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PDF issue: 2025-09-07

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(Citation)
The Kobe journal of the medical sciences,42(3):207-217

(Issue Date)
1996-06

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/E0000961

Kobe J. Med. Sci. 42, 207/217, June 1996

CLINICAL SIGNIFICANCE OF TELOMERASE ACTIVITY IN HEPATOCELLULAR CARCINOMA

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INDEXING WORDS

hepatocellular carcinoma; telomerase; TRAP assay

SYNOPSIS

Telomerase is a ribonucleoprotein enzyme that elongates telomeric DNA. It has been reported that most immortal cell lines express telomerase, whereas in adult normal tissues telomerase activity is not detected. So, in malignant tumors telomerase is thought to be activated to maintain their immortality. In this study, we examined telomerase activity in 12 cases of hepatocellular carcinoma (HCC) with the use of PCR-based assay and analyzed the relationship of telomerase activity to clinicopathological features. In 11 of 12 HCC nodules telomerase activity was detected, of which 9 cases showed strong activity. There was no significant correlation between telomerase activity and clinicopathological features of HCC. Telomerase activity among

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This article is the dissertation submitted by Kyosuke Ohta, Kobe University School of Medicine for the requirement of Doctor of Medical Sciences.

Received for publication : June 21, 1996

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6 well-differentiated HCCs was strong in 3 (50%), weak in 2 (33%), and undetected in 1 (17%). All of 6 moderately-differentiated HCCs (100%), however, showed strong activity. As regards tumor size, 4 of 5 HCCs (80%) less than 3 cm in diameter showed strong telomerase activity. On the other hand, weak telomerase activity was detected in only 2 of 12 (17%) noncancerous liver tissues surrounding HCC nodules. The assay of telomerase activity may be a useful diagnostic marker of HCC regardless of tumor size, and the activity may be expressed even at early stage.

INTRODUCTION

Normal somatic cells have limited life-span.[9] Telomeres are located at the end of eukaryotic chromosomes and consist of repeated sequences $(TTAGGG)_n$. The length of telomere decreases with cell division and represents the replicative history of somatic cells.[1,7,14] Normal somatic cells stop dividing when the telomeres are shortened to a critical length. In most immortalized cells derived from human malignant tumors, however, the telomere length is stabilized by the expression of telomerase, the ribonucleoprotein that can add TTAGGG nucleotide repeats onto the ends of telomeric DNA.[2,12,16] Telomerase can compensate for telomere losses caused by cell division and prolong the cellular life-span beyond the limit. Normal somatic cells, with the exception of germline cells, have strictly repressed telomerase activity. Therefore, it has been proposed that activation of telomerase is necessary for a cell to become immortal.[8] Telomerase activity in human tumor tissue was first demonstrated in ovarian carcinoma.[4] Several reports have shown that telomerase activity was detected in various human cancer cells.[12,19]

In this study, we examined telomerase activity in 12 HCCs and noncancerous liver tissues and investigated the relationship of telomerase activity to clinicopathological features.

MATERIALS AND METHODS

Tissues

All samples were obtained by surgical resection at Kobe University Hospital, Kobe National Hospital and Himeji Heart and Brain Center from 12 HCC nodules and noncancerous liver tissues surrounding HCC nodules at a distance of at least 2 cm from the tumor. The samples were frozen immediately after resection and stored at -80°C until use.

Telomerase assay

Telomerase activity was assayed by the TRAP method.[12] Frozen materials from HCC nodules and noncancerous liver tissues were finely ground into powders in liquid nitrogen, and each 10mg of the samples was homogenized with 500 μl of cold lysis buffer. After 30 min on ice, the lysate was centrifuged at 16,000 × *g* for 30 min at 4°C. The supernatant was poured into a microtube and quickly frozen in liquid nitrogen and stored at -80°C. The protein concentration of the extract was measured with the Coomassie Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). For each TRAP assay an aliquot of the extract containing 6 μg of protein was incubated with 50 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 μM dNTPs, 0.5 mM T4-gene 32 protein, 0.1 μg of TS oligonucleotide (5'-AATCCGTCGAGCAGAGTT-3') and 1 μg of T4-gene 32 protein (Boehringer-Mannheim) at 23°C for 30 min. The mixture was then heated at 90°C for 90 s to inactivate the telomerase activity when 0.1 μg of CX primer [5'-$(CCCTTA)_3$CCCTAA-3'] and 2 units of Taq DNA polymerase were added, and the reaction mixture was subjected to 31 PCR cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 60s. Fluorescently labeled TS primer was then added and the mixture was subjected to an additional 5 PCR cycles under the same conditions. Gel electrophoresis, data collection, and analysis were carried out with the ALF red™ DNA sequencer and Fragment Manager (Pharmacia Biotech). In the fluorescence intensity curve, the increment of DNA

