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Expression of telomerase component genes in hepatocellular carcinomas

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Abstract

The aim of the study was to clarify the role of telomerase component genes in hepatocarcinogenesis and to examine both the relationship between the expression of telomerase component genes and histological differentiation in hepatocellular carcinoma (HCC) and the relationship between expression levels of telomerase component genes and telomerase activity in HCCs. Telomerase is a ribonucleoprotein enzyme composed of a template RNA and several proteins. Recently, three such telomerase component genes have been identified: human telomerase reverse transcriptase (hTERT); human telomerase RNA component (hTERC); and telomerase-associated protein 1 (TEP1). The expression of these components was evaluated in 34 HCCs and 24 non-cancerous liver tissues by reverse transcriptase–polymerase chain reaction (RT–PCR). Expression of hTERT mRNA was detected in most HCCs, but not in the non-cancerous tissues ($P < 0.01$). Expression of hTERC was detected in both HCCs and non-cancerous tissues, but the expression level in HCCs was higher than that in non-cancerous tissues ($P < 0.01$) and tended to increase as histological differentiation became less marked. The expression level of hTERT mRNA correlated with relative telomerase activity ($P < 0.01$). These results suggest that telomerase reactivation during hepatocarcinogenesis might be regulated by only hTERT and an increase in telomerase activity level in tumour progression might be regulated by both hTERT and hTERC.

Keywords: hTERT; hTERC; TEP1; Telomerase; Hepatocellular carcinoma

1. Introduction

Telomeres, the natural ends of linear eukaryotic chromosomes, consist of tandem arrays of TTAGGG and help to maintain the normal chromosomal structure and function [1–4]. Since conventional DNA polymerase cannot replicate the chromosomal ends completely, human somatic cells undergo loss of telomeric DNA with each round of cell division [5]. However, germ line stem cells and cancer cells do not exhibit telomeric shortening, because of the presence of telomerase, the reverse transcriptive DNA polymerase that synthesises telomeric DNA [6,7]. Thus, reactivation of telomerase may represent an important step for unlimited proliferation and indefinite survival of stem and cancer cells.

Telomerase is a ribonucleoprotein enzyme composed of a template RNA and several proteins. Recently, the genes specifying the RNA subunit and the protein subunits of telomerase from a wide variety of species, including humans, have been cloned. In humans, three such subunits have been identified and extensively analysed, human telomerase RNA component (hTERC), encoding the RNA component of telomerase [8,9], telomerase-associated protein 1 (TEP1), encoding a telomerase-associated protein of unknown function [10,11] and human telomerase reverse transcriptase (hTERT), encoding the catalytic subunit of telomerase [12,13]. Interestingly, it has been reported that the expression of hTERT mRNA, in particular, significantly correlates with telomerase activity [12]. Moreover, some studies have revealed that the expression of hTERC also has a weak correlation with telomerase activity [14,15].

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Previously we have reported that hepatocellular carcinomas (HCCs), even well-differentiated HCCs, show a high incidence of telomerase activity, whereas non-cancerous liver tissues have weak or no detectable activity [16,17]. Therefore, testing for telomerase activity may be a useful tool for precise and early diagnosis of small differentiated HCCs [17]. Moreover, Nakayama and colleagues have reported that the expression level of hTERT mRNA significantly correlates with telomerase activity in HCCs [18]. However, no detailed quantitative studies on the expression of each telomerase component gene in HCCs and the relationship between the expression of these telomerase component genes and histological differentiation of HCCs have been reported.

2. Patients and methods

2.1. Tissue samples

Thirty-four HCC nodules, 22 adjacent non-cancerous chronic liver disease tissue specimens (6 chronic hepatitis and 16 liver cirrhosis) and two normal liver tissue specimens were obtained from 39 patients who underwent hepatic resection in our university hospital during the past 8 years. The HCC patients consisted of 26 males and 8 females, with ages ranging from 43 to 77 years (mean 61.4 ± 8.5 years). Of the 34 HCC patients, 5 were positive for HBs antigen and 22 were positive for HCV antibody. All samples were rapidly frozen and stored at -80°C until used. Histopathologically, HCCs were classified into well, moderately and poorly differentiated HCCs, according to the Edmondson Steiner grading system [19]. Of 34 HCC nodules, seven were well differentiated, 22 were moderately differentiated, and five were poorly differentiated (Table 1).

