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Cancer Genetics and Cytogenetics 187 (2008) 34–38

CANCER GENETICS AND CYTOGENETICS

Telomere length in Hepatitis C

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Received 29 July 2008; accepted 13 August 2008

Abstract

Telomeres are nucleoprotein structures located at the termini of chromosomes that protect the chromosomes from fusion and degradation. Hepatocyte cell-cycle turnover may be a primary mechanism of telomere shortening in hepatitis C virus (HCV) infection, inducing fibrosis and cellular senescence. HCV infection has been recognized as potential cause of B-cell lymphoma and hepatocellular carcinoma. The present study sought to assess relative telomere length in leukocytes from patients with chronic HCV infection, patients after eradication of HCV infection (in remission), and healthy controls. A novel method of manual evaluation was applied. Leukocytes derived from 22 patients with chronic HCV infection and age- and sex-matched patients in remission and healthy control subjects were subjected to a fluorescence-in-situ protocol (DAKO) to determine telomere fluorescence intensity and number. The relative, manual, analysis of telomere length was validated against findings on applied spectral imaging (ASI) in a random sample of study and control subjects. Leukocytes from patients with chronic HCV infection had shorter telomeres than leukocytes from patients in remission and healthy controls. On statistical analysis, more cells with low signal intensity on telomere FISH had shorter telomeres whereas more cells with high signal intensity had longer telomeres. The findings were corroborated by the ASI telomere software. Telomere shortening in leukocytes from patients with active HCV infection is probably due to the lower overall telomere level rather than higher cell cycle turnover. Manual evaluation is an accurate and valid method of assessing relative telomere length between patients with chronic HCV infection and healthy subjects. © 2008 Elsevier Inc. All rights reserved.

1. Introduction

Telomeres are nucleoprotein structures located at the termini of chromosomes. In humans, telomeres consist of tandem repeats of the DNA sequence TTAGGG extended over an area of 10-15 kb. Telomeres protect the chromosome ends from fusion and degradation. They are also essential for the normal segregation and maintenance of chromosomes, compensating for the DNA loss and consequent chromosomal shortening that occur during mitotic and meiotic cell division [1]. To maintain chromosomal integrity, telomeric repeats are added to the chromosome via the telomerase enzyme, a specialized reverse transcriptase consisting of an RNA template (hTR) in addition to a catalytic subunit (hTERT). Expression of hTERT is the rate-limiting determinant of telomerase activity [2,3]. Recent studies have shown that several other, still undiscovered, factors may also be involved in the regulation of telomeric function [1-3].

In humans, telomerase is normally active in germ cells but not in somatic cells, where telomeric DNA shortens progressively with time as a result of the end-replication mechanism [4]. According to the telomere theory of aging, based on studies on yeast and in vitro human fibroblast models [3-7], at a critical point in this process, human somatic cells enter a nonreplicative but viable state, known as senescence (cellular growth arrest; mortality stage 1). This is followed by a second cell proliferation barrier, mitotic crisis (mortality stage 2 of senescence), and cell apoptosis [8].

Upregulation/activation of telomerase in human aged somatic cells may help stabilize the telomeres or cap them as functional. In this manner, genetically unstable cells that

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0165-4608/08/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.cancergencyto.2008.08.006

bypass crisis are immortalized by telomere elongation. Almost all cancer cells (85-90%) are characterized by abnormal re-expression of telomerase and drastically shorter telomeres compared to those in surrounding healthy tissue [3,5,6]. Dysfunctional telomeres may recombine and fuse, initiating random chromosome breakage and the formation of dicentric chromosomes, thereby increasing chromosome instability and the risk of oncogenesis [9,10] Dysfunctional telomeres support the survival of aneuploid cells, a characteristic of many human and murine cancers [11].

Chronic hepatitis B and C viral infections, with or without cirrhosis, are major preneoplastic conditions [12,13]. Studies have shown that the majority of hepatocellular carcinomas arise in this pathological setting [14]. Hepatitis C virus (HCV) has recently been recognized as a potential cause of B-cell lymphoma. Its association with non-Hodgkin lymphoma (NHL) was first suggested in studies of patients with essential mixed cryoglobulinemia, a chronic autoimmune disease with underlying bone marrow B-cell clonal proliferation [12,14].

