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Telomere dysfunction in peripheral blood mononuclear cells from patients with primary biliary cirrhosis

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ABSTRACT

Background: Chromosomal instability in peripheral blood mononuclear cells has a role in the onset of primary biliary cirrhosis. We hypothesized that patients with primary biliary cirrhosis may harbour telomere dysfunction, with consequent chromosomal instability and cellular senescence.
Aim: To evaluate the clinical significance of telomerase activity and telomere length in peripheral blood mononuclear cells from patients with primary biliary cirrhosis.
Study design: In this population-based case control study, 48 women with primary biliary cirrhosis (25 with cirrhosis), 12 with chronic hepatitis C matched by age and severity of disease, and 55 age-matched healthy women were identified. Mononuclear cells from the peripheral blood of patients and controls were isolated. Telomere length and telomerase activity were measured.
Results: Telomere length and telomerase activity did not differ between cases (5.9 ± 1.5 kb) and controls (6.2 ± 1.4 kb, $p_c = 0.164$). Telomere shortening and advanced-stage disease strongly correlated with telomerase activity. Patients with advanced disease retained significantly less telomerase activity than those with early-stage disease (0.6 ± 0.9 OD vs. 1.5 ± 3.7 OD, $p = 0.03$). Telomere loss correlated with age, suggesting premature cellular ageing in patients with primary biliary cirrhosis.
Conclusion: Our data strongly support the telomere hypothesis of human cirrhosis, indicating that telomere shortening and telomerase activity represent a molecular mechanism in the evolution of human cirrhosis in a selected population of patients.

1. Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by progressive destruction of intrahepatic bile ducts with cholestasis, portal inflammation, and fibrosis [1,2]. The origin of PBC may be associated with sex chromosome defects mainly based on the observation that X chromosome contains a considerable number of sex- and immune-related genes [3–6]. It has been suggested that environmental factors may trigger immune-mediated aggression against small and medium intrahepatic bile ducts in genetically predisposed individuals [7,8]. The chronic immune characteristic of PBC, similarly to the majority of autoimmune disorders, can affect the stability, replication and function of chromosomes [5,7,8].

Telomere dysfunction is a hallmark of chromosomal instability [9–11]. Telomeres are chromatin structures formed by kilobases of tandem repeats of the sequences TTAGGG and specific proteins, which cap and protect the end of chromosomes [12]. These telomeric repeats are elongated by telomerase, a ribonucleoprotein enzyme that adds TTAGGG repeats into pre-existent telomeres and extends the 3′ end of telomeres. In humans the telomerase is expressed only in germ and stem cells, and prevents critical shortening of their telomeres. Studies in somatic cells have demonstrated that there is a telomere progressive shortening with every cell division and it is believed to be a biological clock, which leads to cellular senescence. Stably capped telomeres contribute greatly to genome stability and a healthy lifespan. Telomerase can counteract replicative senescence by maintaining telomeres and genomic stability. Telomerase also plays a role in ageing and longevity of organisms via its telomere stabilizing function as demonstrated in a cancer-protected mouse model [13].

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Telomere dysfunction has been described in many human pathologies [12,14] mainly in cancers but also in inflammatory disorders such as autoimmune [15,16] and liver diseases [17]. Indeed, telomerase activity is constantly present in malignant cells which are able to escape senescence and to have unlimited replication potential [10,14]. Accelerated telomere loss (with a reactive, but ineffective, enhanced telomerase activity) has also been observed in different lymphocyte subset from patients with autoimmune diseases, reflecting premature ageing of the immune system [15,16]. To this end, it is important to note that women with PBC and other autoimmune diseases are characterized by an enhanced X monosomy rate in peripheral blood mononuclear cells (PBMCs), particularly T and B lymphocytes [3,18], possibly due to chromosomal instability. Finally, a number of reports showed that telomere dysfunction is a disease-dependent sign of liver cirrhosis thus suggesting that hepatocellular telomere shortening and senescence represent a molecular mechanism in the evolution of human cirrhosis [17]. Similarly, a significant decrease in telomere length was recently found in the biliary epithelial cells of patients with PBC, the target organ of the disease [19]. Remarkably, there are several studies analysing telomeres in liver tissue of patients with liver disorders of different origin but hardly any studies analysing telomere function in PBMC. In this regard, Hoare and colleagues showed the relation between lymphocyte telomere length, age and clinical outcome in non-hereditary liver disorder such as chronic hepatitis C virus (HCV) infection [20].

Since telomere shortening may be a trigger of senescence of immune-related cells and autoimmunity, we here tested the hypothesis that patients with PBC bear telomere dysfunction in PBMCs, by estimating telomere length and telomerase activity.