2.2. Telomeric repeat amplification protocol (TRAP) assay and hybridisation protection assay

Tissue samples were homogenised in CHAPS lysis buffer. After 20 min of incubation at 4°C, the lysate was centrifuged at 16 000*g* for 20 min at 4°C. The supernatant was rapidly frozen and stored at -80°C. The protein concentration of the extract was measured using a DC protein kit (Bio-Rad, Hercules, CA, USA). Telomerase activity was assayed by the telomeric repeat amplification protocol (TRAP) assay with some modifications [20,21]. In brief, an aliquot of extract containing 0.6 μg tissue protein was incubated with 20 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.005% Tween-20, 50 μM dNTPs, 0.1 μg of TS primer, 5 attogram of Internal Telomerase Assay Standard (ITAS) and 2U of AmpliTaq DNA polymerase at 20°C for 30 min. The reaction mixture was heat-inactivated at 90°C for 3 min and 0.1 μg of CX primer was added. Polymerase chain reaction (PCR) was performed for 31 cycles at 94°C for 45 s, 50°C for 45 s and 72°C for 90 s (2 min for final step). PCR products of telomerase were detected by hybridisation protection assay (HPA), which is a rapid, useful and quantitative method to measure telomerase activity, using an acridinium ester labelled probe [22]. Telomerase activity was inferred from the value measured by HPA using a dose–response curve obtained with serially diluted samples of MKN-1, which is a gastric cancer cell line, and represented as number of MKN-1 cells containing an equivalent activity.

2.3. RNA preparation and expression of telomerase component gene

Tissue samples were rapidly frozen and stored at -80°C until used. To minimise experimental deviation, RNA isolation and protein extraction were performed from the same tissue samples. Frozen tissue (approximately 20 mg) was powdered in liquid nitrogen. RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) and Qiashredder (Qiagen). The expression of telomerase component genes was assayed by RT–PCR [18]. RT–PCR was performed with total RNA (0.1 μg) using the GeneAmp EZ rTth RNA PCR kit (Perkin-Elmer, Foster City, CA, USA). The primers used for amplification were as follows: for hTERT, TRT/U1513 (5′-ACT TTG TCA AGG TGG ATG TGA CGG-3′) and TRT/L253 (5′-ACG GCT GGA GGT CTG TCA AGG TAG-3′); for hTERC, hTR-F (5′-CCT AAC TGA GAA GGG CGT AGG C-3′), hTR-R (5′-CTA GAA TGA ACG GTG GAA GGC G-3′); for TEP1, TLP1/U4792 (5′-CTT GGA ATT GGG TCT GGT CTC TCG-3′) and TLP1/L5102 (5′-CAC AGC AGT AGG GGA TGA GGA AAC-3′); and for G3PDH, G3PDH-F (5′-ACC ACA GTC CAT GCC ATC AC-3′) and G3PDH-R (5′-TCC ACC ACC CTG TTG CTG TA-3′). Total RNA (0.1 μg) from each sample was added to 10 μM of each primer in a buffer containing final concentrations of 5× EZ buffer, 25 mM $Mn(OAc)_2$, 2.5 mM dNTPs and 1U rTth DNA polymerase in a final volume of 20 μl. The PCR thermal cycles were: 94°C for 20 s and 62°C for 45 s for 30 cycles for hTERT, 94°C for 30 s and 65°C for 60 s for 25 cycles for hTERC and 94°C for 30 s and 60°C for 60 s for 25 cycles for TEP1 and G3PDH. The amplified products were fractionated on a 2% agarose gel. Gels were stained with SYBR GREEN I (FMC Bio Products, Rockland, ME, USA) and analysed. The RT–PCR products of hTERT mRNA, hTERC and TEP1 mRNA were quantified using a fluorescence imaging analyser (FUJI FLA-2000, Tokyo, Japan), normalised to the G3PDH RT–PCR signal. The expression level of hTERT mRNA, hTERC and TEP1 mRNA was inferred from the normalised RT–PCR signals using a dose–response

