Pergamon

European Journal of Cancer, Vol. 34, No. 12, pp. 1946–1949, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00237-8

Original Paper

Telomerase Activity and Telomere Length in Human Hepatocellular Carcinoma

G.-T. Huang, H.-S. Lee, C.-H. Chen, L.-L. Chiou, Y.-W. Lin, C.-Z. Lee, D.-S. Chen and J.-C. Sheu

Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, Taiwan

Telomerase activity is activated and telomere length altered in various types of cancers, including hepatocellular carcinoma (HCC). A total of 39 HCC tissues and the corresponding non-tumour livers were analysed and correlated with clinical parameters. Telomere length was determined by terminal restriction fragment assay, and telomerase activity was assayed by telomeric repeat amplification protocol. Telomerase activity was positive in 24 of the 39 tumour tissues (1.15–285.13 total product generated (TPG) units) and in six of the 39 non-tumour liver tissues (1.05–1.73 TPG units). In the 28 cases analysed for telomere length, telomere length was shortened in 11 cases, lengthened in six cases, and unaltered in 11 cases compared with non-tumour tissues. Neither telomore length nor telomerase activity was correlated to any clinical parameters. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: hepatocellular carcinoma, telomere, telomerase
Eur J Cancer, Vol. 34, No. 12, pp. 1946–1949, 1998

INTRODUCTION

TELOMERES ARE specialised structures at the ends of eukaryotic chromosomes that are important in maintaining the stability and integrity of chromosomes [1]. In humans, telomeres are composed of hundreds to thousands TTAGGG sequence repeats [1]. The shortening of telomeres is the 'clock that times' cellular senescence [2]. Tumours with shorter telomeres than in the original tissue have been detected in many cancer types, although longer telomeres have also been described [3]. The length of telomeres can be determined by analysis of chromosome terminal restriction fragments (TRFs), composed of telomeric TTAGGG repeats and subtelomeric DNA [4].

Telomere length in cancer cells appears to depend on a balance between the loss of telomere repeats at each cell cycle and the telomere elongation mediated by telomerase activity [1]. Telomerase activity is repressed in somatic cells and tissues, but activated in immortal cells and cancers [2]. Telomerase activity can be measured by telomeric repeat amplification protocol (TRAP) and a more reliable method with internal control and quantification has been reported [5].

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world [6], and it is the leading cause of cancer deaths in Taiwan [6, 7]. Telomere length and telomerase activity in HCC have been reported in Japan [8–14]. We studied telomere length by TRF analysis and telomerase activity by TRAP assay in HCC tissues and their corresponding non-tumour liver tissues from Taiwan patients.

MATERIALS AND METHODS

Thirty-nine pairs of surgically resected HCC tissues and the corresponding non-tumour liver tissues were obtained for telomere and telomerase studies. All samples were collected within 1 h after resection, frozen in liquid nitrogen immediately, and stored at − 80°C until use.

TRF analysis

Before TRF, DNA degradation was excluded by electrophoresis of undigested DNA. TRF analysis, modified from the method of Allsopp and colleagues [15], was performed in 28 of the 39 cases. Two micrograms of genomic DNA was digested with RsaI and then electrophoresed on 0.7% agarose gels and blotted on to Nylon membranes (Hybond-N,

Correspondence to J.-C. Sheu.
Received 31 Jul. 1997; revised 16 Apr. 1998; accepted 6 May 1998.

Amersham, Buckinghamshire, U.K.). The membrane was hybridised with the telomeric probe $^{32}P(CCCTAA)_3$ at 37°C and washed at 42°C in 2×standard saline/citrate (SSC). The filters were autoradiographed on Kodak XAR-5 X-ray films with an intensifying screening at −70°C for 12–24 h. Autoradiographs were scanned with a densitometer. The TRF lengths were estimated at the peak position of the hybridisation signal. Shortening or lengthening of TRFs were arbitrarily defined if the TRF length of tumour tissues was more than 20% shorter than or more than 20% longer than the corresponding non-tumour tissues, respectively [11].

TRAP assay

Telomerase activity was assayed by the TRAP method [5] using TRAPEZE™ telomerase detection kit (Oncor, Gaithersburg, Maryland, U.S.A.). Frozen tissue samples, 50–100 mg, were minced with a knife and then washed in ice-cold wash buffer (10 mM Hepes–KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol), then homogenised with 200 µl ice-cold 1×3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) lysis buffer supplied in the kit. After 30 min of incubation on ice, the lysate was centrifuged at 13 000 rpm for 1 h at 4°C and the supernatant was frozen and stored at −70°C. The concentrations of protein were measured using the BSA binding assay kit (Bio-rad, Hercules, California, U.S.A.), and an aliquot of extract containing 10 mg of protein was used for each TRAP assay. The protocol suggested by the manufacturer was followed, except that the conditions for step 3 were modified to 27 cycles of 30 sec at 94°C, 30 sec at 55°C. After polyacrylamide gel electrophoresis, a phosphorimager (Fujix BSA 1000, Fuji Photo Film Co., Tokyo, Japan) was used and quantification was performed according to protocol. All samples were checked by Western blot and Coomassie blue staining to exclude the degradation of protein. Only the presence of typical ladder bands with total product generated (TPG) > 1.0 unit was regarded as positive.

