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Telomerase is strongly activated in hepatocellular carcinoma but not in chronic hepatitis and cirrhosis

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Abbreviations: TRAP, telomeric repeat amplification protocol; HCC, hepatocellular carcinoma

Abstract

Telomerase is highly activated in human immortal cell lines and tumor tissues, whereas it is not activated in primary cell strains and many tumor-adjacent tissues. It is suggested that telomerase activation is one of the critical steps in malignant transformation. In the present study, the telomerase activity was investigated in hepatocellular carcinoma tissues and non-tumor liver tissues from Korean patients with chronic hepatitis and cirrhosis. Eighty two liver tissues (24 chronic hepatitis specimens, 34 cirrhosis specimens, and 24 hepatocellular carcinomas) were obtained from 23 chronic viral hepatitis patients, 19 cirrhosis patients (including 7 liver transplants), and 24 patients with hepatocellular carcinoma, of which the surrounding non-tumor liver tissues were available in 16 patients (1 chronic hepatitis and 15 cirrhosis). As negative controls, 3 normal liver tissues were included. Protein from liver specimens was purified by a detergent lysis method as described elsewhere, and telomerase activity was measured in 2 diluents of each sample (1:1 and 1:100) by a telomeric repeat amplification protocol (TRAP). Telomerase was strongly activated in 79% (19/24) of the hepatocellular carcinomas, while weakly in 8% (2/24) of the chronic hepatitis tissues and in 24% (8/34) of the cirrhosis tissues. All of 3 normal control livers showed no telomerase activation. No relationship could be observed between the enhancement of telomerase activity and tumor nature. None of the chronic hepatitis or cirrhosis patients with mild telomerase activation in the liver have developed hepatocellular carcinoma for at least 2 years of follow-up period. These results suggest that the strong enhancement of telomerase activity may be a critical part of hepatocarcinogenesis, although the exact mechanism of such high activation in hepatocellular carcinoma is not clear. In addition, further study will be necessary to clarify the reason why no telomerase activity detectable by a conventional TRAP can be seen in some hepatocellular carcinoma.

Keywords: telomerase activity, TRAP, hepatocellular carcinoma, HCC, chronic hepatitis, cirrhosis

Introduction

Telomeres, specialized structures at the ends of all eukaryotic chromosomes, which are composed of 10-15 kilobases of TTAGGG repeats, play a critical role for protecting termini of the chromosomes from the devastating attack of exonuclease and ligase (Blackburn and Gall, 1978; Moyzis *et al.*, 1988; Blackburn, 1991). In addition, it has been considered that these telomeres are the mitotic clock of normal human somatic cells, because the shortening in length of these telomeric DNAs is associated with the decrease of *in vivo* division of the cells (Harley *et al*., 1990; Lindsey *et al*., 1991; Allsopp *et al*., 1992), and, by such a mechanism, cells count their division cycles. All immortal cells have no net loss of telomeric length with cell division, suggesting that the maintenance of telomeres is required for the cells to escape from replicative senescence and to proliferate indefinitely (Counter, 1992; Counter *et al*., 1994; Counter *et al*., 1994).

It has been found that the preservation of the telomeric length is associated with the activation of telomerase, a ribonucleoprotein, which synthesizes the telomeric DNA onto chromosomal ends using a segment of its RNA component as a template (Greider and Blackburn, 1985). Reportedly, the telomerase is activated in many human tumor tissues and immortal cell lines (Greider and Blackburn, 1985; Counter *et al*., 1994; Kim *et al*., 1994). Like other malignant cells, most hepatocellular carcinomas also had the enhancement of the telomerase activity (Tahara *et al*., 1995; Nouso *et al*., 1996; Kojima *et al*., 1997). In addition, it was suggested that telomere shortening might play an important role in the associated generation of hepatocellular carcinoma (Ohashi *et al*., 1996), although there was a controversial result (Kojima *et al.*, 1997). In the present study, telomerase activity of

hepatocellular carcinoma was analyzed and compared to that of non-tumor liver tissues from Korean patients with chronic hepatitis or cirrhosis which was the precancerous diseases of hepatocellular carcinoma to investigate the role of telomerase in hepatocarcinogenesis.