There is evidence suggesting that hepatocyte cell-cycle turnover is a primary mechanism of telomere shortening in HCV infection, inducing fibrosis and cellular senescence [15]. Hepatocyte cycling is accelerated by upregulation of mitogenic pathways, in part through epigenetic mechanisms. This leads to the production of monoclonal populations of aberrant and dysplastic hepatocytes, which are characterized by telomeric erosion and telomerase re-expression and, occasionally, microsatellite instability and structural genetic or chromosomal aberrations [15,16]. In addition, senescence may be accelerated by oxidative stress, especially in advanced stages of fibrosis.

The aim of the present study was to assess relative telomere length in leukocytes from patients with chronic HCV infection, HCV patients after eradication of the virus (in remission), and healthy controls. A novel method of manual evaluation is described.

2. Materials and methods

2.1. Patients

The study sample consisted of 45 patients with a diagnosis of HCV infection attending a major medical center. Twenty-two patients, 16 females and 6 males (mean age 55.2 years) had chronic hepatitis, defined as levels of alanine aminotransferase (ALT)/aspartate aminotransferase (AST) $\geqslant$37 IU/L, gamma glutamyl transferase (GGT) $\geqslant$ 40 IU/L, and alkanine phosphatase (AP) $\geqslant$120 IU/L. Twenty-three patients, 17 females and 7 males (mean age 52.5 years), had achieved remission from HCV infection after treatment. Remission was defined as maintenance of normal liver enzyme levels for at least 6 months after interferon treatment. The duration of remission in our patients ranged from 6 to 10 years. Twenty-five healthy, age-matched subjects (mean age 50.6 years) served as the control group.

2.2. Lymphocyte culture

Lymphocytes derived from peripheral blood were cultured with RPMI 1640 supplemented with phytohemagglutinin (PHA), 0.2 heparin, and 1% antibiotics. Colchicine at a final concentration of 0.1 μg/mL was then added to the cultures for 1 hour. This was followed by hypotonic treatment with 0.075-M KCl at 37 °C for 15 minutes and 4 washes with a fresh cold 3:1 methanol:acetic acid solution. The lymphocyte suspensions were stored at 4 °C.

2.3. Flow cytometry analysis

For DNA content analysis, 100 μl of whole blood were lysed with 2 ml of ammonium chloride lysing reagent (cat. no. 555899, PharmLyse; BD PharMingen, San Jose, CA) at room temperature for 15 min and washed once with 2 mL of phosphate-buffered saline (PBS). The samples were processed with a DNA-PREP reagent kit (cat. no. 6607055, Beckman-Coulter, Fullerton, CA) according to the manufacturer's instructions and then transferred to a flow cytometer (Epics-XL, Beckman-Coulter, Fullerton, CA). A total of 10,000 events were accumulated for each sample. The histogram was analyzed with multicycle software.

2.4. Fluorescence in situ hybridization (FISH)

The FISH protocol used here has been described previously [17]. The fluorescence intensity in the nuclei was measured with a C3Y-labeled telomere-specific peptide nucleic acid probe (cat. no. 5326; DAKO, Glostrup, Denmark). The slides were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (1000 ng/mL, Vysis-32-804830; Abbott Molecular/Vysis, Des Plaines, IL) and overlaid with a glass coverslip for observation of the number of telomere signals.

2.5. Applied Spectral Imaging (ASI)

Lymphocytes (83-161 cells) from 5 patients with HCV and 5 control subjects were analyzed with ASI software (Beith Haemek, Israel).

2.6. Study design

Telomere length was quantified as a function of the signal intensity and telomere number. The cells were first categorized as having high (strong) or low (weak) fluorescence, and then further categorized by number of telomere signals: $0 \leqslant 10$, $11 \leqslant 30$, or $\geqslant$31. This yielded a total of six categories, as shown in Table 1. This analysis was done for each of the study groups (patients with active HCV, patients in remission, and controls), and the findings were compared. The manual analysis was validated against the ASI results.

