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LIVER CANCER

Dynamic alteration of telomerase expression and its diagnostic significance in liver or peripheral blood for hepatocellular carcinoma

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Abstract

AIM: To investigate the dynamic alteration of telomerase expression during development of hepatocellular carcinoma (HCC) and its diagnostic implications in liver tissues or peripheral blood mononuclear cells for HCC.

METHODS: Dynamic expressions of liver telomerase during malignant transformation of hepatocytes were observed in Sprague-Dawly (SD) rats fed with 0.05% of 2-fluoenyacetamide (2-FAA). Total RNA and telomerase were extracted from rat or human liver tissues. The telomerase activities in livers and in circulating blood were detected by a telomeric repeat amplification protocol-enzyme-linked immunosorbent assay (TRAP-ELISA), and its diagnostic value was investigated in patients with benign or malignant liver diseases.

RESULTS: The hepatoma model displayed the dynamic expression of hepatic telomerase during HCC development. The telomerase activities were consistent with liver total RNA levels (r = 0.83, P < 0.01) at the stages of degeneration, precancerosis, and cancerization of hepatocytes. In HCC patients, the telomerase levels in HCC tissues were significantly higher than in their adjacent non-cancerous tissues, but liver total RNA levels were lower in the former than in the latter. Although the circulating telomerase of HCC patients was abnormally expressed among patients with chronic liver diseases, the telomerase activity was a non-specific marker for HCC diagnosis, because the incidence was 15.7% in normal control, 25% in chronic hepatitis, 45.9% in liver cirrhosis, and 85.2% in HCC, respectively when absorbance value of telomerase activity was more than 0.2. If the value was over 0.6, the incidence was 60% in HCC group and 0% in any of the others (P < 0.01) except in two cases with liver cirrhosis. However, the combination of circulating telomerase with serum alpha-fetoprotein level could increase the positive rate and the accuracy (92.6%, 125 of 135) of HCC diagnosis.

CONCLUSION: The overexpression of telomerase is associated with HCC development, and its abnormality in liver tissues or in peripheral blood could be a useful marker for diagnosis and prognosis of HCC.

Key words: Hepatocellular carcinoma; Telomerase; Peripheral blood mononuclear cells; Telomeric repeat amplification protocol

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and rapidly fatal malignancies worldwide[1], and has been ranked as the 2nd cancer killer in China, particularly in the eastern and southern areas, including the inshore area of the Yangtze River[2]. Major risk factors for HCC in these areas are exposure to aflatoxin B1 (AFB1) and infection with hepatitis viruses (HBV and HCV)[3]. Its prognosis is poor and early diagnosis is of utmost importance[4]. Treatment options are severely limited by the frequent presence of metastases. Although serum alpha-fetoprotein (AFP) is a useful tumor marker for the detection and monitoring of HCC development, the false-negative rate with AFP level alone may be as high as 40%

for patients with small size HCC. If hepatocyte-specific mRNAs are detected in the circulation, it is possible to infer the presence of circulating, presumably malignant liver cells and to predict the likelihood of haematogenous metastasis[5-7]. The use of telomerase assay for cancer diagnosis might be possible because telomerase is expressed in malignant lesions but rarely in normal somatic cells[8].

Telomerase, an RNA-dependent DNA polymerase, can maintain the telomeric length by acting as a reverse transcriptase[9]. In humans, tumor cells escape programmed cell senescence through reactivation of telomerase[10]. These immortalized cells can compensate for telomeric shortening at each cell division, leading to progressive neoplastic evolution[11]. Telomerase re-expression is found in 85% of human malignant tumors[12]. The expression of telomerase is important to cell proliferation, senescence, immortalization and carcinogenesis[13]. However, early expression and kinetic alteration of hepatic telomerase during development of HCC remain unclear. The objectives of this study were to observe the dynamic expression of liver telomerase in rat hepatoma model induced by oral administration of 0.05% of 2-fluoenyacetamide (2-FAA), and investigate the telomerase activities in liver tissues and peripheral blood mononuclear cells (PBMCs) by a telomeric repeat amplification protocol-enzyme-linked immunosorbent assay (TRAP-ELISA), as well as its diagnostic implications for HCC in patients with benign or malignant liver diseases.