Table 1
Expression of telomerase component genes and telomerase activity in hepatocellular carcinomas (HCCs) and adjacent liver tissues

		HCC					Adjacent liver tissue				
No.	Size (mm)	Histology	Relative telomerase[a] activity	hTERT[b] expression	hTERC[b] expression	TEP1[b] expression	Histology	Relative telomerase[a] activity	hTERT[b] expression	hTERC[b] expression	TEP1[b] expression
1	10	Well	16	0.103	0.101	0.086	LC	< 1	< 0.005	0.038	0.061
2	10	Well	187	4.436	0.037	0.146	LC	< 1	< 0.005	0.064	0.035
3	13	Well	43	2.259	0.011	0.174	LC	< 1	< 0.005	0.006	0.04
4	16	Well	1	0.032	0.014	0.12	LC	< 1	< 0.005	0.024	0.237
5	18	Well	< 1	< 0.005	0.046	0.161	CH	< 1	< 0.005	0.01	0.01
6	18	Well	52	0.009	0.048	0.046	CH	< 1	< 0.005	0.016	0.045
7	20	Well	< 1	0.017	0.024	0.011	LC	< 1	< 0.005	0.005	0.041
8	15	Moderately	35	0.01	0.095	0.026	CH	< 1	< 0.005	0.031	0.043
9	16	Moderately	35	0.047	0.006	0.132	LC	< 1	< 0.005	0.037	0.025
10	18	Moderately	471	0.389	0.095	0.303					
11	20	Moderately	4	0.007	0.026	0.01					
12	22	Moderately	530	1.102	0.057	0.067	LC	< 1	< 0.005	0.02	0.071
13	25	Moderately	376	0.108	0.097	0.093	CH	< 1	< 0.005	0.026	0.08
14	25	Moderately	281	0.077	0.052	0.251	LC	< 1	< 0.005	0.062	1.446
15	30	Moderately	3	0.147	1.23	2.485					
16	30	Moderately	153	0.03	0.007	0.042					
17	30	Moderately	265	0.413	0.403	0.146					
18	32	Moderately	293	0.061	0.008	0.007	LC	< 1	< 0.005	0.012	< 0.005
19	33	Moderately	< 1	< 0.005	0.036	0.006	LC	< 1	< 0.005	0.023	0.147
20	33	Moderately	112	0.084	0.199	0.328					
21	35	Moderately	63	0.15	0.087	0.422					
22	38	Moderately	7	0.007	0.052	0.066					
23	43	Moderately	95	0.239	0.703	0.205					
24	45	Moderately	799	0.673	0.02	0.034					
25	53	Moderately	< 1	< 0.005	0.061	0.206	LC	< 1	< 0.005	0.008	0.062
26	53	Moderately	623	0.047	0.053	0.014					
27	70	Moderately	161	0.048	0.023	0.013					
28	90	Moderately	13	0.005	0.46	0.081	CH	< 1	< 0.005	0.029	0.043
29	130	Moderately	563	0.109	0.117	0.015	LC	< 1	< 0.005	0.063	0.211
30	28	Poorly	199	9.556	0.008	0.079					
31	30	Poorly	179	2.082	0.603	0.046	CH	< 1	< 0.005	0.068	0.056
32	51	Poorly	564	0.527	0.128	0.877					
33	65	Poorly	849	0.374	0.194	0.219	LC	2	< 0.005	0.029	0.082
34	70	Poorly	446	0.047	1.04	0.103					
							LC	< 1	< 0.005	0.075	0.501
							LC	< 1	< 0.005	0.016	0.131
							LC	< 1	< 0.005	0.044	0.093
							normal	< 1	< 0.005	0.007	0.037
							normal	< 1	< 0.005	0.007	0.013

LC, liver cirrhosis; CH, chronic hepatitis; normal, normal liver tissue; well, well differentiated; moderately, moderately differentiated; poorly, poorly differentiated hepatocellular carcinoma.

