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Numerical Aberrations of Chromosome 17 and the p53 Locus in Small Hepatocellular Carcinomas

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Abstract. *To investigate numerical aberrations of chromosome 17 and the p53 locus in early stages of hepatocellular carcinoma (HCC), 12 fresh-frozen specimens of small HCCs (less than 30 mm in size) were examined by dual-color fluorescence in situ hybridization. We used a chromosome 17 alpha-satellite DNA probe and a p53 locus-specific DNA probe. We also performed immunohistochemical analysis for p53 protein in the same cases. Gain of chromosome 17 was the most frequently observed anomaly, present in 58% of cases, and deletion of the p53 locus was observed in 50% of cases. The combination of chromosome 17 gain and p53 locus deletion was observed in 33.3% of cases. However, overexpression of p53 protein was not observed in any specimens. Our results suggest that gain of chromosome 17 and deletion of the p53 locus could represent early genetic events, prior to overexpression of p53 protein due to mutation, in early stage HCC.*

The development of accurate imaging techniques has led to earlier recognition and diagnosis of hepatocellular carcinoma (HCC) at a stage when tumors are quite small. Detection of such small tumors means that genetic abnormalities associated with early stages of tumor progression might be identified, by virtue of the presence of undifferentiated components such as "nodules in nodules".

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In early or small-size HCC, loss of heterozygosity (LOH) of chromosome 16q has frequently been observed (1-5). Recent advances in biotechnology and molecular biology, including cytogenetic analysis techniques, such as fluorescence *in situ* hybridization (FISH) (6) and comparative genomic hybridization (CGH) (7), have allowed easy detection of these abnormalities in cancer tissues. FISH analysis has revealed frequent numerical aberrations of chromosome 17 and p53 locus deletion on chromosome 17p in many solid tumors, including HCC, even in early stage carcinomas (8-12). These anomalies have been implicated in the progression of cancers and the prognosis of patients with malignancies (13,14).

To our knowledge, however, aberrations of p53 copy number detected by FISH in early stage and small HCCs have not yet been reported. We hypothesized that aberrations of chromosome 17 and the p53 locus might be related to genetic activities and tumor behavior in the early stages of HCC. In this study, we used FISH to determine numerical aberrations of chromosome 17 and the p53 locus (17p13) in samples of small HCC (measuring < 30 mm in diameter). We also analyzed overexpression of p53 protein by immunostaining to identify p53 mutation.

Materials and Methods

Materials. We examined 12 specimens of small HCC measuring less than 30 mm in diameter, which were surgically resected at the Division of Surgical Oncology, Nagasaki University Graduate School of Biomedical Sciences, Japan, between May 1991 and February 1999. Table I summarizes the clinicopathological features of the patients. They ranged in age between 44 and 74 years (mean, 60 years) and included 9 males and 3 females. Nine patients had solitary tumors and the others had multiple tumors, which were thought to represent multicentric carcinogenesis. The background liver diseases included chronic hepatitis (n=3) and liver cirrhosis (n=9), and the types of viral infection included hepatitis B virus (HBV; n=5), hepatitis C virus (HCV; n=6) and non-B, non-C

Table I. *Clinicopathological features of patients with small hepatocellular carcinoma.*

No.	Age (years)	sex	tumor diameter (mm)	HBs Ag	HCV Ab	underlying liver disease	Child-Pugh classification	histological type	Edmondson grading	AFP* (ng/ml)
1	71	F	15	–	+	LC	B	trabecular	II	27.9
2	44	M	20	+	–	LC	A	trabecular	II	1475
3	62	M	10	–	+	CH	A	trabecular	II	588
4	65	M	15	–	+	LC	A	trabecular	II	1.6
5	50	M	26	+	–	CH	A	trabecular	II	**98.8
6	57	F	20	+	–	LC	A	trabecular	II	5237
7	60	M	25	–	+	LC	A	trabecular	II	16.7
8	53	M	21	+	–	LC	A	scirrhous	I	96.2
9	74	M	18	–	+	LC	A	trabecular	II	17.6
10	61	M	20	–	+	LC	A	trabecular	II	35.9
11	63	F	26	+	–	LC	B	trabecular	I	240.6
12	60	M	29	–	–	CH	A	trabecular	II	2.9

LC: liver cirrhosis; CH: chronic hepatitis.
* serum alpha-fetoprotein (normal < 8.9 ng/ml), ** post operation

hepatitis virus (n=1). Tumor size ranged between 10 and 29 mm in maximal diameter (mean, 20.4 mm). All tissue samples were examined histopathologically. Tumors were either trabecular (n=11) or scirrhous type (n=1) and grade I (n=2) or grade II (n=10) according to Edmondson's histopathological differentiation of tumors. All cases were T1 or T2 and stage I or II (15). None of the patients had received adjuvant chemo- or radio-therapy prior to surgery for HCC.