MATERIALS AND METHODS

Hepatoma rat models

Forty-two closed colony male Sprague-Dawly (SD) rats weighing 120-160 g were obtained from the Experimental Center of Medical Animals, Nantong University, China, and divided randomly into 7 groups, one group (n = 6) as normal control and 6 experimental groups (n = 6 in each group). All rats were housed under bio-clean conditions at a temperature-controlled (22℃ ± 2℃) environment with a 12-h light/dark cycle and 55% humidity. The control group was given basal foodstuffs and the feed of experimental rats contained 0.05% 2-FAA (Sigma, USA). Rats were then monitored daily of survival and weight loss, recorded of their clinical signs and sacrificed at different stages of hepatoma development. One group of the experimental rats and one control rat were killed in every two weeks. All surgical procedures were conducted under deep ether anaesthesia. Blood was drawn from rat's heart. The livers were collected, and histological examination was performed with hematoxylin and eosin (HE) staining. All procedures performed on the rats were conducted in accordance with the Guidelines for Experimental Animals approved by the Animal Care and Use Committee of Nantong University, China.

Patient selection and human HCC tissues

All patients with liver diseases came from the Affiliated Hospital, Nantong University, China. We evaluated 135 HCC patients (103 males and 32 females, age between 35-76 years, mean 49). Ninety-five patients (70.3%) had a history of cirrhosis, and 52 (38.5%) had a history of chronic hepatitis. Moreover, 85.2% (115/135) were hepatitis B surface antigen (HBsAg) carriers, 10.4% (14/135) had antibodies to hepatitis C virus (anti-HCV, second-generation antibody) and 8.9% (12/135) had antibodies to hepatitis G virus by an enzyme-linked immunosorbent assay (ELISA), respectively. Other cases studied included 80 patients with chronic hepatitis (CH, 58 males and 22 females), 37 patients with liver cirrhosis (LC, 30 males and 7 females), and 70 healthy individuals (35 males and 35 females, mean age 35 years) with negative hepatitis B virus markers (HBsAg, HBcAb, and HBV-DNA) and anti-HCV, and normal levels of serum alanine aminotransferase (ALT) from the Nantong Central Blood Bank as normal controls (NC). The venous blood of patients and normal controls were collected with heparin anticoagulant and stored on ice. The diagnosis of HCC and viral hepatitis was based on the criteria proposed at China National Collaborative Cancer Research Group[14] and the China National Viral Hepatitis Meeting[15], respectively.

Fifteen specimens of HCC and their corresponding non-tumor tissues as self-control were obtained from HCC patients who had undergone curative hepatectomy. These specimens were immediately frozen in liquid nitrogen and kept at -85℃. All specimens were hepatocellular cancer confirmed by pathological examination. Eleven non-cancerous tissues from those patients were atypical proliferation. The patients' ages ranged between 31-64 years (mean 51.3 years). There were 12 specimens with a tumor size larger than 3 cm, and 3 specimens smaller than 3 cm.

Isolation of total RNA

Fifty mg of each liver tissue (rat liver, human HCC, and their self-controlled non-tumor tissues) was homogenized with a Polytron homogenizer after the addition of 1.0 mL of TRIzol reagent (Promega). Then 0.2 mL of chloroform was added into the tubes, mixed by vortexing for 15 s, and placed at -20℃ for 5 min. Samples were centrifuged at 12000 r/min for 15 min at 4℃. The supernatants were collected, and equivalent isopropanol was added and then they were put into another tube and mixed gently, placed at -20℃ for 15 min, then centrifuged at 12000 r/min for 15 min at 4℃. The supernatants were removed, the RNA pellets were washed twice with 0.5 mL of 75% ethanol, mixed and centrifuged at 8000 r/min for 8 min at 4℃. The RNA pellets were air dried 5 min at room temperature and reconstituted in 20 μL of RNase-free DEPC water and incubated at 60℃ for 10 min. The purity and concentration of the RNA was estimated by the ratio of absorbance readings at 260 and 280 nm, with an $A_{260/280}$ ratio between 1.8 and 2.0 indicating sufficient purity. RNA samples were kept frozen at -85℃ until use.