Materials and Methods

Liver tissues

Liver tissue specimens were collected from 66 patients with chronic liver diseases, consisting of 23 chronic hepatitis, 19 cirrhosis (including 7 liver transplants), and 24 hepatocellular carcinoma, while 3 controls without any evidences of liver diseases, on whom operation was performed for the treatment of early gastric cancer. They were from the Department of Internal Medicine, Kangnam St. Mary's Hospital, The Catholic University, Seoul, Korea, from 1991 to 1995. Out of these patients, 85 liver tissues were obtained by blind liver biopsy, laparoscopic biopsy, ultrasono-guided biopsy, or operative biopsy. In 16 of the 24 patients with hepatocellular carcinoma, a pair of both tumor and surrounding non-tumor tissues were available, so that the liver tissues consisted of 24 chronic hepatitis specimens (1 from a patient with hepatocellular carcinoma), 34 cirrhosis specimens (15 from patients with hepatocellular carcinoma), 24 hepatocellular carcinomas. And six hepatoma cell lines originating from liver cancers, HuH7, Hep G2, 2.2.15., Hep3B, HLE and HLF, were also examined.

The 66 patients consist of 14 females and 52 males and ranged from 19 to 65 years (mean 40 years). Hepatitis B surface antigen (HBs Ag) was positive in 55 patients (83 %), antibody to hepatitis C virus (anti-HCV) was positive in 3 patients (4.5%), and both HBs Ag and anti-HCV tests were negative in 8 patients (12%). In accordance with the WHO classification for chronic hepatitis and the Edmondson-Steiner criteria for hepatocellular carcinoma (Edmondson and Steiner, 1954), liver tissues were histologically interpreted.

Serological markers

HBsAg, antibody to HBsAg (anti-HBs), Hepatitis B e antigen (HBe Ag), antibody to HBeAg (anti-HBe), and total antibody to hepatitis B core antigen (anti-HBc) were assayed using commercially available radioimmunoassay kits (Abbott Laboratories, Chicago, IL, USA). Anti-HCV was assayed with a second generation enzyme immunoassay kit (Abbott Laboratories).

Extraction of protein

By a detergent lysis method (Counter *et al*., 1994; Kim *et al*., 1994), both protein and nucleic acids from tissue specimens were obtained simultaneously. Briefly, liver tissues were homogenized with 50 μl of cold lysis buffer (10 mM Tris HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N,N-tetraacetic acid, 0.1 M phenylmethylsulfonyl fluoride, 5mM β-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl)-dimethyammonio]-1-propanesulfate, and 10% glycerol. After incubation for 25 min on ice, the lysate was centrifuged at 10,000 *g* for 30 min. The supernatant was quickly frozen and stored at -80˚C. And protein concentration was measured by a Bradford method (Sharma *et al*., 1980).

Measurement of telomerase activity

Telomerase activity in an aliquot (6 μg of protein) of the extracts isolated from each tissue sample was measured by the telomeric repeat amplification protocol (TRAP) (Kim *et al*., 1994). Briefly, assay tubes were prepared by lyophilizing 0.1 μg of CX primer sequence [5'-$(CCCTTA)_3$ CCCTAA-3'] onto the bottom of the tube and sealing it with 10 μl of molton wax (Ampliwax, Perkin Elmer, Norwalk, CT, USA). The assay mixture (50 μl) containing 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM ethyleneglycoltetraacetic acid, 50 μM deoxynucleoside triphosphate, 0.1 μg of TS primer (5'-AATCCGTCGAGCAGAGTT-3'), 1 μg of T4g32 protein (Boehringer Mannheim, Mannheim, Germany), bovine serum albumin (BSA; 0.1 mg/ml), 2 U of *Taq* DNA polymerase (Boehringer Mannheim), 0.3 μl of [α-^{32}P]-deoxycytidine triphosphate (3000 Ci/mmol; Amersham, UK) and 2 μl of extracted samples were placed in the prepared tube . After incubation at 23˚C for 30 min, the tubes subjected to 31 polymerase chain reaction (PCR) cycles at 94˚C for 30 sec, 52˚C for 30 sec, 72˚C for 45 sec. The final extension reaction was performed at 72˚C for 2 min. PCR products were analyzed by electrophoresis on 10% (19:1) polyacrylamide nondenaturating gels in 0.5X Tris-borate EDTA buffer (pH 8.0). After the gel was dried, autoradiogram (Hyperfilm™-MP, Amersham) was processed with an exposure at -80˚C for 24 h. The data were collected in a blind fashion and decoded later.