Cell preparation. HCC tissue samples were obtained from viable areas of the largest section in the tumors. All fresh specimens were stored at -80˚C until use. After thawing to room temperature, each specimen was stamped on a glass slide. The slides were fixed with 3:1 methanol/acetic acid solution at -20˚C.

Fluorescence in situ hybridization. Dual-color fluorescence *in situ* hybridization (FISH) was performed as described previously (16). A p53 locus-specific DNA probe labeled with Spectrum-Orange (LSI p53 SpectrumOrange; Vysis Inc., Downers Grove, IL, USA) and a chromosome 17-specific alpha-satellite DNA probe labeled with Spectrum Green (CEP 17 SpectrumGreen; Vysis Inc.) were used in this study. The target DNA was denatured with 70% formamide/2 x SSC (standard saline citrate) at 73˚C for 3 min and treated with 1.5 mg/ml of proteinase K at 37˚C for 7.5 min, followed by dehydration using serial concentrations of 70, 85 and 100% ethanol solutions. The DNA probe mixtures were denatured at 70˚C for 5 min and incubated on glass slides at 37˚C for hybridization. After hybridization for 16 h, the samples were washed three times with 50% formamide at 45˚C and twice with 2 x SSC at room temperature. The nuclei were counterstained with 0.2 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) in antifade solution (Oncor Inc., Gaithersburg, MD, USA). At least 150 non-overlapping interphase nuclei were examined under a fluorescence microscope with a dual bandpass filter and a 1000x lens (Olympus, Tokyo, Japan), as shown in Figure 1. Each signal was counted with a single bandpass filter. The figure was acquired with a fluorescence microscope equipped with a cooled charge-coupled device camera. Color image processing was carried out using a digital image analysis system (FineStar CGH-FISH analysis software, Toyobo, Tokyo).

Immunohistochemistry. Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded primary tumor specimens. The sections (5 μm thick) were mounted onto glass slides. An immunoperoxidase method, using a LSAB kit (Dako, Glostrup, Denmark), was used to detect p53 and PCNA in deparaffinized tissue sections. Tissue sections were microwave-processed (10 min) in 10 mM citric acid buffer (pH 6.0). Anti-human p53 protein (clone DO7) (Dako) was used as the primary antibody. The peroxidase reaction was carried out using diaminobenzidine as the chromogen. Tissue sections were counterstained with hematoxylin. We observed at least 1000 tumor cells in the section including the largest tumor diameter. Tumors were scored positive for p53 protein when at least 10% of tumor cells displayed strong nuclear staining. We used specimens of liver metastasis lesions of colon cancer as positive controls for p53 staining.

Data processing and statistical analysis. Anomalies of chromosome 17 (gain or loss) and p53 loci were expressed by comparing the percentages of cells with gains or losses in tumors over 20 mm or under 20 mm in diameter. Control hybridization using normal peripheral blood lymphocytes was performed to confirm that the hybridization efficiency using the test and reference probes was similar. Cell populations with a gain or loss of copy number of chromosome 17, respectively, were determined when they constituted over 15% or 20% of the tumor cells, respectively. These values were selected because the mean+3SD values of gain and loss observed in normal peripheral blood lymphocytes were less than 15% or 20% of the total cells, respectively (17). On the other hand, cell populations with a gain (p53 copy number greater than that of chromosome 17) or deletion (p53 copy number less than that of chromosome 17) of p53 copy number were determined when they constituted over 15% and 30% of the total tumor cells, respectively. Similarly, these values were determined because the mean+3SD values of gain and deletion of p53 copy number observed in normal peripheral blood lymphocytes were 15% and 30%, respectively (17). Differences in the incidence of chromosome aberrations between groups were tested using the Chi-square test and Mann-Whitney's *U*-test. A p value less than 0.05 denoted the presence of a statistically significant difference.

