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# Shortened Telomere Length in Hepatocellular Carcinomas and Corresponding Background Liver Tissues of Patients Infected with Hepatitis Virus

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**The telomere length in 20 surgically resected human hepatocellular carcinomas (HCCs) and adjacent non-cancerous livers with hepatitis virus infection were investigated. All the HCC samples examined demonstrated shorter telomere length than the corresponding non-cancerous liver tissues, the respective average values being 5.4 kbp and 8.8 kbp ($P < 0.001$). The shortening of telomere length was most prominent in HCCs larger than 30 mm in diameter, and in both tumors and non-cancerous livers it was more marked with hepatitis B virus as compared with hepatitis C virus infection. These results indicate that telomere shortening is associated with not only progression, but also development of HCC, and there is a possible difference in the nature of the association in patients with hepatitis viruses of B and C types.**

Key words: Telomere length — Hepatocellular carcinoma — Hepatitis virus

It is generally accepted that hepatocellular carcinomas (HCCs) commonly develop in patients suffering from chronic active liver disease, where death and regeneration of hepatocytes occur in a continuous cycle.[1)] This suggests that hepatitis-related proliferative change may itself play some role in virus-associated hepatocarcinogenesis. Among the various kinds of chromosome changes caused by repeated cell division, shortening of telomeric DNA has recently been a focus of interest in relation to cellular senescence and malignant transformation.[2–4)] Telomeric DNA consists of guanine-rich repetitive TTAGGG motifs, which may be reiterated in tandem for 10 to 15 kb in human somatic cells,[5,6)] and is recognized as being important in protecting chromosomes from recombination and end-degradation.[5–7)] With numerous cell divisions, the length of telomeric DNA gradually decreases due to an inability of cells to achieve complete DNA replication at the ends of telomeres, the so-called "end replication problem."[8,9)] This shortening can ultimately lead to loss of telomeric function and chromosome destabilization, with possible links to malignant transformation or cellular senescence. Alterations in the length of telomeric DNA have been reported in a subset of human neoplasms, including colorectal carcinoma, renal cell carcinoma, childhood leukemia and neuroblastoma.[8,10–12)] The aim of the present study was to evaluate the telomeric DNA status of human HCCs and corresponding non-cancerous liver tissues in order to assess any possible link with hepatocarcinogenesis.

Twenty surgically resected HCC samples together with adjacent non-cancerous liver from patients suffering from hepatitis B or C virus infections (positive for hepatitis B surface antigen or showing antibodies against hepatitis C virus) were available for the study. The non-cancerous liver was histologically diagnosed as cirrhotic in 8 cases and as demonstrating features of chronic hepatitis in the remaining 12 cases. Tumor (HCC) samples and non-cancerous liver tissues were immediately frozen after surgical resection and stored at −80°C until use. High-molecular-weight genomic DNA was prepared from samples and its integrity was confirmed by electrophoresis in 0.4% agarose gels. Samples were then digested with *Hin*fI (Toyobo Co., Tokyo) and DNA fragments were separated by electrophoresis on 0.5% agarose gels, followed by transfer to nylon membranes (Hybond-N; Amersham, Buckinghamshire, England) using the protocol described by Church and Gilbert.[13)] The membranes were hybridized with a 5′-$^{32}$P-labeled oligonucleotide probe $(TTAGGG)_4$, and subsequently autoradiographed using X-ray films. Telomere length was assessed quantitatively with the aid of a Bio-Imaging Analyzer (Fuji Photo Film Co., Ltd., Tokyo). Terminal restriction fragments (TRF) were estimated as indicators of mean telomere length at the peak position of the hybridization signals and their lengths were expressed in kilo base pairs (kb). The obtained data were used to

