Table I. *Anti-FasL mAb Did Not Deplete HBsAg-specific CD8⁺ T Lymphocytes*

Treatment	CD8⁺	CD8⁺IFN-γ⁺ stimulated with: HBV S28–39	CD8⁺IFN-γ⁺ stimulated with: LCMV NP
Control IgG	15.4 ± 0.5	0.18 ± 0.02	<0.02
Anti-FasL	16.1 ± 0.8	0.13 ± 0.02	<0.02
Unmanipulated	9.1 ± 1.1	<0.02	<0.02

IHLs were isolated from control IgG or anti-FasL mAb treated transgenic mice 7 d after the splenocyte transfer, or from unmanipulated transgenic mice. The numbers give the proportion of CD8⁺ cells ([%] = [number of CD8⁺ cells]/[number of total cells] × 100) or CD8⁺IFN-γ⁺ cells ([%] = [number of CD8⁺IFN-γ⁺ cells]/[number of CD8⁺ cells] × 100) ± standard deviation.

of the IFN-γ–producing cells in the total CD8⁺ cells was ~0.18%. This percentage is similar to the percentage of CD8⁺ cells that stained with the phycoerythrin-labeled tetramer consisting of MHC class I molecule (H-2L^d) and the peptide epitope of HBsAg (data not depicted). In contrast, the percentage of IFN-γ–producing CD8⁺ cells specific for LCMV NP peptide was less than 0.02%. The percentage of HBsAg peptide-induced IFN-γ–producing CD8⁺ cells in unmanipulated transgenic mice was also less than 0.02%. Importantly, the anti-FasL mAb treatment did not significantly affect the percentage of IFN-γ–producing CD8⁺ cells. Therefore, the liver protective effect of anti-FasL mAb was not a result of CTL depletion, and thus it was likely due to its neutralizing effect against FasL.

Prevention of Chronic Liver Disease Including Hepatocarcinogenesis by the Administration of Anti-FasL mAb. To evaluate the effect of anti-FasL mAb treatment on the development of chronic liver disease, including HCC, we histopathologically examined the livers of mice that were treated with a control substance or with anti-FasL mAb and assessed tumor development by autopsy. 9 mo after splenocyte transfer, the control mice, which received PBS, displayed portal lymphocytic infiltrates, lobular disarray, and marked variation in the size and shape of hepatocytes, reflecting long-term, persistent hepatitis (Fig. 2 A, left). In contrast, the mice treated with anti-FasL mAb showed minimal inflammatory infiltrates and no dysplastic or preneoplastic changes in the liver (Fig. 2 A, right). After one year or later, all the control animals displayed marked hepatic atrophy and developed multiple liver tumors, most of which were larger than 4 mm in diameter with the largest ranging up to 20 mm in diameter (Fig. 2 B, and Table II). The tumor specimens illustrated the classical histological features of HCC, consisting of relatively poorly differentiated hepatoma cells (Fig. 2 C, left). The surrounding hepatic parenchyma displayed focal lobular inflammatory infiltrates associated with degenerating hepatocytes and marked lobular disarray. In contrast, most of the anti-FasL mAb-treated livers had an almost normal appearance

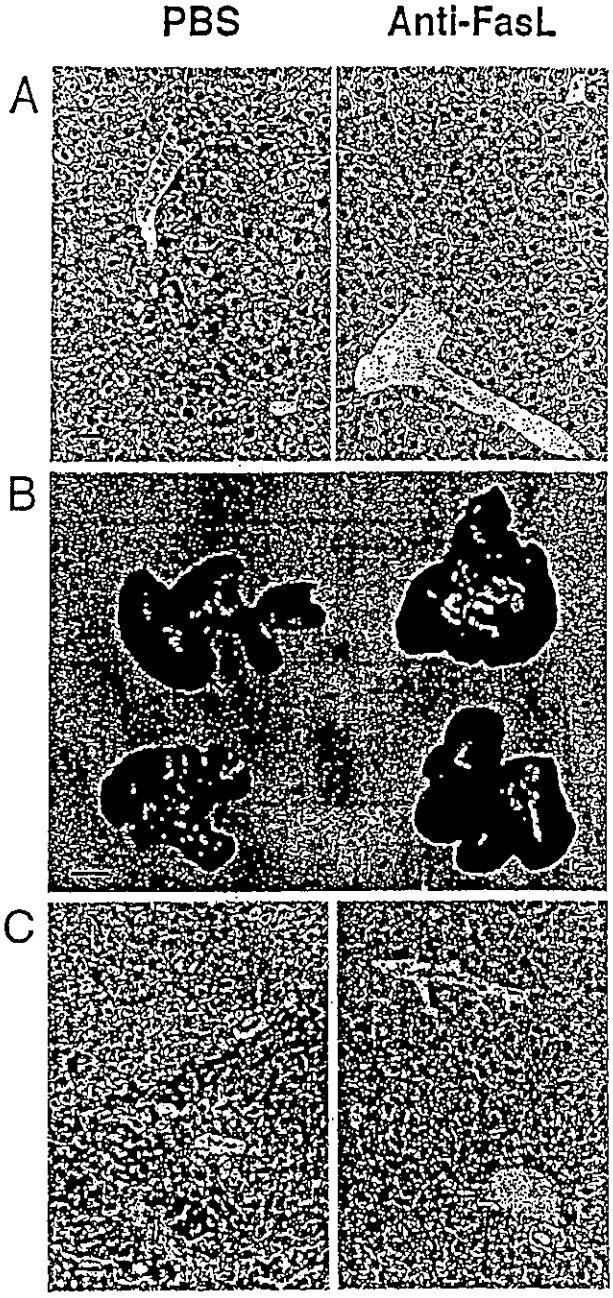

Figure 2. Prevention of progressive liver dysplasia and HCC development by anti-FasL mAb treatment. The transgenic mice described in the legend to Fig. 1 were killed 9 (A) and 15 (B and C) mo after the splenocyte transfer. (A) A 9-mo liver specimen with PBS (left) or anti-FasL mAb treatment (right). (B and C) 15 mo after the splenocyte transfer, livers from PBS-injected animals displayed marked atrophy and multiple liver tumors (arrows) up to 11 mm in diameter (arrowheads; B, left). A representative specimen illustrates the classical histological features of HCC (arrowheads), and the surrounding hepatic parenchyma displays focal lobular inflammatory infiltrates associated with degenerating hepatocytes (arrows; C, left). Most of livers from anti-FasL mAb-injected animals did not show apparent atrophy or liver tumors (B, right). A representative specimen demonstrates minimal portal infiltrates and very mild lobular disarray (C, right). Liver sections were stained with hematoxylin and eosin. The bars represent 40 μm (A and C), and 10 mm (B).

both macro- and microscopically (Fig. 2, B and C, right). Only two of the 15 animals developed solitary liver tumors, one of which was histologically classified as HCC (Table II). Collectively, these results demonstrated that the hepatocarcinogenesis was greatly suppressed by the administration of anti-FasL mAb in this chronic hepatitis model, suggesting that FasL expressed on liver-infiltrating $CD8^+$ T cells activated the caspase cascade in the hepatocytes and induced hepatocellular apoptosis, liver inflammation, regeneration, and the eventual development of HCC.

Discussion

In this model, the major mechanism of hepatocyte injury seemed to be apoptosis, because we observed massive apoptotic hepatocytes associated with an elevation of the serum ALT level. The anti-FasL mAb treatment markedly attenuated the hepatocyte apoptosis. However, because the proportion of HBsAg-reactive $CD8^+$ T cells to the total population of infiltrates in the liver was so low (Table I), it is unlikely that FasL expressed on the surface of CTLs induced all the apoptosis. Recent studies have established that FasL has a proinflammatory activity (21–23). We have previously demonstrated that FasL induces the release of the activated form of proinflammatory cytokines such as IL-1β and IL-18 from neutrophils and/or macrophages (19, 20). Consistent with this, we found strong inflammatory infiltration in the liver and an elevated serum IL-18 level in this model, and anti-FasL mAb treatment reduced them (Fig. 1, B and D). Thus, it is more likely that the massive apoptosis was induced by inflammation that was exaggerated by FasL.

Table II. *Prevention of Hepatocarcinogenesis by Anti-FasL mAb Treatment*

Mouse ID	Mo after spl. transfer	Age (mo) at killing	No. of tumors	Largest tumor (mm)[a]	Tumor histology
Treated with PBS or control hamster IgG (intraperitoneally)					
110	9	16	1	2	Adenoma
113	8	15	2	2	Adenoma
189	9	15	1	4	Adenoma
266	13	17	1	11	HCC
276	15	18	1	5	HCC
323	15	18	2	11	HCC
341	15	18	4	3	HCC
343	14	17	11	3	HCC
391	15	17	3	7	HCC
6	17	19	1	10	HCC
55	17	19	3	10	HCC
59	20	22	2	20	HCC
Treated with anti-FasL mAb (intraperitoneally)					
33	7	17	0	0	
93	7	12	0	0	
96	7	12	0	0	
135	9	16	0	0	
136	13	20	1	3	Adenoma
147	13	20	0	0	
179	12	16	0	0	
287	18	20	0	0	
243	17	23	1	12	HCC
258	18	21	0	0	
283	17	19	0	0	
349	12	14	0	0	
351	15	17	0	0	
360	15	17	0	0	
78	23	25	0	0	

spl., splenocyte.
[a]Diameter in the major axis.

It is widely believed that apoptosis is a mechanism for preventing oncogenesis. Therefore, one might be concerned that inhibition of FasL, an apoptosis-inducing factor, may increase the risk of cancer. However, our observations indicate that anti-FasL mAb prevented both apoptosis and HCC development. The precise mechanism for the HCC prevention by anti-FasL mAb is not perfectly clear at this moment. However, it is likely that inhibition of persistent inflammation is the major mechanism for it, because it has been suggested that persistent inflammation is a strong risk factor for virus-induced hepatocarcinogenesis (8–11), and HCC development in the animal model used here is strictly dependent on an immune response to HBsAg (12). Additional experiments involving other anti-inflammatory treatment are necessary to confirm this notion. In addition, inhibition of hepatocyte proliferation by anti-FasL mAb treatment may be an important factor, because hepatocyte proliferation may enhance the chance of oncogenesis. We found many $PCNA^+$ hepatocytes after the acute-phase injury, and anti-FasL antibody treatment reduced it. It is likely that massive cell loss by inflammation induced the regenerative proliferation of hepatocytes, and FasL might indirectly cause the latter by inducing the former. Alternatively, FasL may be directly involved in the inflammation-induced hepatocyte proliferation, because it was recently reported that FasL is involved in hepatocyte proliferation in the regenerating liver after partial hepatectomy (24).

