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Surgical Significance of Telomerase Activity in Noncancerous Liver Tissue from Patients with Hepatocellular Carcinoma

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Telomerase activity has been detected in tissue from noncancerous liver of patients with chronic liver disease, but its functional significance remains to be elucidated. We therefore evaluated the telomerase activity in surgically obtained noncancerous liver tissue from 20 hepatocellular carcinoma (HCC) patients. Two samples of noncancerous liver tissue were obtained from each patient: one from the parenchyma adjacent to the HCC nodules of the resected specimen; the other from the parenchyma distant from the HCC nodules of the remnant liver. Telomerase activity was assayed by a non-radioisotope quantitative system based on "TRAP-eze." Five samples from the noncancerous liver tissue adjacent to the HCC nodules (25.0%) were telomerase-positive; all such cases showed high-grade malignant potential, such as intrahepatic metastasis and/or portal vascular invasion and infiltration of the fibrous capsule in the corresponding HCC nodules, and telomerase positivity showed neither a relationship with the histological activity index scores nor a correlation with liver function. Interestingly, no telomerase activity was detected in any of the 20 samples obtained from the parenchyma of the remnant liver. These results indicate that telomerase in noncancerous liver tissue is associated not with the hepatic condition accompanying HCC, but with the biological characteristics of the tumor itself, and may derive from infiltrating cancer cells. Determination of telomerase status may aid in designing more effective surgical procedures.

Key words: Telomerase activity — Hepatocellular carcinoma — TRAP — Noncancerous liver tissue

Telomere structures are necessary for maintaining chromosomal integrity. In vertebrate organisms, telomerase is the main mechanism presently known that can stabilize the loss of telomeres.[1)] Over essentially the last 2 years, screening of most types of human cancers has established a very strong association between the presence of telomerase activity and malignancy, making this enzyme one of the most common tumor markers.[2)] Since the development of the sensitive TRAP assay,[2, 3)] some normal somatic cells such as germ, hematopoietic, and cervical epithelial cells, as well as epidermal keratinocytes, have been shown to be weakly positive for telomerase activity. In some cases, liver tissue, especially from patients with viral hepatitis or cirrhosis, also expresses weak telomerase activity.[4, 5)] The reasons for this may be that, first, continuous necrosis and regeneration due to chronic persistent inflammation cause excessive division of hepatocytes beyond normal limits; in a small number of cells, telomerase might compensate for the loss of telomeres.[6–8)] Second, a small number of clinically unrecognized cancer cells may already have arised from these telomerase-positive cells in conjunction with some other events of carcinogenesis.[9)] Another explanation may be that large numbers of infiltrating lymphocytes contained in the liver tissue express telomerase activity; in viral hepatitis the inflammatory cells, such as activated lymphocytes, plasma cells and a variety of antigen-presenting cells, infiltrate the portal tracts and the acini to various degrees.[10)]

Noncancerous liver tissue in patients with HCC also expresses telomerase activity.[11–13)] In connection with this, we have previously shown that infiltrating cancer cells might be contained in the noncancerous liver tissue adjacent to the HCC nodules of the resected liver.[13)] Recent efforts to determine the optimum surgical treatment for HCC have generally yielded satisfactory results.[14)] But, the rate of recurrence after surgery still remains high, and most recurrence is seen in the remnant liver. Intrahepatic metastasis and portal vascular invasion were considered to be the most important factors leading to recurrence.[15, 16)] However, there is a possibility that some recurrent nodules after surgery are not derived from the main tumor but are instead independent occurrences due to multicentricity.[17)] Understanding the functional significance of telomerase activity in noncancerous liver tissue could be

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Abbreviations: TRAP, telomerase repeat amplification protocol; TPG, total products generated; HCC, hepatocellular carcinoma; HBs, hepatitis B virus surface antigen; HCV, hepatitis C virus; HAI, histological activity index; ICG-R15, indocyanine green retention rate at 15 min; AFP, α-fetoprotein; PCR, polymerase chain reaction; hTRT, human telomerase reverse transcriptase.

helpful in designing optimum surgical procedures to improve the long-term prognosis of patients with surgically treated HCC.

In this study, we employed a quantitative system based on the "TRAP-eze" kit to evaluate synchronously telomerase activity in HCC nodules, in the noncancerous liver tissue adjacent to the HCC nodules, and in the liver tissue distant from the HCC nodules of the remnant liver.