2. Materials and methods

2.1. Study population

Blood samples were obtained from 48 women with PBC. The diagnosis of PBC was based on the internationally accepted criteria, which include the presence of cholestatic liver disease for at least six months, a liver biopsy compatible with the diagnosis, a positive test for anti-mitochondrial antibodies (AMA), serum alkaline phosphatase levels of at least one and a half times more than the upper normal limit, and the absence of biliary obstruction as assessed by ultrasonography, computed tomography or endoscopic cholangiography [2,21]. The characteristics of PBC patients are shown in Table 1. Serum autoantibodies were determined using indirect immunofluorescence [22]. All PBC patients were AMA positive [23]. The following were considered disease-related symptoms: pruritus, jaundice, major complications of portal hypertension, i.e. hepatic encephalopathy, variceal bleeding or ascites requiring diuretic therapy. Disease duration was calculated as the time from the date of the earliest suspected evidence of liver disease to the date of blood collection. Twenty-three patients with no fibrosis on liver biopsy fibrosis (Ludwig's stage I and II) [24] were considered as having early-stage disease, while the remaining 25 with fibrosis or cirrhosis (stage III or IV) as having advanced disease. At the time of enrolment, 38 (79%) of the PBC patients were receiving ursodeoxycholic acid (UDCA) treatment.

The control populations consisted of 12 women with chronic hepatitis C (CHC) who attended our Liver Unit during the same enrolment period as the PBC patients, and 55 age-matched healthy women. The CHC patients were matched with the PBC patients in terms of age (10 five-year age classes ranging from 34 years or younger to 75 years or older) and the severity of liver disease (absence of cirrhosis, presence of compensated cirrhosis, development of major complications of portal hypertension). CHC patients underwent blood sampling before receiving any anti-viral medication.

All subjects provided written consent after being informed about the nature of the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a priori approval by the institution's human research committee.

2.2. Cell separation

PBMCs obtained from a total of 48 female PBC patients were used for this study. PBMCs were isolated from 40 ml of blood with ethylenediaminetetraacetic acid (EDTA) by density-gradient centrifugation using Histopaque-1077 (Sigma). After centrifugation, cells were washed with phosphate-buffered saline (PBS) and the viability of cells was determined using trypan blue. Part of these PBMCs was stored for the study on telomerase activity.

2.3. Telomere length measurement

DNA was purified from PBMC using the QIAamp DNA blood Midi Kit (Qiagen, and stored in aliquots at −20 °C. The mean terminal restriction fragment (TRF) length was determined by the use of the TeloTAGGG Telomere Length Assay (Roche). Briefly, 2 μg of purified DNA was digested in a final volume of 20 μl reaction mixture with *Hin*fI/*Rsa*I enzyme mixture (1 U/μl for each enzyme) at 37 °C for 2 h and electrophoresed on 0.8% agarose MP gel in 1× Tris–acetate–EDTA buffer (0.04 M Tris–acetate, 0.001 M EDTA, pH 8) for 2.30 h at 150 V cm. Gels were then denatured, neutralized, and Southern transferred to a positively charged nylon membrane, according to the protocol described by the supplier (Roche). The blotted DNA fragments were hybridized to a digoxigenin (DIG)-labelled probe specific for telomeric repeats and incubated with a DIG-specific antibody covalently coupled to alkaline phosphate. To determine the TRF length, the hybridized probe was visualized by chemiluminescent detection which detected TRF DNA on Hyperfilm (Amersham Biosciences). The exposed X-ray film was scanned and analysed densitometrically by Gel Doc with QuantityOne software (Biorad). The amount of telomeric DNA was calculated by integrating the area highest signal intensity of each smear. The TRF length was estimated relative to molecular weight standards that was run in each gel. For quantitative measurement and valid comparison of telomere length in different samples, control-DNA with short telomeres (L) and long telomeres (H) were included in each gel. Fig. 1 shows a representative Southern blotting gel.

2.4. Telomerase activity assay

Telomerase activity was measured in PBMCs from PBC patients and controls using the TeloTAGGG Telomerase PCR ELISA kit (Roche), which combines a telomere repeat amplification protocol (TRAP) assay with detection by ELISA. In brief, 2×10^5 PBMCs were added to an elongation/amplification mixture and distilled water in a total volume of 50 μl. The resulting elongation products were amplified by polymerase chain reaction (PCR), and PCR products were hybridized to a DIG-labelled telomeric repeat-specific probe bound to a streptavidin-coated 96-well plate. The binding reaction was detected with an anti-DIG-peroxidase antibody, visualized by a colour reaction product, and quantified photometrically. The absorbance of each sample was measured at 450 nm reading against the blank (reference wavelength 690 nm) using an ELISA microplate reader (Labsystems iEMS) within 30 min after addition of the stop reagent. Each sample was measured in duplicates. Each negative sample was obtained by heat treatment (10 min at 80 °C). The samples were regarded as telomerase-positive if the difference