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TELOMERE SHORTENING IN CHRONIC LIVER DISEASES

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Summary: We measured the telomere length in patients with chronic hepatitis or liver cirrhosis and found a significant telomere shortening in the liver with chronic liver disease compared to that in the normal liver. The telomere length tended to decrease with the progression of chronic liver disease.

The structures of human chromosome ends, called telomeres, may play an important role in protecting the chromosome ends from aberrant recombination and end to end fusion (1). The length of the telomere gradually shortens during rounds of cell divisions, aging of cultured fibroblasts (2), and other somatic cells including blood cells and colon mucosal cells (3,4). Although the biological role of this reduction is not well defined, telomere shortening (3,5-7) and age related decreases in telomeric repair capacities (8) may lead to chromosome instability and genetic alterations of possible significance for tumor development.

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers in the world, and usually develops in close association with preexisting chronic liver disease (9,10), and the accumulation of genetic alterations during the stages of chronic liver disease is considered to play a major role in multistage hepatocarcinogenesis (11-13). Although alterations of telomere repeat length

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have been examined in various tumors including HCC (3, 14-17), little is known about changes in telomere length during chronic liver disease.

In this study, we measured the telomere length in the liver with chronic liver disease (chronic hepatitis, liver cirrhosis) and in the histologically normal liver by Southern blot analysis using $(TTAGGG)_4$ as a probe.

Materials and Methods

Samples: Tissue samples were obtained from 28 patients with liver disease (10 with chronic hepatitis, 12 with liver cirrhosis, 6 with hepatocellular carcinoma) either at surgery or using a Silverman needle at peritoneoscopy. Diagnoses were based upon clinical and histological findings. Five histologically normal liver tissues were also collected during surgery from patients without chronic liver disease. All samples were kept frozen at -80℃ until DNA preparation.

DNA extraction and Telomeric Repeat Analysis: High molecular weight DNA was prepared by digestion with 0.2mg/ml proteinase K and extraction with phenol/chloroform. After extraction, genomic DNA was digested overnight with 5 μg/ml of Hinf I under conditions recommended by the manufacturer (Takara Shuzou Co., Kyoto). DNA samples (5 μg each) were loaded onto a 0.5% agarose gel and electrophoresed for 15h at 60V. The separated DNA fragments were depurinated in 0.25N HCl for 15min, denatured in 0.5N NaOH/ 1.5M NaCl, and transferred to positively charged nylon membranes (Hybond-N^+: Amersham Japan, Tokyo). Hybridization and wash were performed as described previously (14) with some modifications. Breifly, the filter was hybridized to a digoxigenin (DIG)-labeled $(TTAGGG)_4$ oligonucleotide probe at 48℃ in 5 x standard saline citrate (SSC), 5 x Denhardt's solution, and 0.1% sodium dodecyl sulfate (SDS). Washing was performed at room temperature in 4 x SSC, then at 48℃ in the same solution. Next, DIG-labeled probes were detected by a DIG Luminescent Detection Kit (Boehringer Mannheim, Tokyo), and the filter was exposed to Fuji XR film (Fuji Film Co., Tokyo) at room temperature. The telomere length was assessed using a densitometer, and we determined the peak of telomere length in kilobases as the average telomere length in each patient. DNA loading and integrity were checked by prehybridizing to a $(CAC)_5$ microsatellite probe.

Statistical analysis: We used the data analysis system; DANS V4.0 for statistical analysis of telomere length.

Results and Discussion

To confirm that our data were reproducible, we repeated the experiment with restriction enzyme Rsa I and obtained similar results to those obtained with Hinf I (data not shown).

Table 1 shows the average telomere length in normal liver, liver with chronic hepatitis, and liver with cirrhosis obtained by Southern blot analysis

Table 1 Telomere length (Kb) in the livers with chronic liver disease and in normal livers

	sample	age, sex	diagnosis	telomere length (Kb)
NL	1	53 M	meta.(colon*)	9.4
	2	54 M	meta.(colon)	10.6
	3	62 M	meta.(colon)	9.2
	4	64 M	meta.(stomach)	8.7
	5	70 F	cholangioma	8.2
CH	6	33 M	CAH(C**)	10.8
	7	37 M	CAH(C)	9.3
	8	40 M	CPH(C)	10.6
	9	41 M	CPH(B)	11.4
	10	43 M	CAH(C)	8.4
	11	48 M	CAH(C)	9.3
	12	50 F	CAH(B)	6.8
	13	56 M	CAH(C)	8.8
	14	56 M	CAH(C)	7.4
	15	62 M	CAH(C)	6.2
LC	16	56 M	LC(B+C)	6.6
	17	60 M	LC(C)	6.8
	18	62 M	LC(B)	5.6
	19	62 M	LC(unknown)	6.1
	20	63 F	LC(C)	5.4
	21	63 M	LC(B)	6.2
	22	64 F	LC(C)	6.3
	23	65 M	LC(C)	5.2
	24	67 M	LC(C)	5.8
	25	68 M	LC(C)	5.1
	26	68 M	LC(C)	5.4
	27	70 M	LC(C)	5.2

NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; CAH, chronic active hepatitis; CPH, chronic persistent hepatitis; meta., metastatic liver cancer; *primary cancer; **hepatitis virus.