Separation of PBMCs

Five milliliters of peripheral blood were collected with heparin anticoagulant from each patient. Ficoll (2.5 mL) was added to each sample. After centrifugation at 2000 r/min for 20 min, the PBMCs were collected from the Ficoll/plasma interface. Then they were washed three times in normal saline and pelleted by using low-speed cen-

trifugation. The cells were collected at 2×10^5/tube, then stored at -85℃ for telomerase assays.

TRAP reaction

A commercial telomerase TRAP-ELISA kit (Boeheringer Mannhein), based on TRAP introduced by previous methods[16], was used to assay telomerase activity in all specimens. Fifty mg liver tissue or 2×10^5 cells were suspended in 200 μL lysis reagent and incubated for 30 min, then centrifuged at 16000 r/min for 20 min at 4℃. Positive control cell extract was from immortalized telomerase-expressing human kidney cells (293 cells). Negative control cell extract was as follows: heat-treatment of the cell extract for 10 min at 65℃ prior to the TRAP reaction. The supernatant was removed to other EP tubes and kept frozen at -85℃. Telomeric repeats were added to a biotin-labeled primer for 30 min at 25℃ during the first reaction. The mixture was incubated at 94℃ for 5 min to induce telomerase inactivation, then subjected to 30 PCR cycles of 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 90 s, and finally at 72℃ for 10 min. The PCR products were used for analysis of telomerase activity and gel electrophoresis.

Telomerase detection

An aliquot of the PCR product was denatured and hybridized to a digoxigenin-(DIG)-labeled, telomeric repeat-specific detection probe. The resulting product was immobilized *via* the biotin labeled primer to a streptavidin-coated microtiter plate. The detection probe and the hybridization conditions have been optimized to obtain the highest specificity and sensitivity. The immobilized PCR products were then detected with an antibody against digoxigenin (anti-DIG-POD) that was conjugated to peroxidase. Finally, the probe was visualized by use of peroxidase metabolizing tetramethyl benzidine (TMB) to form a colored reaction product. The absorbance (A) of the samples was measured at 450 nm (reference wavelength 620 nm) within 30 min of addition of the stop reagent. Telomerase activities were expressed according to absorbance difference (△A) between sample and negative control.

Telomerase by TRAP silver staining

Fifteen microliters of the PCR products were loaded to 12% polyacrylamide gel and resolved by electrophoresis at 160-180 V for 1-2 h. Then the gel was immobilized to 10% ethanol for 5 min, soaked in 1% $AgNO_3$ for 5 min, dyed in silver dye solution for 10 min, and then in developing dye for 10 min. The amplified products were of heterogeneous length and created a ladder pattern of bands each representing the addition of a hexanucleotide telomeric repeat by telomerase, and photographed by Delphin-Doc Digital Imaging System (Wealtec, USA). All samples were done in duplicate and the reproducibility was confirmed.

Statistical analysis

The livers of experimental rats according to pathological examination were divided into 4 groups: degeneration of hepatocytes (Exp-1), precancerosis of hepatocytes (Exp-2), cancerization of hepatocytes (Exp-3), and normal controls (Con). The telomerase activities in PBMCs were investigated in patients with HCC, chronic hepatitis (CH), liver cirrhosis (LC), and normal controls (NC). Human HCC tissues were divided into cancerous and their non-tumor (non-HCC) groups. Levels of telomerase and total RNA were expressed as mean ± SD. Differences between groups were assessed by the Student's *t* test or the χ^2 test, and the Fisher's exact probability test. Probability values less than 0.05 were considered significant.

Table 1 Histopathological alterations of hepatocytes at different stages of rat hepatomas induced with 2-FAA

Group	*n*	Pathological feature (HE staining)		
		Degeneration	Precancerosis	Cancerization
Control	6	0	0	0
2nd wk	6	6	0	0
4th wk	6	3	3	0
6th wk	6	0	5	1
8th wk	6	0	4	2
10th wk	6	0	1	5
12th wk	5[1]	0	0	5
Total	41	9	13	13

[1]One rat died during the experimental process; 2-FAA: 2-fluoenylacetamide.

