Gene 593 (2016) 162–166

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Aneuploidy and asynchronous replication in non-alcholic fatty liver disease and cryptogenic cirrhosis

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ARTICLE INFO

Article history:
Received 21 February 2016
Received in revised form 24 July 2016
Accepted 8 August 2016
Available online 09 August 2016

Keywords:
Telomeres
FISH
Fatty liver
Cryptogenic cirrhosis

ABSTRACT

Background/aims: Non-alcoholic fatty liver disease (NAFLD) and cryptogenic cirrhosis (CC), which is largely a late sequela of NAFLD, are considered pre-neoplastic conditions that might progress to hepatocellular carcinoma. Aneuploidy, telomere aggregates and synchronization of replication were evaluated as markers of genetic instability in these patients.

Methodology: Peripheral blood lymphocytes from 22 patients with NAFLD, 20 patients with CC and 20 age-matched healthy controls were analyzed. To determine random aneuploidy, we used the fluorescence in situ hybridization (FISH) with probes for chromosomes 9 and 18. The rate of aneuploidy was inferred from the fraction of cells revealing one, three or more hybridization signals per cell. Aggregate size was divided into three fusion groups of 2–5, 6–10 and 11–15 telomeres, relative to the size of a single telomere. The replication pattern was determined by FISH in two pairs of alleles, 15qter and 13qter. Asynchrony was determined by the presence of one single and one set of double dots in the same cell.

Results: Significantly higher random aneuploidy rate was found in the CC patients than in the control group, and to a lesser degree in NAFLD patients. Telomere aggregates were insignificantly higher in both groups. Only patients with CC showed significantly higher rate of asynchronous replication with proportionately more cells with two single dots among the normal cells ($p < 0.001$).

Conclusions: These results likely reflect changes in gene replication and cell cycle progression in these conditions, possibly correlating with their malignant potential.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease in the Western world and represents a spectrum of clinicopathologic conditions, including simple steatosis, nonalcoholic steatohepatitis (NASH) with or without fibrosis, and cirrhosis (Chalasani et al., 2012; Vernon et al., 2011). Major risk factors for NAFLD are metabolic derangements that constitute the metabolic syndrome, namely obesity, diabetes type 2 and dyslipidemia. Despite its name, cryptogenic cirrhosis (CC) is largely caused by fatty liver disease, supported by the fact that patients with CC have disproportionally high prevalence of the metabolic derangements typical of patients with NAFLD (Vernon et al., 2011). In addition, patients with NAFLD are at increased risk for hepatocellular carcinoma (HCC), but this risk is probably limited to those with advanced fibrosis and cirrhosis (Chalasani et al., 2012; Ascha et al., 2010). Still, there is some evidence for an increased incidence of HCC even in the absence of significant fibrosis (Paradis et al., 2009).

Telomeres are nucleoprotein structures containing 10–15 kb repeats of the DNA sequence TTAGGG. They are located at the termini of chromosomes and protect their ends from fusion and degradation. DNA polymerase is unable to replicate the 3′ end primer of the chromosomes and therefore, telomeres become shorter with each cell division, leading to genetic instability and senescence (Harley et al., 1990; Counter et al., 1992; Preston, 1997). Short, dysfunctional telomeres lead to chromosomal breakage and reformation of dicentric chromosomes, which are genetically unstable and thereby increase the prevalence of aneuploid

Abbreviations: CC, Cryptogenic cirrhosis; CEP, Chromosome enumeration probes; FISH, Fluorescence in situ hybridization; HCC, Hepatocellular carcinoma; NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; PBS, Phosphate buffered saline; TA, Telomere aggregates.

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http://dx.doi.org/10.1016/j.gene.2016.08.017
0378-1119/

cells, as well as the risk of pre-malignant and malignant transformations (Preston, 1997; Pathak & Multani, 2006).

Aneuploidy refers to a chromosomal constitution of cells that deviates from the normal number of chromosomes or chromosome segments, generated by gain or loss of specific chromosomes or genes. According to the aneuploidy-cancer theory, carcinogenesis is initiated by a random aneuploidy, which causes chromosomal destabilization due to imbalance of highly conserved proteins that segregate, synthesize and repair chromosomes (Duesberg & Li, 2003; Duesberg et al., 2004). Thus, it is predicted that the chromosomal and genetic instability is proportional to the degree of aneuploidy (Pathak & Multani, 2006; Duesberg & Li, 2003).

Another marker of chromosomal instability, seen in various malignant and pre-malignant states, is the tendency to form telomere aggregates (TA), in contrast to the non-overlapping nature of telomeres in normal nuclei (Chuang et al., 2004). This can be apparent when a three-dimensional (3D) imaging approach is applied. It signifies not just a transient aberration in the 3D organization of the nucleus, but a true end-to-end fusion (Chuang et al., 2004). Telomere aggregates are formed during a breakage-bridge-fusion (BBF) cycle that contributes to deletions, gene amplification and overall genetic changes that are associated with tumorogenesis (Mai & Garini, 2006).

Another genetic aspect that can potentially lead to aneuploidy and genetic instability is the timing of DNA synthesis during the S-phase of cell division (McNairn & Gilbert, 2003; Woodfine et al., 2004). There is usually a close association between the specific time interval during S-phase at which a particular DNA sequence replicates in a given tissue and its transcriptional status: expressed loci usually replicate early, while unexpressed ones replicate late. A simple method for evaluating the temporal order of allelic replication uses the fluorescent in situ hybridization (FISH) assay, described by Selig et al., 1992. Using this method, several studies have clearly shown that concomitantly expressed alleles (biallellic expression) replicate synchronously, while alleles subjected to an allele-specific mode of expression (monoallelic expression) replicate asynchronously (Amiel et al., 1999a; Kitsberg et al., 1993).

