Interferon Can Block Telomere Erosion and in Rare Cases Result in Hepatocellular Carcinoma Development with Telomeric Repeat Binding Factor 1 Overexpression in Chronic Hepatitis C

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ABSTRACT

Purpose: The purpose of this study was to evaluate whether IFN therapy for chronic hepatitis C could overcome telomere reduction in the liver, a possible risk factor for hepatocellular carcinoma (HCC) development.

Experimental Design: Relative telomeric repeat content (RTC) in the liver was measured before and after IFN therapy in 21 chronic hepatitis C cases. Liver samples were obtained at average intervals of 12, 75, and 32 months in eight complete responders (CRs) and one biochemical responder (BR), four CRs in whom HCC developed after an eradication of hepatitis C virus, and eight nonresponders, respectively. Telomeric repeat binding factor 1 (TRF1) was immunostained in specimens from CRs and a BR.

Results: Although the average RTC of 0.96 ± 0.14 (mean ± SD) significantly decreased to 0.85 ± 0.12 after IFN therapy in nonresponders (P = 0.023), the value of 0.91 ± 0.14 before IFN therapy in CRs and a BR increased significantly to 1.0 ± 0.085 (P = 0.031). TRF1 expression was barely detectable and attenuated after IFN therapy, except in CRs developing HCC, in which frequent staining appeared, and the RTC evidently decreased from 0.97 ± 0.11 to 0.63 ± 0.0092 in corresponding noncancerous liver tissues.

Conclusions: It is strongly suggested that successful IFN therapy blocks telomere erosion, except in rare cases in which telomere reduction continues with overexpression of TRF1. Successive RTC evaluation in the liver may distinguish a risky case from a clinically cured one.

INTRODUCTION

HCV2 is the main causative agent of chronic liver injury in the world and is implicated in the increasing incidence of HCC (1). Administration of IFN is used to resolve chronic inflammation and eradicate HCV (2). Epidemiological studies reveal that IFN therapy reduces HCC development not only by successfully eradicating HCV but also by prolonging suppression of the serum levels of ALT and AST in the presence of HCV viremia (3, 4). In some cases, however, HCC develops in patients successfully treated with IFN (5, 6). Clarification of the molecular basis of how HCC development is reduced by IFN therapy is eagerly sought to establish a useful marker that could identify cases at risk of developing HCC among cases that are clinically cured.

Telomeres are composed of tandem arrays of a short DNA sequence, d(GTTAGG)n in vertebrates (7), and associated proteins (7–10). They are essential genetic elements to stabilize the natural ends of linear eukaryotic chromosomes (7). Telomeres confer steady chromosomal segregation by forming a special circular structure termed a t-loop (11) involving telomere binding proteins such as TRF1, which has a function to reduce telomere length by overexpression (8). Recent reports suggest that abrogation of telomere integrity triggers a telomere-based crisis and genome-wide chromosomal instability (12), a critical process that leads to cancer development (13, 14). Telomeres shorten as a result of successive cell divisions based on the end replication problem (15). Liver biopsies from patients with chronic liver diseases at various stages reveal that telomere length gradually decreases during progression of chronic hepatitis toward development of HCC (16, 17).

In this report, we used liver specimens before and after IFN therapy in the same donor to determine whether (a) telomeres in the liver shorten in an individual during progression of chronic hepatitis C, (b) reduction in liver inflammation using IFN inhibits telomere erosion, (c) telomere length is reduced in cases developing HCC after eradication of HCV, and (d) telomere

2 The abbreviations used are: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TRF1, telomeric repeat binding factor 1; NCL, corresponding noncancerous liver; CR, complete responder; BR, biochemical responder; NR, nonresponder; PBL, peripheral blood leukocyte; HAI, histology activity index; RTC, relative telomeric repeat content; CR-HCC, CR in whom HCC developed after successful eradication of HCV.

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alteration and histological activity are correlated. We estimate the critical telomere length in the liver for HCC development and evaluate TRF1 expression as a candidate molecule leading to telomere shortening in the absence of chronic liver inflammation. The implications of measuring telomere length in chronic hepatitis C are discussed.

MATERIALS AND METHODS

Methods conform to ethical guidelines of the 2001 Declaration of Helsinki (18) as reflected in a priori approval by Niigata University Medical and Dental School human research committee.