[a] MKN-1 cell equivalent activity in 0.6 μg protein.

[b] μg-MKN-1 RNA equivalent expression in 0.1 μg total RNA.

curve obtained with serially diluted MKN-1 samples, and represented as MKN-1 RNA producing an equivalent RT–PCR signal.

2.4. Statistical methods

Mann–Whitney U test and Spearman's rank correlation were used to evaluate the statistical significance of differences. A P value of <0.05 was considered significant.

3. Results

3.1. Expression of hTERT mRNA in HCCs and non-cancerous liver tissues

Some examples of telomerase activity and expression of telomerase component genes in HCCs and non-cancerous liver tissues are shown in Fig. 1. The RT–PCR signals of hTERT mRNA were quantitated as previously described. The RT–PCR reaction of hTERT mRNA was confirmed

to be linear when more than 0.005 μg-MKN-1 RNA was used. When RT–PCR signals below this range were tentatively designated as negative, 31 of the 34 HCCs were positive, but all of 24 non-cancerous liver tissues were negative (Fig. 2a left). The expression level of hTERT mRNA in HCCs was 0.68 ± 1.80 μg-MKN-1 RNA equivalent, whereas it was not detected in the non-cancerous tissues, a significant difference ($P < 0.0l$). The expression level of hTERT mRNA in well, moderately and poorly differentiated HCCs was 0.97 ± 1.73, 0.17 ± 0.26 and 2.52 ± 4.01 μg-MKN-1 RNA equivalent, respectively (Fig. 2a right). This result indicated that expression of hTERT mRNA occurred at an early stage in hepatocarcinogenesis and that the expression level of hTERT mRNA did not always correlate with the degree of histological differentiation. There was no relationship between hTERT mRNA expression and tumour size.

3.2. Expression of hTERC in HCCs and non-cancerous liver tissues

Similarly, the RT–PCR reaction of hTERC was confirmed to be linear when more than 0.005 μg-MKN-1 RNA was used. When RT–PCR signals below this range were tentatively designated as negative, all of HCC and non-cancerous liver tissues were positive. Thus, hTERC was expressed in both HCC and non-cancerous tissues. Whilst the expression level of hTERC in HCC varied (0.18 ± 0.30 μg-MKN-1 RNA equivalent), that in all non-cancerous tissues was below 0.1 μg-MKN-1 RNA equivalent (0.03 ± 0.02 μg-MKN-1 RNA equivalent), a significant difference ($P < 0.01$) (Fig. 2b left). The expression level of hTERC tended to increase as the degree of histological differentiation became less marked. The expression level of hTERC in well differentiated HCC was similar to that in non-cancerous tissues, whereas that in moderately or poorly differentiated HCC was often higher than that observed in non-cancerous tissues (Fig. 2b right).

3.3. Expression of TEP1 mRNA in HCCs and non-cancerous liver tissues

Similarly, the RT–PCR reaction of TEP1 was confirmed to be linear when more than 0.005 μg-MKN-1 RNA was used. When RT–PCR signals below this range were tentatively designated as negative, all HCC and 23 of 24 non-cancerous liver tissues were positive with no significant difference between them (0.20 ± 0.43, 0.15 ± 0.30 μg-MKN-1 RNA equivalent) (Fig. 2c left). Thus, it was thought that TEP1 was mostly expressed in HCCs and non-cancerous tissues, as reported previously [23]. There was no correlation between expression of TEP1 and histological differentiation (Fig. 2c right).