with a certain length was observed as a signal peak at the position of corresponding length, the height of a signal peak correlating with the amount of DNA. All samples exhibited two signal peaks at about 18 bp (TS primer) and 40 bp (primer-dimer formation between TS and CX primers). If a sample expressed no telomerase activity, the data showed no more than these two peaks. If the sample expressed telomerase activity, several additional peaks would appear with increments of 6 bp over 40bp, such as 46 bp, 52 bp, 58 bp, 64 bp and so on.

Histological examinations

HCC was histologically examined for tumor size, capsule formation (fc), capsule infiltration (fc-inf), portal vascular invasion (vp), intrahepatic metastasis (im) and classified as well-, moderately-, and poorly-differentiated HCC, according to the criteria of the Liver Cancer Study Group of Japan.[15] Histologic diagnosis of chronic hepatitis was done according to international criteria and graded according to the numerical scoring of Knodell.[6,13]

Statistical analysis

Statistical analysis was carried out using both the Mann-Whitney *U* test and the chi-square (χ^2) test. *P* values less than .05 were considered statistically significant.

RESULTS

Telomerase activity was detected in 11 of 12 HCC nodules. Telomerase activity was strong in some positive cases and weak in others (Fig.1). The 11 telomerase positive HCC cases were divided into two groups according to the strength of telomerase activity determined by the TRAP assay: group A, high signal peak at 46bp and several repeated peaks at longer than 70 bp were observed; group B, low signal peak at 46 bp and a few peaks at shorter than 70 bp were

