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Immuno-histochemical detection of human telomerase reverse transcriptase in human liver tissues

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Although telomerase activity in hepatocellular carcinoma (HCC) increases in accordance with degree of histological undifferentiation, it is unknown whether the level of telomerase activity in HCC reflects of the degree of activity in individual cells or the frequency of telomerase-positive HCC cells. Non-cancerous liver tissues exhibit low but significant levels of telomerase activity, but the nature of telomerase-positive cells in these tissues is unclear. In this study, we performed immunohistochemical staining using specific antibody against telomerase reverse transcriptase (hTERT) protein in 15 HCC samples and 13 adjacent non-cancerous liver tissues. There were hTERT-positive hepatocytes, though very low frequency, in non-cancerous liver tissues. The frequencies in hTERT positive hepatocytes were very well correlated with clinicopathological parameters and telomerase activity levels: the average frequencies of chronic hepatitis was 0.2%, liver cirrhosis 0.2%, well-differentiated HCC 3.0%, moderately differentiated HCC 28%, and poorly differentiated HCC 95%. The intensity of staining varied among cells within a given specimen, and correlation with degree of histological undifferentiation was less obvious. Portions of migrating lymphocytes and biliary epithelial cells were also hTERT-positive. These findings indicate that the up-regulation of telomerase activity with degree of undifferentiation of HCC is mainly due to the increase in frequency of hTERT positive HCC cells. *Oncogene* (2000) **19,** 3888–3893.

Keywords: human telomerase reverse transcriptase; hepatocellular carcinoma; immunohistochemical detection

Introduction

Telomeres are specialized structures at the ends of eukaryotic chromosomes and appear to function in chromosome stabilization, positioning, and replication (Blackburn *et al*., 1984; Zakian, 1989; Blackburn, 1991). Telomeric DNA is shortened at each cell division. When the length of telomeric TTAGGG repeats is shortened, cells stop dividing. Normal human somatic cells correspondingly have a limited proliferative capacity. Telomerase, a reverse-transcriptive DNA polymerase that synthesizes telomere DNA to compensate for the loss of telomeric DNA that occurs at each cell division, plays a major role in maintaining telomere length and in indefinite replicative life span (Blackburn, 1992; Counter *et al*., 1992; Harley *et al*., 1991). Telomerase is expressed in most cancer cells and in a few types of somatic cells of cell renewal systems such as lymphocytes, intestinal mucosal cells, and skin basal cells, but not in the majority of somatic cells (Kim *et al*., 1994; Greider, 1998; Weng *et al*., 1998). Generally, malignant tumors have strong telomerase activity, while the activity in surrounding non-cancerous lesions is weak or below detectable level. Thus, reactivation of telomerase represents an important step toward unlimited proliferation and indefinite survival of cancer cells. We previously reported that hepatocellular carcinomas (HCC) exhibited a high incidence of telomerase activity and that telomerase activity increased in accordance with degree of histological undifferentiation of HCC (Tahara *et al*., 1995; Nakashio *et al*., 1997). The level of expression of hTERT mRNA determined by RT–PCR was very well correlated, in both non-cancerous tissues and HCC tissues with telomerase activity level (Nakayama *et al*., 1998; Takahashi *et al*., 2000). Since these previous studies were based on the analyses in whole tissue extracts, it was unclear whether the level of telomerase activity observed reflected that in individual cells or the frequency in telomerase-positive cells. We also previously reported that such non-cancerous liver tissues as chronic hepatitis and liver cirrhosis exhibited low but significant levels of telomerase activity (Tahara *et al*., 1995; Nakashio *et al*., 1997; Takaishi *et al*., 2000). We did not know whether the low telomerase activity in these tissues was derived from hepatic cells or migrating lymphocytes. *In situ* detection of telomerase activity at the individual cell level could answer this question. However, the *in situ* TRAP method is very difficult, and has been applied only to limited specimens such as free cells or fresh-frozen tissues (Ohyashiki *et al*., 1997). Immunostaining of hTERT protein can now be used as applicable for formalin-fixed paraffin-embedded tissue sections. Recent *in situ* studies have revealed that hTERT protein is expressed only in cells and tissues positive for telomerase activity, i.e., tumor cells and such normal somatic cells as presumptive stem cells and progenitor cells in physiological cell renewal systems, and that hTERT protein is not detected in normal somatic cells that lack

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telomerase activity (Tahara *et al*., 1999; Yasui *et al*., 1999). In the present study, it was clearly observed that the frequency of hTERT-positive cells was low in well-differentiated HCC and high in moderately or poorly differentiated HCC, while no significant difference was found in variation in staining intensity among histologically different HCC cells. We also found, though at low frequency, hTERT-positive hepatocytes and biliary epithelial cells in non-cancerous liver tissues.