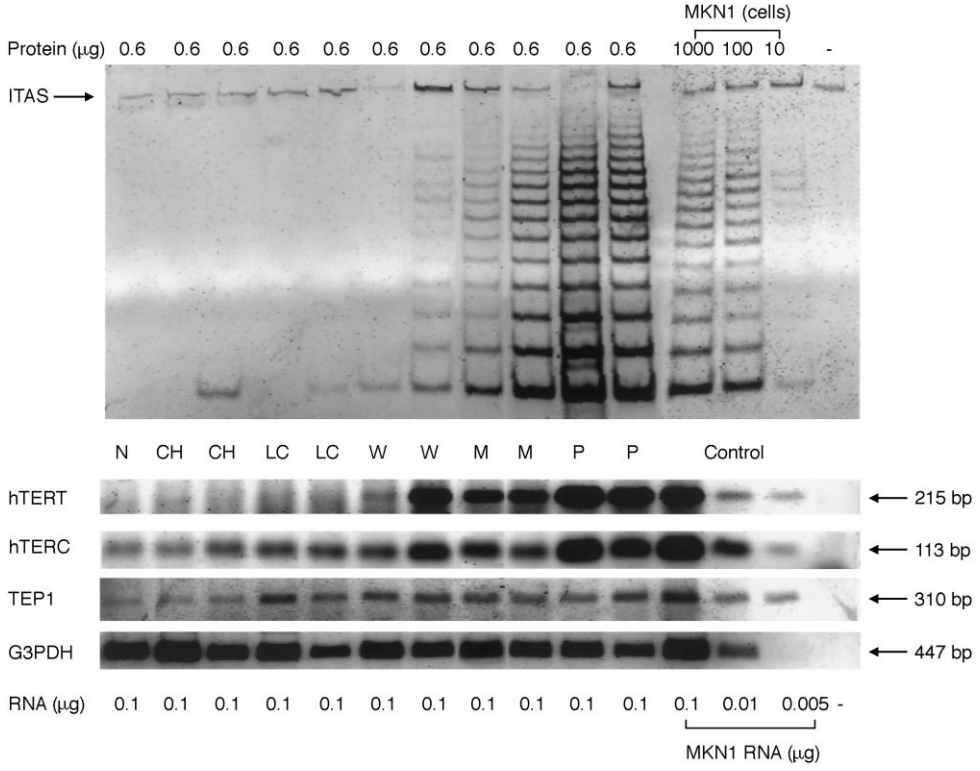

Fig. 1. Expression of telomerase component genes and telomerase activity in various liver tissues. N, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; W, well; M, moderately; P, poorly differentiated hepatocellular carcinoma; MKN-1, gastric cancer cell line as positive control; (−), negative control without sample extract; ITAS, internal telomerase assay standard.

