

Expression of telomerase activity and oxidative stress in human hepatocellular carcinoma with cirrhosis

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Abstract

AIM: To study the expression and significance of telomerase activity and oxidative stress in hepatocellular carcinoma (HCC) with cirrhosis.

METHODS: In this study, TRAP-ELISA assay was used to determine telomerase activity in 21 cases of HCC as well as in 23 cases of hepatic cirrhosis. Malondialdehyde (MDA), glutathione S-transferase (GST) and total anti-oxidative capacity (T-AOC) were also examined in the same samples with human MDA, GST and T-AOC kits.

RESULTS: Eighteen of 21 cases of HCC were found to have increased telomerase activity, whereas only three of the 23 non-cancerous cirrhotic samples were found to have weak telomerase activity, and the difference was significant ($P < 0.001$). No significant difference in telomerase activity was detected according to different tumor size, tumor stage, histological grade, HBsAg, contents of albumin, bilirubin, ALT, AFP, r-GT and platelet. There were significant differences between HCC and cirrhosis in the expression of MDA, GST and T-AOC respectively. Telomerase activity correlated positively with the content of MDA ($P < 0.05$).

CONCLUSION: Telomerase activation is the early event of carcinogenesis, which is not correlated with clinicopathological factors of HCC. The dysfunction of the anti-oxidative system is closely correlated with the progression from cirrhosis to hepatocellular carcinoma. Oxidative stress may contribute partly to telomerase activation.

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INTRODUCTION

Telomeres correspond to the ends of eukaryotic chromosomes and are specialized structures containing unique (TTAGGG) $_n$ repeats^[1]. Telomeres protect the chromosomes from DNA degradation, end to end fusions, rearrangements, and chromosome loss^[2]. Because cellular DNA polymerases cannot replicate the 5' end of the linear DNA molecule, the number

of telomere repeats decreases (by 50-200 nucleotides/cell division) during aging of normal somatic cells. Shortening of telomeres may control the proliferative capacity of normal cells^[3]. Telomerase, a ribonucleic acid-protein complex, adds hexameric repeats of 5'-TTAGGG-3' to the end of telomeres to compensate for the progressive loss^[4]. Although normal somatic cells do not express telomerase, immortalized cells such as tumor cells express this enzyme^[5]. More recently, HeLa cells transfected with an antisense human telomerase were found to lose telomeric DNA and to die after 23 to 26 doublings^[6]. Pertersen *et al.* found that the rate of shortening of telomere restriction fragments in human fibroblasts could be accelerated significantly by oxidative stress^[7]. Importantly, after treatment of cells with short single stranded telomeric G-rich DNA fragments, glioblastoma cells recovered from the arrest and showed enhanced telomerase activity and elongated telomeres^[8].

However, the mechanisms of activation and regulation of telomerase have not been established. Cell line data indicate that a telomere length-dependent mechanism is the major pathway. On the other hand, normal lymphocytes up-regulate telomerase activity upon antigen and mitogen stimulation *in vitro* and *in vivo*. This indicates that telomere length-dependent mechanisms may be important or specific to different cell types for regulation of telomerase activation^[9]. Only a few studies have specifically examined the relationship between telomerase activity in tumors and the status of oxidative stress. Our data implied that genetic defects in HCC facilitated the reactivation of telomerase activity, a process that might be associated with the increased expression of oxidative stress.

MATERIALS AND METHODS

Sample collection and processing

All 21 HCC specimens were sampled from patients who had undergone curative hepatectomy. Patients who had received radiotherapy or chemotherapy before operation were excluded. Liver cirrhosis tissues were obtained from those who had received hepatic biopsy in the operation for hypersplenism. Informed consent was obtained from all patients for subsequent use of their resected tissues. These specimens were immediately dissected into small pieces under aseptic condition within half an hour after removed, snap frozen in liquid nitrogen and stored at -80 °C until extracts for telomerase activity analysis and determination of oxidative stress.