PATIENTS AND METHODS

Tissue samples The samples of HCC nodules and noncancerous liver tissue were synchronously obtained from twenty HCC patients by surgical resection at the First Department of Surgery, Kobe University. Two samples of noncancerous liver tissue were obtained from each patient at different sites of the liver; one from the liver parenchyma at a distance of 2 cm from the edge of HCC nodules, and the other from the lobe of the remnant liver opposite to that with HCC nodules. Three patients underwent preoperative transcatheter arterial embolization. All samples were frozen immediately and stored at −80°C until use. The HCC patients comprised 18 males and 2 females, with ages ranging from 45 to 73 years (mean; 61). Of the 20 HCC patients, 7 were positive for HBs antigen and 13 for HCV antibody. The preoperative ICG-R15 and the AFP level were evaluated. Histopathological examinations were carried out for tumor size, degree of differentiation, portal vascular invasion, intrahepatic metastasis, infiltration of fibrous capsule, and stage, according to the Liver Cancer Study Group of Japan.[18] The inflammatory state and/or fibrous progression in the noncancerous liver tissue was evaluated according to the HAI.[19] The profiles of the patients are listed in Table I. Informed consent for the experimental use of the samples was obtained from all patients.

Table I. Clinicopathologic Features in Patients with Hepatocellular Carcinoma

Case no.	Age (years)	Sex	Hepatitis virus markers[a]	Tumor size (cm)	Stage	Modified HAI[b]		AFP[c] (ng/ml)	ICG-R15[c] (%)	Telomerase activity (TPG)[d]			im[e] and/or vp	fc-inf[e]	Degree of differentiation[f]
						Grading score	Staging score			HCC nodules	Noncancerous liver tissues				
											Resected	Remnant			
1	55	M	C	4.0	III	14	5	4	12.5	78.4	28.3	negative	+	+	moderate
2	73	M	C	3.0	II	13	6	3	18.3	negative	21.4	negative	+	+	necrosis
3	45	M	B	2.2	II	11	5	12	5.9	61.6	18.1	negative	+	+	moderate
4	48	M	B	4.1	II	11	6	917	16.3	10.3	6.1	negative	+	+	moderate
5	65	F	C	6.7	II	8	3	12,558	15.9	87.7	3.5	negative	+	+	moderate
6	64	M	C	3.7	II	10	3	303	8.6	246.0	negative	negative	+	−	poor
7	51	M	B	5.1	II	16	6	49,293	11.7	157.1	negative	negative	+	+	poor
8	64	M	C	6.9	III	14	6	88	30.4	81.8	negative	negative	+	−	moderate
9	58	M	B	3.2	II	14	6	519	29.2	77.3	negative	negative	−	−	moderate
10	58	F	B	14.0	III	6	3	64,930	5.7	61.9	negative	negative	+	+	moderate
11	61	M	C	1.5	I	13	3	7	15.8	40.0	negative	negative	−	−	well
12	60	M	B	3.4	II	12	5	3	10.2	31.3	negative	negative	−	+	moderate
13	69	M	C	1.8	I	10	3	24.8	10.6	9.8	negative	negative	−	−	well
14	54	M	C	2.4	II	10	3	30	12.5	7.4	negative	negative	−	−	well
15	73	M	C	1.4	I	12	3	5	19.2	4.3	negative	negative	−	−	well
16	59	M	C	1.6	III	14	6	370	32.2	negative	negative	negative	+	+	well
17	62	M	C	3.4	III	7	3	5	23.5	negative	negative	negative	+	+	well
18	67	M	C	2.5	II	14	6	86	30.0	negative	negative	negative	+	−	well
19	58	M	C	3.1	III	13	5	8	18.3	negative	negative	negative	−	+	necrosis
20	67	M	B	2.5	II	8	3	640	8.0	negative	negative	negative	−	+	necrosis

a) B, hepatitis B virus surface antigen (HBsAg) was positive; C, antibody to hepatitis C virus was positive.
b) HAI, histological activity index by Ishak *et al.*[19]
c) AFP, α-fetoprotein; ICG-R15, indocyanine green retention rate test at 15 min.
d) TPG, total product generated (units/μg protein).
e) im, intrahepatic metastasis; vp, portal vascular invasion; fc-inf, infiltration of fibrous capsule.
f) well, well-differentiated type; moderate, moderately differentiated type; poor, poorly differentiated type; necrosis, because of preoperative transcatheter arterial embolization.