For statistical analysis, Cox's regression model was used to analyse the significance of telomere length and telomerase activity on clinical parameters. The software used was Statview 4.5 (Abacus Concepts, California, U.S.A.). Probability < 0.05 was regarded as significant.

Table 1. *Patient characteristics*

	Number patients ($n = 39$)
Gender	
Male	29
Female	10
Mean age (years) (range)	56.3 (20–82)
Tumour size (cm)	
1–2.9	10
3–4.9	13
5–9.9	11
10–15	5
Histology—non-tumour tissues	
Non-specific/fatty change	4
Chronic persistent hepatitis or portal fibrosis	7
Primary biliary cirrhosis	1
Liver cirrhosis	27
AFP (ng/ml)	
< 20	16
20–400	11
> 400	12
Recurrence after surgery*	
Within 6 months	11
7–12 months	5
13–18 months	1
19–24 months	2
25–30 months	2
> 30 months	3†
Free of recurrence (for 39–126 months)	12
Edmondson's grading	
Grade II	14
Grade II–III or III	19
Grade III–IV	3
Unclassified	3

*3 other patients died 1 month, 27 months and 30 months after surgery with no sign of recurrence. †1 patient, 32 months; 1 patient, 38 months, 1 patient, 115 months. AFP, alpha-fetoprotein.

RESULTS

Patient characteristics are shown in Table 1. In the 28 cases analysed, the TRFs lengths were shortened in 11 cases, lengthened in six cases, and unaltered in 11 cases. The average length of TRFs of the tumour tissues was 7.19 kb (range 2.5–15.6 kb), while the average length of TRFs of the non-tumour tissues was 7.76 kb (range 4.8–10.6 kb). The change in TRF length was not related to tumour size, time of recurrence, Edmondson's grade or alpha-fetoprotein (AFP) level at diagnosis.

Telomerase activity was positive in 24 of the 39 (61.5%) HCC tissues, and in six of the non-tumour liver tissues

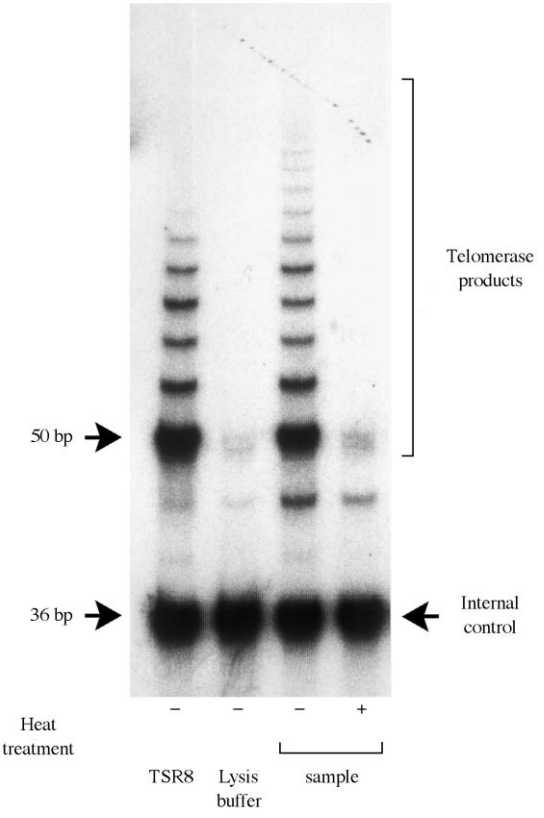

Figure 1. Determination of telomerase activity in hepatocellular carcinomas (HCCs) using the telomeric repeat amplification protocol (TRAP) assay. TSR8 was used as a quantitation control and lysis buffer as a negative control. A heat treated sample was also assayed as a negative control.