The model used in this study is of obvious value in terms of the similarity of the disease process to human viral hepatitis. Meanwhile, there may be a need to tone down the view that the model is a faithful representation of the natural disease because these mice do not carry full HBV genome. However, HBV transgenic mouse lineages that contain full viral genome develop neither inflammation nor tumors in the liver spontaneously (25). Additional studies may be of great interest to examine whether adoptive transfer of splenocytes primed with HBsAg or other viral antigens induces persistent liver inflammation and reproduces the process of hepatocarcinogenesis in these lineages.

In any case, we have demonstrated here that the inhibition of FasL activity not only ameliorated acute liver injury but also chronic liver dysplasia and HCC development. These results provide a rationale for developing a therapy for hepatitis using anti-FasL antibody or inhibitors for the Fas signal transduction pathway.

We thank Dr. F.V. Chisari for kindly providing us the HBsAg transgenic mice and for his helpful comments on the manuscript. We also thank Ms. I. Hashitani for secretarial and technical assistance, and Ms. A. Nakano, Ms. Y. Hashizume, and Ms. Y. Hashimoto for technical assistance.

This study was supported in part by Special Coordination Funds for Promoting Science and Technology, and Grants-in-Aid for Scientific Research on Priority Areas (Diagnosis and Treatment of Cancer) from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

Submitted: 19 April 2002
Revised: 12 August 2002
Accepted: 29 August 2002

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Oncogene (2002) 21, 5946–5950

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Identification of serum anti-human telomerase reverse transcriptase (hTERT) auto-antibodies during progression to hepatocellular carcinoma

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Human telomerase reverse transcriptase, hTERT, is the catalytic component of human telomerase. Expression of hTERT confers telomerase activity, indicating that hTERT is the rate-limiting component of human telomerase. Here we report the detection of anti-hTERT auto-antibodies in the sera derived patients with hepatocellular carcinoma using recombinant, purified hTERT as an antigen in an enzyme linked immunosorbent assay (ELISA). The levels of anti-hTERT antibodies in serum correlated with progression to hepatocellular carcinoma. In contrast, we detected only low levels of anti-hTERT auto-antibodies in the sera derived from 18 normal volunteers. The observation of hTERT auto-antibodies in the sera derived from cancer patients suggests that such auto-antibodies constitute novel and specific tumor marker.
Oncogene (2002) **21,** 5946–5950. doi:10.1038/sj.onc.1205788

Keywords: human telomerase reverse transcriptase; auto-antigen; hepatocellular carcinoma

Auto-antibodies against intracellular components can be found in cancer patients (Imai *et al.*, 1992; Nelson, 1977; Schattner *et al.*, 1983; Tan, 1991; Wasserman *et al.*, 1975). For example, auto-antibodies to p53 and cyclin B1 have been reported in certain malignancies (Winter *et al.*, 1992; Covini *et al.*, 1997). Development of antibodies against p53 appears to be dependent on p53 mutations (Winter *et al.*, 1992). Missense mutations of p53 gene result in increased stability, prolong the half-life of the p53 and the resistance to degradation gives rise to the immune response. In the case of auto-antibodies against cyclin B1, dysregulation of cyclins may result in such proteins stimulating autoantibody responses (Covini *et al.*, 1997).

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Received 25 March 2001; revised 6 June 2002; accepted 17 June 2002

Telomerase, multi-subunit specific reverse transcriptase, maintains the ends of eukaryotic chromosomes, termed telomeres. Without a new synthesis of telomeres at chromosome ends the chromosomes shorten with progressive cell division, eventually triggering either replicative senescence or apoptosis when telomere length becomes critically short. The catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), is the rate-limiting factor of enzyme telomerase. Ectopic expression of hTERT is sufficient to restore telomerase activity in cells that lack the enzyme and can immortalize many cell types (Bodnar *et al.*, 1998). Telomerase is tightly repressed in the vast majority of normal human somatic cells but becomes activated during cellular immortalization and in cancers (Shay and Wright, 1996; Kim *et al.*, 1994). The ectopic expression of hTERT with two oncogenes, Ras and SV40 large T antigen, results in the direct tumorigenic conversion of several types of normal human epithelial cells (Hahn *et al.*, 1999; Elenbaas *et al.*, 2001), indicating that hTERT plays an important role in the process of malignant transformation. While the mechanisms for telomerase activation in cancers have not been fully defined, they include telomerase catalytic subunit gene (hTERT) amplification. In humans, telomerase confers immortality upon incipient cancer cells, and telomerase activity is required for the continued cancer growth. Thus hTERT is one of the important proteins involving in the cellular malignant transformation (Hahn *et al.*, 1999; Elenbaas *et al.*, 2001) and might be a potential auto-antigen to induce the auto-antibodies.

Recently we succeeded in producing a recombinant catalytic subunit of telomerase, hTERT, in insect cells (Masutomi *et al.*, 2000). This recombinant hTERT is functional and active biochemically. In this study, we attempted to detect the auto-antibodies against hTERT in cancer patients' sera using this unique reagent.

We used recombinant purified hTERT as the antigen to detect auto-antibodies against hTERT in cancer patients' sera (Masutomi *et al.*, 2000) (Figure 1a). At first, we set out to detect the insect expressed protein by Western blotting analysis with patients' sera. Fractions from several purification steps of cFLAG-hTERT (S1 and S2 fractions) were reacted with

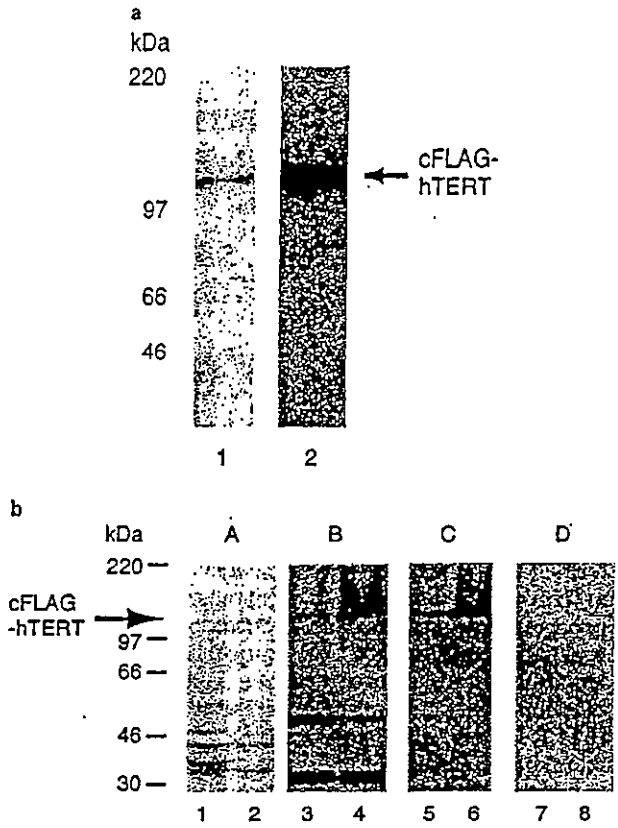


Figure 1 (a) Purified C terminal FLAG tagged hTERT used as an antigen. We purified the recombinant hTERT according to the protocol as we previously reported (Arai *et al.*, 2002; Masutomi *et al.*, 2000). Purified C terminal FLAG tagged hTERT (cFLAG-hTERT) was fractionated by 8% SDS–PAGE and stained with Coomassie brilliant blue (CBB) (lane 1). Western blotting was performed by the standard method with anti-FLAG M2 monoclonal antibody (Sigma Co. Ltd.) (lane 2) (Masutomi *et al.*, 2000). The predicted molecular weight of cFLAG-hTERT is about 127 kDa. Arrow indicates cFLAG-hTERT. (b) Fractions of cFLAG-hTERT protein during the purification procedures. S1 and S2 fractions of High5 cells infected with baculovirus were fractionated (Masutomi *et al.*, 2000). Fractions stained with Coomassie Brilliant Blue (CBB) (A). Same samples were Western blotted against anti-FLAG M2 antibody (B), against serum from a cancer patient serum treated with non-infected High5 cell lysate to absorb nonspecific antibodies (C), and against serum from a healthy volunteer (D), respectively. We obtained the sera from a total of 65 patients (age range, 40–82 years) including 30 patients with hepatocellular carcinoma (HCC), 16 patients with liver cirrhosis (LC), seven patients with chromic hepatitis (CH), 12 patients with other malignancies. As a negative control, we obtained the sera from 18 normal healthy volunteers. These patients gave written informed consent for blood sampling. No significant differences in age and sex were found between the two groups. Proteins transferred onto nitrocellulose membranes were reacted with patient's serum diluted 50 times with TBST buffer (Tris-HCl, pH 7.4, 150 mM NaCl, 1% Tween 20) and anti-human horseradish peroxidase linked whole IgG (Amersham) was diluted 10 000-fold with TBST buffer. Lanes 1, 3, 5 and 7: S1 soluble fraction with buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.1% Nonidet P-40, 150 mM NaCl, 10 mM β-mercaptoethanol). Lanes 2, 4, 6 and 8: S2 soluble fraction with buffer B (20 mM Tris-HCl, pH 7.5, 50% glycerol, 0.5% MEGA-9, 300 mM NaCl, 10 mM β-mercaptoethanol). Arrow indicates cFLAG-hTERT

patients' sera and negative controls (Figure 1b). We examined the same fractions by Western blotting using anti-FLAG M2 monoclonal antibody as a positive control (Figure 1b panel B). The reaction with patient's serum and fraction from purification steps of approximately 127 kDa was specific, whereas specific signals were undetectable in serum from healthy volunteers (Figure 1b panels C and D). We then screened the sera from patients and healthy volunteers by Western blotting with recombinant, purified hTERT as an antigen. The antigen was detected in serum samples from patients with HCC and from patients with colon cancer, stomach cancer and lung cancer but not in patients with liver cirrhosis, chronic hepatitis and normal healthy volunteers (Figure 2a and data not shown). The specific antibodies in the patient's sera were absorbed with purified hTERT (Figure 2b), indicating that the signals detected by Western blotting in serum from cancer patients were specific. These results indicate the presence of circulating anti-hTERT antibodies in the cancer patients' sera. These auto-antibodies are specific in cancer patients since we could not find the reaction in the normal sera and the specific antibodies in the cancer patients' sera were absorbed with the purified hTERT. Western blotting showed that the signals were diminished in serum samples by this procedure.