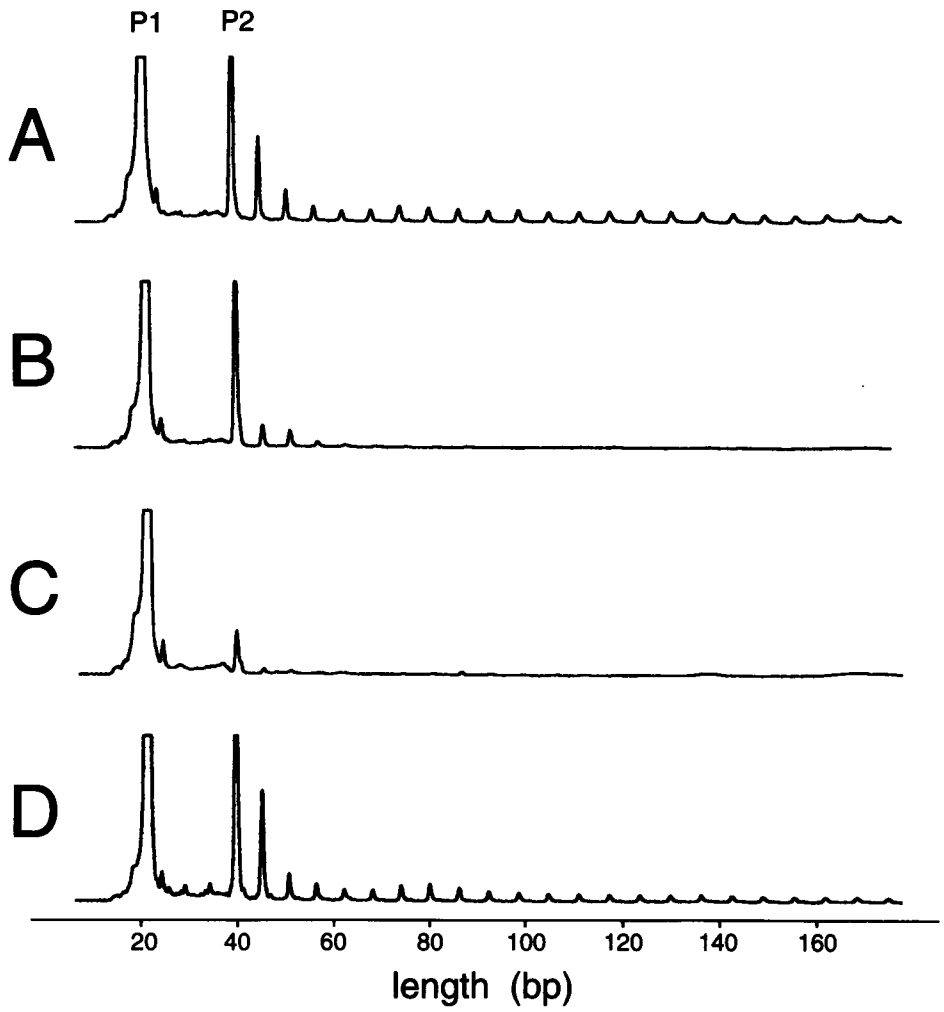

Fig. 1. Fluorescence intensity curve of TRAP assay.
(A) Strong telomerase activity. (B) Weak telomerase activity. (C) Telomerase activity was not detected. (D) HepG2 was tested as positive control. (P1) TS primer peak; (P2) primer-dimer formation between TS and CX primers.

Table I. Clinicopathological features in patients with hepatocellular carcinoma.

Case No.	Age (yr)	Sex	Hepatitis virus markers	Tumor size (cm)	Number of liver tumors	Stage	Serum AFP (ng/ml)	Associated liver disease	HAI score	Degree of differentiation	Telomerase activity	
											HCC	noncancerous liver tissue
1	55	M	C	4	2	3	4	LC	15	well	strong	weak
2	67	M	C	2.3	2	3	431	CH	10	well	strong	negative
3	64	M	C	3.8	1	2	9.1	LC	13	well	strong	negative
4	43	F	B	13	1	4A	105000	CH	6	moderate	strong	weak
5	73	F	C	1.7	1	1	1732	LC	11	moderate	strong	negative
6	59	M	C	2.4	1	2	10	LC	12	moderate	strong	negative
7	63	M	C	3.5	2	3	20.8	CH	10	moderate	strong	negative
8	62	M	B	3.9	1	2	0	CH	6	moderate	strong	negative
9	72	M	NBNC	2	3	2	14.7	CH	9	moderate	strong	negative
10	39	M	C	7	1	2	6.7	CH	10	well	weak	negative
11	47	M	B	13.5	>3	4A	5	CH	4	well	weak	negative
12	67	M	C	1.9	1	1	7	LC	11	well	negative	negative

AFP, alfa-fetoprotein; CH, chronic hepatitis; LC, liver cirrhosis; HAI, histological activity index.

Table II. Telomerase activity and characteristics of HCC.

	Telomerase activity[a]			Total positive rate ++,+ / total (%)
	group A ++	group B +	negative -	
Differentiation of tumor				
well-differentiated	3	2	1	5/6 (83)
moderately-differentiated	6	0	0	6/6 (100)
Total	9	2	1	11/12 (92)
Diameter of tumor				
≤ 3cm	4	0	1	4/5 (80)
> 3cm	5	2	0	7/7 (100)
Total	9	2	1	11/12 (92)

[a] ++, strong telomerase activity; +, weak; -, negative.

observed. Nine (75%) of 12 HCCs showed strong telomerase activity and 2 (17%) showed weak activity. Only 1 cases (8%) showed no telomerase activity (Table I). Between the two groups there were no statistically significant differences regarding age, sex, virus markers, tumor size, serum α-fetoprotein level, associated liver disease, Histological Activity Index (HAI) score, and other pathological features, such as fc, fc-inf, im, vp and vv. Telomerase activity in 6 well-differentiated HCCs was relatively weaker (3 strong, 2 weak, 1 none) than in 6 moderately-differentiated HCCs (6 strong) (Table II). There was no correlation between tumor size and telomerase activity since telomerase activity was detected in 4 of 5 HCCs (80%) less than 3 cm in diameter, and all of 7 HCCs (100%) more than 3 cm in diameter.