Telomerase assay

Frozen tissue samples (100 µg) were homogenized in 500 µL of freshly made ice-cold lysis buffer. After 30 min incubation on ice, the lysate was centrifuged for 20 min at 16 000 g, and the supernatant was transferred to fresh tubes and used as tissue extracts for the telomerase assay. The protein concentrations were determined. Telomerase activity was assayed by the TRAP-ELISA kit, a polymerase chain reaction (PCR)-based on an improved version of the original method described by Kim *et al.*^[10]. In brief, aliquots of tissue extract containing 40 µg protein were added to 50 µL reaction mixtures containing

0.1 µg substrate oligonucleotide (TS) primer, TSK (internal control) template. The reaction mixtures were incubated at 25 °C for 20 min and then amplified for 33 cycles of PCR at 94 °C for 30 s, at 50 °C for 30 s, and at 72 °C for 90 s, then preserved at 4 °C for ELISA reaction process. 5 µg PCR product was taken for ELISA reaction. The value at A450 was read within 30 min. Telomerase activity equaled A450 for experimental well minus A450 for control well. The strength of telomerase activity was defined as follows: ++, >0.4; +, >0.2; -, <0.2.

MDA, GST and T-AOC determination

Frozen tissue samples (100 mg) were homogenized in 1.0 mL, the homogenized samples were centrifuged for 15 min at 3 000 r/min, and the supernatant was transferred to fresh tubes. After the protein concentrations were determined, MDA, GST and T-AOC were assayed with human MDA, GST and T-AOC kit (Jiancheng Biological Technical Institute, Nanjing, China).

Statistics analysis

Contingency table methods were used to analyze the univariate association between telomerase activity and clinicopathological data (age, sex, tumor grade, tumor size, and liver status). Significance was confirmed by Fisher's exact test. The association between telomerase activity and oxidative stress was investigated by Pearson correlation analysis test. All calculations were performed using the SPSS version 10.0 statistical software package, and the results were considered statistically significant at $P < 0.05$.

RESULTS

Telomerase activity in HCC and hepatic cirrhosis

Telomerase positive cells were used as a positive control for assessing telomerase activity in clinical specimens. We measured telomerase activity in surgically resected specimens from 21 cases of HCC and 23 cases of liver cirrhotic tissue. Telomerase activity was detected in 18 of the 21 HCC specimens (85.7 %), but it was detected only in 3 of 23 samples of liver cirrhosis (13.4 %). There was a significant difference in telomerase activity between HCC and hepatic cirrhosis ($P < 0.001$). There was no significant difference in telomerase activity in regard to different tumor size, tumor stage, histological grade, HBsAg, contents of albumin, bilirubin, ALT, AFP, r-GT and platelet (Table 1).

Table 1 Relationship between telomerase activity and clinicopathologic factors in HCC

	Telomerase activity		
	High	Low or loss	P value
Tumor size (cm)	7.7±3.5	7.1±3.6	0.664
Tumor grade (high/low)	9/4	4/4	0.245
Tumor stage (early/ advanced)	6/7	3/5	0.327
ALT (normal /abnormal)	9/2	6/4	0.212
Bilirubin (normal /elevated)	9/2	9/1	0.414
Platelet (normal/ decreased)	9/2	7/3	0.324
HbsAg (positive/negetive)	6/5	7/3	0.272
AFP (>400U/<400U)	7/5	5/5	0.309
Albumin (g/L)	39.2±5.6	39.3±4.2	0.946
Globulin (g/L)	28.6±5.3	26.8±6.5	0.423
γ-GT(U)	115.2±98.7	108.9±69.5	0.814

Expression of MDA, GST and T-AOC in HCC and hepatic cirrhosis

The content of MDA was 84.76±26.98 nM/ml in HCC, while

it was 49.49±23.03 nM/ml in hepatic cirrhosis, and the difference was significant between them ($P < 0.001$). Nevertheless, the contents of GST and T-AOC were lower in HCC than those in hepatic cirrhosis ($P < 0.001$).