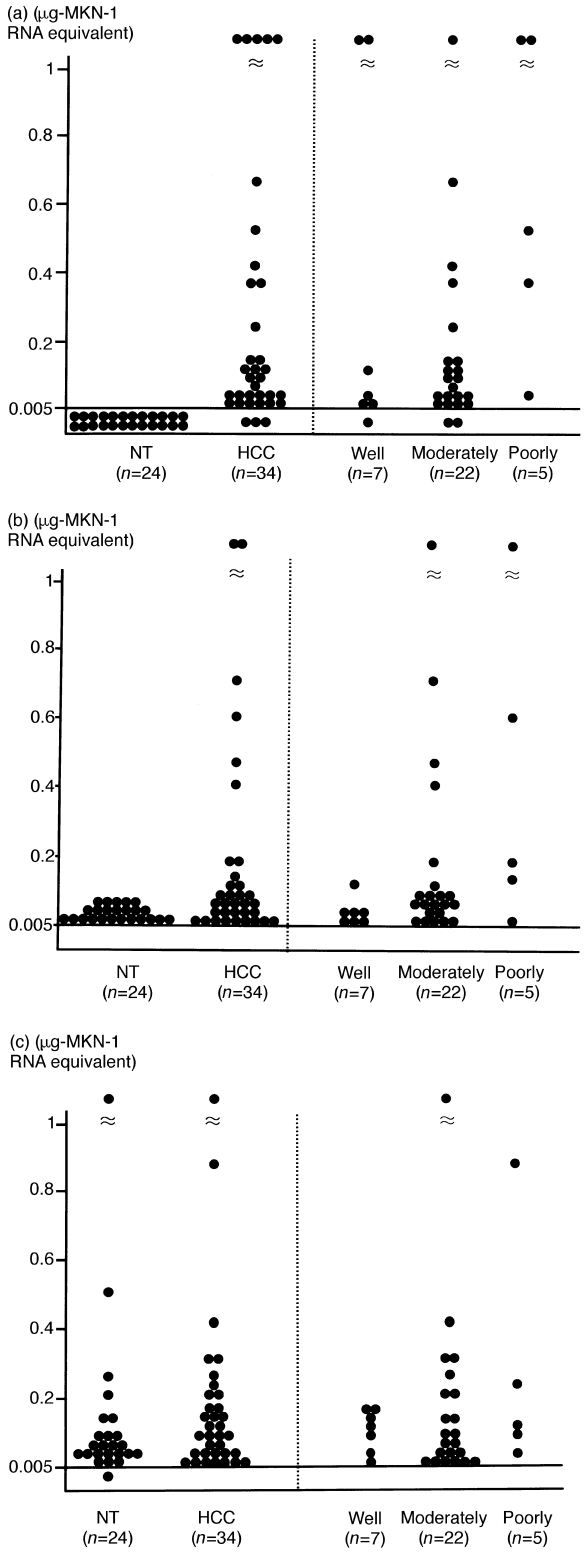

Fig. 2. Expression level of telomerase component genes in hepatocellular carcinomas (HCC) and non-cancerous liver tissues and the relationship between telomerase component genes and histological differentiation in HCC. (a) hTERT mRNA expression; (b) hTERC expression; (c) TEP1 mRNA expression. The expression level of telomerase component genes was represented as the amount of MKN-1 RNA producing an equivalent RT–PCR signal. NT indicates non-tumorous liver tissues.

3.4. Telomerase activity in HCCs and non-cancerous liver tissues

The mean value for relative telomerase activity expressed as cell equivalent activity of MKN-1 was 20.3 cells in non-cancerous liver tissues including chronic liver diseases and 2248 cells in HCC at 0.6 μg tissue protein per assay. There was a significant difference between HCCs and non-cancerous tissues ($P < 0.01$), as previously reported [17]. Telomerase activity significantly increased as the degree of histological differentiation of HCCs became less marked.

3.5. Correlation between expression levels of telomerase component genes and relative telomerase activity in HCC and non-cancerous liver tissues

The expression levels of telomerase component genes and telomerase activity in the 34 HCC were quantified and plotted. The expression of hTERT mRNA and relative telomerase activity in HCCs showed a good correlation in most samples, and the correlation coefficient was 0.63 (Fig. 3). All but one of 24 non-cancerous liver tissues were in the bottom left corner of Fig. 3, i.e. hTERT mRNA expression was below 0.005 μg-MKN-1 RNA equivalent and relative telomerase activity was below one MKN-1 cell equivalent, although the relationship between hTERT mRNA and telomerase activity in non-cancerous tissues are not indicated in Fig. 3. There was no relationship between hTERC expression and relative telomerase activity, nor between TEP1