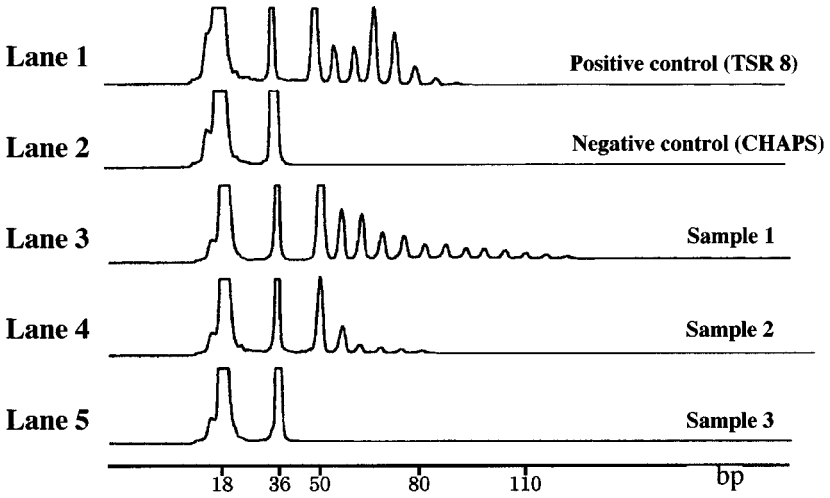

Fig. 1. Electrophoresis pattern of telomerase activity. The first peak is Cy-5-labeled TS primer. The second peak is the internal control (36 bp). The PCR product of telomerase extension yielded a six-nucleotide peak (50, 56, 62, 68, 74 bp...), from the third peak (50 bp), the first amplifiable telomerase product. Lanes 3, 4, and 5 show the telomerase activity in an HCC nodule (sample 1) (TPG; 61.6 units/μg), noncancerous liver tissue adjacent to the HCC nodule of the resected liver (sample 2) (TPG; 18.1 units/μg), and noncancerous liver tissue of the remnant liver (sample 3) (TPG; 0 unit/μg) obtained from case 3, respectively.

Telomerase assay "TRAP-eze" was used according to the manufacturer's instructions, and telomerase activity was determined as described.[20)]

Briefly, 50 mg frozen samples were homogenized in ice-cold wash buffer (10 m*M* HEPES-KOH (pH 7.5), 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* DTT), pelleted again, resuspended in 200 μl of ice-cold CHAPS Lysis buffer ("TRAP-eze") and incubated for 30 min on ice. The lysates were then centrifuged at 12,000*g* for 20 min at 4°C. The supernatants were rapidly frozen and stored at −80°C. TSR8 ("TRAP-eze") was used as the positive control. The concentration of protein was determined by using "Coomassie" Protein Assay Reagent (Pierce Chemical Co., Rockford, IL), and an aliquot of the extract containing 1 μg protein was used for each TRAP assay; the aliquots were incubated with 0.1 ng of Cy-5 labeled TS primer (5′-AATCCGTCGAGCAGAGTT-3′) in Master Mix ("TRAP-eze"). After 30 min incubation at 30°C, PCR was carried out at 94°C/30 s, 60°C/30 s and 72°C/45 s for 30 cycles. The products were diluted with an equal volume of formamide dye solution, heated at 94°C for 5 min, and applied (5 μl/lane) to 10% denaturing gel containing 6 *M* urea fitted to an automated DNA sequencer ("ALF red" DNA Sequencer: Pharmacia Biotech). During electrophoresis at 45 W, the temperature of the gel was maintained at 45°C. The data from the "ALF red" DNA Sequencer were collected and analyzed automatically by Fragment Manager V1.1 (Pharmacia Biotech). Telomerase activity was detected in a shark-tooth pattern with a periodicity of about 6 nucleotides (Fig. 1). Each peak was quantified in terms of size, peak height, and peak area. The quantification of telomerase activity was calculated by use of the following formula:

TPG units/μg protein=[(*A*/*B*)/(*A* in positive control/*B* in positive control)]×100; *A*, total area of telomerase activity (50 bp, 56 bp, 62 bp, 68 bp....); *B*, area of internal control (36 bp).