For the quantitative measurement of telomerase activity, each aliquot (6 μg of protein) from individual extracts were diluted to and 1:100 (0.06 μg of protein) by the published method (Tahara *et al*., 1995; Kojima *et al*., 1997; Yashima *et al*., 1997) and the telomerase activity was examined. Final results obtained from individual samples were compared to those from a positive control by visual observation and a densitography (ImageMaster VDS and image master software, Pharmacia Biotech Asia Pacific Ltd., Hong Kong, China), and the telomerase activity was arbitrarily defined into 3 groups; 'strong' if the multiple ladder-like amplification bands of individual sample were similar to those of positive control in densities of the sixth to seventh bands from the first band at the base line, 'weak' if they were seen only in an undiluted sample, and 'moderate' if they were between strong and

weak results. Samples which showed no bands or only one or two faint bands in the undiluted sample were defined as negative results. In all experiment, at least 3 controls were included; Huh7 cell line as a positive control, a piece of normal liver as a negative tissue control, and distilled water as a negative control to differentiate the carryover contamination during the experiments.

Statistical Analysis

Statistical analyses were performed with Student's *t* test and χ^2 test.

Results

Telomerase was strongly activated in 19 of 24 hepatocellular carcinomas (79%) and in all of 6 hepatoma cell lines. Intensities of the telomerase activity from these 19 positive samples could not be differentiated from those of hepatoma cell lines and each other. On the contrary, telomerase activity was weakly detected in 10 of 59 non-tumorous liver tissues (16.9%); 2 of 24 chronic hepatitis specimens (8%) and 8 of 35 cirrhosis specimens (24%). All of 3 negative control liver tissues had no telomerase activity. (Figure 1 and Table 1)

Regarding 5 hepatocellular carcinomas negative for TRAP, no cases had a paradoxical phenomenon which shows higher enhancement level of the telomerase activity in serially diluted sample than in undiluted sample. So, a serial dilution method was not beneficial to identify further cases with telomerase activation.

Comparing with that in chronic hepatitis without hepatocellular carcinoma (1/23, 4%), the frequency of telomerase activity in cirrhosis without hepatocellular carcinoma was significantly high (5/19, 26%) (p=0.049). However, no patients with telomerase activation have

Table 1. Results of telomerase activity measurement in liver specimens from chronic hepatitis, cirrhosis, and hepatocellular carcinomas.

	No. of cases tested	Telomerase activation No. of cases	(%)	(Weak/Strong)[b]
Chronic hepatitis	24	2	(8%)	(1/1)
without HCC	23	1	(4%)	(1/ -)
with HCC	1	1	(100%)	(- /1)
Liver cirrhosis	34	8	(24%)	(8/0)
without HCC[a]	19	5	(26%)	(5/ -)
with HCC	15	3	(20%)	(3/ -)
Hepatocellular carcinoma	24	19	(79%)	(0/19)
Normal Liver	3	0	-	(- / -)

HCC: Hepatocellular carcinoma.

[a] Liver tissues from 7 transplantation recipients were included; 2 of them were weak positive in TRAP assay.

[b] It was arbitrarily considered for the telomerase activity to be 'strong' if the multiple ladder-like amplification bands of individual sample are similar to those of positive control in densities of the sixth to seventh bands from the first band at the base line, 'weak' if they are seen only in an undiluted sample, and 'moderate' if they were between strong and weak results. Samples which showed no bands or only one or two faint bands in the undiluted sample were defined as negative results.