Weak telomerase activity was detected in only 2 of 12 samples (17%) of noncancerous liver tissue (case 1 and 4). Strong telomerase activity was detected in the HCC of both cases. Telomerase activity showed no relationship to liver disease and HAI score.

DISCUSSION

Telomerase is thought to be activated frequently in human malignant tumors, including HCC. There have been several reports demonstrating telomerase activity in clinical specimens.[5,10] In our study, telomerase activity was found in 11 (92%) of 12 HCCs. Among 11 cases, 9 cases showed strong and 2 cases showed weak telomerase activity. This difference may be explained by the hypothesis that a tumor consists of mortal cells and immortal cells at a certain rate. The larger the population ratio of immortalized cancer cells to mortal ones, the stronger would telomerase activity be. In our study, although there was no statistically significant relationship between telomerase activity and clinicopathological features, weak tendency that telomerase activity in well-differentiated HCCs was relatively weaker than in moderately-differentiated HCCs was observed. The stronger telomerase activity in a sample might mean worse malignant potential of the tumor, because the population ratio of immortalized cancer cells is very high. These results may

support the theory proposed by Kenmochi *et al.* that de novo HCC arises in the well-differentiated type and is then gradually replaced by less differentiated HCC that has a worse biologically malignant potential during progression.[11] Thus, a malignant tumor may increase telomerase activity parallel with the progression of Cancer.

It is clinically important that 4 of 5 tumors less than 3 cm in diameter showed strong telomerase activity. In Japan, high risk patient suffering from chronic viral hepatitis are carefully observed and frequently screened by serologic examination and diagnostic imaging, such as ultrasonography and computerized tomography. So, the detection of solitary liver tumors less than 2 cm in diameter has increased. These cases are often diagnosed histologically with tiny tissues obtained by echo-guided needle biopsy. Small-sized, well-differentiated hepatocellular carcinoma, however, is difficult to diagnose differentially from non-neoplastic liver tissue by histological features alone. Our data have shown that telomerase activity were detected in HCCs at high rate regardless of tumor size and clinical stage, indicating that the assay of telomerase activity in biopsy specimens may provide a useful diagnostic information.

Weak telomerase activity was detected in 2 of 12 noncancerous liver tissues. HCCs of both cases showed strong telomerase activity. Three possible reasons for this result could be proposed.

1.A small number of immortalized cancer cells might be contained in the noncancerous liver samples, although at the time of operation there were no macroscopically apparent microsatellites in the surrounding noncancerous liver tissues.

2.It is well known that most HCCs in Japan are associated with chronic viral hepatitis or cirrhosis.[17] Continuous necrosis and regeneration due to chronic persistent inflammation would cause excessive division of hepatocytes beyond the normal limit. A small number of cells whose telomeres are critically short might express telomerase activity.[2,18]

3.It has been reported that normal leukocytes in peripheral blood show telomerase activity.[3] So, a small number of leukocytes contained in the liver tissues suffering from chronic viral hepatitis might show telomerase activity. Our data showed no correlation between HAI score and telomerase activity in

noncancerous liver tissues.

Thus, although there is no precise explanation for our result in noncancerous liver tissues at present, the assay of telomerase activity in noncancerous liver tissues may be of some clinical value in further study.

In conclusion, telomerase activity was detected in 92% of HCC nodules and high positivity was also observed in small HCCs less than 3 cm in diameter. However, telomerase activity in noncancerous liver tissue was weakly positive in only 17%. We propose that the assay of telomerase activity may become a useful diagnostic marker of HCC.

ACKNOWLEDGMENTS

We thank Dr. Syuichi Okumura, Kobe National Hospital, Dr. Junichi Sugihara and Dr. Seiji Haji, Himeji Heart and Brain Center for supplying samples and clinical data.

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