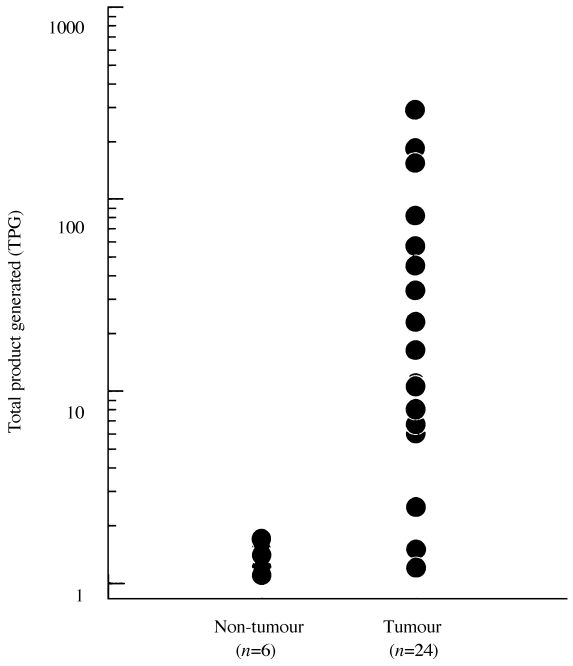

Figure 2. Scattergram of telomerase activity levels in hepatocellular carcinomas (HCCs) and non-tumour livers positive for telomerase activity. The mean level of the HCCs was higher than that of non-tumour livers.

(Figure 1). The range of telomerase activity was 1.15–285.13 TPG units for tumours and 1.05–1.73 TPG units for non-tumour livers (Figure 2). The difference between tumours and non-tumours of the 39 patients was significant (student *t* test, $P < 0.002$). The histology of six telomerase positive non-tumour specimens was liver cirrhosis in four, chronic hepatitis in one and non-specific change in one. There was no significant relationship between telomerase activity and tumour size, AFP level, Edmondson's grade or recurrence of HCC. The TRF lengths and telomerase activity also had no significant relationship.

DISCUSSION

Telomerase activity has been extensively studied [8–12]. It has been reported to have been detected in all HCCs [10, 12], and at least an 80% positive rate has been noted [9]. Using the TRAP method [5], only 61.5% was detected in our series. Differences in sampling may be one important factor. The criteria of quantification with TPG > 1. 0 unit, in addition to the typical ladder bands, may be another. Similar to previous studies, tumour size had no significant relationship to telomerase activity [8]. We also found that tumour recurrence and AFP value were not correlated with telomerase activity. Differentiation of HCC has been reported to have good correlation with telomerase activity, with poorly differentiated HCCs 100% positive for telomerase activity [8]. However, negative cases in poorly differentiated HCC have also been reported [9]. Although tumour necrosis has been suspected as an important influencing factor [9], our study again showed a negative correlation, but inadequate sampling was excluded in our series by DNA and protein checks before experimentation. Therefore, telomerase activity might not be an important predictor for prognosis of HCC.

The exact mechanism of telomerase activation in malignancy has not been defined. The shortened telomere may lead to genomic instability, contribute to the loss of heterozygosity, and result in reactivation of telomerase [2]. There may be a selection for telomerase positive cells [16]. No matter what the mechanism, the presence of telomerase activity in HCCs suggests that anticancer agents based on telomerase inhibition [17] might be potentially effective.

Telomerase activity although very weak, was detected in six of non-tumour liver tissues in our study and has been detected in others [8–12]. Micrometastasis leading to positive telomerase activity might be a possibility. However, long term observation and detailed histological examination of the 6 patients with detectable telomerase activity in non-tumour livers did not completely support this hypothesis. Although 2 had early recurrence 3 and 5 months after the operation, there were also 3 patients without evidence of recurrence for 27, 49 and 84 months respectively. Another possibility was that some of the cells in the non-tumour liver were truly telomerase positive. Adenomatous hyperplasia has been reported to have telomerase activity [12], and other chronic liver diseases with telomerase activity do exist [8–11]. Telomerase positive non-tumour livers might be precancerous lesions, as reported for adenomatous hyperplasia; and, thus, the development of HCC in the telomerase positive livers may be higher than telomerase negative livers. However, further observation is needed. Finally, telomerase activity may be due to infiltration of lymphocytes into the liver [18], which might be clarified by an *in situ* TRAP assay [19].