(Fig. 1). The average telomere length ranged from 8.2 (kilobase pairs) to 10.6 in the normal liver group, 6.2 to 11.4 in the chronic hepatitis group, and 5.1 to 6.8 in the liver cirrhosis group. We found a significant correlation between telomere length and age by regression analysis in all groups (Fig. 2). To separate the effect of age on telomere length, we used analysis of covariance and found that the group with normal livers had longer telomere than the group with chronic hepatitis (p= 0.002), and than the group with cirrhosis

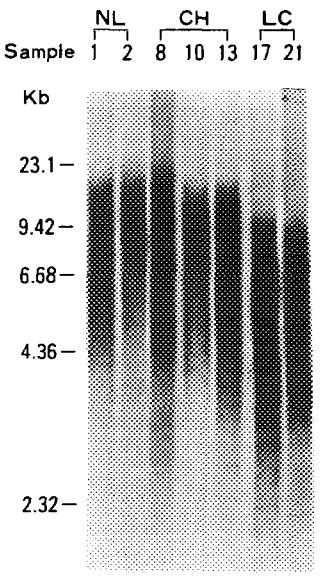

Fig. 1. Southern blot analysis of telomere repeat arrays in the normal liver and liver with chronic liver disease using a digoxigenin-labeled $(TTAGGG)_4$ as a probe. Size markers are indicated on the left (kb). NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; kb, kilobases.

($p < 0.001$). Although the difference in telomere length between the chronic hepatitis group and liver cirrhosis group was not significant, the telomere length tended to decrease with progression of liver disease. The integrity of the DNA obtained from the liver with chronic liver disease showed no difference from that obtained from the normal liver (data not shown).

Hepatocytes of normal adult liver rarely divide. However, in the liver with chronic liver disease, cell death and proliferation occur repeatedly(18). So it is possible that this repetation is the main reason for telomere reduction in chronic liver disease. We can not determine whether telomere reduction is specific to the liver with chronic liver injury, because there are few reports about changes in telomere length in other organs with chronic inflammation.

We also examined alterations in telomere length in HCC. Table 2 shows the clinical data and average telomere length in HCC. All HCC samples we examined showed a reduction in telomere length compared to that in non-cancerous surrounding liver, ranging from 3.4 to 5.4 kilobase pairs. In a

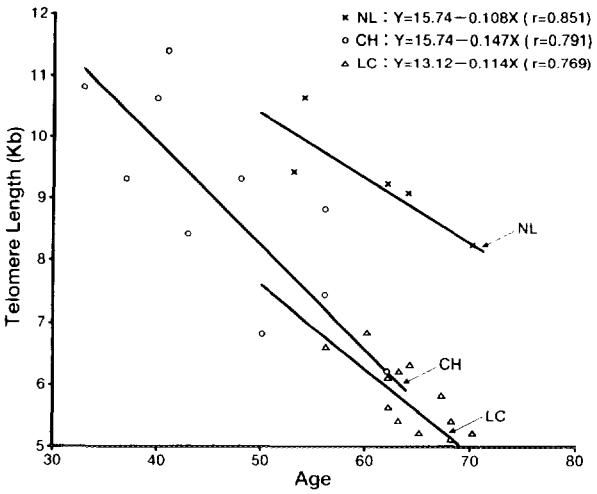

Fig. 2. Telomere shortening with age in normal liver (NL), chronic hepatitis (CH), liver cirrhosis (LC). The straight lines were obtained by regression analysis.

previous report (16), the frequency of telomere reduction in HCC was lower (28.5%) than that of our data (100%). The reason for this difference is uncertain, but it may be due to the differences in clinical stage including tumor size, age of the patient, and preexisting liver disease.

Recent studies have suggested that progressive telomere shortening and reactivation of telomerase (a ribonucleoprotein that maintains telomere

Table 2 Average telomere length in HCC and in the noncancerous surrounding liver (background)

Sample	Age, Sex	Tumor size (mm)	Grade* / Background	Telomere length (Kb)
1	60 M	30	Ed. II / LC (C)	3.4 / 6.4
2	62 M	80	Ed. III / LC (B)	4.8 / 6.1
3	62 F	40	Ed. II / CAH (C)	5.0 / 8.2
4	67 M	20	Ed. II / LC (unknown)	5.1 / 5.7
5	68 M	10	Ed. I / LC (C)	5.4 / 5.6
6	71 M	20	Ed. III / LC (C)	4.6 / 5.3

* Grade; grade of Edmondson and Steiner (21). Abbreviations are the same as in Table 1.

length) may be important in carcinogenesis and cellular immortalization (5-7, 19). Kim et al. suggested that telomerase activities may be required to maintain tumor growth in various malignancies (20). Based upon these reports and our findings, telomere shortening during the stages of chronic liver disease may possibly play a role in hepatocarcinogenesis. Further studies are needed to examine this hypothesis.

In summary, we demonstrated a significant telomere shortening during stages of chronic liver disease. And it would be of much interest to investigate whether telomere reduction can predict HCC generation.

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