Table 1
Mean number of cells in the different categories of signal intensity (low, high) /telomere number (⩽10, 11 ⩽ 30, ⩾31)

Signal	Signal intensity/ telomere number Low	Low	Low	High	High	High
Group	⩽10	11 ⩽ 30	⩾31	⩽10	11 ⩽ 30	⩾31
Control	7.65 ± 8**	23.12 ± 15	20.99 ± 11	23.37 ± 7**	20.22 ± 10**	4.62 ± 5**/***
Eradicated HCV	9.02 ± 8**	22.19 ± 8	15.69 ± 8	19.29 ± 10	25.5 ± 11**	8.28 ± 8**
Chronic HCV	18.71 ± 10*	26.33 ± 8	26.38 ± 10**	17.56 ± 7*	10.35 ± 7*	1.17 ± 1.6***

* Significance $p < 0.05$ vs. control.
** Significance $p < 0.05$ vs. chronic HCV.
*** Significance $p < 0.05$ vs. eradicated HCV.

2.7. Statistics

Pearson correlation analysis was applied to determine the relationship of cell signal intensity with mean number of telomere signals for each of the study groups per category. The correlation coefficient (r) calculated for each group. Data were further analyzed by one-way ANOVA and the nonparametric Mann-Whitney test. A p value of <0.05 was considered statistically significant. All analyses were performed with SPSS software, version 14.

3. Results

As shown in Fig. 1, the distribution of the cells among the categories was nearly equal in the control group ($r = 0.138$) and in the patients in remission ($r = 0.074$). These findings indicate that there was no correlation of telomere number with signal intensity. By contrast, in the active-HCV group, there were significantly more telomeres per cell in cells of low than high intensity ($r = 0.8$; Fig.1), yielding a positive inverse correlation of high telomere number and low signal intensity.

The results for the ANOVA study are shown in Table 1. At the lowest intensity, there were significantly more telomeres per cell in the lymphocytes from the chronic HCV group than in those from the remitted HCV group or the controls ($p < 0.001$). At high intensity, there were significantly more telomeres per cell in lymphocytes from the control and remitted HCV groups than in lymphocytes from the chronic HCV group ($p < 0.001$). Furthermore, at high intensity, the number of telomere signals per cell was significantly higher for the remitted group than for the control group ($p < 0.05$).

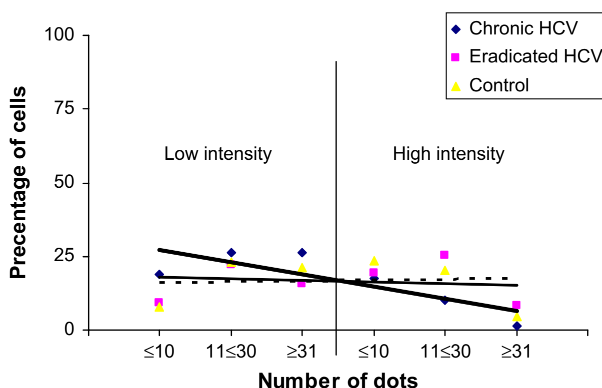

Fig. 1. Mean percentage of cells and telomere length in patients with chronic HCV versus patients in remission and control group.

We then combined the three subcategories of low and high intensity for comparison. We found that the samples from the chronic HCV group contained significantly more cells of low intensity than the samples from the other two groups ($p \leqslant 0.001$; Table 2). Accordingly, samples from the control and remitted HCV groups had significantly more cells of high intensity than the chronic HCV group ($p < 0.001$; Table 2).

Similar results were obtained with the nonparametric Mann-Whitney test.

ASI analysis yielded significantly higher cell intensity values for the 5 tested patients with chronic HCV than the 5 control individuals ($p = 0.001$), corroborating the results achieved with standard methods.

4. Discussion

Previous studies have reported telomere shortening in hepatocytes from patients with active HCV, but not individuals with normal ALT levels [5]. On the basis of correlation analyses, the authors concluded that cell-cycle turnover is the primary mechanism of telomere shortening and can induce the progression of fibrosis and cellular senescence [5].