Results

Nuclei of poorly differentiated HCC were stained with anti-hTERT antibody (Figure 1a), but not with antibody pre-absorbed with hTERT peptide used as immunogen (Figure 1b), indicating that positive signal in carcinoma cells was specific. Pre-immune antibody did not give positive staining (data not shown). Intensity of staining was not uniform among cells: some cells were stained strongly, but others weakly or not all (Figure 1a). In addition, nuclei of moderately differentiated HCC were stained with specific antibody against hTERT (Figure 2a). The frequency of nuclei stained with the specific antibody was low in well-differentiated HCC (Figure 2b). To obtain quantitative estimations of the expression of hTERT in HCC, we assessed hTERT-positive cells with respect to both frequencies in positive cells and intensity of the positive signal. The frequency in hTERT positive cells was determined by random evaluation of 500–1000 cell nuclei in several microscopic fields. Clinicopathological parameters and frequencies of telomerase-positive hepatocytes are summarized in Table 1. The frequency of hTERT-positive cells was very well correlated with clinicopathological parameters: CH (0.2%), LC (0.2%), well-differentiated HCC (3.0%), moderately-differentiated HCC (28%), and poorly-differentiated HCC (95%). The frequency of hTERT-positive cells was well correlated, with a few exceptions among well-differentiated HCCs, with telomerase activity level in tissue extracts (Figure 3), although not all samples used for immuno-histological examination were preserved for assay of telomerase activity. On the other hand, the signal intensity varied from weak to strong among HCC cells within a given specimen, as shown in Figures 1a and 2a. The signal intensity of hTERT-positive cells in the well-differentiated HCC shown in Figure 2b was strong. Generally speaking, however, the more malignant the HCC, the more intensely was hTERT expressed at the individual cell level.

Non-cancerous tissues unexpectedly contained a significant number of immuno-reactive hepatocytes (Figure 4). Some infiltrating lymphocytes were also stained (Figures 4 and 5d). In addition to hepatocytes and lymphocytes, a significant number of biliary epithelial cells were also moderately stained as shown in Figure 5. The expression of hTERT was observed more frequently in biliary epithelial cells than in

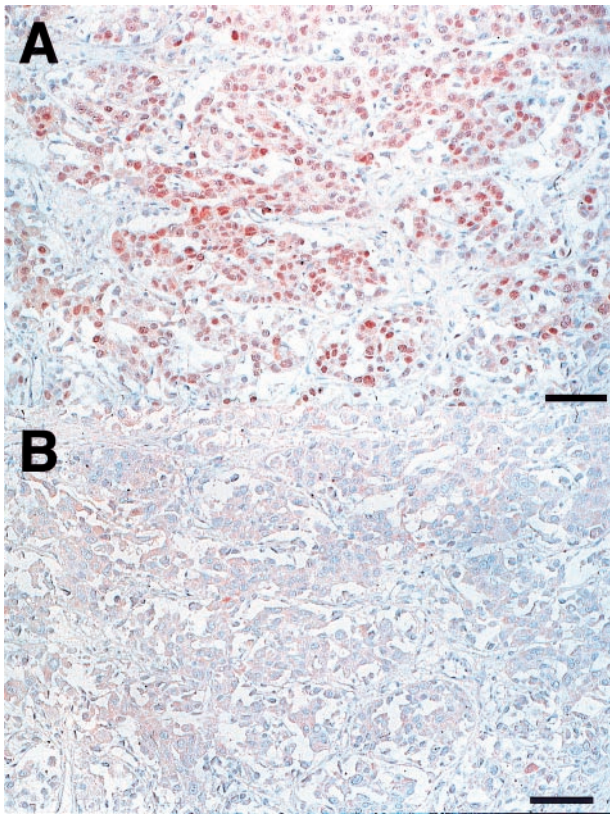

Figure 1 Immunohistochemical detection of hTERT protein in poorly differentiated HCC. A tissue section (case 27) was immunostained with anti-TERT antibody (**a**) or with anti-TERT antibody pre-absorbed with TERT protein-antigen (**b**). Many hTERT-positive cells were found with moderate staining (**a**). Positive reaction was not detected with pre-absorbed antibody (**b**). Bar indicates 100 μm