Next we set up the enzyme linked immunosorbent assay (ELISA) to screen the sera and statistical analyses. Since the optical density increased dose-dependently according to the concentrations of the anti-M2 and anti-mouse IgG antibodies, reactions between the antigen and the M2 antibody were specific (data not shown). The mean optical densities (OD) of serum samples from 30 patients with HCC, 16 with liver cirrhosis, seven with chronic hepatitis, 18 normal healthy volunteers, and 12 patients with other types of cancer were 0.365 ± 0.021, 0.325 ± 0.019, 0.256 ± 0.020, 0.239 ± 0.013 and 0.319 ± 0.027, respectively (Figure 3). The difference of O.D.s between HCC patients' sera and normal sera, and other malignancies and normal sera was statistically significant ($P < 0.0001$ and $P < 0.01$ respectively) (Figure 3). Moreover, we noted that the concentration of auto-antibodies to hTERT increased as the disease progressed in chronic liver disease (Figure 3). In contrast, we could not find antibodies in patients' and normal sera to bovine serum albumin (BSA) to be an irrelevant protein (data not shown). The result that we also could not find antibodies in sera to an irrelevant protein just proves to specificity of the reaction.

In a number of malignant tumors such as hepatocellular carcinoma, colon carcinoma, cervical carcinoma or certain leukemia, the progression of disease has been reported to clearly correlate with the extent of telomerase activity (Nakayama *et al.*, 1998; Kojima *et al.*, 1997; Nouso *et al.*, 1996; Kitamoto and Ide, 1999; Kolquist *et al.*, 1998). Therefore, it would be important to investigate whether the progression of a malignant disease correlates with the amount of hTERT auto-antibodies. The results of the present

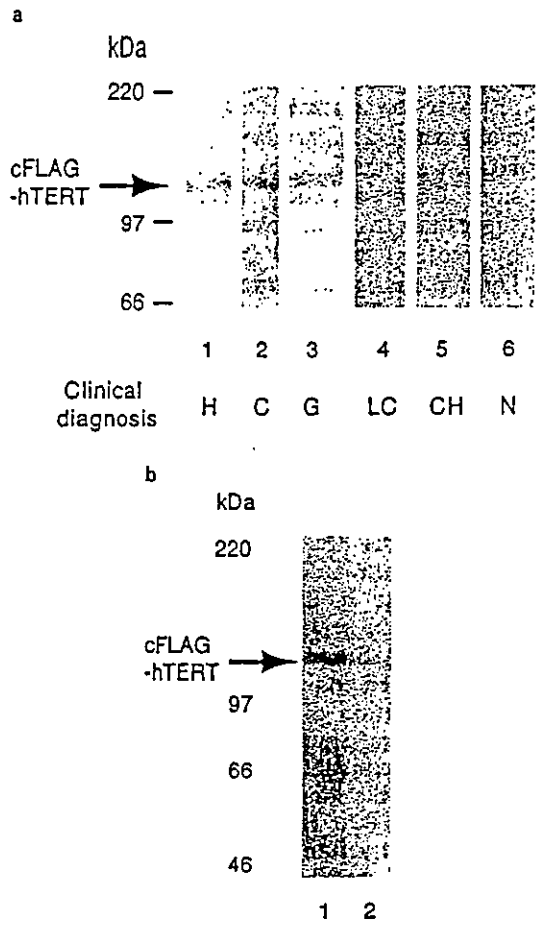


Figure 2 (a) Screening of sera from patients by Western blotting. A total of 83 non-treated sera from patients and healthy volunteers were Western blotted. Only typical results are shown. Lanes 1–3, sera from patients with cancer (H, C and G indicate HCC, colon and gastric cancer, respectively). Lanes 4 and 5, sera from patients with chronic liver disease (LC and CH indicate liver cirrhosis and chronic hepatitis, respectively). Lane 6, sera from healthy volunteers (N indicates sera from normal healthy volunteers). (b) Western blots with non-treated serum and absorbed serum. Lane 1, Western blotting by non-treated cancer patient serum; Lane 2, Western blotting by serum incubated with purified hTERT to absorb anti-hTERT auto-antibodies before blotting. In order to absorb patient's sera with purified protein, patient sera (100 μl) was added to 35 μg of purified hTERT and incubated at room temperature for 2 h, then centrifuged at 10000 g for 10 min. The supernatant was diluted 1:50 with TBST buffer and Western blotted

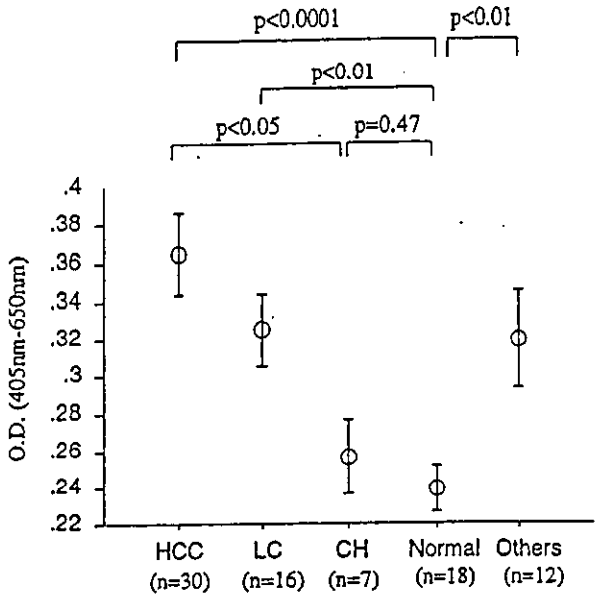
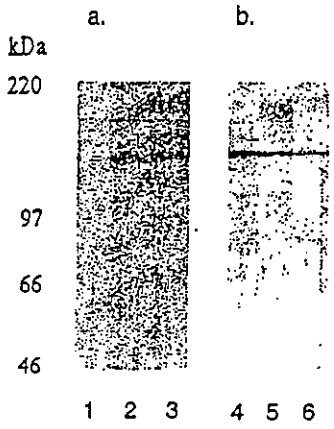


Figure 3 Comparison of optical density (OD) measured by ELISA. Wells of 96-well plates (Nunc) coated with 0.2 μg of C-terminal FLAG tagged hTERT were incubated overnight at 4°C. The residual binding capacity of the plate was blocked with 100 μl of blocking buffer (9.6 mM PBS, pH 7.3, 1% BSA) (PBSB buffer) for 1 h at 37°C. To measure the OD of the reaction, 100 μl of patient's serum diluted 1:100 with PBSB buffer was added to each well and incubated for 1 h at 37°C. After five washes with PBST buffer, 100 μl of anti-human Ig (horseradish peroxidase linked whole antibody) diluted 1:2000 dilution with PBSB buffer was added to the wells. ABTS substrate was added and the OD was measured using a microtiter plate reader with a 405 nm filter according to the manufacturer's instructions (Nakalai). Comparison of OD measured by ELISA in sera from patients with hepatocellular carcinoma (HCC), liver cirrhosis (LC), chronic hepatitis (CH), normal control (normal) and other types of cancer is shown. A total of 12 patients with other types of cancer are seven of gastric cancer, two of lung cancer, one of pancreatic cancer, and two of colon cancer. All data are expressed as means ± standard error of the mean (s.e.m.). Results were statistically analysed using Student's t-test and the χ^2 test

study indicate that auto-antibodies in the serum of some patients with HCC are directed against antigens with functional properties associated with malignant transformation. The difference of optical density (OD) between the patients with HCC and patients with CH was significant. Since the amount of positive telomerase activity increased during hepatocarcinogenesis, we concluded that the significantly increased levels of anti-hTERT were due to the hTERT expression level (Nakayama *et al.*, 1998; Kojima *et al.*, 1997; Nouso *et al.*, 1996). On the other hand, although the OD of anti-hTERT antibodies in patients with LC tended to be lower than that of patients with HCC, the difference was not significant. A previous study, did not detect telomerase activity in most patients with LC or CH, but did identify the activity in several patients without cancer (12.5–42.1%) (Nouso *et al.*, 1996; Kitamoto and Ide, 1999). Conceivably, some regenerative precancerous hepatic nodules or some populations of clonally expanded cells already had telomerase activity even at the LC stage, and this could have induced the immune responses against hTERT. Patients with LC who are anti-hTERT positive may quickly develop HCC, since some clones with early telomerase activity and have high potential to undergo malignant transformation combined with other genetic events.

For one individual who eventually developed HCC, we obtained serum samples and stored at successive office visits. Changes in anti-hTERT auto-antibodies during progression from the non-cancerous to the



c.

Lane (Panel a.)	1	2	3
O.D. (405nm-650nm)	0.31	0.39	0.45

Figure 4 Western blots and ELISA of sera sampled at various stages of disease progression. (a) Patient developed liver cirrhosis and HCC. Western blots of serum sampled at LC (lane 1) and HCC (lanes 2 and 3) stages, respectively. (b) Western blots with anti-FLAG-M2 antibody to confirm equivalent loading of hTERT (lanes 4, 5 and 6). (c) OD measured with ELISA at different stages of disease

cancerous stage of the patient were detected (Figure 4a). We find that a faint band was detected in the patient during the LC stage. The hTERT signal increased after this patient developed HCC. We confirmed with anti-FLAG M2 antibody that the same amount of protein was loaded in each lane (Figure 4b). The specific signal could be recognized clearly at the first time when the patient was diagnosed as HCC. This result was confirmed by ELISA, demonstrating a significant increase in auto-antibodies against hTERT (Figure 4c). Furthermore, a patient with stomach cancer and a patient with lung cancer were tested positive for anti-hTERT, and their sera before discovery of malignancy (before 2 years, and 1 year before cancer appearance, respectively) were analysed. Both sera were negative for anti-hTERT antibodies during the period of no malignancies (data not shown). These results suggest that at least in some patients, a novel immune response to hTERT is driven by the process of malignant transformation, and that specific circulating anti-hTERT auto-antibodies are produced.