In the present study, we found, in agreement with earlier reports, that patients with active HCV had shorter telomeres than both healthy control subjects and patients in whom HCV infection had been eradicated. On statistical analysis, more cells with low signal intensity on telomere FISH had

Table 2
Mean number of cells by two intensity groups

Signal	Signal intensity Low	High
Group	Low	High
Control	51.77 ± 16**	48.22 ± 16**
Eradicated HCV	46.91 ± 16**	53.08 ± 16**
Chronic HCV	70.9 ± 12*/***	29.09 ± 12*/***

* Significance $p \leqslant 0.001$ vs. control.
** Significance $p \leqslant 0.001$ vs. chronic HCV.
*** Significance $p \leqslant 0.001$ vs. eradicated HCV.

shorter telomeres whereas more cells with high signal intensity had longer telomeres. These findings have important diagnostic implications, because in future readings using our microscope, we can define the number of telomeres per cell (<10 to >31) on the basis of fluorescence intensity (high, low). Microscopes at other centers may be even more sensitive. The use of ASI to directly measure telomere signal intensity indicated that our novel manual method of assessing relative telomere length (longer or shorter) between two groups is accurate and valid.

In disagreement with earlier studies, however, the similarity in cell-cycle turnover noted here between the patients with active HCV disease and the healthy, age-matched controls indicates that lymphocyte telomere shortening in HCV is not biologically attributable to shorter cell cycles. Our observations may be partly explained by previous reports of lower-than-normal levels of telomerase activity in peripheral lymphocytes from patients with HCV and HBV infection, quantified by real-time polymerase chain reaction studies of TERT DNA levels [18]. These authors suggested that the catalytic subunit of telomerase may be involved in the immunopathogenesis of chronic hepatitis B and C infection, given that lower telomerase activity may represent premature aging of the immune system [16,18].

Another potential explanation for the high rate of hepatocarcinogenesis in the natural history of these chronic liver diseases is provided by the persistently high telomerase levels in patients with remitted disease relative to the age-matched control subjects, as shown here. This finding suggests that the virus somehow affects the down regulation of telomerase synthesis. The diversity of telomere lengths in this group may have been due to the presence of residual virus particles in the lymphocytes [19-21].

Shorter telomere length has been found to correlate with higher random aneuploidy rate. In a previous study, we found significantly higher rates of random aneuploidy, including triploidy, in patients with active HCV compared to a control group [22]. This observation was not unexpected given reports that dysfunctional (short) telomeres can recombine and fuse, initiating random chromosome breakage and the formation of dicentric chromosomes and, thereby, increasing chromosome instability and the probability of oncogenesis [9,10]. High random aneuploidy rates have been reported in other malignant and premalignant conditions [17,23,24], high telomere aggregates rate and an asynchronous replication pattern was reported in patients with non-Hodgkin lymphoma and HCV [17,25,26]. Surprisingly, however, the patients in remission also had higher than normal rates of random aneuploidy, which ranged between those of the control and the chronically infected groups [22]. The failure of the random aneuploidy to disappear despite normalization of the ALT level indicates that the telomere-shortening characteristic of the active disease is irreversible. It also dovetails with recent findings of some HCV particles in lymphocytes and hepatocytes in patients after a sustained response to treatment[19-21]. Thus, taking the earlier data together with the present study of markers of disturbed cell-cycle progression and gene replication in HCV disease, we suggest that genomic instability persists in cells during remission. Patients with HCV may retain some of the components that initiate the cascade of events leading to malignancies, and their risk of developing hepatocellular carcinoma is life-long [19-21].

A search for additional molecular cytogenetic parameters and additional telomere and telomerase studies in larger samples are needed to reach definitive conclusions regarding the epigenetic mechanisms underlying HCV infection. The prognostic value of telomeres in non-Hodgkin lymphoma and other malignancies and premalignant conditions also warrants further investigation.

Acknowledgments

This research was performed as part of L. Goldberg-Bittman's Ph.D. thesis.

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