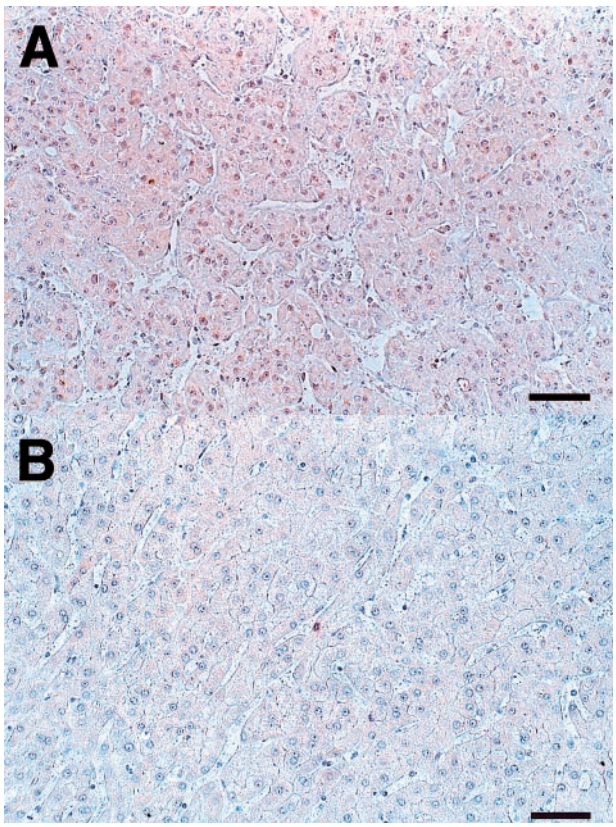

Figure 2 Immunohistochemical detection of hTERT protein in moderately-differentiated (**a**) and well-differentiated (**b**) HCC. In moderately differentiated HCC (case 26), many hTERT positive cells were found with strong staining (**a**). In well-differentiated HCC (case 18), a few hTERT positive cells were found with strong staining (**b**). Bar indicates 100 μm

Table 1 Frequencies of expression of hTERT protein in liver tissues

No	*Histology*[a]	*Tumor size* (mm)	*Frequency of telomerase-positive hepatocytes* (%)	(mean)
1	CH	–	0.1	
2	CH	–	0.2	
3	CH	–	0.2	0.17
4	CH	–	0.4	
5	CH	–	0.1	
6	CH	–	0	
7	LC	–	0.1	
8	LC	–	0.1	
9	LC	–	0.5	
10	LC	–	0.3	0.2
11	LC	–	0.1	
12	LC	–	0.2	
13	LC	–	0.1	
14	Well	10	6	
15	Well	10	8	
16	Well	10	0.8	
17	Well	22	0.8	3
18	Well	26	1.3	
19	Well	30	1.3	
20	Moderately	15	18	
21	Moderately	20	15	
22	Moderately	20	33	
23	Moderately	26	12	28.3
24	Moderately	30	20	
25	Moderately	30	20	
26	Moderately	60	80	
27	Poorly	30	90	
28	Poorly	51	100	95

[a]CH, chronic hepatitis; LC, liver cirrhosis. W, well-differentiated; M, moderately differentiated; P, poorly differentiated hepatocellular carcinoma