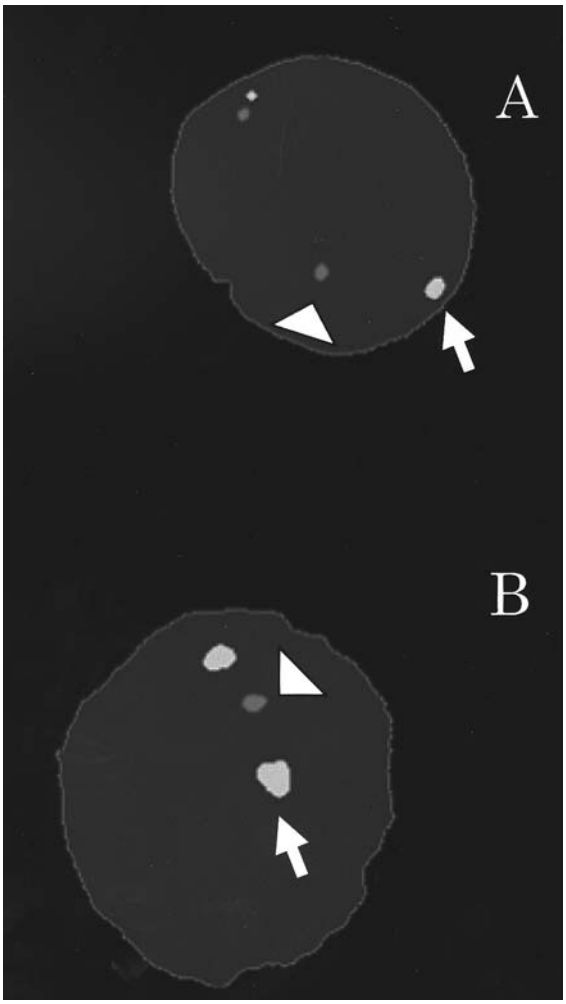

Figure 1. *Dual-color FISH of hepatocellular carcinoma cells, using a p53 gene-specific probe (arrow) and chromosome 17 centromeric probe (arrowhead); counterstained with 4',6-diamidino-2-phenylindole (DAPI) (x1000). A: Two p53 loci were observed in the cell. B: One p53 locus was lost in the cell.*

Table II. *Ratio of centromere 17 and p53 locus in small hepatocellular carcinoma was determined by dual-color fluorescence in situ hybridization.*

Case	centromere 17*	p53 locus**
1	gain	deletion
2	gain	deletion
3	gain	deletion
4	gain	deletion
5	gain	normal
6	gain	normal
7	gain	gain
8	normal	deletion
9	normal	normal
10	normal	normal
11	normal	normal
12	loss	deletion

* Gain indicates a tumor with more than 15% of tumor cells containing three or more centromere 17, and loss indicates a tumor with more than 20% of tumor cells containing one centromere 17. Normal indicates others.
** Gain indicates a tumor with more than 15% of tumor cells in which the p53 copy number was greater than the number of chromosome 17, and deletion indicates a tumor with more than 30% of tumor cells in which p53 copy number was less than the number of chromosome 17.

Table III. Percentages of cells with p53 loci or centromere 17 aberrations using fluorescence in situ hybridization. Amplification of p53 loci was significantly greater in tumors over 20 mm than in tumors under 20 mm.

	Tumor diameter (mm)		*P* value
	≤20	>20	
cells with gain of centromere 17 (%)	39.8±39.9	36.5±30.1	0.81
cells with loss of centromere 17 (%)	1.8±2.2	9.2±7.6	0.19
cells with gain of p53 loci (%)	2.7±2.3	11.2±6.5	0.03
cells with deletion of p53 loci (%)	40.0±30.1	37.9±23.5	0.94

$p<0.05$ denoted the presence of significant difference.

Results

Our findings of numerical aberrations of chromosome 17 and p53 loci are shown in Table II. The predominant aberration of chromosome 17 was a gain (7 out of 12 samples, 57.9%) and one sample showed loss of chromosome 17. On the other hand, deletion of p53 loci was observed in 6 out of 12 samples (50.0%) and a gain was observed in one (8.3%). With respect to the pattern of aberration of copy number of chromosome 17 and p53, the combination of gain/deletion was observed in 4 cases (33.3%) and disomy/deletion in one (8.3%). Amplification of p53 loci was significantly greater in tumors over 20 mm than in tumors under 20 mm ($p=0.03$) (Table III). In this study, the status of chromosome 17 and p53 did not correlate with any clinicopathological parameters.

Overexpression of p53 protein was not observed in any specimens, whereas positive control slides of colon cancer cells were strongly stained for p53 (Figure 2).

