Advances in Brief

Telomerase Activity in Human Liver Tissues: Comparison between Chronic Liver Disease and Hepatocellular Carcinomas[1]

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Abstract

Telomerase activity was examined in 105 frozen samples from human normal liver tissues, chronic liver disease, and hepatocellular carcinoma (HCC). Telomerase activity was positive in 28 of 33 HCC tissues regardless of tumor stage or size. Telomerase was expressed in 15 of 18 differentiated HCC nodules smaller than 3 cm. HCC tissues from all eight hepatitis B virus-positive patients were telomerase positive, while telomerase activity was not detected in normal liver tissues (0 of 4). Weak telomerase activity was only detected in 1 of 22 nontumor liver tissues from HCC patients. Interestingly, in 19 of 38 hepatitis tissues and 6 of 8 cirrhotic liver tissues from apparently cancer-free patients, very weak telomerase activity was detected. These results indicate that the expression of telomerase may play a crucial role in hepatocarcinogenesis.

Introduction

The development of human cancer results from multiple independent genetic changes that activate proto-oncogenes or negate the action of tumor suppressor genes (1). It has also been established that malignant transformation of normal cells is accomplished through several steps, including distinct phenotypic changes regarding transformation and immortalization (2, 3). However, specific oncogenes or tumor suppressor genes, the changes of which efficiently immortalize human somatic cells *in vitro*, have not been reported, and the difficulty in establishing cell lines from tumor tissues has raised a question of whether human cancer cells *in vivo* are really immortalized. Several recent advances in understanding the mechanisms regulating cell mortality (limited division potential of somatic cells) are providing the information needed to understand the development of the immortal phenotype (unlimited cell division potential) in human cells (reviewed in Ref. 4). A number of studies have shown that telomere DNA length decreases with both *in vivo* and *in vitro* division of human somatic cells (5–7). Progressive telomere shortening has been proposed to be the major mitotic clock mechanism by which cells know how many times they have divided and how many times they will be able to divide. In cells immortalized *in vitro*, telomere length is stabilized, probably due to activation of telomerase, the enzyme which elongates telomeres *de novo* (8, 9). Although telomerase activity is assumed to be directly involved in telomere maintenance and cell immortality, the conventional telomerase assay is a very difficult procedure and has severely limited wide surveys of primary tumor tissues (10). Recently, an improved method (TRAP[3] assay) was developed by which telomerase activity was detected in

a wide variety of human tumors and tumor-derived cell lines, whereas it was absent in both normal cells *in vitro* and normal somatic tissues *in vivo* (11). We have been interested in telomerase activity in HCC because the incidence is relatively high in Japan; about 20,000 persons die of HCC every year. In addition, diagnosis is sometimes difficult and ambiguous when hepatomas are small and of the differentiated type. Since only one hepatoma example was reported in the previous report (11), we assayed telomerase activity in malignant and nonmalignant human liver tissues to determine whether malignant progression may correlate with the expression of telomerase. We report that expression of high levels of telomerase activity correlated with HCC. Unexpectedly, however, very low activity was also detected in about one-half of noncancerous chronic liver disease tissues.

Materials and Methods

Tissues. The samples were obtained by needle biopsy or surgical resection during 5 years at our institution from 4 normal livers, 7 resolving acute hepatitis, 31 chronic hepatitis, 8 liver cirrhosis, and 33 HCC nodules. These samples were frozen immediately in liquid nitrogen and stored −80°C until used. Of 33 HCC nodules, we also examined 22 apparently nontumor tissues surrounding HCC nodules. Diagnosis of chronic hepatitis was performed according to the international criteria (12, 13). HCC was histologically classified as well, moderately, and poorly differentiated HCC, according to the Edmondson Steiner criteria (14).

Telomerase Assay. Surgical materials containing small hepatomas were sliced with a razor blade so as to contain the hepatoma nodule and were homogenized with 250 μl of cold lysis buffer (11) in Kontes tube with matching pestles rotated at 450 rpm. Needle biopsy samples were homogenized with 50 μl of cold lysis buffer in Kontes tube with matching pestles rotated at 450 rpm. After 20 min at 4°C, the lysate was centrifuged at 16,000 × g for 20 min at 4°C. The supernatant was poured into two microtubes and quickly frozen in liquid nitrogen and stored at −80°C. Then the protein concentration of the extract was measured by Coomassie Brilliant Blue assay. An aliquot (6 μg protein) of the extract was used for each telomerase assay. Telomerase activity was assayed by the TRAP method (11). In brief, the cell extract (0.5 to 4 μl) was incubated with 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 mM deoxynucleoside triphosphates, 0.1 μg of TS primer sequence (5'-AATCCGTCGAGCA-GAGTT-3'), 1 μg T4g32 protein (Boehringer-Mannheim), and BSA (0.1 mg/ml) at 20°C for 30 min. Then the mixture was incubated at 90°C for 3 min to inactivate the telomerase activity. During this step, 0.1 μg of CX primer sequence [5'-(CCCTTA)$_3$CCCTAA-3'] was 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 90 s (2 min for the final step). One-half of the PCR products was analyzed by electrophoresis in 0.5× Tris-borate EDTA buffer on 10% polyacrylamide nondenaturing gels. The gel was dried and processed for autoradiography with an exposure at −80°C for 8 h. The data were collected in a blind fashion and decoded later.