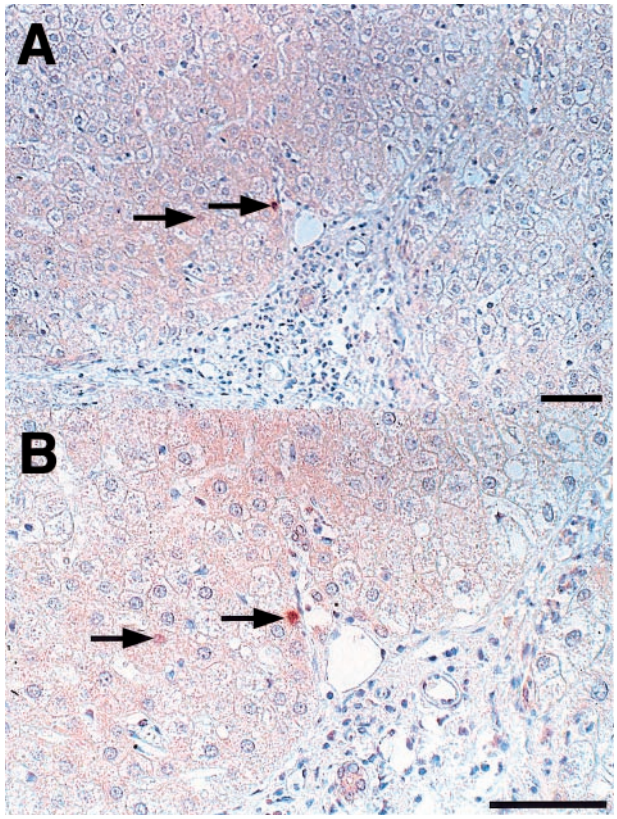

Figure 4 Immunohistochemical detection of hTERT protein in hepatocytes (case 3, CH). Tissue sections were immunostained with anti-TERT antibody. A few hTERT positive hepatocytes and migrating lymphocytes were detected (**a**). (**b**) Higher magnification of (**a**). Bar indicates 100 μm

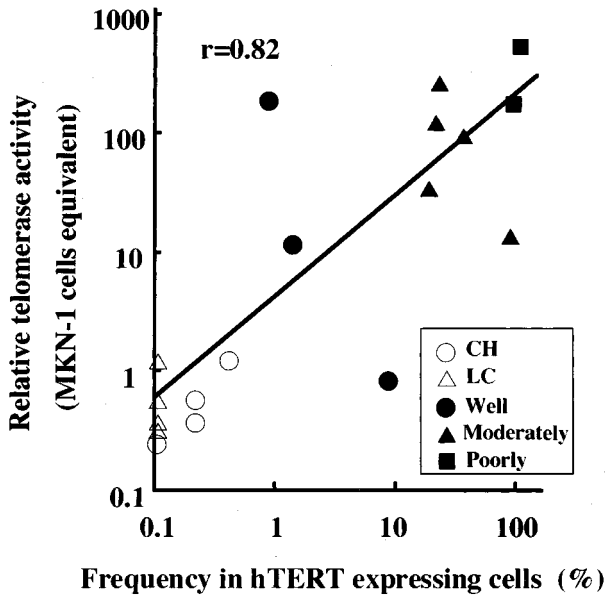

Figure 3 Correlation between telomerase activity and frequency of hTERT-positive cells. Abscissa: percentage of hTERT-positive cells. Ordinate: telomerase activity (number of MKN-1 cells giving telomerase activity equivalent to that in 0.6 μg protein extracted from liver tissue). Open circle, CH; open triangle, LC; closed circle, well-differentiated HCC; closed triangle, moderately-differentiated HCC; and closed square, poorly-differentiated HCC

hepatocytes. Biliary cells were mainly stained in the nuclear periphery or nuclear membrane. Since signal disappeared following pre-absorption of antibody with hTERT peptide used as immunogen, staining was specific for hTERT protein (Figure 5b). As we have previously reported (Tahara *et al*., 1995; Nakashio *et al*., 1997), chronic liver diseases such as chronic hepatitis and liver cirrhosis exhibited low but significant levels of telomerase activity. We did not know at the time of publication of those reports whether the weak telomerase activity was derived from telomerase-positive hepatocytes or from infiltrating lymphocytes. We now know that expression of hTERT protein was detected in hepatocytes in addition to lymphocytes and biliary epithelial cells.

Discussion

We have previously reported that HCCs exhibit a high incidence of telomerase activity, that non-cancerous liver tissues have feeble or no detectable activity, and that telomerase activity increases in accordance with degree of histological undifferentiation of HCC (Tahara *et al*., 1995; Nakashio *et al*., 1997). While the level of expression of hTERT mRNA and level of telomerase activity were not always well correlated in other tissues (Yasui *et al*., 1998, 1999; Ouellette *et al*., 1999; Nakamura *et al*., 1999), they were well correlated in liver tissues, as we previously reported (Nakayama *et al*., 1998; Takahashi *et al*., 2000). It remained unclear, however, whether the increases in the level of telomerase activity with degree of