The fact that the temporal order of replication of two allelic counterparts is preserved even in cells in which these alleles are not expressed (Ensminger & Chess, 2004), enabled researchers to study replication patterns of a large repertoire of genes in peripheral blood lymphocytes. It was then found that among patients with hematological malignancies (Amiel et al., 1998a; Korenstein-Ilan et al., 2002) or solid tumors, such as renal cell carcinoma or prostate cancer (Dotan et al., 2000; Dotan et al., 2004), as well as in pre-malignant conditions [23, 16], genes that normally replicate synchronously (e.g. tumor-suppressor gene TP53 and the oncogene C-MYC), lose their synchronous mode and replicate asynchronously.

Patients with NAFLD and CC have both hepatocyte telomere shortening (Nakajima et al., 2006; Carulli et al., 2012) and cellular senescence (Aravinthan et al., 2013; Wiemann et al., 2002), which attest to telomere dysfunction, probably reflecting the underlying metabolic derangements and pre-malignant potential. The current study aimed to determine whether markers of genetic instability – aneuploidy, telomere aggregates and replication patterns in peripheral blood lymphocytes – are present in patients with NAFLD and CC compared to healthy controls.

2. Patients and methods

2.1. Patients

The study included 3 patient groups: 22 with NAFLD, 20 with a diagnosis of cryptogenic cirrhosis (CC) and 20 healthy, age-matched controls. The average age and standard deviation in each group was 62.8 ± 9.82 in the NAFLD patients, 60.9 ± 8.64 in the CC patients, and 61.45 ± 11.37 in the control group. The number of male patients in each group was 11/22 (50%), 10/20 (50%) and 8/20 (45%), respectively.

Non-alcoholic fatty liver disease was defined as the presence of fatty liver in imaging and accompanying metabolic risk factors (diabetes, obesity and dyslipidemia) in the absence of significance alcohol consumption. Only 2 patients with NAFLD underwent liver biopsy to rule out other liver diseases.

Cryptogenic cirrhosis (CC) was diagnosed by the characteristic imaging of cirrhosis in the appropriate clinical context of accompanying metabolic risk factors and after other liver diseases were ruled out serologically. Seven patients in this group had liver biopsy, which showed the characteristic pathology of nodules separated by fibrotic septa, varied level of macrovesicular steatosis and the absence of significant inflammation.

2.2. Lymphocyte culture

Phytohemagglutinin, 0.2 ml heparin (1000 IU) and 1% antibiotics were added to RPMI 1640 culture medium. After incubation, colchicine (final concentration 0.1 μg/ml) was added to the cultures for 1 h, followed by hypotonic treatment (0.075 mol/l KCl at 37 °C for 15 min) and four washes each with a fresh, cold 3:1 methanol-acetic acid solution. The lymphocyte suspensions of the three samples were stored at −4 °C.

2.3. Fluorescence in situ hybridization (FISH) technique

As previously described (Dotan et al., 2000), fresh slide spreads were incubated for 10 min in 2× standard saline citrate (2× SSC) at 37 °C, followed with fixation in formamide (diluted 1:40 in phosphate buffered saline (PBS) and 0.18 g $MgCl_2$) for 15 min. The slides were washed in PBS for 5 min and incubated in pepsin solution (75 g lyophilized pepsin dissolved in 50 ml HCl 0.01 N) followed by a wash in PBS for 5 min. The slides were then dehydrated in a graded ethanol series and dried on a hot plate (40 °C) for 3 min.

2.4. Cytogenetic evaluation of random aneuploidy

Random aneuploidy was visualized with the chromosome enumeration probes (CEP) chromosome 18 Spectrum green (Catalog no. 5J10-18) and CEP chromosome 9 Spectrum orange (catalog no. 6J36-09) (Abbott Molecular-Vysis, Des Plaines, IL. USA). Probes for chromosomes 9 and 18 were used to detect aneuploidy. For each cell, we recorded the number of hybridization signals. The rate of aneuploidy was inferred from the fraction of cells with one, three or more hybridization signals per cell. Triploidy was defined as both probes (green and red signals) showing three signals in the same nucleus. At least 200 nuclei were scored from each sample and the slides were scored blindly.

2.5. Telomere aggregate count

As described in detail previously (Amiel et al., 2009) aggregate size was divided into three groups relative to the size of a single telomere: 1) Fusion of 2–5 telomeres; 2) fusion of 6–10 telomeres; and 3) fusion of 11–15 telomeres (Fig. 1). We used one filter for three colors, automatic exposure for both images, and ×100 magnification on an AX70 Olympus Provis microscope.

2.6. Cytogenetic evaluation for replication status

Two directly-labeled commercial probes were used: 15qter (Cytocell, UK; Cat No. C-LPU005) and 13qter (Cytocell, UK; Cat No. C-LPU006). The cells were classified into three categories according to the replication status of the two homologous loci: two singlets (SS), two doublets (DD) and singlet and doublet (SD), as reviewed by Selig et al., 1992. Accordingly, an unreplicated DNA sequence reveals a single fluorescent signal (singlet; S) at interphase, while a replicated sequence gives rise to a doubled signal (doublet; D). Thus, in a population of replicating cells, a high frequency of nuclei with two similar hybridization