22% of cases in HCCs. In contrast, LOH was not detected on any loci in cirrhotic nodules. On chromosome 13q, the common region of allelic loss was mapped to the region including the retinoblastoma (RB) locus, by using 8 polymorphic probes. Furthermore, one case with 13q loss had an interstitial deletion of the RB gene, indicating the involvement of inactivation of the RB gene in hepatotumorigenesis. Losses were associated with portal-vein thrombosis or intrahepatic metastasis, increased tumor size, a poorly differentiated phenotype and clinical stage. Losses occurring together on 13q, 16q and 17p were significantly higher in patients in clinical stage IV or histologically poorly differentiated tumors, suggesting that the accumulation of allelic loss occurs in advanced tumors and that patients with multiple allelic losses may have a worse prognosis than those with a single loss.

Hepatocellular carcinoma (HCC) is one of the most common types of tumor found, especially in Asia and Africa. Infection with hepatitis B or C virus, chronic liver inflammation and hepatic regeneration seem to be important risk factors for HCC (Beasley *et al.,* 1981; Sakamoto *et al.,* 1988; Saito *et al.,* 1990). However, little is known about their role in HCC tumorigenesis.

Loss-of-function mutations of tumor-suppressor genes may be very important in the carcinogenesis of a variety of human cancers (Lee *et al.,* 1988; Vogelstein *et al.,* 1988; Fong *et al.,* 1989; Windle *et al.,* 1990). The mutation of a tumor-suppressor gene and subsequent loss of the corresponding normal allele have been observed as loss of specific chromosomes or chromosomal regions in tumor cells. An excellent model of the genetic alterations occurring in the course of the development and progression of colorectal tumors has been proposed (Fearon and Vogelstein, 1990), in which a preferred order in each step of tumorigenesis was suggested. In tumors, such as renal-cell carcinoma, transitional-cell carcinoma of the bladder and ovarian carcinoma, deletions of specific chromosomal regions were also correlated well with clinical and pathological parameters (Olumi *et al.,* 1990; Ogawa *et al.,* 1991; Zheng *et al.,* 1991).

In human HCC, several studies showed frequent loss of the chromosomes 1p, 4q, 5q, 10q, 11p, 13q, 16q and 17p (Wang and Rogler, 1988; Buetow *et al.,* 1989; Zhang, W. *et al.,* 1990; Fujimori *et al.,* 1991; Simon *et al.,* 1991; Walker *et al.,* 1991). Furthermore, the loss of chromosome 16 and changes in tumor-suppressor genes such as retinoblastoma (RB) and p53, were associated with tumor progression (Tsuda *et al.,* 1990; Murakami *et al.,* 1991).

To determine how allelic losses on certain chromosomes are related to the initiation and/or progression of HCC, we examined 56 human HCCs for loss of heterozygosity (LOH) on chromosomes 4p, 11p, 13q, 16q and 17p and analyzed the relationship between LOH and the clinicopathological characteristics of HCC.

Since the majority of HCCs were detected in cirrhotic liver and this condition is considered as a premalignant state, tissues from cirrhotic regenerative nodules obtained from 42 HCC patients were also analyzed to determine whether or not they had any LOH on these chromosomes.

Some reports showed LOH on chromosome 13q in human HCC. As observed in RBs, osteosarcomas and lung cancers, the target of this 13q loss in HCC may be the RB gene, since LOH in the RB locus was detected by using polymerase chain-reaction–single-strand conformation polymorphism (PCR–SSCP) and the breakpoints of mitotic recombination or chromosomal deletion within the RB locus were also observed by using restriction fragment length polymorphisms (RFLPs) on the chromosome 13 (Wang and Rogler, 1988; Walker *et al.,* 1991; Murakami *et al.,* 1991). To confirm this, we deletion-mapped chromosome 13q using 8 polymorphic probes, and also analyzed the structural alteration of the RB gene from 25 patients by Southern hybridization using the RB cDNA.

MATERIAL AND METHODS

Samples

HCC tissues and peripheral lymphocytes were obtained from 56 HCC patients with informed consent. In 42 of these cases, regenerative nodules from cirrhotic tissues were also obtained from neighboring tumor tissues resected at the time of surgery. The demographic data of the patients are summarized in Table I. The clinical stage of each patient was determined according to the classification given in the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (Liver Cancer Study Group of Japan, 1987). Operations were performed at the Kyoto University Hospital, and tumor and cirrhotic tissue samples were frozen immediately after surgical removal and stored at –80°C until DNA isolation. Histological studies were performed at the Clinical Pathology Department of the hospital and the histological grade of tumor differentiation was assigned according to the Edmondson and Steiner grading system (1954). Peripheral lymphocytes were isolated using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation from heparinized blood obtained immediately before or after the operation.

DNA isolation and Southern blot analysis

High-molecular-weight DNA was isolated from tumor tissues, cirrhotic non-cancerous tissues and peripheral lymphocytes, as previously described (Sambrook *et al.,* 1982). DNA samples were digested with appropriate restriction endonucleases and electrophoresed in 0.8 or 1.2% agarose gels. The

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主論文2

Role and Mutational Heterogeneity of the *p53* Gene in Hepatocellular Carcinoma[1]