Auto-antibodies directed against intracellular antigens located in either nuclear or cytoplasm have been identified in patients with cancer. The prevalence of antinuclear antibodies was significantly increased in hepatocellular carcinoma (HCC) compared with precancerous conditions such as cirrhosis or chronic hepatitis (31 *vs* 13%) indicating that a novel immune response to some cellular antigens develops during malignant transformation (Imai *et al*., 1992; 1993a,b). HCC was preceded by or coincided with seroconversion from antibody negative to positive status, suggesting that the novel immune responses are driven by proteins involved in the transformation process. During malignant transformation of normal cells, many proteins might be over-expressed and involved in the cellular immortalization. Such newly up-regulated proteins might drive the novel immune response as auto-antigens. Many of these auto-antigens are intracellular components, involved in important cell functions (Tan, 1991). Recent evidence suggests that the induction of auto-antibodies against such intracellular components may be relevant to the cancer.

Our results are the first so far reporting that anti-hTERT auto-antibodies could be detected with recombinant hTERT, and that anti-hTERT auto-antibodies in sera are elevated during progression of malignancy. Since the detection of telomerase activity has generated considerable interest as a diagnostic tool, the observation of hTERT autoantibodies in the sera derived from cancer patients suggests that such auto-antibodies constitute novel and specific tumor markers as a possible impact diagnostic tool (Hiyama and Hiyama, 2002). In this sense, these results strongly suggest that serum anti-hTERT auto-antibodies would become a possible novel early tumor marker in hepatocellular carcinoma. Furthermore, since several groups have initiated vaccination strategies to induce anti-hTERT immunity, these studies suggest that cancer patients may well have on-going humoral immune responses to hTERT (Saeboe-Larssen *et al*., 2002; Treon *et al*., 2000; Vonderheide *et al*., 2001; Vonderheide, 2002).

Although the basic mechanisms underlying induction of anti-hTERT auto-antibodies during malignant transformation are unknown at this point, the over-expression of hTERT in the cancer tissues might be involved in the stimulation of novel immune response. Another possibility is that auxiliary factors of telomerase, such as hsp90, p23 and/or hsp70, which are activated during process of malignancy, may positively modulate immunogenicity of hTERT by changing subcellular localization and/or degradation process of hTERT.

Further studies are necessary to clarify the molecular mechanism of induction of the auto-antibodies and to apply this potential novel tumor marker, anti-hTERT antibody, for clinically diagnostic applications.

Acknowledgements
We thank Dr T Yamashita for critical comments and Ms K Kuwabara for excellent technical assistance.

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Journal of Hepatology 37 (2002) 231–239

Journal of Hepatology

www.elsevier.com/locate/jhep

Mutation of p53 gene in regenerative nodules in cirrhotic liver

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Background/Aims: Mutations of p53 gene have been detected in precancerous stages of several cancers, and the possible role in multistep carcinogenesis is suggested. The aim of this study was to examine the mutation profile of p53 gene in regenerative nodules in cirrhotic livers.

Methods: Ninety eight tissue specimens of regenerative nodules obtained from 15 cases of cirrhosis were used for analysis. Twenty cases of chronic hepatitis and two cases of fatty liver were used as controls. DNA was extracted from each of manually demarcated regenerative nodules, and nucleotide sequence analysis was performed on p53 gene exon 5.

Results: Direct sequencing detected p53 mutations in seven of 98 DNA samples (7.1%) from regenerative nodules in six cases of cirrhosis. Subcloning analysis revealed that mutation sites differed in each subclone and the incidences of the mutation varied from 7.7 to 58.8% depending on individual nodules. The mutation was not detected in any of chronic hepatitis and fatty liver. There were inconsistent p53 sequence with regenerative nodules and accompanied hepatocellular carcinomas in six cases.

Conclusions: Mutations of p53 gene were frequently found in cirrhotic livers compared with livers of patients with chronic hepatitis ($P < 0.01$), suggesting that p53 mutations at the stage of cirrhosis may be a causative factor that may potentially lead to hepatocellular carcinoma.

Keywords: Carcinogenesis; Tumor suppressor gene; Hepatitis virus; Hepatocellular carcinoma; Liver cirrhosis

1. Introduction

Mutations or loss of tumor suppressor gene p53 are found in many different kinds of tumors of somatic origin, and are the most frequent molecular alterations in human cancers. The prevalence of p53 mutation is reported from several countries including Japan as approximately 50% in cases of advanced hepatocellular carcinoma (HCC) [1–5].

p53 mutations can be detected in the precancerous stages of affected tissues in colorectal cancers and esophageal cancers, suggesting that inactivation of p53 is involved in a multistep process leading to carcinoma [6,7]. In countries where people are exposed to food contamination by aflatoxin B_1 (AFB_1), high incidences of point mutation of p53 gene at codon 249 are reported for HCCs [8,9] as well as for non-cancerous hepatic lesions [10]. In countries including Japan where such AFB_1 contamination is rare, p53 mutations are associated with poorly differentiated HCC, but generally not well differentiated HCC. Thus, in hepatocellular carcinogenesis, it is accepted that p53 mutations are not associated with precancerous or hyperproliferative hepatic lesions, but are associated with progressive and later stages of HCC [1]. However, to our knowledge, in cirrhosis which may lead to HCC and in adenomatous hyperplasia, a precancerous state of HCC, p53 mutations have not been rigorously examined.

In the present study, we examined mutations in p53 gene exon 5 by direct sequencing of polymerase chain reaction (PCR) products using genomic DNAs extracted from individual regenerative nodules as templates. We further examined the mutation profile using subcloned DNAs.

Received 9 July 2001; received in revised form 15 April 2002; accepted 30 April 2002

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0168-8278/02/$20.00
PII: S0168-8278(02)00144-7

2. Materials and methods

2.1. Tissue preparation

Ninety eight autopsy specimens of regenerative hepatic nodules were obtained from 15 Japanese cases of cirrhosis, 14 of which were complicated by HCC (Table 1). Five cases were seropositive for hepatitis B virus (HBV) antigen, and nine were seropositive for antibodies to hepatitis C virus (HCV). To avoid micro metastasis of HCC, sample regenerative nodules were at least 3 cm distant from HCC, and tissue specimens were diagnosed by at least two pathologists specialized in liver disease. Control samples were obtained by needle biopsy from patients with chronic active hepatitis secondary to HBV (10 cases), HCV (10 cases) or with fatty liver (two cases). All samples were obtained and used with patients' informed consent. Specimens of regenerative nodules in cirrhotic liver, tumor specimens of accompanied HCC, chronic hepatitis lesions, and fatty liver lesions that had been fixed in formalin and embedded in paraffin were cut on a cryostat to obtain serial sections.

Table 1
Patient characteristics

Case	Histology	Age (yrs)	Sex	HBsAg[a]	HCV Ab[b]	HCC[c]
1	Cirrhosis	76	F	+	−	−
2	Cirrhosis	75	F	−	+	+
3	Cirrhosis	58	M	−	+	+
4	Cirrhosis	67	M	−	+	+
5	Cirrhosis	71	M	−	+	+
6	Cirrhosis	62	M	−	−	+
7	Cirrhosis	69	F	−	+	+
8	Cirrhosis	63	M	−	+	+
9	Cirrhosis	72	M	−	+	+
10	Cirrhosis	60	M	−	+	+
11	Cirrhosis	61	M	−	+	+
12	Cirrhosis	50	F	+	−	+
13	Cirrhosis	61	F	+	−	+
14	Cirrhosis	48	M	+	−	+
15	Cirrhosis	73	M	+	−	+
16	Chronic hepatitis	51	M	+	−	−
17	Chronic hepatitis	24	M	+	−	−
18	Chronic hepatitis	59	F	+	−	−
19	Chronic hepatitis	40	F	+	−	−
20	Chronic hepatitis	45	M	+	−	−
21	Chronic hepatitis	36	M	+	−	−
22	Chronic hepatitis	30	F	+	−	−
23	Chronic hepatitis	45	F	+	−	−
24	Chronic hepatitis	46	M	+	−	−
25	Chronic hepatitis	40	M	+	−	−
26	Chronic hepatitis	50	M	−	+	−
27	Chronic hepatitis	50	M	−	+	−
28	Chronic hepatitis	55	M	−	+	−
29	Chronic hepatitis	57	M	−	+	−
30	Chronic hepatitis	53	F	−	+	−
31	Chronic hepatitis	47	F	−	+	−
32	Chronic hepatitis	61	M	−	+	−
33	Chronic hepatitis	48	F	−	+	−
34	Chronic hepatitis	54	M	−	+	−
35	Chronic hepatitis	44	F	−	+	−
36	Fatty liver	42	F	−	−	−
37	Fatty liver	58	F	−	−	−

[a] HBsAg, hepatitis B virus surface antigen; +, presence of serum HBsAg; −, absence of serum HBsAg.
[b] HCVAb, antibodies against hepatitis C virus; +, presence of serum HCVAb; −, absence of serum HCVAb.
[c] Complication of HCC, complication of hepatocellular carcinoma; +, presence of HCC; −, absence of HCC.

2.2. Immunohistochemistry

Three micro meter-thick sections prepared from formalin-fixed tissue specimens derived from six cases of liver cirrhosis were immunohistochemically examined for p53 protein using p53 ES Immunohistochemistry system (OSI, Cambridge, MA, USA). In brief, after deparaffinization, sections placed on slides were pretreated with microwaves. For a reaction with primary antibodies, sections were incubated overnight at 4°C with monoclonal antibodies against p53 (DO-1, 1:10 dilution). Expression of p53 was graded positive when more than 10% of cells showed positive signals for p53. As a negative control, primary antibodies were replaced by phosphate-buffered saline (PBS) in the reaction procedure. As a positive control, sections prepared from formalin-fixed hepatocellular carcinoma specimens were used.

2.3. DNA extraction

For DNA extraction from regenerative nodules, two to five serial sections (4 μm thick) were prepared from each formalin-fixed tissue specimen. After deparaffinization, sections placed on slides were stained with hematoxylin. Under a microscope at a magnification of 40 x, materials within the regenerative nodules were carefully collected from serial sections using a 27G needle. Using this procedure, sharply demarcated regenerative nodules containing sufficient tissue materials in the central region were collected (Fig. 1). DNA extraction was performed using a QIAamp Tissue DNA Kit (QIAGEN, Hilden, Germany).