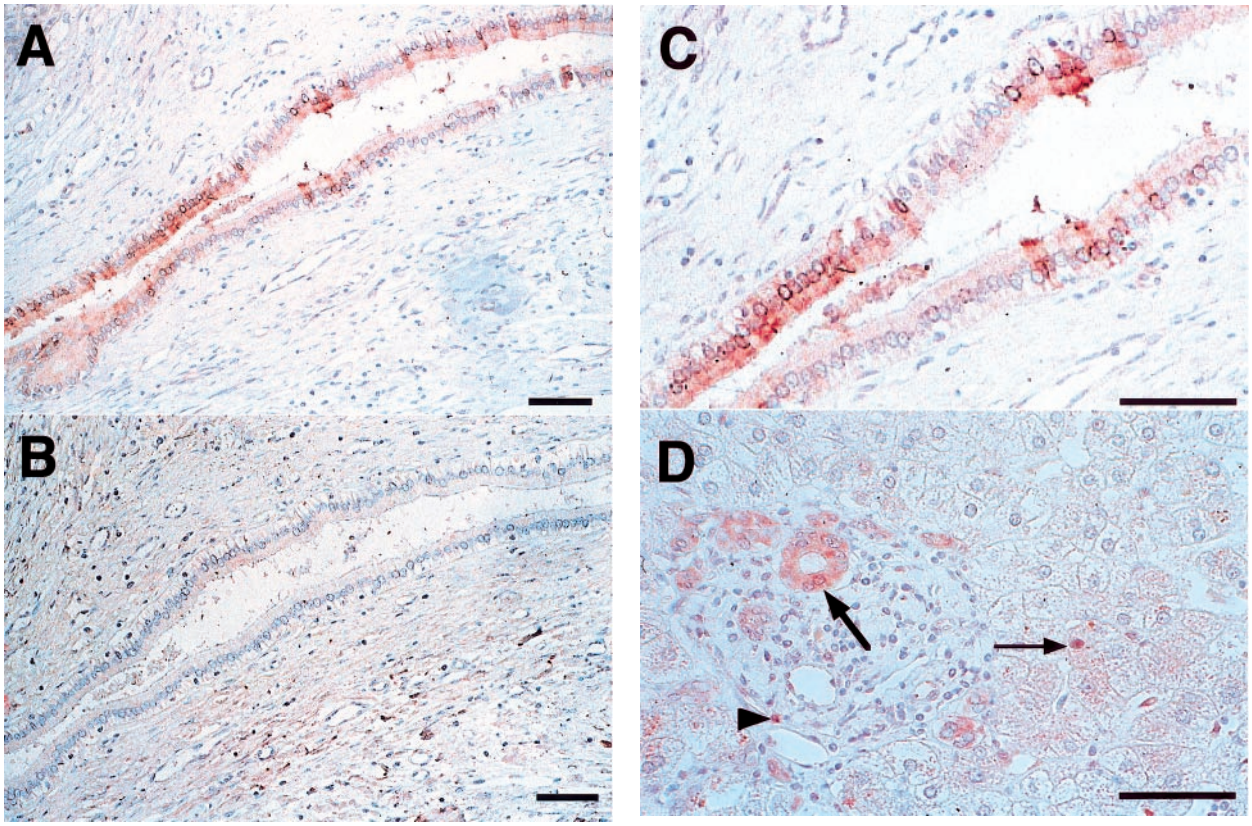

Figure 5 Immunohistochemical detection of hTERT protein in biliary epithelial cells (case 11, LC). A tissue section was immunostained with anti-TERT antibody (**a**,**c**,**d**) or with anti-TERT antibody pre-absorbed with TERT protein-antigen (**b**). (**c**,**d**) are higher magnifications of a longitudinal section and a cross-section of a biliary ductule, respectively. Thick arrow indicates biliary epithelial cell. Thin arrow indicates hepatocyte. Arrowhead indicates migrating lymphocyte. Bar indicates 100 μm

histological undifferentiation of HCC correlates with the increase in telomerase activity in individual carcinoma cells or with the increase in frequency of telomerase-positive carcinoma cells. In the present study, it was clearly observed that the frequency of hTERT-positive cells was correlated with the increase in telomerase activity level (Figure 3). Although the intensity of immunostaining in moderately or poorly differentiated HCC was often higher than that in well-differentiated HCC or non-cancerous liver tissues, the signal intensity varied widely among cells even within one tissue sample, and the correlation between the signal intensity and degree of histological undifferentiation was less obvious. These findings indicated that the increase in telomerase activity level with degree of undifferentiation of HCC was mainly due to the increase in frequency of hTERT-positive cells. In well-differentiated HCC nodules, the majority of HCC cells were telomerase-negative and only a very small number of cells were telomerase-positive. This might be explained by two different ways. First, many, if not all, cells in an HCC nodule had the potential to express telomerase activity but were negative at the time of examination due to arrest of proliferation. Alternatively, all HCC cells in the nodule had progressed more malignant phenotype as diagnosed by cytological examination, but only a small portion of cells had an additional mutation resulting in expression of telomerase and had indefinite proliferative capability. Since, that more malignant part of HCC sometimes arose as a 'nodule-in nodule' within the less malignant part of a nodule, the latter explanation appears more likely, although it does not apply in all cases of HCC.