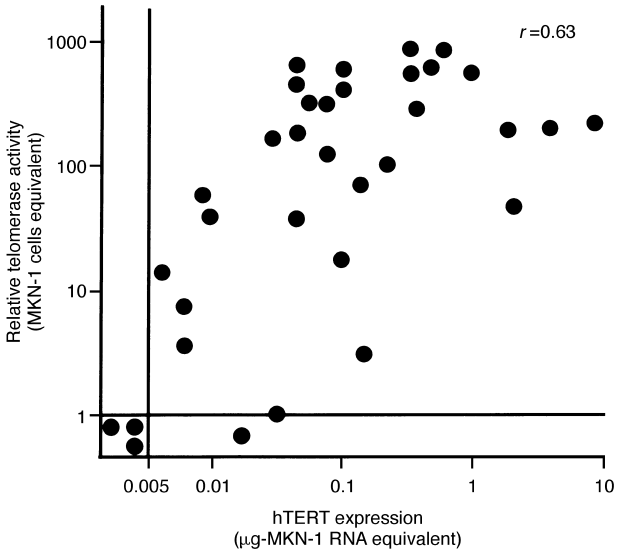

Fig. 3. Correlation between hTERT mRNA and telomerase activity in hepatocellular carcinomas (HCCs). The expression level of hTERT mRNA below the 0.005 μg-MKN-1 RNA-equivalent and relative telomerase activity below the one MKN-1 cell-equivalent were considered as negative or very weak. The expression level of hTERT mRNA correlated with relative telomerase activity in most cases.

mRNA expression and relative telomerase activity (data not shown).

4. Discussion

hTERT has been identified as the catalytic subunit of telomerase. Recently it was reported that hTERT mRNA is expressed in several cancers such as HCC, cervical cancer, urothelial cancer, gastric cancer, colon cancer and leukaemia [18,24–29]. These studies demonstrated that expression of hTERT mRNA is high in cancerous lesions but not in non-cancerous tissues. Some studies also demonstrated the expression of hTERC and TEP1 mRNA in addition to hTERT mRNA, but detailed quantitative studies have not been done [18,23,24,26,29]. In a parallel assay using the gastric cancer cell line MKN-1 as a positive control, we could semi-quantify the expression of telomerase component genes. Thus, our study is the first report that revealed the expression of three such telomerase component genes quantitatively in liver tissues.

Our data demonstrated that hTERT mRNA was expressed in most HCCs but not in non-cancerous liver tissues. The results also demonstrated that the expression level of hTERT mRNA correlated with relative telomerase activity in most cases. Therefore, these results supported the fact that hTERT is the primary determinant regulating telomerase activity in HCC [12,13]. We demonstrated that hTERT mRNA was frequently expressed even in well differentiated HCC, indicating that expression of hTERT mRNA occurred at an early stage in hepatocarcinogenesis. Our previous study showed that telomerase activity significantly increased as the differentiation status of HCC decreased [17]. Our present study demonstrated the correlation between the expression level of hTERT mRNA and relative telomerase activity. These observations taken together suggest that the expression level of hTERT mRNA tended to increase as the degree of histological differentiation of HCCs became less marked, although there were a few exceptions in this study. Whilst the induction of hTERT expression and appearance of telomerase activity clearly coincided with the occurrence of HCC, the increase in the level of telomerase activity during tumour progression was regulated not only by the expression of hTERT but also by that of hTERC.

hTERC was expressed in both HCC and non-cancerous liver tissues, as reported previously [9], but the expression level of hTERC in HCC was significantly higher than that observed in non-cancerous tissues. The expression level of hTERC increased as the HCC tissue became less differentiated. It should be emphasised that the expression level of hTERC in well differentiated HCCs was similar to that observed in non-cancerous tissues, whereas that observed in moderately or poorly differentiated HCCs was often higher than that detected in non-cancerous tissues. These results allow us to speculate that the expression of hTERC, in addition to hTERT, might also increase telomerase activity level during tumour progression of HCCs. TEP1 mRNA was also expressed in both HCCs and non-cancerous liver tissues. The expression level of TEP1 mRNA correlated with neither relative telomerase activity nor with histological differentiation, as reported previously [10,11]. A recent study has suggested the possibility that post-translational modification of TEP1 may regulate telomerase activity [11], but the expression level of TEP1 mRNA seems to be independent of telomerase activity in HCC.

In conclusion, we have demonstrated that hTERT mRNA was expressed not in non-cancerous liver tissues but in HCCs, even in well-differentiated HCC, and that the expression level of hTERT mRNA correlated with relative telomerase activity in most cases. We also demonstrated that hTERC expression increased as the degree of histological differentiation of HCCs decreased. These results suggest that the onset of telomerase reactivation in hepatocarcinogenesis might be regulated by the expression of hTERT and an increase in telomerase activity level might be regulated by both hTERT and hTERC in tumour progression of HCCs.

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