Tissues. Liver samples were obtained from 21 patients with chronic hepatitis C before and after IFN therapy. Samples were obtained by laparoscopic or ultrasound-guided liver biopsy, except for 4 cases with HCC (cases 18–21), from whom HCC and NCL tissues were obtained by surgical resection (Table 1). All specimens were stored as formalin-fixed and paraffin-embedded sections. Natural IFN-α was administered at a dose of 6–10 megaunits/day for the first 2–4 weeks and then administered 3 times a week for a maximum duration of 6 months in total, depending on cases. The therapeutic outcome was evaluated for 6 months after IFN therapy, and patients were classified into three groups according to their response. CRs were those patients with undetectable levels of HCV RNA in their serum as determined by reverse transcription-PCR 6 months after completion of IFN therapy. BRs were defined as cases in which ALT and AST were within normal limits in the presence of HCV viremia at least 6 months after completion of IFN therapy. NRs were defined as those patients with HCV viremia and abnormal values of ALT and/or AST within 6 months after completion of IFN therapy. In CR and BR cases, liver biopsies after IFN therapy were performed 6 months after completion of IFN therapy, whereas in NR cases, the second liver samples were obtained at various times when a second-line therapy was considered. PBLs were collected around the time of each liver biopsy from all patients by centrifugation at 5000 g after disrupting RBCs by suspending whole blood in 0.2% NaCl. The degree of chronic inflammation in the liver was classified by expert histologists according to the HAI scoring system (19).

For an immunohistochemical study of TRF1, eight additional sets of control liver samples were used, including four sets of HCC and NCL tissues obtained by surgical resection and four samples without chronic liver diseases but diagnosed as fatty liver in two cases and nonspecific reaction in two cases.

RTC Assay. Genomic DNA was extracted from paraffin-embedded sections as described previously (20). Briefly, three 5-μm sections were digested with 2 μl of 10 mg/ml protease K (Sigma) in 100 μl of 0.5% Tween 20 for 3 h at 55°C after deparaffinization. In HCC cases, HCC and NCL tissues were separated under a microscope. After adding 100 μl of TE [10 mM Tris-HCl (pH 8.3), 1 mM EDTA] and heating at 95°C for 10 min, any hardened wax was removed by placing it on ice after

### Table 1 Summary of cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Intervala</th>
<th>Resultb</th>
<th>RTCa</th>
<th>RTC</th>
<th>I + II + IIIC</th>
<th>IVb</th>
<th>TRF1c</th>
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<td>1</td>
<td>51</td>
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<td>0.81</td>
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<tr>
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<td>0.86</td>
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<tr>
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<tr>
<td>6</td>
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<td>1.1</td>
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<td>3</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>21</td>
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<td>CR-HCC</td>
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<td>0.63</td>
<td>−0.042</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

a Months between histological examinations of the liver.  
b Effects of IFN administration judged at 6 months after completion of IFN therapy.  
c 1st, results using the first biopsy specimen; 2nd, results using the second biopsy specimen; Alt rate, alteration rate of RTC; Pre, before interferon therapy; Post, after interferon therapy; ND, not determined.  
d RT duration in a year, − means decrease.  
e Activity score of histology activity index, HAI (Ref. 19).  
f Fibrosis grade of HAI (Ref. 19).  
g Immunoreactivity was graded using a scale from 0–3 at ×200 magnification; 0 represents no detectable stain, 1 represents detectable stain in only 1 cell in a field, 2 represents detectable stain in 2–4 cells in a field, and 3 represents detectable stain in ≥5 cells in a field.
centrifugation at 10,500 × g for 15 min. The samples were re-spun at 10,500 × g for 15 min, and supernatant was recovered. Genomic DNA in PBLs was extracted, and the RTC in the liver was calculated as described previously (16). In brief, approximately 20 ng of genomic DNA from PBLs and three consecutive sections were blotted (Slot Blotter; Schleicher & Schuell) onto Hybond-N+ membrane (Amersham) after alkalization with 0.2 M NaOH and cross-linked by UV irradiation. The filter was first hybridized with [γ-32P]ATP-end-labeled d(TTAGGG)3 in 5 × SSC (1 × SSC is 0.15 M NaCl plus 0.0015 M sodium citrate) at 40°C and washed in 2 × SSC twice at room temperature and once at 40°C for 30 min. Signal intensities were estimated by ImageJ software (NIH; Fig. 1). After stripping the probe, the membrane was rehybridized to [γ-32P]ATP-end-labeled d(CCT)7 under the same conditions. Signal intensities for the microsatellite probe were estimated in the same way. Telomeric repeat content was calculated by dividing the count due to d(TTAGGG)3 by that of d(CCT)7. For standardization of differences in telomere length among individuals, RTC in the liver was calculated by dividing the telomeric repeat content in the liver by that in PBLs from the same donor. To avoid variation of RTC among experiments, genomic DNA from the liver and PBLs from the same donor were blotted on the same membrane.