Table 2 Expression of MDA, GST and T-AOC ($\bar{x} \pm s$)

	HCC	Cirrhosis	P value
MDA (nM/ml)	84.76±26.98	49.49±23.03	<0.001
GST (U/mg)	8.18±5.59	18.70±5.20	<0.001
T-AOC (U/mg)	0.257±0.241	0.689±0.302	<0.001

Telomerase activity and content of MDA

The relationship between telomerase activity and oxidative stress was investigated by Pearson correlation analysis. Tumor specimens with a higher level of MDA expressed increased telomerase activity ($r = 0.496$, $P < 0.05$).

DISCUSSION

HCC is the most common solid tumor worldwide, being responsible for more than 1 million deaths annually, especially in Eastern Asia and South Africa^[11], which ranks eighth in frequency among cancers in the world^[12]. It is one of the few human cancers in which an underlying etiology can be identified in most cases, and has a background of chronic inflammatory liver disease caused by viral infection that induces cirrhosis^[13]. However, it is not clear how these disorder results in HCC. The reactivation of telomerase activity may play a significant role in hepatocarcinogenesis.

Telomerase is a ribonucleoprotein complex^[14] that is thought to add telomeric repeats onto the ends of chromosomes during the replicative phase of the cell cycle. Telomeres have classically been regarded as a simple linear structure, possibly capped by specific proteins. However, this simple structural view was challenged. Recent data have shown that the structure of human telomeres might be more complicated than originally thought^[15]. Three different mechanisms were currently thought to contribute to telomere shortening: the so-called end replication problem, the C-strand degradation model and single-strand damage^[16]. Both the end replication problem and the C-strand degradation model of telomere shortening do not take into account the possibility that the shortening rate of telomeres depends on external influences, especially oxidative stress-dependent DNA damage. von Zglicnicki *et al* demonstrated that the telomere shortening rate could be either accelerated or decelerated by a modification of the amount of oxidative stress^[17].

Recently, a highly sensitive PCR based TRAP assay for measuring telomerase activity that also includes an improved method of detergent lysis has been developed^[10]. This assay allows more uniform extraction of telomerase from a small number of cells than conventional techniques, in which telomerase first synthesizes extension products that then serve as templates for PCR amplification. The simplicity and increased sensitivity of this assay have resulted in a dramatic increase in the investigation of telomerase expression. In this study, telomerase activity was positive in 18 of 21 HCC specimens (85.7 %), which suggested that telomerase activation was a universal event in human hepatocellular carcinoma. However, undetectable telomerase activity has been reported by others in about 10 % of tumors samples^[18,19]. Some immortal cell lines without detectable telomerase activity have been described that were characterized by long and heterogenous telomeres^[20,21]. These observations might indicate the presence of a telomerase-independent mechanism for telomere length maintenance in these tumors.

It is well documented that telomerase activity is detectable in the majority of cancers but rarely in normal somatic tissue. Some studies have demonstrated that some types of somatic cells express low levels of telomerase activity^[22-24]. In particular physiologically regenerating somatic cells, such as hematopoietic cells, epithelial cells of skin or intestine, and endometrial cells, have been shown low levels of telomerase activity. In this study, low telomerase activity was detected in 3 of 23 cirrhotic specimens. One possible explanation for this finding was that these cirrhotic tissue samples may also contained probable cancer cell, infiltration of lymphoid cells, or dysplasia cells. Finally, demographic and clinical information of patients, such as tumor size, tumor stage, histological grade, HBsAg, albumin, bilirubin, ALT, AFP, r-GT and platelet were not correlated with the telomerase activity.