Statistical analysis All data are expressed as the mean with standard deviation, and comparisons among mean values were made by using the Mann-Whitney U or Anova test. The χ^2 test was used to compare the incidence among groups. All *P* values less than 0.05 were considered statistically significant.

RESULTS

Telomerase activity in HCC nodules TPG ranged from 0 to 246.0 units/μg protein as quantitated by telomerase assay in 20 HCC nodules. Telomerase activity was not detected in 6 HCC nodules. Three telomerase-negative cases were preoperatively treated with transcatheter arterial embolization (case 2, 19, and 20), and, histologically, the other three were well-differentiated HCC (case 16, 17, and 18). The mean values in well-, moderately, and poorly differentiated HCC cases were 8.7, 61.3, and 201.5 units/μg, respectively. There was a statistically significant difference among the three groups (Fig. 2).

Telomerase activity in noncancerous liver tissue Telomerase activity in noncancerous liver tissue adjacent to the HCC nodules was positive in 5 of 20 (25.0%) sam-

ples and ranged from 3.5 to 28.3 units/μg protein. The relationship of telomerase activity to clinicopathological features is shown in Table II. The positivity rate with both portal vascular invasion and/or intrahepatic metastasis, and infiltration of the fibrous capsule was higher than the rate without these events (P<0.05). Telomerase positivity demonstrated neither a relationship with the HAI scores nor a correlation with serum AFP level or ICG-R15 level.

Interestingly, none of the 20 samples from the parenchyma of the remnant liver showed telomerase activity (Table I).

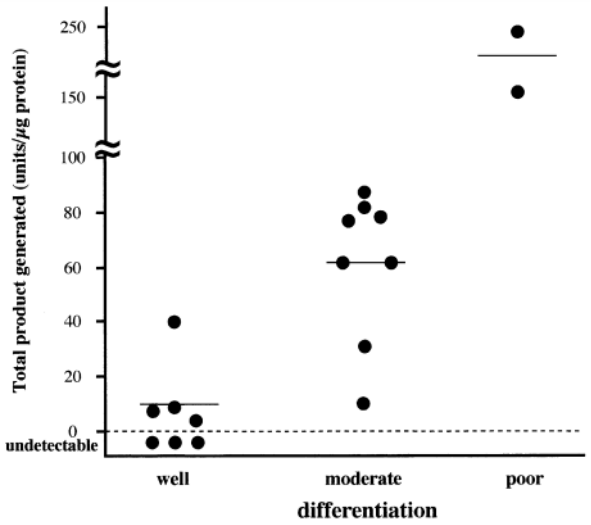

Fig. 2. Relationship of TPG to histologic grade in HCC nodules. The mean values in well-, moderately, and poorly differentiated HCC cases were 8.7, 61.3, and 201.5 units/μg, respectively. There are statistically significant differences among the three groups (P<0.01).

Table II. Comparison of Telomerase Activation in Resected Noncancerous Liver Tissue with Clinicopathological Features

Variables[a)]	Telomerase activity		Statistics
	Positive	Negative	
Age	57±12	62±6	NS
Virus marker (B/C)	2/3	5/10	NS
Tumor size	57±12	62±6	NS
AFP	2,699±5,526	7,754±20,257	NS
ICG-R15	13.8±4.9	17.7±9.2	NS
Stage (I/II/III)	0/4/1	3/7/3	NS
TPG in HCC nodule	59.5±34.5	55.9±73.6	NS
Histological activity index			
grading	11.0±1.4	11.3±3.1	NS
staging	4.8±1.3	4.0±1.6	NS
Differentiation (well/moderate/poor)[b)]	0/4/0	7/4/2	NS
im and/or vp (positive/negative)	5/0	7/8	P<0.05
fc-inf (positive/negative)	5/0	7/8	P<0.05

a) See Table I.
b) Cases with preoperative transcatheter arterial embolization were excluded.