**Immunohistochemistry of TRF1.** For all BR and CR cases, TRF1 expression level was immunohistochemically evaluated in samples taken before and after IFN therapy. Specimens were hydrated by immersing in a 90% to 40% serial ethanol gradient after deparaffinization and digested with 0.4% trypsin (Sigma) in 0.01 N HCL at 37°C for 30 min after washing twice with PBS. Endogenous peroxidase activity was inhibited by immersing the slides in 3% hydrogen peroxide for 45 min. After re-equilibration with PBS, the slides were incubated with goat polyclonal anti-TRF1 antibody sc-1977 or antibody sc-6165 (Santa Cruz Biotechnology), which recognizes the COOH or NH2 terminus of TRF1, respectively, for 60 min at room temperature and then incubated with a biotinylated rabbit antigoat IgG for 30 min. The reaction was developed with diaminobenzidine solution after incubation with avidin-biotin peroxidase for 30 min. Signal intensities were calculated by dividing the telomeric repeat content in the liver by that in PBLs from the same donor. To avoid variation of RTC among experiments, genomic DNA from the liver and PBLs from the same donor were blotted on the same membrane.

**RESULTS**

**Decrease of RTC Values in the Liver During Chronic Hepatitis C.** For the eight NR patients (cases 1–8), RTC values in the liver were evaluated before and after IFN therapy at various intervals, from 14 to 51 months, as shown in row 1 of Fig. 1 (32 ± 14, mean ± SD; Table 1). Although 210–710 megunits of IFN were administered in total during the 10–24 weeks after the first histological examination, HCV was detected in the blood soon after completion of IFN therapy. Average RTC values before IFN therapy were 0.96 ± 0.14 (mean ± SD), and they decreased significantly at second biopsy to 0.85 ± 0.12 (P = 0.023; Fig. 2A). The rate of reduction of the RTC value was 0.040 ± 0.033/year. The first RTC value in case 4 and the first and second RTC values in case 8, in which the histological examination revealed a cirrhotic liver, were 1.0, 0.84, and 0.77, respectively.

**Increase in RTC Value with Resolve of Chronic Hepatitis Using IFN Therapy.** IFN therapy led to biochemical response in the one case (case 9) and complete response in eight cases (cases 10–17) without HCC development (Table 1). In BR and CR cases, the second biopsy was performed just 12 months after the first histological examination. The RTC in the liver was evaluated before and after IFN therapy as shown in row 2 of Fig. 1. The average RTC value before IFN therapy in BR and CR cases was 0.91 ± 0.14, and it was not significantly different from the value before IFN therapy in NR cases (P = 0.48). The average RTC value at the second biopsy, however, increased significantly to 1.0 ± 0.085 compared with that before IFN therapy (P = 0.031, Fig. 2B). The RTC value decreased from 0.96 to 0.93 after IFN therapy only in case 10. The rate of increase of the RTC value over 12 months was 0.11 ± 0.14/ year.

**Successive Telomere Erosion in the Liver in Which HCC Developed After Eradication of HCV.** In cases 18–21, HCC developed 60, 79, 58, and 102 months after the first histological examination (Table 1). ALT and ASL levels stayed within normal limits in all four cases until HCC was detected.
RTC in the liver was evaluated before IFN therapy and at surgical resection of HCC as shown in row 3 of Fig. 1. The RTC value before IFN therapy was 0.97 ± 0.11 and was not different from the value before IFN therapy in other cases. In NCL tissues, the RTC value decreased to 0.63 ± 0.0092. The average rate of decrease of the RTC value was 0.057 ± 0.025/year.

**Histological Changes with IFN Therapy.** The extent of chronic inflammation was evaluated using the HAI scoring system. In this system, categories I, II, and III correspond to inflammation activity, and category IV indicates the level of fibrosis (19). Before IFN therapy, the activity score was 8.9 ± 2.4 in NR cases and 5.9 ± 2.0 in CR and BR cases without HCC. The score for CR and BR cases was significantly lower than that for NR cases (P = 0.027). The activity score in NR cases was 7.5 ± 1.7 at second biopsy, which is not significantly different from the score before IFN therapy (P = 0.13). In CR and BR cases, this score was significantly lower at second biopsy (2.9 ± 2.0; P = 0.0039). The fibrosis grade at first biopsy was 2.8 ± 1.2 for NR cases and 1.9 ± 1.1 for CR and BR cases without HCC, which are not significantly different from each other (P = 0.17). The fibrosis grade did not significantly improve at second biopsy in both NR cases (2.6 ± 1.1) and CR and BR cases [1.4 ± 0.88 (P = 1.0 and P = 0.50, respectively)]. There was no significant correlation between RTC values and HAI activity stage or between RTC values and HAI fibrosis grade (P = 0.13 and P = 0.18, respectively).