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Table I. Clinical Data and TRF Lengths in HCC and Adjacent Non-cancerous Liver Samples

| Patient No. | Age/sex | Tumor diameter (mm) | Histological type | AFP (ng/ml) | Virus infection | Adjacent liver | TRF length (kbp) in HCC/adjacent liver |
|---|---|---|---|---|---|---|---|
| 1 | 66/M | 17 | MD | 22 | HCV | CH | 2.8/8.8 |
| 2 | 54/M | 40 | MD | 124 | HBV | LC | 3.3/9.8 |
| 3 | 47/M | 80 | MD | 29 | HBV | LC | 4.2/11.2 |
| 4 | 65/M | 38 | MD | 92 | HCV | LC | 3.8/9.2 |
| 5 | 73/M | 40 | MD | 4 | HCV | CH | 3.0/6.7 |
| 6 | 41/M | 20 | PD | 3 | HBV | LC | 3.4/6.7 |
| 7 | 68/M | 20 | WD | 39 | HCV | CH | 5.0/9.2 |
| 8 | 64/M | 21 | WD | 4 | HCV | CH | 6.5/11.5 |
| 9 | 75/M | 20 | MD | 8 | HCV | CH | 5.5/9.3 |
| 10 | 62/M | 21 | PD | 38 | HCV | CH | 6.0/8.9 |
| 11 | 65/M | 30 | MD | 45 | HCV | LC | 5.0/7.9 |
| 12 | 29/M | 55 | MD | 10 | HBV | CH | 5.0/7.6 |
| 13 | 44/M | 70 | MD | 38 | HBV | LC | 3.3/5.6 |
| 14 | 50/M | 10 | MD | 43 | HBV | CH | 3.9/5.6 |
| 15 | 64/M | 24 | MD | 17 | HCV | CH | 9.2/11.3 |
| 16 | 58/M | 25 | PD | 808 | HCV | CH | 7.7/9.5 |
| 17 | 72/M | 27 | MD | 43 | HCV | CH | 9.0/10.8 |
| 18 | 63/F | 18 | WD | 5 | HCV | CH | 6.8/8.1 |
| 19 | 58/F | 20 | MD | 10 | HCV | LC | 5.0/7.6 |
| 20 | 60/F | 14 | MD | 286 | HCV | LC | 9.0/10.0 |

M, male; F, female; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; CH, chronic hepatitis; LC, liver cirrhosis.

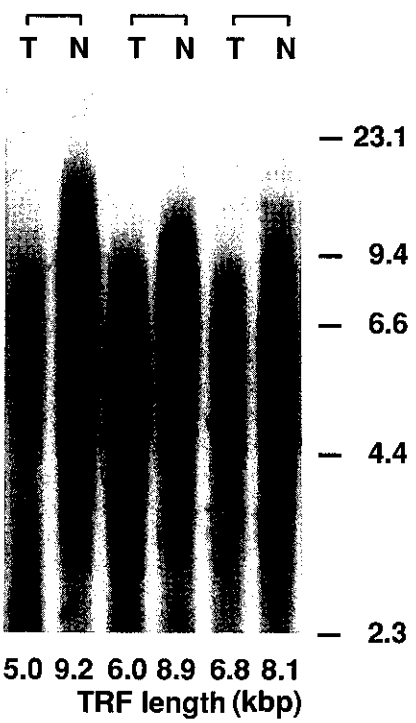


Fig. 1. Southern blot analysis, with a $^{32}$P-labeled telomere-specific probe $(TTAGGG)_4$, of TRFs in DNAs isolated from HCC and adjacent non-cancerous liver tissues. TRF lengths for each lane are shown below. Note the consistent reduction in TRF length in tumor samples in this series of HCCs. T, HCC samples; N, adjacent non-tumorous liver tissues.

calculate mean±SD values. Comparisons between groups were performed using Student's $t$ test and a probability value of $P<0.05$ was considered significant.