RESULTS

Histopathological findings

The pathological changes of experimental rat livers with HE staining are shown in Table 1. The rats were administered with 0.05% of 2-FAA for two weeks. The appearance of livers turned to be grey-yellow and scabrous. The hepatocytes showed granular degeneration. Most of normal liver tissue structures remained. A few of hyperplastic oval cells, and the tendency toward nodule formation were seen at the early stage of hepatoma development. At the end of four weeks, precancerous lesions appeared in half of liver tissues. Histologically, normal liver follicles existed in most areas but hyperplastic small round cells or oval cells or hyperplastic nodules were found locally. After the 6th wk, all livers progressed into canceration stage. The structures of liver lobules were completely destroyed, and necrosis was spread over the whole livers. Large numbers of small oval cells, and cancer nests were observed. The histological features of livers showed diffuse patchy necrosis and high degree differentiation of HCC.

Expression of telomerase during rat hepatoma development

The dynamic expression of liver telomerase in rat hepatocyte degeneration (Exp-1), hepatocyte precancerosis (Exp-2), and hepatocyte cancerization (Exp-3) groups during development of hepatomas in comparison with controls (Con) are shown in Figure 1. After rats were given 2-FAA, the specific activities (△A/mg protein) of liver telomerase were significantly higher than that of controls ($P < 0.01$) at the stage of hepatocyte degeneration, kept rising at the stage of hepatocyte precancerosis, and peaked at the formation stage of hepatoma. Telomerase activities were consistent with the levels of liver total RNA ($r = 0.83$,

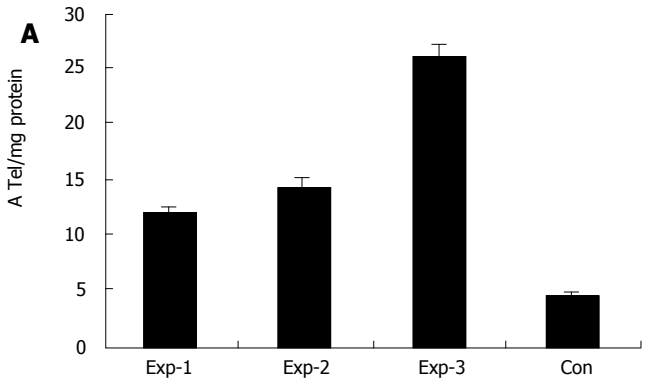

Figure 1 Alterations of hepatic telomerase at different stages of rat hepatoma model. The feed of experimental rats contained 0.05% of 2-fluoenylacetamide. Exp-1: Hepatocyte degeneration group; Exp-2: Hepatocyte precancerosis group; Exp-3: Hepatocyte cancerization group; and Con, rats given basal feed without 2-fluoenylacetamide as normal control group.

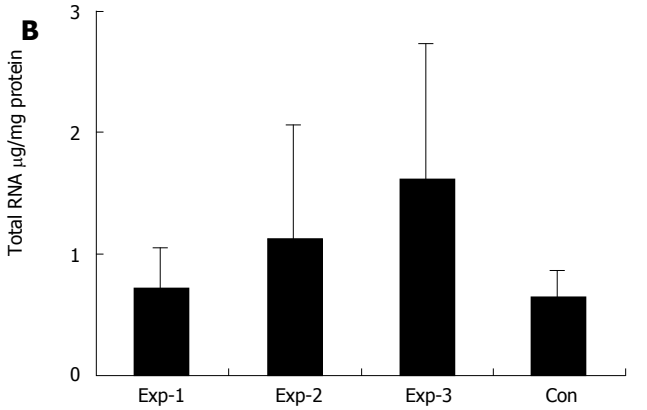

Figure 2 Alterations of liver total RNA at different stages of rat hepatoma model. The feed of experimental rats contained 0.05% of 2-fluoenylacetamide. Exp-1: Hepatocyte degeneration group; Exp-2: Hepatocyte precancerosis group; Exp-3: Hepatocyte cancerization group; and Con, rats given basal feed without 2-fluoenylacetamide as normal control group.