2.4. PCR amplification

PCR amplification of a portion of DNA sequences covering p53 gene exon 5 was specified by a sense primer (5′-CTCTTCCTACAG-TACTCCCCTGC-3′) and an antisense primer (5′-GCCCCAGCTGCT-CACCATCGCTA-3′) using extracted DNA as a template and Taq polymerase (TaKaRa Ex Taq, TaKaRa, Otsu, Shiga, Japan).

2.5. Sequence analysis

Nucleotide sequence was determined by an automated sequencer Model 373 (PE Applied Biosystems, Foster, CA). Direct sequencing was carried out using a pair of above PCR primers after PCR products were purified with the aid of a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Results of sequencing were examined by naked eye as well. The nucleotide sequence of plasmid DNA was determined using a sense primer (5′-CTAATACGACTCACTATAGGG-3′) and an antisense primer (5′GTTTTCCCAGTCACGACGT-3′). Mutation occurring in p53 gene exon 5 was determined by comparing the sequence of interest with that of the wild-type p53 gene available from sequence data submitted in GenBank U94788.

2.6. Subcloning and sequencing of PCR products

When DNA sequencing revealed that a single regenerative nodule contained mutations of p53 gene, DNA samples from such nodules were all subjected to subcloning. When accompanied HCC contained the mutations, those samples were all subjected to subcloning. As controls, DNA samples from three different regenerative nodules, all of which contained only wild-type of p53 gene, and two different fatty liver lesions were subjected to subcloning.

PCR products purified with the aid of a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) were inserted into pT7 Blue T-vector (Novagen, Madison, WI) and the resulting recombinant plasmids were subjected to transformation. Nine colonies were randomly picked up from transformant colonies derived from each control DNA samples that contained only wild-type of p53 gene. Seventeen colonies were randomly picked up from

Fig. 1. Histological apperance of representative regenerative nodules. (A) nodule 3-A ; (B) nodule 3-B. Boxes indicate the area of DNA exracted. (Case 3, hematoxlylin and eosin, original magnification × 12.5).

transformant colonies derived from each DNA sample that contained mutants of p53 gene. Plasmid DNAs were recovered from subcultured bacteria, and subjected to direct sequencing by the method described above.

2.7. Statistical analysis

Data were analyzed using the χ^2 test for independence, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Immunohistochemistry

When sections from hepatocellular carcinoma were tested as a positive control, brown immunochemical signals for p53 were clearly observed in nuclei of tumor cells (Fig. 2B). Regarding sections from the regenerative nodules in cirrhotic liver, less than 1% of hepatocytes showed weak signals within the nuclei (Fig. 2A). When primary antibodies were replaced by PBS as a negative control, none of the nuclei showed positive signals.

3.2. Direct sequence analysis

Sharply demarcated regenerative nodules were collected from 4 to 17 nodules for each cirrhotic case (Table 2). Ninety eight regenerative nodules from 15 cirrhotic cases, 14 accompanied HCCs, 20 samples with chronic hepatitis, and two samples with fatty liver were examined for the presence of p53 gene exon 5 mutation using direct sequencing (Table 2). DNAs from 91 of 98 nodules (92.9%) contained only wild-type sequence, however, DNAs from the remaining seven nodules (7.1%) obtained from six cases (cases 3–6, 10, 13) contained mutant p53 sequences (Fig. 3). Six out of 15 cirrhotic cases (40.0%) had p53 mutations in their regenerative nodules while none of 20 livers with chronic hepatitis had ($P < 0.01$). Histopathological features of regenerative nodules containing mutations of p53 gene did not differ from those containing only wild-type of p53 gene under a light microscope. On the other hand, DNAs from six of 14 accompanying HCCs (42.9%) found in cirrhotic liver contained mutant p53 sequences (cases 2–4, 7, 11, 13). Three cases (cases 3, 4, 13) had p53 mutations in both regenerative nodules and accompanied HCC.

3.3. Authenticity of direct sequence analysis

To ascertain the authenticity of direct sequence analysis, all seven genomic DNAs from regenerative nodules (nodule nos. 3-B, 3-D, 4-A, 5-A, 6-A, 10-A, 13-A) and all six DNAs from HCCs which showed the presence of p53 mutations by direct sequence analysis, underwent subcloning together with DNAs from one nodule (2-A) of liver without p53 mutations, two nodules (3-A and 3-C) of liver with p53 mutaions and two fatty livers (Table 3). Seventeen transformants were obtained from each DNA sample that contained mutants of p53 gene, and 17 subclones were available for subcloning in all samples except nodule 6-A and HCC sample of Case 3. Nine transformants were obtained from each DNA sample that did not contain the mutants, and nine subclones available in all samples. All 13 genomic DNAs containing p53 mutations by direct sequence analysis demonstrated the presence of subclones with p53 mutations. On the other hand, all five DNAs negative for p53 mutations by direct sequence analysis did not contain subclones with

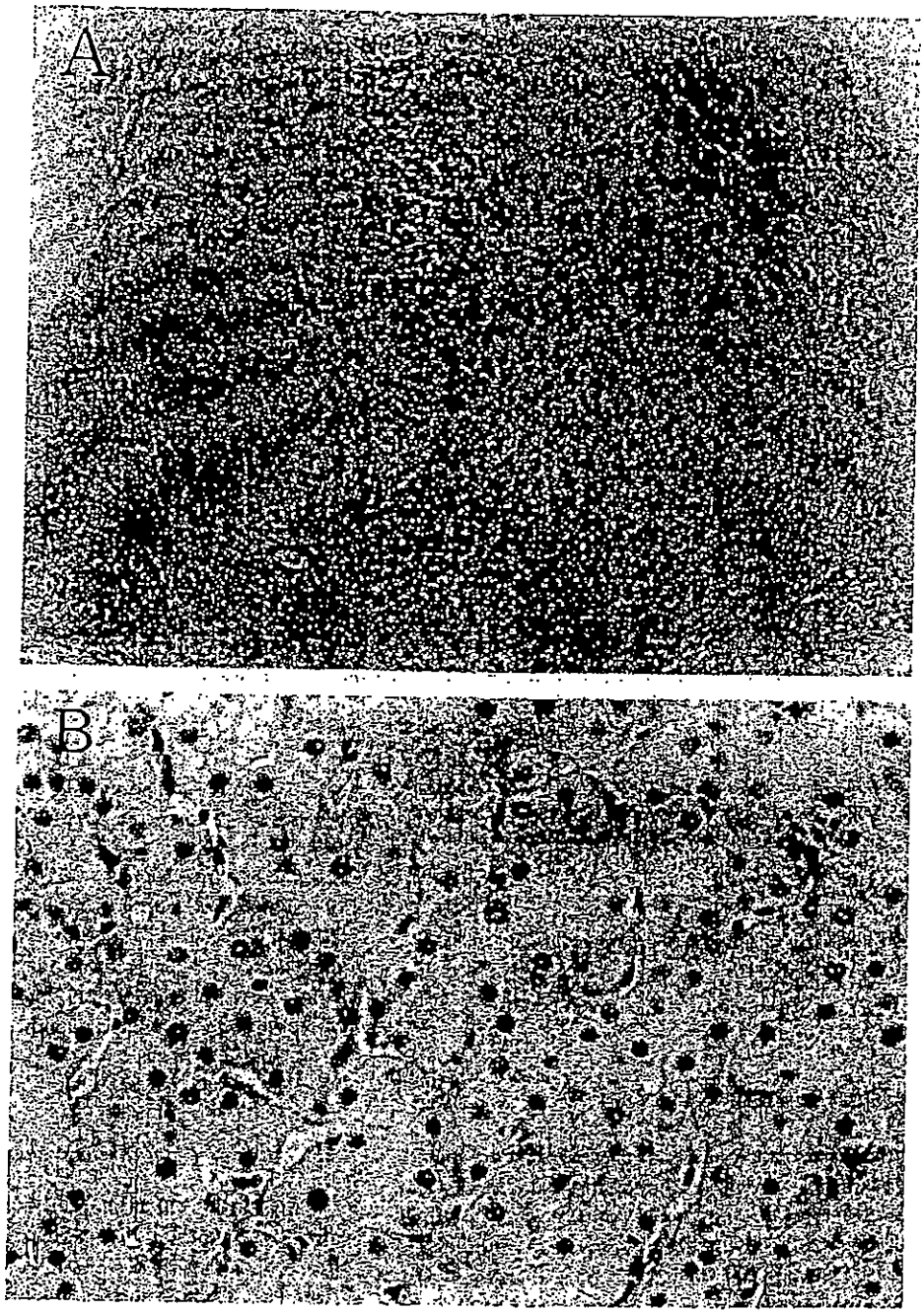

Fig. 2. Immunostaining of p53. A cirrhotic nodule 6-A (A) and a hepatocellular carcinoma were stained with hematoxylin and immunostained with anti-p53 antibody (original magnification A × 25, B × 100).

the mutation. Thus, the direct sequence analysis was found to be accurate.

3.4. Mutation profile of p53 gene

A total of 115 subclones were obtained from the seven regenerative nodules with p53 mutations, and 33 subclones (28.7%) had p53 gene exon 5 mutations. This value varied from 7.7 to 58.8% when assessed for each nodule. Interestingly, all regenerative nodules except nodule 6-A contained multiple p53 mutation sites in a nodule, and 38 mutations, 33 substitutions and five deletions were obtained from the seven regenerative nodules (Table 4). Thus, these results indicated that variety of p53 mutation is occurring in some of regenerative nodules in cirrhotic liver.

Various different nucleotide changes resulting in amino acid replacement were detected in subclones. Fourteen of the 33 substitutions (42.4%) resulted in silent mutation and 10 (30.3%) were C:Gto T:A mutations. There were 21 transitions and 12 transversions. One base deletion from five

Table 2
Summary of direct sequence of p53 gene mutations in cirrhotic liver and others[a]

Histology	No. of nodules or samples	Direct sequence of p53 Exon 5		Direct sequence of accompanying HCC
		Wild-type	Mutant	
Cirrhosis	98	91	7	–
Case 1	11	11	0	–
Case 2	5	5	0	Mutant
Case 3	17	15	2	Mutant
Case 4	5	4	1	Mutant
Case 5	10	9	1	Wild-type
Case 6	5	4	1	Wild-type
Case 7	5	5	0	Mutant
Case 8	5	5	0	Wild-type
Case 9	4	4	0	Wild-type
Case 10	4	3	1	Wild-type
Case 11	5	5	0	Mutant
Case 12	7	7	0	Wild-type
Case 13	5	4	1	Mutant
Case 14	5	5	0	Wild-type
Case 15	5	5	0	Wild-type
Chronic hepatitis	20	20	0	–
Fatty liver	2	2	0	–

[a] All cirrhotic cases except Case 1 were complicated with hepatocellular carcinoma (HCC).

regenerative nodules resulted in frame shift mutation. It is also noted that mutations occurred at a codon resulted in different amino acid replacement depending on the kind of base changes or site of base substitution within a codon.