**Immunohistochemical Detection of TRF1 Expression.** The expression level of TRF1 was evaluated by immunohistochemistry in CR and BR cases with and without HCC because TRF1 overexpression leads to telomere reduction. Four other sets of HCC and NCL tissues were examined, and four samples of liver without chronic liver diseases were also examined as a control. Immunoreactivity against TRF1 was similar between two antibodies, which recognized the NH2 or the COOH terminus of TRF1, and rarely observed in control liver tissues, irrespective of HCC development, as shown in Fig. 3, A and B. In CR and BR cases without HCC, reactivity was observed at an index of 1.9 ± 0.60 (see “Materials and Methods”) before IFN therapy (Table 1; Fig. 3C). As shown in Fig. 3, C–F, immunoreactivity was essentially localized to the cytoplasm and rarely seen in the nucleus. After IFN therapy, the reactivity was significantly reduced in CR and BR cases to 0.67 ± 1.1 (P = 0.016; Fig. 3, C, D, and G). Although reactivity against TRF1 was maintained after IFN therapy in cases 14 and 16 at a level of 2 and 3, respectively, positive hepatocytes were seen in only one or two fields of a specimen. In contrast, TRF1 was extensively expressed in NCL tissues at a level of 3 in all four HCC cases (cases 18–21), in whom HCC developed after eradication of HCV (Fig. 3, E and F). In these cases, positive hepatocytes were located diffusely throughout the specimens. Before IFN therapy, TRF1 staining was observed at a level of 0, 0, 1, or 0 in cases 18–21, respectively.

**DISCUSSION**

We have reported that RTC value decreases significantly during the progression of chronic liver injury using liver samples from various diseases in various clinical stages (16), and the RTC value was lowest in the NCL tissues, with an average value of 0.66 ± 0.15. In this study, time-dependent telomere reduction was confirmed through the course of chronic hepatitis C using liver samples obtained from the same donor at sequential time intervals. Intriguingly, HCC developed in one NR case (case 8) 24 months after the second biopsy. The RTC value at the time of HCC development was estimated to be 0.68 from the regression rate between two biopsies [0.77 – (0.044/year) × 2]. ALT and AST values were maintained at the same high level during the course. This RTC value is consistent with the average value obtained for NCL tissues in the previous report.

A telomere-based crisis is induced when the average terminal restriction fragment length is reduced to around 3500 bp in normal human mammary cells (12), and the net telomere length at the time of crisis is estimated as 1500 bp (21). The average regression rate of the RTC value in NR cases was 0.040 ± 0.033/year, and HCC developed when the RTC value in the liver decreased approximately 0.66 (16). Thus, the telomere length in NR cases is estimated to be reduced by 91 bp every year [(1500 bp/0.66) × 0.04]. This means that the liver cells divide 2.3 times every year, if telomeres shorten 40 bp every population doubling (22). Because NCL tissue is estimated to divide an additional 35–50 times from our previous study, (16) it would take 15–22 years for HCC to develop as a result of chronic liver cell damage [(35–50)/2.3]. This estimation is consistent with clinical observations because HCC usually develops several decades after the outbreak of chronic...
hepatitis C (1, 23). Taken together, these observations strongly suggest that liver cells enter a telomere-based crisis when the RTC level reaches around 0.66.

Although eradication of HCV and normalization of ALT/AST levels decrease the risk of HCC development (3, 4, 24), the molecular basis for this decreased risk is unknown. Sustained inflammation due to chronic hepatitis C could result in a telomere-based crisis after several decades as discussed above. Therefore, therapies that inhibit the shortening of telomeres may reduce the risk of HCC development in chronic hepatitis. The present study shows the first direct evidence that IFN therapy could not only inhibit telomere reduction but also lead to telomere elongation. Although there was only one BR case in this study, it appears that normalization of ALT/AST levels inhibits telomere reduction, even in the presence of HCV viremia. The RTC level reveals the ratio of telomeric repeat content in the liver against that of peripheral blood leukocytes; therefore, an increase in the RTC level does not necessarily equate to telomere elongation. However, a reduction rate of RTC in PBLs from normal volunteers is reported to be 0.62% every year (16). Thus, an increase in the RTC value from 0.91 ± 0.14 to 1.0 ± 0.085 during 12 months in CRs without HCC indicates absolute telomere elongation in the liver. Further investigations are required to understand the mechanism of telomere elongation after improvement of inflammation.