Many lines of evidence indicate that telomerase is reversibly regulated^[25]. Resting lymphocytes express little telomerase activity, but stimulation of specific antigen receptors on the cell plasma membrane markedly increases telomerase activity^[26-29]. High-level sun light exposure of normal human skins results in an increased incidence of telomerase activation^[30]. Human hematopoietic cells with γ -rays^[31], or human carcinoma cell lines with X-rays^[32] induce the activation of telomerase. Activated telomerase in cancer cells is repressed when the cells leaves the cell cycle and become quiescent^[33-37]. Nevertheless, the mechanisms of telomerase regulation, such as its suppression in normal human somatic cells and activation in neoplastic cells, are far from established.

Telomeres are believed to protect the ends of chromosomes against exonuclease and ligases, to prevent the activation of DNA-damage checkpoints, and to counteract loss of terminal DNA-segments that occurs when linear DNA is replicated^[2,38,39]. Oikawa *et al* demonstrated that oxidative stress induced DNA damage at the 5' site of 5' -GGG-3' in the telomere sequence, and the telomeric G triplet was especially sensitive to cleavage by oxidative damage^[40,41]. Moreover, it was shown that oxidative stress increased the frequency of S1 nuclease-sensitive sites, especially in telomeres^[42,43]. However, it was unknown whether oxidative stress was associated with the telomerase activity in human tissue specimens. In this study, the expression of malondialdehyde, glutathione S-transferase and total anti-oxidative capacity were examined in the same samples. There were higher levels of the expression of glutathione S-transferase and total anti-oxidative capacity in hepatic cirrhosis specimens, while enhanced expression of malondialdehyde was found in HCC specimens. The difference between HCC and hepatic cirrhosis was significant ($P < 0.05$). These findings suggested that the dysfunction of the anti-oxidative system was closely correlated with the progression from hepatic cirrhosis to hepatocellular carcinoma. Other studies also showed that HCC patients with higher anti-oxidative capacity levels survived longer after hepatectomy^[44].

Henle *et al.* found that the telomeric G triplet was especially sensitive to cleavage by oxidative damage. In MRC-5 fibroblasts and U87 glioblastoma cells, oxidative stress-mediated production of single-strand damage in telomeres was concomitant to cell cycle arrest. This response can be modeled by treatment of cells with short single stranded telomeric G-rich DNA fragments. Recovery from it is accompanied by up-regulation of telomerase activity and elongation of telomeres^[45]. The gene transcription of TERT is an essential rate-limiting step in telomerase activation and may be subjected to multiple levels of control and regulated by different factors in different cellular contexts^[46]. In the present study we found that telomerase activity correlated positively with the content of MDA ($P < 0.05$). One possible explanation for this observation was that telomere shortening might be accelerated by oxidative stress when telomere reached critical length, which caused the gene

transcription of TERT, and telomerase was activated. Buchkovich *et al*^[47] first demonstrated that in primary human leukocytes stimulated with phytohemagglutinin, telomerase activity was increased by more than 10-fold as naturally quiescent cells entered the cell cycle. In an animal model, treatment with an antagonist of growth hormone-releasing hormone dramatically decreased telomerase activity in xenografted U-87-MG human glioblastoma cells^[46]. Their research was the first demonstration of a signaling pathway in normal cells that regulated telomerase, and paved the way for experimental analysis of "upstream" regulators. The possibility of a relationship between upstream regulators and oxidative stress is an important issue for future experimental studies on control of telomerase activity.

Although tumor cells have a much shorter telomere length, telomeres shorten with rates between 15 and 76 bp/PD in different culture. Too much time^[48] is needed to reach the critical length of telomeres in tumor cells. However, oxidative stress may increase the rate of telomere shortening by the site-specific DNA damage in the telomere sequence. Thus, combination treatment^[7] of oxidative stress and telomerase inhibitor in cancer cells will accelerate greatly the telomere shortening. Telomeres might shorten quickly to the point which is no longer able to divide.

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