On the other hand, subclones from six patients with accompanying HCCs that showed p53 mutations comprised 41.6% (42/101) (Table 3). None of 38 p53 mutations found in seven regenerative nodules was found in the accompanying HCCs except one mutation (nodule 3-D, codon 152) indicating that there were inconsistent with subclones of

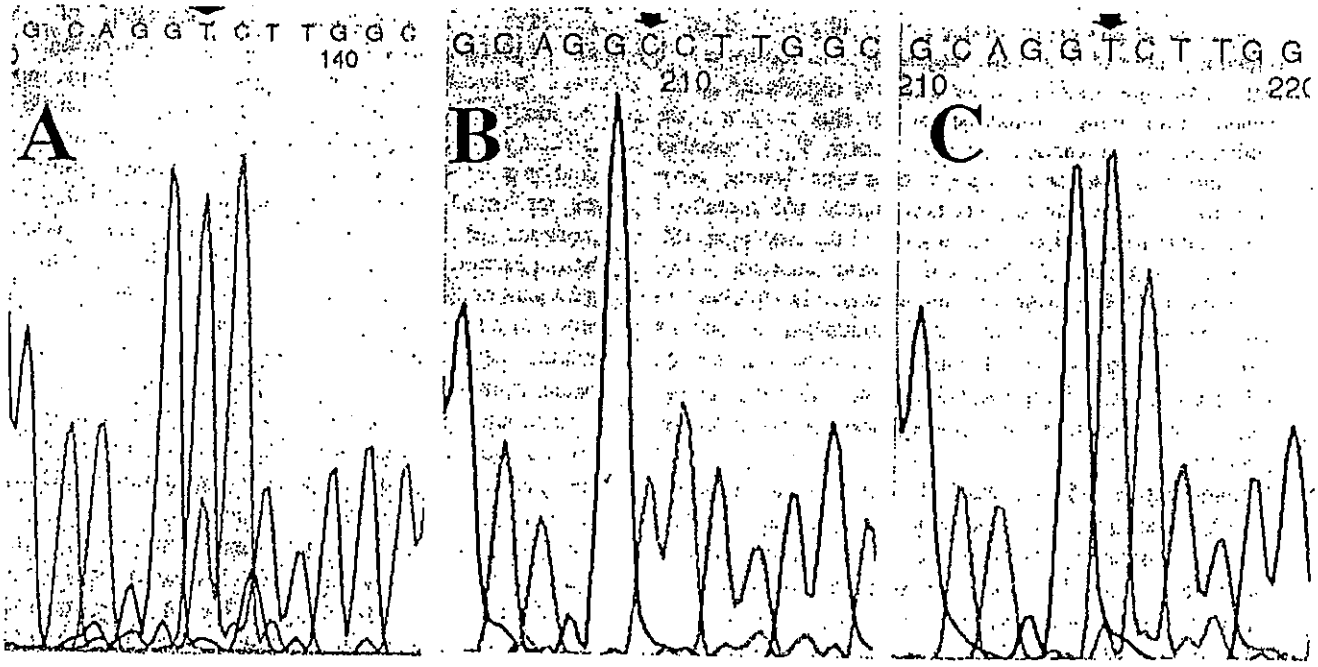

Fig. 3. Sequencing of p53 gene exon 5 (nodule 3-B). Direct sequencing was carried out. An automated sequencer read 'T' which is wild type sequence of p53 gene as indicated an arrow, however, there was a small peak of 'C' by naked eye (A). The DNA sample was subjected to subcloning, and purified DNAs were sequenced. Sequencing revealed the presence of 'C' (B) as well as 'T', and p53 mutation from T to C of the sample was detected.

Table 3
Summary of subcloning analysis

Case	Histology	Nodule No.	Direct sequence of p53 Exon 5	No. of clones	Sequence of p53 Exon 5	
					Wild-type	Mutant (%)
Case 2	Cirrhosis	2-A	Wild-type	9	9	0 (0)
Case 3	Cirrhosis	3-A	Wild-type	9	9	0 (0)
		3-B	Mutant	17	12	5 (29.4)
		3-C	Wild-type	9	9	0 (0)
		3-D	Mutant	17	7	10 (58.8)
Case 4	Cirrhosis	4-A	Mutant	17	13	4 (23.5)
Case 5	Cirrhosis	5-A	Mutant	17	12	5 (29.4)
Case 6	Cirrhosis	6-A	Mutant	13	12	1 (7.7)
Case 10	Cirrhosis	10-A	Mutant	17	12	5 (29.4)
Case 13	Cirrhosis	13-A	Mutant	17	14	3 (17.6)
Case 36	Fatty liver	–	Wild-type	9	9	0 (0)
Case 37	Fatty liver	–	Wild-type	9	9	0 (0)
Case 2	HCC	–	Mutant	17	12	5 (23.5)
Case 3	HCC	–	Mutant	16	12	4 (25.0)
Case 4	HCC	–	Mutant	17	11	6 (35.3)
Case 7	HCC	–	Mutant	17	4	13 (76.5)
Case 11	HCC	–	Mutant	17	12	5 (29.4)
Case 13	HCC	–	Mutant	17	8	9 (52.9)

regenerative nodules and HCCs (Table 5). These results support that p53 mutations found in regenerative nodules did not come from the mutations of metastatsis of accompanied HCC.

4. Discussion

A multistep process involving a series of genetic changes including those of p53 gene, originally proposed for colorectal tumorigenesis [11], is increasingly recognized as the general underlying molecular events that may explain tumorigenesis in various types of tumor. Thus, possible p53 mutations in precancerous dysplastic lesions have been examined in various diseases. Indeed, p53 mutations were reported for Barrett's epithelium of the esophagus [6], colorectal adenoma [7,12], and gastric adenoma [13], implying that p53 mutations are causatively involved in carcinogenesis. In addition, p53 mutations in benign prostatic hyperplasia and keratinocytes in normal human skin are a focus of interest with respect to the potential role of p53 mutations in tumorigenesis in these tissues [14–16].

Regarding the role of mutant p53 in HCC, there are many reports describing p53 mutations in progressive HCC [17,18]. Murakami et al. reported that p53 mutations were associated with HCC in later progressive stages of carcinoma, but were not associated with well or moderately differentiated HCC collected from Japanese patients [1]. Moreover, p53 mutations in lesions of chronic hepatitis, cirrhosis, or potentially precancerous adenomatous hyperplasia have not been reported except a study performed in countries where people are exposed to food contamination by AFB_1[10]. Five of 15 cirrhotic cases were cigarette smokers, and six were alcohol drinkers. There are no relation with the habits and the incidence of p53 mutations. Also, special occupation relating with carcinogenesis was not found in the cases. In this study, p53 mutations were demonstrated by nucleotide sequence analysis in regenerative nodules in cirrhotic liver, collected from a geographic region not contaminated by AFB_1. The previously unexpected results may be due to the methods used for the detection of p53 mutation. Manual microdissection of slides were used to remove only hepatocytes in a cirrhotic nodule. Furthermore, the nucleotide sequencing was carried out instead of screening methods such as single strand conformation polymorphism (SSCP) . In the present study, overexpression of p53 was basically not demonstrated in any cirrhotic lesions of the liver including those associated with p53 mutations. These inconsistent findings concerning p53 protein levels in cirrhotic lesions may be due to different paratopes, binding affinity to epitopes of anti-p53 antibodies used as primary antibodies, or sensitivity to detect small population of cells with p53 mutations.

Fourteen of 15 cirrhosis cases were accompanied by HCCs, and the exclusion from the possibility of micro metastasis in regenerative nodules was important. Obtaining enough materials of cirrhotic livers without HCC is difficult in Japan, since most cirrhotic cases are known to be complicated with HCC at autopsy [19]. Therefore, we were most careful to sample regenerative nodules at a site distant to the HCC. Moreover, tissue specimens were diagnosed by at least two pathologists, and no carcinoma cells were seen under the light microscope. Furthermore, we sequenced p53 gene of accompanying 14 HCCs. HCC found in cases 5, 6, 10 did not have p53 mutations, however, the regener-

Table 4
p53 gene mutations detected by subcloning analysis in regenerative nodules

Nodule no.	Codon	Nucleotide change	Amino acid change	No. of reports[a]	No. of reports on HCC[b]
3-B	138	GCC → GTC	Ala → Val[c]	81	0
	170	ACG → ACA	Thr → Thr[c]	25	1
	139	AAG → TAG	Lys → Stop	37	2
	140	ACC → GCC	Thr → Ala	28	1
	147	GTT → GCT	Val → Ala	31	1
	157	GTC → GCC	Val → Ala	177	13
3-D	130	CTC → CCC	Leu → Pro	41	3
	143	CTG → GTT	Val → Val	54	1
	143	GTG → GTT	Val → Val	54	1
	152	CCG → CCT	Pro → Pro	103	3
	153	CCC → CTC	Pro → Leu	31	0
	155	ACC → ACT	Thr → Thr	58	0
	165	CAG → CCG	Gln → Pro	45	0
	176	TGC → TAC	Cys → Tyr	265	9
	179	CAT → TAT	His → Tyr	228	5
	183	TCA → TCG	Ser → Ser	22	0
4-A	131	AAC → ACC	Asn → Thr[c]	22	2
	176–177	1 base pair deletion (frame shift)		265	9
	140	ACC → ACT	Thr → Thr	28	1
	165	CAG → CTG	Gln → Leu	45	0
	184	GAT → GAC	Asp → Asp	15	0
5-A	139	AAG → AGG	Lys → Arg[c]	37	2
	158	CGC → CGT	Arg → Arg	182	2
	140	ACC → ACA	Thr → Thr[c]	28	1
	151–152	1 base pair deletion (frame shift)		149	2
	148	GAT → GAC	Asp → Asp	22	0
	162	ATC → ATG	Ile → Met	35	1
	183	TCA → GCA	Ser → Ala	22	0
	184	GAT → GGT	Asp → Gly	15	0
6-A	176–177	1 base pair deletion (frame shift)		265	9
10-A	140	ACC → ACT	Thr → Thr	28	1
	165	CAG → CTG	Gln → Leu	45	0
	171	GAG → GTG	Glu → Valu	35	1
	176–177	1 base pair deletion (frame shift)		265	9
	178	CAC → CGC	His → Arg	60	0
13-A	140	ACC → ACA	Thr → Thr	28	1
	151–152	1 base pair deletion (frame shift)		149	2
	158	CGC → CGT	Arg → Arg	182	2

[a] Number of reports which described mutation at the codon, was cited from International Agency for Research on Cancer (IARC) data base. (http://www.iarc.fr/p53/).
[b] Number of mutant reports which analyzed hepatocellular carcinoma (HCC).
[c] 2 mutations in a clone.

ating nodules found in the cases had p53 mutation. This finding clearly indicated that the p53 mutation found in the regenerative nodules did not come from tumor cells. HCC in cases 3, 4, 13 had p53 mutations, however, the mutations were different from those found in the regenerative nodules. Thus, there were inconsistent sequence with regenerative nodules and HCCs. Therefore, we thought these results were of significance.