We have previously reported that tissues of non-cancerous liver diseases such as chronic hepatitis and liver cirrhosis exhibited low but significant levels of telomerase activity (Tahara *et al*., 1995; Nakashio *et al*., 1997; Takaishi *et al*., 2000). We did not know whether the low telomerase activity in these tissues was derived from precancerous immortalized hepatic cells or migrating lymphocytes. It was previously reported that hTERC was detected in chronic liver lesions by *in situ* staining, mainly in migrating lymphocytes and sinusoidal cells (Kolquist *et al*., 1998; Ogami *et al*., 1999), but no report was published on *in situ* detection of hTERT in liver tissues. We found here that non-cancerous liver tissues contained hTERT-positive hepatocytes, though at only 0.2% in frequency, in addition to hTERT-positive biliary epithelial cells and migrating lymphocytes. We do not know, however, whether the hTERT-positive hepatocytes in non-cancerous tissues were precancerous immortalized cells or normal hepatic stem cells. Hepatocytes are usually in resting state in normal liver, following injury, however, they regenerate extensively. It was reported that proliferating hepatocytes were found mainly in periportal areas in chronic active hepatitis (Seki *et al*., 1990; Kawakita *et al*., 1992). In experimental animals such as rats, oval cells arose in the periportal area of the liver after treatment with hepatocarcinogens or hepatotoxins, and these cells had the ability to proliferate and differentiated into both mature hepatocytes and biliary epithelial cells (Dunsford *et al*., 1985,

1989; Evarts *et al*., 1989; Elmore *et al*., 1991; Novikoff *et al*., 1996; Golding *et al*., 1995). The presence of oval cells in human liver has been reported, these cells proliferated in the periportal region and were suggested to be derived from a stem cell compartment (De Vos and Desmet, 1992; Haque *et al*., 1996; Demetris *et al*., 1996; Roskams *et al*., 1996; Ruck *et al*., 1996). The largest number of stem cells was observed in the periportal region in fulminant hepatic failure (Baumann *et al*., 1999). The hTERT-positive hepatocytes we reported here were mostly found at or around the periportal region, and they might be candidates for dormant stem (oval) cells. In this study, the expression of hTERT was also observed in biliary epithelial cells. Oval cells, presumptive stem cells, were found among biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis (Crosby *et al*., 1998a,b) and biliary epithelial cells were stained with antiDNA polimerase-α antibody (Seki *et al*., 1991). The hTERT-positive biliary epithelial cells observed in our study might include epithelial stem cells and their progeny. It is unlikely that hTERT-positive biliary epithelial cells are precancerous immortal cells, since they are observed too frequently in non-cancerous tissues. However, the present observations do not provide direct evidence of, but are consistent with, the existence of a human hepatic stem cell. Determination whether such stem cells exist will require a specific marker of such cells.

Materials and methods

Samples

Specimens from 15 nodules of HCC were obtained from 13 patients who had undergone surgical resection at Hiroshima University Hospital and related facilities. Subsequently, samples from 13 adjacent non-cancerous liver tissues were also obtained as six chronic hepatitis and seven liver cirrhosis. Histologic grades of HCC were classified into well, moderately, and poorly differentiated HCC according to the Edmondson–Steiner grading system (Edmondson & Steiner, 1954). Of 15 HCC nodules, six were well-differentiated, seven moderately differentiated, and two poorly differentiated (Table 1). Of 13 HCC patients, two were positive for HBs antigen and 11 positive for HCV antibody. For immunohistochemistry, tissues were fixed in 10% buffer formalin and embedded in paraffin. To examine telomerase activity and expression of hTERT mRNA, tissues were frozen in liquid nitrogen immediately after removal and stored at -80°C.

Antibodies

A part of hTERT cDNA, corresponding to the nucleotide 1687–2042 of the GenBank accession number AF015950 clone, was fused with the GST (glutathione S-transferase) gene, and the fused recombinant protein was produced in *E. coli*. The hTERT recombinant protein antibody (anti-hTERT1.0) was purified by affinity chromatography and used in this study. Validity of antibodies used here was discussed in the previous reports for Western blotting and immunostaining (Tahara *et al*., 1999).