Data on patient and tumor characteristics are summarized in Table I. Representative autoradiography results are shown in Fig. 1. In all HCC cases examined, the TRF length was shorter in HCC samples (ranging from 2.8 to 9.2 kbp) than in adjacent liver tissues (ranging from 5.6 to 11.5 kbp), as shown in Table I. The mean TRF length in HCCs was 5.4±2.1 kbp, which is significantly smaller ($P<0.001$) than the mean value for non-cancerous liver (8.8±1.7 kbp). The relations between mean TRF length and clinical parameters are shown in Table II. HCCs more than 30 mm in diameter demonstrated significant shortening as compared to their smaller counterparts. No significant differences were seen in terms of histological grade of HCC, the infecting virus type and the presence of chronic hepatitis or cirrhosis in adjacent non-cancerous liver for either HCC or background liver samples. However, the TRF length in hepatitis B virus (HBV)-associated cases tended to be shorter as compared with hepatitis C virus (HCV)-associated values in both cancer and non-cancerous tissues.

In the present investigation, marked TRF shortening was observed in HCC samples as compared with adjacent non-cancerous liver tissues. The reduction in TRF length in tumor samples corresponded well with the results found for various other kinds of tumors such as colon

Table II. Comparison of TRF Lengths of HCC and Adjacent Non-cancerous Liver Samples with Clinicopathological Features[a)]

| Features | No. of samples | TRF length in HCC | | TRF length in non-cancerous liver |
|---|---|---|---|---|
| Tumor size | | | | |
| ≥30 mm | 13 | 6.2±2.2 | * | 9.2±1.9 |
| <30 mm | 7 | 3.9±0.8 | * | 8.3±1.9 |
| Histological grade | | | | |
| Well differentiated | 3 | 6.1±1.0 | | 9.6±1.7 |
| Moderately differentiated | 14 | 4.8±2.1 | | 8.6±1.9 |
| Poorly differentiated | 3 | 5.7±2.2 | | 8.4±1.5 |
| Virus infection | | | | |
| HBV | 6 | 3.9±0.7 | | 7.8±2.3 |
| HCV | 14 | 5.9±2.0 | | 9.3±1.6 |
| Status of non-cancerous liver | | | | |
| Chronic hepatitis | 12 | 6.0±2.1 | | 9.1±1.9 |
| Liver cirrhosis | 8 | 4.7±2.0 | | 8.4±2.0 |
| Total | 20 | 5.4±2.1 | ** | 8.8±1.8 |

*a*) Values are mean±SD (kbp).
* Significant difference between groups, $P<0.05$.
** Significant difference between groups, $P<0.001$.

and renal cell carcinomas.[8, 10–12)] The demonstrated relation with size is in line with the requirement for cell division during HCC progression, during which multistep gene alterations, including chromosome instability, are thought to be key players.[14, 15)] Such sequential changes during HCC progression are consistent with the present results on TRF shortening and with the proposed link to a propensity for genomic instability.[6, 7)]

Despite the existence of overwhelming epidemiological evidence of a role for HBV or HCV in the pathogenesis of HCC,[1)] there is still controversy as to how these viruses actually mediate the development of liver cancers.[16–18)] One postulated mechanism is the expression of HBx transcriptional trans-activator protein or HBV DNA integration into the human genome in HBV-associated hepatocarcinogenesis.[16, 17)] In the case of HCV, formation of oxidative damage may be involved.[18)] In terms of types and sites of alteration in the *p53* gene, the carcinogenic pathways are reported to be independent for HBV- and HCV-related HCC.[19)] Interestingly, the TRF lengths of both HBV-infected non-cancerous liver tissues and the associated tumors tended to be shorter than in the HCV-infected cases. Although the difference did not reach statistical significance, this might have been partly due to the relatively small number of tumors evaluated. From the encountered differences, it may be speculated that HCV-related HCCs develop in livers where less necrosis and compensating regeneration has occurred as compared with HBV-related HCCs.

Recently, activation of telomerase, by which cells maintain the TRF length by *de novo* elongation, has been detected in immortalized cells and human neoplasms, including HCC.[20, 21)] Such activation of telomerase may indeed play important roles in maintaining the growth of malignant cells[22)] and the length of telomeres in cancer cells is now recognized as being regulated by the balance between loss due to cell division and elongation due to telomerase activity. In this context, the results of our ongoing examination of telomerase activation in this series of cases will be of interest. Whatever the result, the present study is the first to systematically document changes in TRF length in HCCs and adjacent non-cancerous liver.

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