Results

HCC. Although telomerase activity was not quantitatively titrated, semiquantitative definition was estimated as described previously (15)

through the standard assay condition as follows (Table 1): strong (A), detectable in ×100 (or more) diluted sample (0.06 μg protein/assay); moderate (B), detectable in ×10 diluted sample (0.6 μg protein/assay); weak (C), detectable in ×1 diluted sample (6 μg protein/assay); trace (D), detectable in ×1 diluted sample (6 μg protein/assay) only after 3–5 times longer exposure than the 8 h-standard; and negative (−), not detectable in ×1 diluted sample (6 μg protein/assay) after 40 h exposure. Telomerase activity was positive in 28 of 33 HCC tissues, as several examples were shown in Fig. 1. The reaction was RNase sensitive and primer dependent. The positive rate of telomerase was 71% (5 of 7) in well-differentiated HCC, 86% (19 of 22) in moderately differentiated HCC, and 100% (4 of 4) in poorly differentiated HCC (Table 1). The high incidence of telomerase in HCC was found even in the nodules less than 3 cm in diameter. The telomerase activity in HCC was high (22 cases), moderate (4 cases) titer, and weak and trace for one case each. The incidence of telomerase in HCC was not related to the histology and tumor size, but HCC tissues from eight HBV-positive patients were all telomerase positive. Of 22 nontumor tissues examined from HCC patients, weak telomerase activity was detected in only one case.

Nontumor Liver Tissues. Telomerase activity was not detected in four biopsy samples of normal liver. Mixing HCC extract with normal liver extract did not interfere with telomerase activity. Unexpectedly, very weak telomerase activity was detected in 55% (25 of 46) of the tissues of chronic liver diseases from apparently nontumor patients (Table 1). Although telomerase activity in these tissues was weak or trace (Fig. 2), it clearly gave a characteristic 6-base ladder after long exposure (data not shown). The high activity of telomerase was not detected in all nontumor liver tissues. In view of the stage of liver diseases, the incidence of telomerase was 71% (5 of 7) in acute resolving hepatitis, 38% (3 of 8) in chronic persistent hepatitis, 33% (5 of 15) in chronic aggressive hepatitis 2A (mild type), 75% (6 of 8) in chronic aggressive hepatitis 2B (severe type), and 75% (6 of 8) in liver cirrhosis. There was no relationship between telomerase activity and age or sex. There was no apparent correlation between telomerase
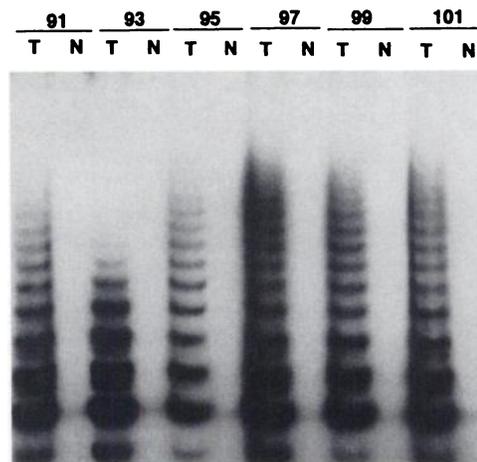

Fig. 1. Telomerase activity in HCC. HCC and noncancerous hepatic tissues of the same patient were assayed for telomerase activity as described in the text. Numbers at the *top* of the figure indicate patient code. *T* and *N*, HCC and noncancerous tissues from the same patient, respectively.
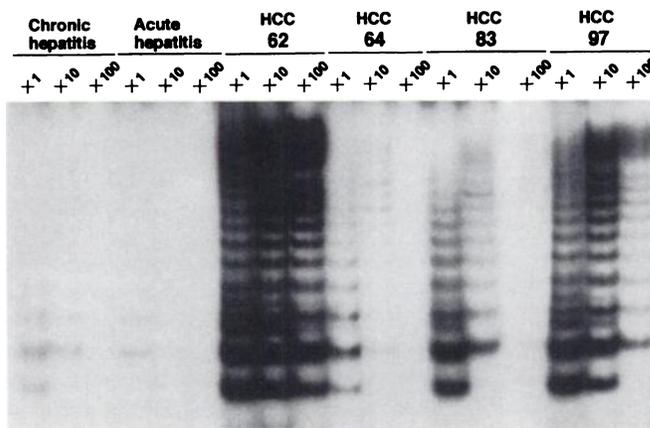

Fig. 2. Telomerase activity in HCC and noncancerous hepatic tissues. Extracts from HCC and noncancerous tissues were diluted and used for telomerase assay. × 1, 6 μg protein/assay; × 10, 0.6 μg protein/assay; and × 100, 0.06 μg protein/assay. Numbers *below* HCC, patient code.

activity and virus markers: 3 of 9 for telomerase positive/HBV positive and 13 of 29 for telomerase positive/HCV positive.