$P < 0.01$) at the different stages of hepatoma development of rats (Figure 2).

Expression of telomerase in human liver tissues

The levels of total RNA and telomerase expression in different human liver tissues are shown in Figure 3. The concentrations of liver total RNA and telomerase were 12.40 ± 7.34 μg/mg liver protein and 18.25 ± 15.02 A/μg RNA in HCC tissues, and 53.77 ± 52.02 μg/mg liver protein and 8.16 ± 6.22 A/μg RNA in their non-cancerous tissues, respectively. The expression of total RNA in HCC tissues was significantly lower than that in their non-cancerous tissues ($P < 0.01$); however, the expression level of telomerase was significantly higher in HCC tissues than in their non-cancerous tissues ($P < 0.05$). Stronger positive expression of telomerase was presented in HCC tissues and not in their non-cancerous tissues (Figure 4).

Significance of telomerase in PBMCs

There were different telomerase activities of PBMCs in NC, CH, LC and HCC, but the activities in HCC were significantly higher than in others ($P < 0.01$, Figure 5). The

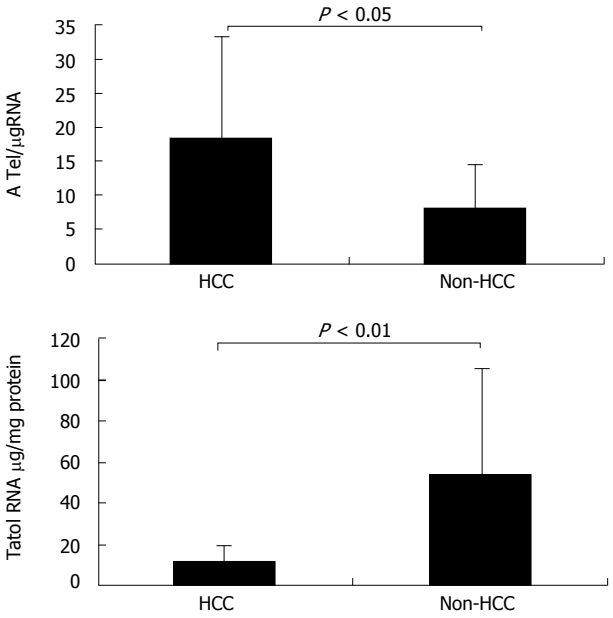

Figure 3 Comparative analysis of total RNA levels and telomerase activities in human hepatoma tissues and their non-cancerous tissues.

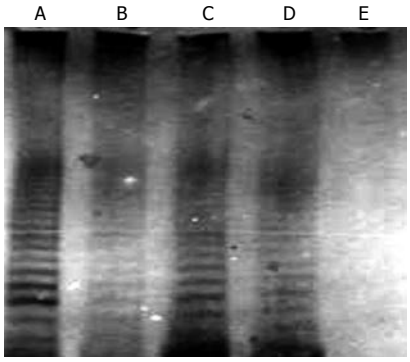

Figure 4 Electrophoresis patterns of human telomerase in polyacrylamide gel. A,C: Human hepatocellular carcinoma tissues; B,D: Non-cancerous tissues of human hepatocellular carcinoma; E: Negative control.

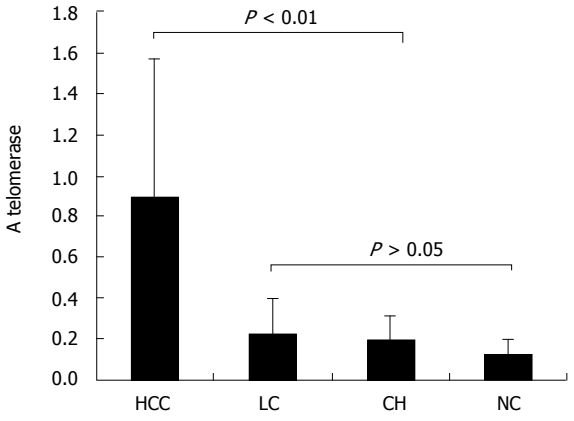

Figure 5 Comparative analyses of telomerase activities in peripheral blood mononuclear cells from patients with benign or malignant liver diseases.