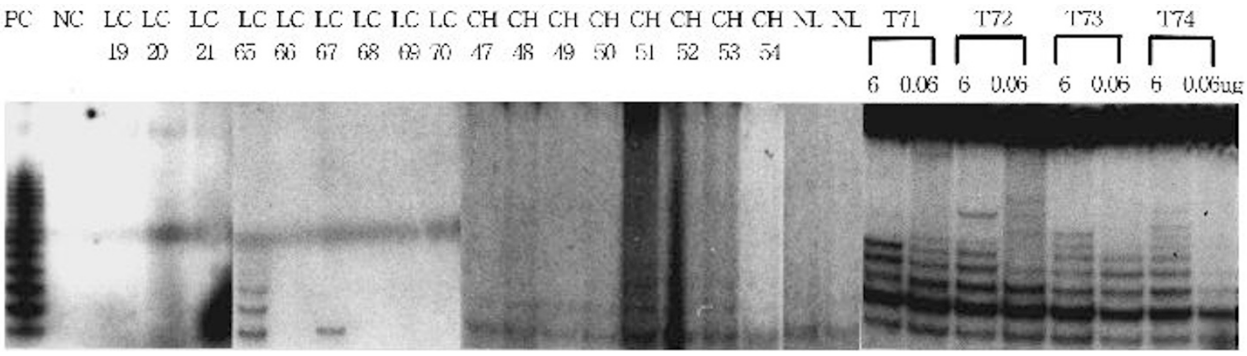

Figure 1. Representative figures of telomerase activity in 17 chronic liver disease, 4 hepatocellular carcinoma, and 2 normal liver tissues. Tumor tissues (T71-T74), which showed detectable telomerase activity by TRAP, had the multiple amplification bands as strong as those of a positive control (PC; Huh7 cell line), so that high telomerase activity could be seen in 1:100 dilution of sample. However, in non-tumor liver tissues like chronic hepatitis (CH) or cirrhosis (LC), telomerase activity was usually negative, although few cases (LC65, LC67, CH51, CH53) showed weak amplification bands. NL, normal liver (negative tissue control); NC, negative control (distilled water).

developed hepatocellular carcinoma, when evaluated at 2 years later after the detection of telomerase acitivity in the tissue specimens.

Among 16 patients with hepatocellular carcinoma, in whom a pair of both tumor and non-tumor were available, the enhancement of the telomerase activity in the surrounding non-tumor liver tissues was identified in 4 cases (25%); 1 chronic hepatitis and 3 cirrhosis. (Table 2)

There was no difference in the frequency of the telomerase activity in accordance with the differentiation grades of the hepatocellular carcinoma; well differentiated (3/4), moderately differentiated (9/10), and poorly differentiated (7/10). Also, no other natures in hepatocellular carcinoma were associated with the telomerase activity. (Table 3)

Discussion

In most immortalized cells, telomeres are stabilized at a length that depends on a balance between the loss of telomeric repeats at each cycle of DNA replication and the telomeric elongation by the enhancement of the telomerase activity. It has been suggested that telomerase is repressed in somatic tissues to reduce the probability of cancer in long-lived organism like human (Allsopp *et al*., 1992; Vaziri *et al*., 1993). A recently developed method to measure the telomerase activity, so-called TRAP, is highly sensitive and reproducible as a PCR-based assay, so that it has been possible to examine this hypothesis

Table 3. Correlation between the enhancement of telomerase activity and clinicopathologic features of hepatocellular carcinoma.

	Increased telomerase activity			
	No. of cases	Number	%	Significance
TNM stage				No
I-II	4	3	75%	
III	3	3	100%	
IV	17	13	76%	
Size (long diameter)				No
smaller than 5cm	5	3	75%	
between 5cm and 10cm	6	6	100%	
larger than 10cm	13	10	77%	
Multiplicity				No
single	7	4	57%	
multiple	17	15	88%	
Differentiation grade				No
well	4	3	75%	
moderately	10	9	90%	
poorly	10	7	70%	
Alpha-fetoprotein in serum				No
lower than 500 ng/ml	9	8	89%	
higher than 500 ng/ml	15	11	73%	
Viral markers				No
HBs-Ag-positive	18	14	78%	
Anti-HCV-positive	2	2	100%	

Table 2. Telomerase activity and tumor nature of patients with hepatocellular carcinoma who were available for both tumor (T) and non-tumor (NT) liver tissues (N=16). HBV, Hepatitis B virus; HCV, Hepatitis C virus; CAH, Chronic active hepatitis; TNM: Cancer staging system recommended by the American Joint Committee (Carr *et al*, 1983).