Immunohistochemical detection of hTERT protein

Immunohistochemical staining was performed as described previously (Yasui *et al*., 1998; Tahara *et al*., 1999). In brief, three to five micrometer-thick sections were prepared from paraffin blocks and deparaffinized with graded ethanol. Deparaffinized sections were immersed in methanol containing 0.03% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. Microwave pretreatment in citrate buffer was performed for 10 min three times to retrieve the antigenicity, and the sections were incubated with blocking solution for 10 min. The sections were treated at room temperature with anti-hTERT antibody (3–5 μg/ml) for 90 min. The immuno-complex was visualized by a modification of the immunoglobulin enzyme bridge technique (ABC method) using the SensiTek HRP kit (ScyTek Laboratories, Logan, UT, USA) (Yasui *et al*., 1996). Since 3,3′-diaminobenzidine tetrahydrochloride was used as substrate, positive reaction was detected as a brown color. The sections were weakly counterstained with 0.1% hematoxylin. The specificity of hTERT-immunostaining was determined by pre-absorption of the antibody with an excess of specific antigen.

Telomerase activity

Tissue samples were homogenized in CHAPS lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.1 mM AEBSF (4-(2-aminoethyl)-bebzensulfonyl fluoride hydrochlorine), 0.5% CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), and 10% glycerol) (Kim *et al*., 1994). 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, California, USA). Telomerase activity was assayed by the TRAP (telomeric repeat amplification protocol) assay with some modifications (Kim *et al*., 1994; Tahara *et al*., 1995; Nakashio *et al*., 1997). 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 2 U of AmpliTaq DNA polymerases 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. PCR (Polymerase chain reaction) 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 analysed by electrophoresis on a 12% polyacrylamide gel. The gel was dried on filter paper and exposed to Fuji X-ray film at 80°C for 8 h with an intensifying screen. In some cases, the exposure period was extended up to 72 h. To estimate the levels of telomerase activity in tissue sample, we compared the intensity of the TRAP assay DNA ladder products with that of the ITAS signal using BioMax ID Software (Kodak, Rochester, NY, USA). Extract of a gastric-cancer cell line, MKN-1, was used as a positive control of telomerase activity (Nakashio *et al*., 1997).

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References

Baumann U, Crosby HA, Ramani P, Kelly DA and Strain AJ. (1999). *Hepatology,* **30,** 112–117.

Blackburn EH. (1991). *Nature,* **350,** 569–573.

Blackburn EH. (1992). *Annu. Rev. Biochem.,* **61,** 113–129.