samples were regarded as telomerase-positive if the difference in absorbance (Table 2) was greater than 0.2 △A. The incidence of telomerase expression was 15.7% in NC, 25% in CH, 45.9% in LC, and 85.0% in HCC (sensitivity 68.4% and positive predictive value 75.7%), respectively. The incidence of telomerase was significantly higher in

Table 2 Diagnostic values of different telomerase levels in peripheral blood mononuclear cells for hepatocellular carcinoma

Group	*n*	Positive (%)		
		△A>0.2	△A>0.4	△A>0.6
HCC	135	115 (85.2)	101 (74.8)	81 (60.0)
Liver cirrhosis	37	17 (45.9)	6 (16.2)[b]	2 (5.4)[b]
Chronic hepatitis	80	20 (25.0)[b]	8 (10)[b]	0 (0)[b]
Normal control	70	11 (15.7)[b]	0 (0)[b]	0 (0)[b]

[b]$P < 0.01$ *vs* the HCC group.

Table 3 Assessment of the diagnostic values of different telomerase levels

Telomerase	Sen (%)	Spe (%)	Predictive value		Acc (%)
			Pos (%)	Neg (%)	
△A > 0.2	85.2	68.4	75.7	80.0	77.4
△A > 0.4	74.8	88.0	87.8	75.2	81.0
△A > 0.6	60.0	98.3	97.6	68.0	77.8

Sen: Sensitivity; Spe: Specificity; Pos: Positive; Neg: Negative; Acc: Accuracy.

HCC group than in any of other groups ($P < 0.01$). If the value rose up to 0.6 △A, the incidence of telomerase was 60% in HCC (sensitivity 98.3% and positive predictive value 97.6%), and 0% in any of other groups ($P < 0.01$) except two cases with liver cirrhosis (Table 3). For combined diagnostic values of telomerase and AFP for HCC, the AFP concentration in HCC patients was 1765 ± 1705 μg/L and the positive rate was 70% for a cutoff value of AFP level 200 μg/L. Total AFP detection in combination with telomerase activity could increase the accuracy of HCC diagnosis (92.6%).

DISCUSSION

Carcinogenesis of hepatocytes is a multi-factor, multi-step and complex process and has many characteristics, such as fast infiltrative growth, metastasis in early stage, high malignancy, and poor therapeutic efficacy[17]. Probably HBV infection is merely a carcinogenic factor, and is not related to the growth, infiltration and metastasis of HCC[18]. It is increasingly clear that oncogenesis is driven by the activation of telomerase, a ribonucleoprotein complex that adds telomeric repeats (hexanucleotide 5'-TTAGGG-3') to the ends of replicating chromosomes. Telomerase is a ribonucleoprotein composed of an essential RNA and a few proteins. It is expressed in embryonic cells and in adult male germ line cells, but is undetectable in normal somatic cells except proliferative cells of renewable tissues. Although the precise processes leading to HCC are still unknown, progression to a cancerous state does require the accumulation of a series of genetic alterations.

To understand the mechanisms and alterations of telomerase expression during carcinogenesis of hepatocytes, rat hepatoma models were induced with chemical carcinogen (2-FAA), and telomerase expression was studied at the different stages of degeneration, precancerosis, and cancerization of hepatocytes (Table 1). Although the histopathological examination found the oval cells and non-differentiation in morphology at the early stage of hepatoma, telomerase was significantly expressed in livers (Figure 1). This has raised two possibilities: (1) telomerase may be an important target for therapy aiming at controlling cell growth[19,20]. Telomerase activation in the critical step of tumor progression during hepatocarcinogenesis might be regulated only by hTERT, whereas increased telomerase activity in tumor progression might be regulated by both hTERT (reverse transcript) and hTERT (RNA component)[21-23]; (2) histological examination in combination with telomerase activity could increase the accuracy of diagnosis at early stage and help the staging of HCC development.