Although the underlying mechanism and factors inducing somatic mutation of p53 gene are not fully understood, several potentially mutagenic factors include nitric oxide, free radicals, and reactive oxygen intermediates. Chronic hepatitis is known to be associated with increased expression of inducible nitric oxide synthetase and 8-hydroxydeoxyguanine [20,21]. Point mutation of p53 is enhanced by hepatic regeneration in AFB_1-induced rat liver tumors and preneoplastic lesions [22]. Thus, repeated persistent inflammation and regeneration of liver tissue under conditions of oxidative stress may be associated with mutation of p53 gene. In this study, a specific hot spot was not found among 38 mutations, and a specific event of substations was not detected. Thus, similar to other reports on mutations of the codons (Table 4), the underlying mechanism which caused the mutations was not directly driven by the mutation profile.

Among 15 cases with cirrhosis in the present study, five cases were associated with HBV infection and nine cases were associated with HCV infection. Five cases with cirrhosis in which p53 mutations were detected were associated with HCV infection. Likewise, only one case was associated

Table 5
p53 gene mutation detected by subcloning analysis in HCC

Case	No. of clone	Codon	Nucleotide change	Aminoacid change
Case 2	1	133	ATG → AAG	Met → Lys
	2	137	CTG → CTA	Leu → Leu
	1	141	TGC → TAC	Cys → Tyr
	1	182	TGC → TAC	Cys → Tyr
Case 3	1	152	CCG → CCT	Pro → Leu
	1	162	ATC → ATG	Ile → Met
	1	164	AAG → AAT	Lys → Asn
	1	167	CAG → CAA	Glu → Glu
Case 4	1	132	AAG → GAG	Lys → Glu
	1	142	CCT → CCC	Pro → Pro
	1	151	CCC → CTC	Pro → Leu
	1	151-152	1 base pair deletion (frame shift)	
	1	155	ACC → GCC	Thr → Ala
	1	159	GCC → GCT	Ala → Ala
	1	163	TAC → CAC	Tyr → His
	1	182	TGC → TAC	Cys → Tyr
Case 7	1	129	GCC → GCT	Ala → Ala
	1	133	ATG → ACG	Met → Thr
	1	138	GCC → GCT	Ala → Ala
	1	140	ACC → GCC	Thr → Ala
	1	152	CCG → CCA	Pro → Pro
	1	157	GTC → ATC	Val → Ile
	1	175	CGC → CAC	Arg → His
	9	183	TCA → TCG	Ser → Ser
Case 11	1	144	CAG → CAA	Gln → Gln
	1	148	GAT → AAT	Asp → Asn
	1	165	CAG → TAG	Gln → Stop
	1	168	CAC → TAC	His → Try
	1	171	GAG → GGG	Glu → Gly
	1	173	GTG → ATG	Val → Met
Case 13	1	130	CTC → GTC	Lru → Val
	1	134	TTT → TCT	Phe → Ser
	3	135	TGC → TGT	Cys → Cys
	1	140	TGC → TGT	Thr → Thr
	1	163	TAC → CAC	Tyr → Thr
	1	166	TCA → CCA	Ser → Pro
	1	169	ATG → GTG	Met → Val
Case 38	9	175	CGC → CAC	Arg → Hois

with HBV infection. Upon infection, HBV X protein demonstrated to inhibit hepatocyte functions by binding to wild-type p53 protein [23]. Absence of p53 mutations in HBV-associated cirrhosis in the present study and infrequent mutation of the p53 gene in HBV-positive HCC may be consistent with this inhibitory nature of HBV X protein [24].

An interesting finding in the present study is that the presence of p53 mutations differed between regenerative nodules in a liver while histopathologic appearance under a light microscope did not. Thirty to 40% of regenerative nodules in cirrhotic liver are known to be consisting of monoclonal cells [25–27]. These findings suggest that individual cirrhotic nodules are independent of each other in terms of genetic background, and some of nodules have p53 mutations. Population of subclones showing p53 mutations differed in each nodule (up to 58.8%). This result indicated that some of nodules consisted of mix population of cells with p53 mutations. Furthermore, the cell population contained multiple p53 mutation sites. Because of the reproducibility in our laboratory, exon 5 of p53 gene was chosen for analysis, however, considering that only the exon 5 was sequenced in this study, higher incidence of p53 mutations may exist in some regenerative nodules. Contributing factor for the mutation in regenerative nodules is not clear, whereas exon 5 of p53 is known to be involved in DNA binding. Accumulation of affected gene mutations including those of p53 in cirrhotic liver may play an integral role in process leading to HCC. Further study is necessary to clarify selective survival and accumulation of particular cells harboring specific spectrum of gene mutations including p53 mutations.

Acknowledgements

We are grateful to Dr M. Kosugi, K. Kiyohara, and T. Sugimoto (Tonami Municipal General Hospital, Tonami,

Japan) and N. Tanaka (Fukui Saiseikai Hospital, Fukui, Japan) for providing samples.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 10, Issue of March 8, pp. 8538–8544, 2002
Printed in U.S.A.

Two Independent Regions of Human Telomerase Reverse Transcriptase Are Important for Its Oligomerization and Telomerase Activity*

Received for publication, November 19, 2001, and in revised form, December 17, 2001
Published, JBC Papers in Press, December 18, 2001, DOI 10.1074/jbc.M111068200

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Human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, contains motifs conserved among reverse transcriptases. Several nucleic acid-dependent polymerases that share a "fingers, palm, and thumb substructure" were shown to oligomerize. Here we demonstrate that hTERT also has this ability using partially purified recombinant hTERTs and mammalian cells co-expressing differently tagged hTERTs. Human template RNA (hTR), by contrast, has no effect on the structural oligomerization of hTERTs. Therefore, hTERT has an intrinsic ability of oligomerization in the absence of hTR. We identified two separate regions as essential for the oligomerization. The regions, amino acids 301–538 (amino-terminal region) and amino acids 914–928 (carboxyl-terminal region), are outside the fingers and palm substructure covering motif T to D and interact with each other *in vivo*. A substituted mutant of hTERT, hTERT-D712A-V713I, which was reported as a dominant negative form of hTERT, bound to the wild-type hTERT and inhibited its telomerase activity transiently expressed in telomerase-negative finite normal human fibroblast. The truncated forms of hTERT containing the binding region to the wild-type hTERT partially inhibited the telomerase activity, probably by preventing the wild-type hTERT from forming an oligomer. Taken together, the oligomerization of hTERT is an important step for telomerase activity.

Telomeres are specialized structures positioned at the ends of linear eukaryotic chromosomes that provide a mechanism for maintaining chromosome length and stability. The termini of telomeric DNA cannot be fully replicated by the conventional replication machinery. Telomerase, a ribonucleoprotein complex composed of template RNA and several proteins, elongates telomeres as one means of end replication (1). Telomerase reverse transcriptase (TERT[1]), the catalytic subunit of telomerase, is a specific type of reverse transcriptase that forms stable complexes with template RNA (TR) (2). Human TERT is the rate-limiting factor for telomerase activity both biologically and biochemically (3, 4). Introduction of hTERT into normal human primary cells overcomes senescence and extends their lifespan (3, 5). We recently reported that hTERT and hTR, are the minimum components required for telomerase activity reconstituted *in vitro* with purified forms (4).

TERT is part of a large family of nucleic acid-dependent nucleic acid polymerases that share a " fingers, palm, and thumb substructure" (2, 6–8). Human TERT contains several motifs conserved among many reverse transcriptases, and additional motifs conserved only among TERTs from species ranging from budding yeasts to humans (9–11). Some polymerases that share a fingers, palm, and thumb substructure, such as HIV reverse transcriptase, polio RNA-dependent RNA polymerases (RdRP), and hepatitis C virus RdRP, oligomerize (12–16). Oligomerization in these enzymes induces conformational changes, which provide active or open forms that are essential for catalytic functions.

In *Saccharomyces cerevisiae*, telomerase forms an active multimer *in vivo* that may contain two active sites. This suggests that *Est2p* can oligomerize (17). Recently, one group reported that the human telomerase complex also forms a homodimer that contains two template RNA molecules (18), and another group found that two separate, catalytically inactive TERT proteins can complement each other in *trans* to reconstitute catalytic activity (19). These results indicated that just one hTERT molecule and one hTR molecule alone could not reconstitute telomerase activity. In other words, hTERT and hTR molecules must form a multimer and reconstitute telomerase activity by working together. However, it was not clear whether disruptions to the oligomeric formation of hTERT reduce telomerase activity.