Blackburn EH and Szostak JW. (1984). *Annu. Rev. Biochem.,* **53,** 163 – 194.
Counter CM, Avilion AA, Lefeuvre CE, Stewart NG, Greider CW, Harley CB and Bacchetti S. (1992). *EMBO J.,* **11,** 1921 – 1929.
Crosby HA, Hubscher SG, Joplin RE, Kelly DA and Strain AJ. (1998a). *Hepatology,* **28,** 980 – 985.
Crosby HA, Hubscher S, Fabris L, Joplin R, Sell S, Kelly D and Strain AJ. (1998b). *Am. J. Pathol.,* **152,** 771 – 779.
De Vos R and Desmet V. (1992). *Am. J. Pathol.,* **140,** 1441 – 1450.
Demetris AJ, Seaberg EC, Wennerberg A, Ionellie J and Michalopoulos G. (1996). *Am. J. Pathol.,* **149,** 439 – 448.
Dunsford HA, Karnasuta C, Hunt JM and Sell S. (1989). *Cancer Res.,* **49,** 4894 – 4900.
Dunsford HA, Maset R, Salman J and Sell S. (1985). *Am. J. Pathol.,* **118,** 218 – 224.
Edmondson HA and Steiner PE. (1954). *Cancer,* **7,** 462 – 503.
Elmore LW and Sirica AE. (1991). *Cancer Res.,* **51,** 5752 – 5759.
Evarts RP, Nagy P, Nakatsukasa H, Marsden E and Thorgeirsson SS. (1989). *Cancer Res.,* **49,** 1541 – 1547.
Golding M, Sarraf CE, Lalani E, Anilkumar TV, Edwards RJ, Nagy P, Thorgeirsson SS and Alison MR. (1995). *Hepatology,* **22,** 1243 – 1253.
Greider CW. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 90 – 92.
Haque S, Haruna Y, Saito K, Nalesnik MA, Atillasoy E, Thung SN and Gerber MA. (1996). *Lab. Invest.,* **75,** 699 – 705.
Harley CB. (1991). *Mutat. Res.,* **256,** 271 – 282.
Kawakita N, Seki S, Sakaguchi H, Yanai A, Kuroki T, Mizoguchi Y, Kobayashi K and Monna T. (1992). *Am. J. Pathol.,* **140,** 513 – 520.
Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL and Shay JW. (1994). *Science,* **266,** 2011 – 2015.
Kolquist KA, Ellisen LW, Counter CM, Meyerson M, Tan LK, Weinberg RA, Haber DA and Gerald WL. (1998). *Nat. Genet.,* **19,** 182 – 186.
Nakamura Y, Tahara Eg, Tahara H, Yasui W, Tahara E and Ide T. (1999). *Mol. Carcinog.,* **26,** 312 – 320.
Nakashio R, Kitamoto M, Tahara H, Nakanishi T, Ide T and Kajiyama G. (1997). *Int. J. Cancer,* **74,** 141 – 147.
Nakayama J, Tahara H, Tahara Eg, Saito M, Ito K, Nakamura H, Nakanishi T, Tahara E, Ide T and Ishikawa F. (1998). *Nat. Genet.,* **18,** 65 – 68.
Novikoff PM, Yam A and Oikawa I. (1996). *Am. J. Pathol.,* **148,** 1473 – 1492.
Ogami M, Ikura Y, Nishiguchi S, Kuroki T, Ueda M and Sakurai M. (1999). *Lab. Invest.,* **79,** 15 – 26.
Ohyashiki K, Ohyashiki JH, Nishimaki J, Toyama K, Ebihara Y, Kato H, Wright WE and Shay JW. (1997). *Cancer Res.,* **57,** 2100 – 2103.
Ouellette MM, Aisner DL, Savre-train I, Wright WE and Shay JW. (1999). *Biochem. Biophys. Res. Commun.,* **254,** 795 – 803.
Roskams T, De Vos R and Desmet V. (1996). *Histopathology,* **28,** 291 – 299.
Ruck P, Xiao JC and Kaiserling E. (1996). *Am. J. Pathol.,* **148,** 321 – 329.
Seki S, Sakaguchi H, Kawakita N, Yanai A, Kim K, Mizoguchi Y and Kobayashi K. (1990). *Human Pathol.,* **21,** 1020 – 1030.
Seki S, Sakaguchi H, Kawakita N, Yanai A, Kuroki T, Mizoguchi Y, Kobayashi K and Monna T. (1991). *Hepatology,* **14,** 781 – 788.
Tahara H, Nakanishi T, Kitamoto M, Nakashio R, Shay JW, Tahara E, Kajiyama G and Ide T. (1995). *Cancer Res.,* **55,** 2734 – 2736.
Tahara H, Yasui W, Tahara EJ, Fujimoto J, Ito K, Tamai K, Nakayama J, Ishikawa F, Tahara E and Ide T. (1999). *Oncogene,* **18,** 1561 – 1567.
Takahashi S, Kitamoto M, Takaishi H, Aikata H, Kawakami Y, Nakanishi T, Shimamoto F, Tahara E, Tahara H, Ide T and Kajiyama G. (2000). *Euro. J. Cancer,* **36,** 496 – 502.
Takaishi H, Kitamoto M, Takahashi S, Aikata H, Kawakami Y, Nakanishi T, Nakamura Y, Shimamoto F, Kajiyama G and Ide T. (2000). *Cancer,* **88,** 312 – 317.
Weng NP, Hathcock KS and Hodes RJ. (1998). *Immunity.,* **9,** 151 – 157.
Yasui W, Akama Y, Kuniyasu H, Yokozaki H, Semba S, Shimamoto F and Tahara E. (1996). *J. Pathol.,* **180,** 122 – 128.
Yasui W, Tahara H, Tahara EJ, Fujimoto J, Nakayama J, Ishikawa F, Ide T and Tahara E. (1998). *Jpn. J. Cancer Res.,* **89,** 1099 – 1103.
Yasui W, Tahara EJ, Tahara H, Fujimoto J, Naka K, Nakayama J, Ishikawa F, Ide T and Tahara E. (1999). *Jpn. J. Cancer Res.,* **90,** 589 – 595.
Zakian VA. (1989). *Annu. Rev. Genet.,* **23,** 579 – 604.