The predominant mechanism for telomere stabilization in tumor cells is the activation of the telomere-synthesizing enzyme telomerase. Both of liver telomerase activity (Figure 1) and total RNA increased (Figure 2) during hepatoma progression. This may be relevant to the cytotoxic effects of liver cells in resisting carcinogenic effect of 2-FAA. In the meantime, the telomerase level of liver tissues and sera of the experimental groups were obviously higher than the normal control group ($P < 0.01$), indicating that the oval cells could express telomerase and secrete it into blood. Telomerase activities increased continuously and reached the highest level in the experimental groups, and livers of the experimental groups in the 6th wk were seen to have canceration. Rat hepatoma is an aggressive malignancy. Both total RNAs and telomerase activity increased during HCC progression. Moreover, in the following weeks, the liver tissues continued to synthesize telomerase, which remained at a high level. These results suggest that the strong enhancement of telomerase activity may be a critical step in hepatocarcinogenesis, although the exact mechanism is not clear.

Telomerase is highly activated in human tumor tissues, whereas it is not activated in primary cell strains and tumor-adjacent tissues[24,25]. With cell division, telomerase is activated. The cells with positive telomerase activity become immortalized and further develop to carcinoma cells[26,27]. The telomerase activities in HCC and their surrounding non-tumor tissues were detected by TRAP-ELISA in this study. The telomerase expression in HCC was negatively correlated with differentiation, but was not related to tumor size or histological grade. The expression level of telomerase was significantly higher in HCC tissues than in their corresponding non-cancerous tissues ($P < 0.05$, Figure 3A). Stronger positive expression of telomerase was present in HCC and not in their non-cancerous tissues (Figure 4). No relationship could be observed between the enhancement of telomerase activity and liver total RNA in human HCC tissues (Figure 3B). In China, major risk factors for HCC are exposure to aflatoxin B1 (AFB1) and infection by hepatitis viruses, especially HBV. HBx, encoded by HBV, could block the function of *p53*, resulting in genome unstablization. Activation of telomerase could make liver cells immortalized, and microvascularization could provide nutrition for their growth and promote metastasis[17,28]. These two factors may induce carcinogenesis and have a key role in occurrence, development, infiltration and metastasis of HCC. They

may also have notable clinical values in diagnosing and treating HCC in early stage.

Telomerase was strongly activated in HCC, while weakly activated in CH and LC. Progressive shortening of telomeres during hepatocellular carcinogenesis was found from normal liver to CH to LC to HCC. The average telomere length of HCC was significantly and consistently shorter than that of adjacent CH or LC. The possible sequel of telomere shortening in regenerative, non-cancerous liver lesions (CH and LC) is that it may eventually lead to reactivation of telomerase, which in turn may contribute to the malignant conversion to HCC. Telomerase may be expressed in lymphocytes[29,30]. Lymphocytic infiltration may occur during tumor necrosis, thus the telomerase activity we measured included a proportion generated by lymphocytes and might be overestimated. Almost all HCCs are preceded by CH and/or LC. There were different degrees of telomerase activities of PBMCs in NC, CH, LC and HCC, but the telomerase activities of HCC were significantly higher than others (Figure 5, Table 2). In addition, combined diagnostic values of telomerase and AFP for HCC were investigated in this study. Detection of telomerase activity in combination with serum AFP could increase the accuracy of HCC diagnosis.

These genetic changes may lead to senescence of hepatocytes occurring in patients with advanced liver diseases probably as a result of degeneration and the subsequent regeneration of hepatocytes[31,32]. Although the mechanisms of carcinogenesis in chronic liver diseases are not known, recessive mutations may result in the reactivation of telomerase and stabilization of telomere length, as well as fixation of the additional mutations required for invasiveness and metastasis[33,34]. Moreover, the patients with telomerase-positive HCC show poorer prognosis than those with telomerase-negative tumors. Clinically although the diagnostic specificity of hepatoma is high, the positive rate of telomerase is at present much lower than that from animal model studies, and the number of bands does not coincide with that of human being either[35]. In addition, the cause of human hepatoma is associated with many factors. Telomerase could become a valuable marker for HCC diagnosis if the sensitivity of detection method is improved[36]. Further research should focus on mechanisms of carcinogenesis by HCV and HBV, development of early detection and more effective treatments. Better understanding of HCC related to hepatitis viruses should also be a major concern in future studies.

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