Although histological progression is accepted as an indicator for HCC development in chronic liver diseases, it is a fact that HCC does not always accompany cirrhotic change (25). Furthermore, HCC has been observed after successful eradication of HCV (5, 26). Four such rare cases, cases 18–21, were enrolled in this study. Although we estimated at first that telomeres had expired at the start of IFN therapy in such cases, RTC values before IFN therapy were not different from those in other cases. On the other hand, the RTC value decreased to 0.63 ± 0.0092 in NCL tissue from the CRs developing HCC. Interestingly, this RTC value is consistent with that reported previously for NCL tissue. It is possible that some other etiology, in addition to HCV, may be involved in case 20 because the HAI activity score was high, even after successful HCV eradication. However, in the other three cases, no significant active hepatitis was observed in NCL tissues, and ALT/AST levels were below the normal limits after IFN therapy, even in case 20. Fibrosis and lobular disorganization were very mild in case 18, and the HAI fibrosis grading was not significantly reduced after successful IFN therapy in CR cases without HCC. Thus, even with histological examination, it is very difficult to differentiate a patient at risk of HCC development among successfully treated cases. Although this study was performed with a very limited sample size, the measurement of RTC values could be useful for HCC risk assessment independent of histological and serological evaluation.

Why did telomere length reduce in four HCC cases after a complete response to IFN therapy? To answer this question, we evaluated TRF1 expression level by immunohistochemical study because this protein has a function to shorten telomere length (8, 27). The immunoreactivity of TRF1 was extensively observed in NCL tissues in all four cases in which HCC developed after successful HCV eradication. Although the reactivity increased with progression of hepatitis, it decreased when inflammation was resolved with IFN therapy in CR cases without HCC. We were concerned that HCC might have caused extensive TRF1 positivity, but there was no such reactivity in NCL tissues in any of the four HCC cases who did not receive IFN.
therapy. Continuous necroregenerative inflammation due to chronic hepatitis is responsible for telomere reduction in the liver as shown in NR cases. In rare cases, however, overexpression of TRF1 may cause critical telomere reduction in hepatocytes without inflammation. Thus, TRF1 may have contradictory roles for hepatocarcinogenesis (carcinogenic and anticarcinogenic roles). Overexpression of TRF1 may force hepatocytes toward tumorigenesis by inducing telomere reduction. Once a telomere-based crisis has emerged, TRF1 overexpression may inhibit proliferation of tumor cells by specifically inducing entry into mitosis and apoptosis with the response to double-strand break repair (28). Similar observations were reported in malignant intracranial tumors and gastrointestinal cancers (29, 30). Because the signals were detected mainly in the cytoplasm and because the reactivity decreased as chronic hepatitis reinitiated with IFN therapy, only excess production of TRF1 could be documented in our setting. The immature hepatocytes induced through active inflammation may lead to an increase in TRF1 expression, as in the case of CD31-positive cells in a myeloid lineage (31).

In contrast to APC, k-ras, and p53 in multistep carcinogenesis of colorectal cancers (32–34), oncogenes or tumor suppressor genes responsible for the majority of HCC have not been identified. On the contrary, telomere reduction is associated with progression of HCC in the vast majority of the patients with chronic hepatitis, and telomere elongation is associated with retardation of HCC development as a result of successful IFN therapy. The critical telomeric repeat content was estimated to be approximately the same between longitudinal and cross-section studies. The time dependence for hepatocarcinogenesis observed in the clinic is consistent with the time requirement for HCC development deduced from the telomere reduction rate. It is possible that TRF1 overexpression is responsible for telomere erosion in the absence of chronic inflammation. Although the regulatory mechanism of TRF1 expression in hepatocarcinogenesis should be investigated further in many cases, all observations strongly suggest that reduction of telomeres to a critical level plays a major role in hepatocarcinogenesis and that the RTC value could digitally indicate the risk of HCC development even after regression of hepatitis.

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REFERENCES


18. World Medical Association Declaration of Helsinki. Ethical prin-


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