Here we demonstrate that two independent regions outside of motifs T to D have an important role in the oligomeric interaction of hTERT *in vitro* using purified recombinant hTERT in the absence of hTR, and in mammalian cells transiently co-expressing various tagged hTERTs. These two independent regions can interact. We also demonstrate that catalytically inactive truncated forms of hTERT, which contain the binding region can inhibit telomerase activity of the wild-type hTERT.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Mammalian expression vectors: The plasmids pNKZ-FLAG, pNCZ-FLAG, and pNKZ-GST derived from pSG5UTPL, are mammalian expression vectors. pNKZ-FLAG or pNC-FLAG vector was used to express amino- or carboxyl-terminal FLAG-tagged protein. pNKZ-GST was used to express amino-terminal GST-fused protein (4, 20, 21). The *EcoRI-SalI* fragment containing the

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[1] The abbreviations used are: TERT, telomerase reverse transcriptase; TR, template RNA; h, human; RdRP, RNA-dependent RNA polymerase; HA, hemagglutinin; GST, glutathione *S*-transferase; MEGA-9, *n*-nonanoyl-*N*-methylglucomide; TRAP, telomerase repeat amplification protocol; ELISA, enzyme-linked immunosorbent assay; aa, amino acid(s); HIV, human immunodeficiency virus.

hTERT cDNA was subcloned into the *EcoRI-SalI* sites of pNKZ-GST. pCI-Neo is derived from pCI-Neo-hTERT. To construct a carboxyl-terminal FLAG-tagged wild-type hTERT plasmid, the *EcoRI-BamHI* fragment containing the truncated hTERT cDNA was also subcloned into the *EcoRI-BamHI* site of pNCZ-FLAG. The *BamHI-BamHI* fragment containing the truncated hTERT cDNA of the carboxyl-terminal side, which was subcloned by PCR using appropriate primers, was subcloned into the *BamHI* site of the plasmid pNCZ-FLAG-hTERT containing the *EcoRI-BamHI* fragment.

All truncated hTERT constructs were subcloned by PCR using the appropriate primers, except ΔCT1 and ΔCT2. *EcoRI-BamHI* fragments containing truncated hTERT cDNA were subcloned into the *EcoRI-BamHI* sites of the mammalian expression vector pNKZ-FLAG. ΔCT1 and ΔCT2 were constructed by inserting the *EcoRI-BamHI* or *EcoRI-XhoI* fragment of the hTERT cDNA derived from pNKZ-FLAG-hTERT into the *EcoRI-BamHI* or *EcoRI-SalI* sites of the plasmid pNKZ-FLAG, respectively. All truncated forms of hTERT tagged with 2xHA epitopes at the carboxyl terminus were derived from pCI-Neo-hTERT-HA (9, 22). Substitutions of aspartic acid with alanine and valine with isoleucine at positions 712 and 713, which was defective in substrate-binding and reported as a dominant negative form of hTERT, were introduced via PCR site-directed mutagenesis (23).

Baculovirus expression vectors: The amino-terminal GST-fused hTERT baculovirus expression vector pBKM-GST-hTERT was constructed by inserting the *NotI-BglII* fragment of the GST-hTERT cDNA derived from pNKZ-GST-hTERT into the *NotI-BglII* sites of the pVL1393 Baculovirus Transfer Vector (PharMingen) (4). The carboxyl-terminal FLAG-hTERT baculovirus expression vector pBKM-cFLAG-hTERT was constructed by inserting the *EcoRI-BglII* fragment of the carboxyl-terminal FLAG-hTERT cDNA derived from pNCZ-FLAG-hTERT into the *EcoRI-BglII* sites of the pVL1393 Baculovirus Transfer Vector. Other hTERT constructs for baculovirus expression were also constructed by inserting the *NotI-BglII* fragment of the hTERT cDNA derived from pNKZ-FLAG into the *NotI-BglII* sites of the pVL1393 Baculovirus Transfer Vector. These hTERT truncations contained a FLAG-epitope at the amino terminus.

Cells and the Generation of Recombinant Baculoviruses—Sf9 and High5 cells were cultured, and recombinant baculoviruses were prepared as described (4). COS-1 cells and TIG-3 cells were cultured by the standard method in Dulbecco;s modified Eagle's medium containing 10% fetal calf serum.

Purification of Carboxyl-terminal FLAG-tagged hTERT and Amino-terminal GST-fused hTERT Proteins from Infected Cells—To express carboxyl-terminal FLAG-tagged hTERT protein and amino-terminal GST fusion protein, carboxyl-terminal FLAG-tagged hTERT and amino-terminal GST-fused hTERT expression recombinant baculoviruses, BVKM-cFLAG-hTERT and BVKM-GST-hTERT, were constructed as described previously (4). High5 cells were infected with BVKM-cFLAG-hTERT or BVKM-GST-hTERT at a multiplicity of infection of about 0.2, incubated for 5 days at 27 °C, then scraped off the plates and centrifuged at 4000 rpm for 10 min. All buffers contained proteinase inhibitors and 1 mM dithiothreitol (4). A total of 5×10^7 High5 cells was resuspended in 5 ml of buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.1% Nonidet P-40, 150 mM NaCl, 10 mM β-mercaptoethanol), and the suspension was sonicated for 10 s three times. After a 10-min centrifugation at 10,000 × *g*, the supernatant (S1) was removed, the pellet was resuspended in 1 ml of lysis buffer B (20 mM Tris-HCl, pH 7.5, 50% glycerol, 0.5% MEGA-9, 300 mM NaCl, 10 mM β-mercaptoethanol), and the suspension was sonicated for 10 s three times. After a 10-min centrifugation at 10,000 × *g*, the supernatant (S2) was diluted with buffer F (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.1% Nonidet P-40) to adjust the NaCl concentration to 150 mM (S5). S5 was mixed with 500 μl of 50% anti-FLAG M2 affinity gel (Sigma Chemical Co.) or glutathione-Sepharose 4B resin (Amersham Biosciences, Inc.). This mixture was rotated for 1 h at room temperature. Protein bound to Sepharose was washed three times with buffer A, then eluted with 250 μl of buffer B, which contained 100 μg/ml of FLAG peptide (Sigma) or 10 mM reduced glutathione, respectively.

Immunoprecipitation—The hTERT constructs were transfected into COS-1 cells by calcium phosphate precipitation. COS-1 cells (1×10^7) were sonicated in 600 μl of buffer A and the cell lysate was stored at −80 °C until use. The lysate was immunoprecipitated with 20 μl of anti-HA monoclonal antibody (Santa Cruz Biotechnology, Inc.) immobilized on Protein A-Sepharose 4FF, rotated for 2 h at 4 °C, and washed three times with washing buffer G (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.5% Nonidet P-40, 300 mM NaCl). The bound proteins were separated by SDS-PAGE then visualized by Western blotting.

The cell lysate was also incubated with 20 μl of anti-FLAG M2 affinity gel for 1 h at 4 °C and washed three times with washing buffer H (20 mM Tris-HCl, pH 7.5, 20% glycerol, 1.0% Nonidet P-40, 300 mM NaCl). The bound proteins were separated by SDS-PAGE then visualized by Western blotting.

GST Pull-down Assays—Fifty microliters of glutathione-Sepharose (Amersham Biosciences, Inc.) was added to ~1 μg of partially purified recombinant GST-fused hTERT or GST and FLAG-tagged hTERT proteins, and the mixtures were constantly rotated in binding buffer (20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.05% Nonidet P-40, 0.25% MEGA-9, 150 mM NaCl, 1 mM dithiothreitol) for 1 h at room temperature. Thereafter, the beads were washed three times with binding buffer, then bound proteins were resolved on SDS-PAGE and visualized by Coomassie Brilliant Blue staining or Western blotting.

Western Blotting—Western blotting was performed by the standard method using anti-FLAG M2 monoclonal antibody (Sigma), anti-HA monoclonal antibody (Santa Cruz Biotechnology, Inc.), and anti-GST monoclonal antibody (Santa Cruz Biotechnology, Inc.) (4, 20, 21).

Telomerase Assay—The hTERT constructs were transiently transfected into telomerase-negative TIG-3 cells by calcium phosphate precipitation or with FuGENE 6 transfection reagent (Roche Molecular Biochemicals). Extracts were prepared from ~2.5×10^4 transiently transfected TIG-3 cells using lysis buffers according to the manufacturer's protocol. Telomerase activity was measured by two methods. First, a PCR-based telomerase repeat amplification protocol (TRAP) assay was carried out with TeloChaser (TOYOBO Co. Ltd.). The products of the PCR were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR-Green I (Molecular Probes). Second, a TRAP enzyme-linked immunosorbent assay (ELISA) was used to quantitatively measure telomerase activity with a TRAPEZE ELISA telomerase detection kit (Intergen Co. Ltd.).

Preparation of hTR RNA—hTR was prepared using the T7 *in vitro* transcription system as described for hTR-cDNA (pGRN164) (4).

RESULTS

Oligomeric Interaction of hTERT in Vitro and in Vivo—To examine the homomeric interaction of hTERT, GST-fused and FLAG-tagged hTERT expressed in insect cells were purified using affinity chromatography (Fig. 1*A*, *lanes 1–3*), and the binding of FLAG-tagged hTERT to GST-hTERT was examined using the GST pull-down assay. Purified FLAG-hTERT was pulled-down by GST-hTERT *in vitro* (Fig. 1*B*, *lane 5*). This binding was specific, because GST alone could not bind FLAG-hTERT (Fig. 1*B*, *lane 6*). Importantly, the presence of human telomerase RNA (hTR) did not positively nor negatively affect this oligomeric interaction (Fig. 1*C*, *lanes 7* and *8*). We also confirmed that the oligomeric interaction occurred *in vivo*, because HA- and FLAG-tagged hTERT proteins transiently co-expressed in COS-1 cells were co-immunoprecipitated by anti-HA (Fig. 1*D*, *lane 13*) or by anti-FLAG M2 antibody (Fig. 1*E*, *lane 20*), respectively. These results indicate that hTERT proteins labeled with different tags interacted with each other in the oligomer form *in vivo* and *in vitro*. This interaction does not require hTR, strongly suggesting that hTERT has an intrinsic ability to oligomerize.

RNase Treatment Does Not Inhibit Homomeric Interaction of hTERT—To demonstrate that hTERT can oligomerize *in vivo* without template telomerase RNA, the lysate of COS-1 cells transiently co-transfected with HA-tagged hTERT and FLAG-tagged hTERT was treated with RNase A, and then the binding of FLAG-hTERT to HA-hTERT was examined by co-immunoprecipitation using anti-HA antibody. Although telomerase activity of cell extract was diminished by RNase treatment (Fig. 2*B*), RNase treatment had no effect on homomeric interaction of hTERT (Fig. 2*A*), indicating that intact telomerase RNA is dispensable in the structural oligomerization of hTERT but absolutely necessary for telomerase activity.

Two Regions Bind to the Wild-type hTERT in Vitro and in Vivo—If the homomeric interaction of hTERT does not require the presence of hTR, then the region(s) necessary for oligomeric interaction can be mapped using truncated forms of hTERT. Four truncations covering the amino-terminal region spanning