Case No.	Age/Sex	Hepatitis virus	Non-tumor liver	TNM stage	Differentiation Grade	Tumor number	Growth pattern	AFP (ng/ml)	Telomerase activity T	Telomerase activity NT	Survival (months)
71	35/M	HBV	Cirrhosis	IVa	well	multiple	nodular	1536	+++	-	3
72	40/M	HCV	Cirrhosis	III	moderate	single	nodular	526	+++	-	-
73	32/M	HBV	Cirrhosis	IVa	moderate	multiple	nodular	8	+++	-	18 (alive)
74	67/M	HCV	Cirrhosis	III	well	single	nodular	868	+++	±	10 (alive)
75	52/M	HBV	Cirrhosis	IVa	moderate	single	infiltrative	1539	-	-	10 (alive)
76	45/M	HBV	Cirrhosis	III	well	single	nodular	1161	+++	-	8 (alive)
77	60/F	HCV	Cirrhosis	III	poor	single	nodular	1507	+++	±	3
78	64/F	HBV	Cirrhosis	IVa	poor	multiple	nodular	53.7	+++	-	-
79	44/M	HBV	Cirrhosis	IVa	moderate	multiple	nodular	1453	+++	-	-
80	50/M	HBV	Cirrhosis	IVa	poor	multiple	nodular	4.2	+++	-	4
81	51/M	HBV	Cirrhosis	III	moderate	multiple	nodular	94	+++	-	5
82	40/M	HBV	Cirrhosis	IVa	poor	single	nodular	660	-	-	10
83	52/M	HBV	Cirrhosis	IVa	poor	single	infiltrative	5	-	-	6
84	54/M	HCV	CAH	IVa	moderate	multiple	nodular	1279	+++	+	6
85	42/M	HBV	Cirrhosis	IVa	well	multiple	infiltrative	1057	+++	±	8
86	66/M	HBV	Cirrhosis	IVa	moderate	multiple	infiltrative	1150	+++	-	9

by measuring the telomerase activity in various cells and tissues *in vitro* and *in vivo* (Kim *et al*., 1994).

It is a current opinion that the telomerase is specifically activated in malignant tumors and germ cells and hematopoietic stem cells (Counter *et al*., 1995). By a series of data reported by Hiyama *et al.*, the telomerase activity was detected by 94% of neuroblastomas (1995), 80% of lung cancers (1995), 85% of gastric cancers (1995), 93% of breast cancers (1996), and 95% of pancreatic cancers (1997). And most colorectal cancers (93%) also showed telomerase activation (Chadeneau *et al*., 1995).

In the present study, telomerase was strongly activated in 79% of the hepatocellular carcinomas, which were similar to the results reported by other groups; 84.8% (Tahara et al., 1995), 80% (Nouso *et al.*, 1996) and 85% (Kojima *et al*., 1997), respectively. On the contrary, the telomerase activity in non-tumorous liver tissues was detectable weakly only by 16.9%. Other groups also reported that some non-tumorous liver tissues without hepatocellular carcinoma might have a weak activation of telomerase; 17% (Nouso *et al.*, 1996) and 9% (Kojima *et al*., 1997). These support strongly that the high enhancement of telomerase expression is an essential part for malignant transformation during hepatocarcinogenesis like other malignant cancers and for the immortality of the transformed cells.

Among our non-cancer patients, telomerase was expressed more frequently in cirrhosis (24%) than in chronic hepatitis (4%), but such observation was not coincidental by other groups (Tahara *et al*., 1995; Nouso *et al*., 1996). Furthermore, hepatocellular carcinoma has not developed among non-cancerous patients with the weak telomerase activity by TRAP for at least 2 years follow-up. Therefore, further studies will be needed to clarify the significance of telomerase expression in such a group of patients with chronic hepatitis or cirrhosis.

It has been suggested by Nouso *et al*. (1996) and Kojima *et al*. (1997) that there is a correlation between differentiation grades in malignant phenotype and telomerase activity. However, in the present study, we could not see such relationship in hepatocellular carcinoma pathology. In addition, we could not find out any tumor natures and laboratory variables correlated with strong telomerase activation in hepatocellular carcinomas.

Some hepatocellular carcinomas had no telomerase expression, which was suggested as a reason by Hiyama *et al*. (1995) that certain tissue factor might inhibit Taq DNA polymerase in such group of the patients. So, the telomerase activity was measured in serially diluted samples as described by other groups (Tahara *et al*., 1995; Yashima *et al*., 1997), but we could not observe such paradoxical phenomena. Additionally, we could see 'all-or-none' phenomenon, "strongly positive or negative", in the results. These indicate that certain other factor(s), not identified as of yet, may influence negatively the TRAP method or telomerase activity. Another possibility might be the inactivation of telomerase during freezing and preparing tissues.

In summary, the present study support that hepatocellular carcinomas usually have strong enhancement in the telomerase activity, whatever the cancer nature is (stage, tumor size, number of tumor nodules, growth patterns, cellular types, vascular involvement, and differentiation grade). These charateristics may be helpful for the differential diagnosis between benign and malignant nodules and also for the development of specific treatment of hepatocellular carcinoma.

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