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ABSTRACT

The mutational spectrum of the *p53* gene was analyzed in 53 hepatocellular carcinomas. Somatic mutations of the *p53* gene were detected in 17 cases (32%). Among these 17 mutations, 9 were missense mutations; the mutations in the other 8 cases were nonsense mutations, deletions, or mutations at the intron-exon junctions. These mutations were found in a wide region stretching from exon 4 to exon 10 without any single mutational hot spot. G:C to T:A transversions were predominant, suggesting the involvement of environmental mutagens in the mutagenesis of the *p53* gene in a subset of the hepatocellular carcinoma cases. Mutations of the *p53* gene occurred frequently in advanced tumors, although several tumors in the early stages also showed mutations. A deletion map of chromosome 17 was constructed by using 10 polymorphic probes and were compared with the *p53* gene mutations in each case. Loss of heterozygosity (LOH) on chromosome 17p was observed in 49% of the cases (24 of 49), and two commonly deleted regions were detected (around the *p53* locus and at 17p13.3 to the telomere). Sixteen of the 17 cases with *p53* gene mutations showed LOH around the *p53* locus, and mutations were rare in hepatocellular carcinomas without LOH. However, no mutations were detected in 8 cases with LOH on 17p, suggesting the possibility that an unidentified tumor suppressor gene(s) located on 17p may have also been involved in hepatocarcinogenesis.

INTRODUCTION

Increasing evidence has supported the fact that the *p53* gene acts as a tumor suppressor gene (1), and mutations of the *p53* gene are frequently found in a variety of cancers (2). Bressac *et al.* (3) reported an abnormal structure and expression of the *p53* gene in HCC[3] derived cell lines. In addition, a mutational hot spot at codon 249 of the *p53* gene was found in patients in China and South Africa, which was considered to be closely associated with dietary aflatoxin B_1 intake (4–6). In contrast, no such mutational hot spot has been reported thus far in Japanese HCC cases (7, 8), suggesting the involvement of different etiological factor(s). Therefore, an intensive analysis with a large number of samples is required to understand the mutational mechanism underlying the *p53* gene during hepatocarcinogenesis.

A study on colorectal carcinomas demonstrated that mutations of the *p53* gene were closely associated with LOH on 17p, and vice versa (9). We have also reported that the LOH on 17p in HCCs was strongly correlated with the aggressiveness of the tumor (10), suggesting a role for *p53* gene inactivation in the progression of HCCs.

Several recent studies, however, have shown that the LOH on chromosome 17p was not necessarily associated with mutations of the *p53* gene in tumors such as malignant gliomas, ovarian cancers, and neuroectodermal tumors (11–13). Allelotypic studies on HCCs and breast cancers have also revealed a common deleted region other than the *p53* locus, which suggests that an unidentified tumor suppressor gene might be located on 17p (14, 15). Furthermore, other tumor suppressor genes such as *NF-1*, *nm23*, and the prohibitin gene were mapped to the proximal region of chromosome 17q (16, 17, 18). Therefore, it may be interesting to compare mutations of the *p53* gene with the deletion map of chromosome 17 obtained from the same HCC samples.

In this present study, we have investigated the mutational profile of the *p53* gene in 53 Japanese HCC cases by PCR-SSCP analysis and by direct genomic sequencing over the entire coding region, and compared these mutations with several clinical parameters. We also deletion mapped chromosome 17 by using 10 RFLP probes in the same tumor samples, and analyzed the relationship between mutations of the *p53* gene and LOH.

MATERIALS AND METHODS

Samples. We used 53 HCC patients, with their informed consent, in this study. The patients underwent surgery at Kyoto University Hospital, and the tumors and surrounding noncancerous tissues were frozen immediately after surgical removal and stored at −80°C until the DNA isolation. The clinical stage of each patient was determined according to the classification scheme in the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (19). Histological studies were performed at the Clinical Pathology Department of the hospital, and histological grades were assigned according to Edmondson's grading system (20). Among the cases analyzed, 40 were males and 13 were females. Furthermore, 7 patients were positive for serum hepatitis B virus surface antigen, and 46 were negative. Eight cases were at clinical stage I, 7 were at stage II, 15 were at stage III, and 23 cases were at stage IV.

DNA Isolation and Southern Blot Analysis. High molecular weight DNA was isolated from the tumor and surrounding noncancerous tissues as previously described (10). Restriction endonuclease digestion, agarose gel electrophoresis, Southern blot hybridization, probe labeling by nick-translation, and autoradiography were also all performed as previously described (10).

RFLP Probes. The polymorphic probes used in this study are shown in Table 1. Probes pYNH37.3, pYNZ22, pMCT35.1, pHF12-1, p10.5, pA10-41, pHHH152, pCMM86, and pTHH59 were a gift from Dr. Y. Nakamura. Probe pR4-2 was a gift from Dr. C. W. Miller. Further details of these probes can be found in Human Gene Mapping 11 (21).

PCR-RFLP Analysis. In addition to *Bgl*II RFLP detected by Southern blot analysis with the pR4-2 probe, we also analyzed the *Bst*U1 (*Acc*II) polymorphism at codon 72, which can be detected by PCR-RFLP analysis (22). The fourth exon of the *p53* gene was amplified by using a pair of primers which were the same as those used in the PCR-SSCP analysis. The amplified DNA fragments were then digested with 5 units of *Bst*U1 for 4 h and electrophoresed on 2% agarose gels.

PCR-SSCP and Direct Sequencing. Each sample was screened for mutations of the *p53* gene by the PCR-SSCP method, as described by Toguchida *et al.* (23). For SSCP, each pair of primers was designed to cover the entire coding region and the intron-exon junctions. One hundred ng of genomic DNA was amplified in a buffer containing 0.1 μl [α-^{32}P]dCTP (10 mCi/ml). Thirty cycles of PCR were performed, with 75 s of denaturation at 94°C, 90 s of annealing at 52 or 60°C, and 120 s of polymerization at 71°C. The PCR products were diluted 8- to 10-fold with 0.1% sodium dodecyl sulfate and 10 mM EDTA, and then mixed with the same volume of dye solution (95% formamide; 20 mM

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.