DISCUSSION

Evidence suggests that detection of telomerase activity could be an important marker for the diagnosis of HCC.[4, 5, 11, 13)] It is becoming clear that the intensity of telomerase activity in HCC nodules is closely associated with tumor differentiation, as shown by quantitative assay in this and another study.[12)] Moreover, telomerase activity has been detected in tissue from noncancerous liver in patients with chronic liver disease, whether or not such tissue is taken from tumor-bearing livers.[4, 5, 11, 12)] We have demonstrated telomerase activity in noncancerous liver tissue adjacent to HCC nodules (7/19; 36.8%) and in samples from liver with chronic hepatitis (1/6; 16.7%), but not in samples from normal liver without hepatitis viral infection.[13)] Presently, there appear to be three possibilities for telomerase activation in noncancerous liver tissue: hidden HCC cells, immortal precancerous cells, and lymphocytes migrating into liver tissue. No conclusion has, however, been reached because first, no standard methods for determining telomerase expression have been established. Second, there have been few reports on telomerase activity in HCC nodules compared with that in the corresponding nontumorous liver.[5, 13)] Also, most of the studies do not mention whether the samples of noncancerous liver tissue were obtained from resected liver parenchyma involving HCC nodules and, if so, how distant they were therefrom, or whether from the remnant liver parenchyma. In this study, consequently, we synchronously evaluated telomerase activity in noncancerous liver tissue samples from two different regions of the liver parenchyma by using a quantitative system based on the "TRAP-eze" kit, which has been proved to be highly reproducible and sensitive.[20)]

In this study, telomerase activity was positive in 25.0% of noncancerous liver tissue at a distance of 2 cm from the edge of HCC nodules, which distance was defined as the surgical-margin free at operation by the Liver Cancer Study Group of Japan.[18)] In contrast, none of the samples obtained from the remnant liver tissue showed telomerase expression. If the telomerase activity in noncancerous liver tissue derives from liver cells or migrating lymphocytes, as mentioned above, it may be detected not only in the liver tissue adjacent to the HCC nodules, but also in the liver tissue distant from the HCC nodules, on the assumption that the liver parenchyma is pathophysiologi-

cally uniform. Moreover, telomerase expression in noncancerous liver tissue adjacent to the HCC nodules was associated not with the hepatic condition accompanying HCC, but with the biological characteristics of the tumor itself, such as intrahepatic metastasis and/or portal vascular invasion and infiltration of the fibrous capsule. At present, two modes of recurrence after liver resection are known; intrahepatic recurrence, and multicentric carcinogenesis.[17] It has been considered that the intrahepatic recurrence occurs via the portal system from the primary tumor to the neighboring liver tissue at a relatively early stage, and that intrahepatic metastasis, portal vascular invasion, and infiltration of the fibrous capsule are predictors for such postoperative intrahepatic recurrence of HCC.[15, 16] It is interesting that telomerase activity in HCC nodules was negative (probably because of preoperative transcatheter embolization), as it was in the remnant liver tissue, whereas it was positive in the noncancerous liver tissue adjacent to the HCC nodules (case No.2). This might suggest that intrahepatic metastasis spreading to the neighboring liver tissue was present prior to hepatectomy. Taken together, our results in this study may suggest that telomerase activity from phenotypically non-malignant tissue adjacent to the tumor may derive from previously undetected infiltrating cancer cells. Because faint telomerase activity was detected in one liver sample with chronic liver disease in our previous report,[13] other possibilities such as immortal precancerous cells, and lymphocytes migrating into liver tissue, may not be entirely ruled out. Further detailed studies in a larger number of patients are necessary to clarify this possibility. Studies on hTRT, which has recently been identified as a putative human telomerase catalytic subunit,[21, 22] may also help to clarify the significance of telomerase expression in noncancerous liver tissue. We may at least suggest that the functional significance of telomerase activation in the liver tissue adjacent to HCC nodules differs from that in the liver tissue distant from HCC nodules.

The most important problem for surgical treatment of HCC is the extremely high frequency of recurrence. When the HCC-bearing liver is damaged by chronic hepatitis and/or cirrhosis, surgeons often hesitate to carry out extended hepatectomy with a large safety margin that would obviate postoperative hepatic failure. Such limited hepatectomy may be associated with a high incidence of postoperative recurrence, if micrometastasis has already spread with some carcinomatous cells left in the remnant liver. Assay of telomerase can provide new information not only for determining more effective surgical procedures, but also for choosing follow-up treatment for postoperative HCC patients.

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