Discussion

Telomerase activity has been detected, without exception, in a wide variety of human tumors, including one case of HCC, and tumor-derived cell lines, whereas it was not detected in both normal cells *in vitro* and normal somatic tissues *in vivo* (10, 11). Our study presented here is the first systemic examination for telomerase activity in liver tissues. Telomerase activity was positive in 28 of 33 HCC tissues (85%), whereas it was negative in normal liver. One possible reason for the absence of telomerase activity in some HCC may be due to inactivation of the enzyme during freezing and preparing samples. It should be emphasized that telomerase was positive in 71% of well-differentiated HCC nodules smaller than 2 cm in diameter and also in 91% of moderately differentiated HCC smaller than 3 cm. These results indicate that, when HCC are detectable at the clinical level, most are already telomerase positive. The development of HCC, similar to other tumors, is thought to be a multiple stepwise progression with multiple genetic changes, and it has been reported that genomic abnormalities in p53 or Rb are late events but still rare in well-differentiated HCC types or small-sized nodules in HCC

Table 1 *Telomerase activity in HCC tissues and in hepatic tissues from noncancer patients*

Tissues	Telomerase activity[a] (no. telomerase positive/no. tested)					
	A	B	C	D	−	Total
HCC						
Stages						
Well differentiated	4	0	1	0	2	5/7
Moderately differentiated	14	4	0	1	3	19/22
Poorly differentiated	4	0	0	0	0	4/4
Total	22	4	1	1	5	28/33
Size (diameter)						
Smaller than 2 cm[b]	4	1	1	0	2	5/8
Between 2 and 3 cm[c]	9	0	0	0	1	9/10
Larger than 3 cm	9	3	0	1	2	13/15
Total	22	4	1	1	5	28/33
Normal	0	0	0	0	4	0/4
Chronic liver diseases						
Acute hepatitis	0	0	1	4	2	5/7
Chronic hepatitis	0	0	3	11	17	14/31
Liver cirrhosis	0	0	1	5	2	6/8
Total	0	0	5	20	21	25/46

[a] Relative telomerase activity levels are as follows: (A) strong, detectable in ×100 (or more) diluted sample; (B) moderate, detectable in ×10 diluted sample; (C) weak, detectable in ×1 diluted sample; (D) trace, detectable in ×1 diluted sample only after longer exposure periods than standard procedure; and (−) negative, not detectable in ×1 diluted sample, even after long exposure.
[b] All but one, which was moderately differentiated, were well differentiated.
[c] All were moderately differentiated.

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(16, 17). HBV infection is major risk factor of HCC, and HBV-positive HCC tissues were 100% telomerase positive. HBV encodes a gene product that can physically bind to p53 (18) and block normal function of p53 (19). Therefore, HBS may sequester p53 function as the earliest event in HBS-positive liver, resulting in genetic instability, an increase in cell growth, and then telomerase reactivation.

Since telomerase activity has not been detected in more than 50 examples of normal somatic tissues and normal cultured cells (11), it is surprising that telomerase is frequently detected, although its activity was very weak, in chronic liver diseases from apparently noncancer patients. Both the intensity of telomerase activity and frequency in telomerase-positive cases appeared to correlate with progression of hepatocarcinogenesis, and telomerase activity was not detected in normal liver. In chronic liver diseases, 55% were very weakly TRAP positive, and in HCC, 85% were strongly positive. The enzyme activity observed in nontumor tissues was not an artifact because the reaction was primer dependent, RNase sensitive, and the assay products gave a characteristic 6-base ladder indistinguishable from that in HCC. We do not know at present whether the telomerase activity in these tissues is shared by many cells with weak activity or by a small number of cells with strong activity. HCC frequently arose in the advanced stage of chronic liver diseases, such as chronic aggressive hepatitis 2B (severe type) or liver cirrhosis. Thus, telomerase-positive cells may occur in the early stage of hepatocarcinogenesis, and frequent development of multiple HCC may be of independent clonal origin and multifocal development (20). The chronic diseased liver may contain small, but significant, numbers of telomerase-positive cells that are immortalized but still precancerous and from which HCC cells may arise. In this regard, it is of interest that a HCC actually arose 4 years later in one of six cirrhotic patients who had been telomerase positive but HCC negative. Prospective observations will be continued for the occurrence of HCC to see if telomerase activity in nontumor patients correlated with HCC development. Other explanations, however, cannot be ruled out at present. These include the possibility that apparently tumor-free tissues contain a small number of HCC cells, that normal liver cells can reactivate telomerase expression during regeneration, or that migrating lymphocytes are telomerase positive (21). However, there was no apparent correlation between telomerase activity and mitotic figures, a marker of regeneration of hepatic cells, and lymphocyte migration was not extensive and did not correlate with telomerase activity.

In summary, the present study demonstrates that telomerase activity is frequently detected in HCC, irrespective of the stage or size of the disease. Thus, telomerase activity determinations may be useful for the diagnosis of early HCC. Although the biological significance of the weak telomerase activity detected in apparently noncancerous liver tissues (such as from patients with hepatitis and cirrhosis) is unclear at present, it is possible that this activity may be an early indication of cancer development.

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