Telomere lengths in HCC tissues are usually shortened when compared with corresponding non-tumour liver tissues [12, 13], but longer or unaltered telomere lengths have also been shown [11, 14], as was seen in the present study. Telomerase activity might be reactivated after critical telomere shortening [3], although, telomerase might also be activated before telomere shortening. Alternatively, telomeres could be shortened and then re-elongated during tumour development [16]. Telomere length was again found not to be informative in telomerase positive cells [2]. However, the TRAP assay is very sensitive and a small fraction of tumour cells with telomerase activity will lead to a positive TRAP assay. In contrast, TRF analysis can only indicate the average telomere length of all tumour cells, so it is difficult to detect any change in the TRF length if only a small fraction of tumour cells have altered telomere length. This might explain the different results in previous reports. Telomeres can also be elongated via telomerase-independent mechanisms [20]. Telomeres from sperm DNA do not decrease with the age of the donor [15], whilst the telomere length of lymphocytes does decrease [20], although both are positive for telomerase activities. Many factors, such as Est 1 [21] or Bcl-2 [22], may also mediate telomere functions. Thus, the relationship between telomere length and telomerase activity is probably more complicated than the original telomere hypothesis [16]. Alternative lengthening mechanisms for the maintenance of telomeres have also been reported, although rare for carcinomas [23], but the contribution of this mechanism to telomere length in HCC needs further investigation.

1. Harley CB, Villeponteau B. Telomeres and telomerase in aging and cancer. *Curr Opin Genet Dev* 1995, **5**, 249–255.
2. Shay JW, Wright WE. The reactivation of telomerase activity in cancer progression. *Trends Genet* 1996, **12**, 129–131.
3. Dahse R, Fiedler W, Ernst G. Telomeres and telomerase: biological and clinical importance. *Clin Chem* 1997, **43**, 708–714.

4. Harley CB, Futcher AB, Greider CW. Telomeres shortening during aging of human fibroblasts. *Nature* 1990, **345**, 458–460.
5. Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res* 1997, **25**, 2595–2597.
6. Chen DS. Hepatitis B virus infection, its sequelae, and prevention in Taiwan. In Okuda K, Ishak KG, eds. *Neoplasms of the Liver*. Tokyo, Springer, 1987, 71–80.
7. Sheu JC, Sung JL, Chen DS, *et al.* Early detection of hepatocellular carcinoma by real-time ultrasonography. *Cancer* 1985, **56**, 660–666.
8. Tahara H, Nakanishi T, Kitamoto M, *et al.* Telomerase activity in human liver tissues: comparison between chronic liver disease and hepatocellular carcinoma. *Cancer Res* 1995, **55**, 2734–2736.
9. Nouso K, Urabe Y, Higashi T, *et al.* Telomerase as a tool for the differential diagnosis of human hepatocellular carcinoma. *Cancer* 1996, **78**, 232–236.
10. Nakashio R, Kitamoto M, Tahara H, Nakanishi T, Ide T, Kajiyama G. Significance of telomerase activity in the diagnosis of small differentiated hepatocellular carcinoma. *Int J Cancer* 1997, **74**, 141–147.
11. Kojima H, Yokosuka O, Imazeki F, Saisho H, Omata M. Telomerase activity and telomere length in hepatocellular carcinoma and chronic liver disease. *Gastroenterology* 1997, **112**, 493–500.
12. Miura N, Horikawa I, Nishimoto A, *et al.* Progressive telomere shortening and telomerase reactivation during hepatocellular carcinogenesis. *Cancer Genet Cytogenet* 1997, **93**, 56–62.
13. Ohashi K, Tsutsumi M, Nakajima Y, Kobitsu K, Nakano H, Konishi Y. Telomere changes in human hepatocellular carcinomas and hepatitis virus infected noncancerous livers. *Cancer* 1996, **77**, 1747–1751.
14. Urabe Y, Nouso K, Higashi T, *et al.* Telomere length in human liver diseases. *Liver* 1996, **16**, 293–297.
15. Allsopp RC, Vaziri H, Patterson C, *et al.* Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci USA* 1992, **89**, 10114–10118.
16. Autexier C, Greider CW. Telomerase and cancer: revisiting the telomere hypothesis. *Trends Biochem Sci* 1996, **21**, 387–391.
17. Parkinson EK. Do telomerase antagonists represent a novel anticancer strategy? *Br J Cancer* 1996, **73**, 1–4.
18. Vaziri H, Schachter F, Uchida I, *et al.* Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am J Hum Genet* 1993, **52**, 661–667.
19. Ohyashiki K, Ohyashiki JH, Nishimaki J, *et al.* Cytological detection of telomerase activity using an *in situ* telomeric repeat amplification protocol assay. *Cancer Res* 1997, **57**, 2100–2103.
20. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995, **14**, 4240–4248.
21. Virta-Pearlman V, Morris DK, Lundblad V. Est 1 has the properties of a single-stranded telomere end-binding protein. *Genes Dev* 1996, **10**, 3094–3104.
22. Mandal M, Kumar R. Bcl-2 modulates telomerase activity. *J Biol Chem* 1997, **272**, 14183–14187.
23. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nature Med* 1997, **3**, 1271–1274.

Acknowledgements—This study was supported by grants from the National Science Council and the Liver Disease Prevention & Treatment Research Foundation, Taiwan.