Discussion

Numerical aberrations of chromosomes or loci, which reflect chromosome instability (18), may be associated with cancer growth. A few studies have examined the gain or loss of chromosome 17 and the amplification or deletion of p53 in HCC using FISH or CGH analysis (8,9,19,20), while LOH of certain chromosomes has been fully examined and

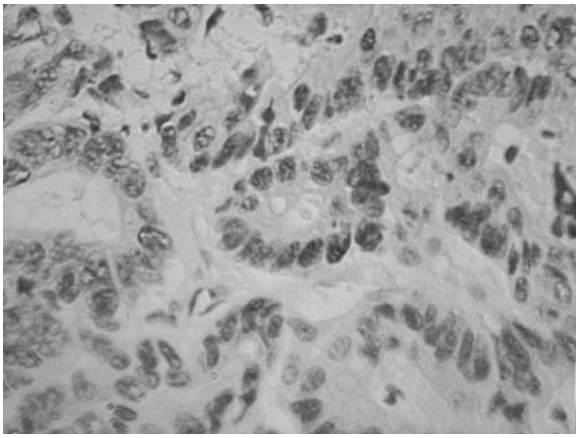

Figure 2. *Immunohistochemistry of p53 in a liver metastasis of colon cancer as a positive control. Positively-stained nuclei appear brown (x400).*

the relationship with tumor progression has been elucidated. We hypothesized that even early stage malignancies harbored a high frequency of genetic abnormalities.

Our results indicated that small-sized HCCs frequently acquire gain of chromosome 17 and deletion of the p53 locus, indicating that these aberrations are early genetic events in hepatocarcinogenesis and progression of HCC. To date, several studies have indicated that LOH is common in HCC (1,3-5,21,22). Yumoto *et al.* used restriction fragment length polymorphism (RFLP) analysis to show that LOH of 17p13.3 occurred in 33.3% of their series of HCCs with a diameter of less than 30 mm, but in 57.9% of HCCs with a diameter exceeding 30 mm. They concluded that the more undifferentiated the tumors were, the higher the frequency of LOH. They also observed that p53 mutation, as detected by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), occurred in no HCCs of less than 30 mm in diameter, but in 25.8% of those with a diameter of more than 30 mm, and that p53 mutation was more frequent in poorly-differentiated than in well- or moderately-differentiated tumors (1).

Our present results suggest a mechanism of HCC progression. In this study, p53 mutation was not observed as all cases were well-differentiated. Comparison of tumors of less than or more than 20 mm in diameter showed that amplification of p53 loci was more common in the latter, suggesting that the p53-amplified cells probably survive by a process of selection. p53 overexpression, which is caused by p53 mutation, was not observed in any specimens in the present study. Taken together, these findings lead us to speculate that the gain of chromosome 17 and deletion of p53 locus occurred initially, and that cells harboring p53 amplification gradually increased in proportion, but that p53-deleted cells were still predominant. Furthermore, p53 mutation had not occurred prior to this because overexpression of p53 was not observed.

With respect to other types of cancer, gain of chromosome 17 was frequently observed in villous adenomas and early cancers. In colon and rectum, the mean frequencies of deletion of p53 in cancers were higher than in adenomas (11). Therefore, we speculate that gain of chromosome 17 and p53 deletion might be associated with carcinogenesis or early progression of digestive cancers in general.

Precancerous lesions such as adenomatous hyperplasia or cirrhosis were not examined in the present study, because a CGH study showed no chromosome 17 aberrations in these lesions (23). These aberrations are therefore thought to occur during the transition from adenomatous hyperplasia to well-differentiated HCC.

Interestingly, gain of the p53 locus was also observed in one sample in our study. Previous studies of colorectal cancers did not identify any gain of p53 locus by FISH (10,11). Furthermore, we could find no studies that reported the gain of p53. While the role of p53 deletion or gain is not clear at present, these aberrations may stimulate or accelerate tumor growth in the early stages of HCC.

In conclusion, gain of chromosome 17 and deletion of the p53 locus were frequently observed, and gain of the p53 locus was also observed in a few cases of HCC measuring less than 30 mm in diameter. Cells with amplification of the p53 loci were more frequent in tumors with a diameter of more than 20 mm, compared with smaller tumors. These findings suggest that gain of chromosome 17 and deletion of the p53 locus represent early genetic events in hepatocarcinogenesis and that p53-amplified cells subsequently gradually increase prior to p53 mutation. We speculate that these changes are associated with carcinogenesis or early progression of HCC. Further studies of a larger number of early or small HCC samples are necessary, based on the results of the present pilot study, in order to confirm the genetic changes in early and advanced stages of HCC.

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