Telomeres in Liver Cancer

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Liver cancer is one of the most common cancer types worldwide and the fourth leading cause of cancer-related death. Liver carcinoma is distinguished by a high heterogeneity in pathogenesis, histopathology and biological behavior. Dysregulated signaling pathways and various gene mutations are frequent in hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA), which represent the two most common types of liver tumors. Both tumor types are characterized by telomere shortening and reactivation of telomerase during carcinogenesis.

The observation that TERT promoter mutations occur early during liver carcinogenesis highlights the importance of telomerase activity for tumor cell survival. Two possible scenarios are conceivable how telomerase contributes to tumorigenesis in liver cancer: On the one side, telomerase reactivation before entering the crisis checkpoint may stabilize critically short telomeres, providing growth advantage for cells with oncogenic mutations. On the other side, early reactivation of telomerase may be related to its non-canonical functions.

Similarities and differences between HCC and iCCA in telomere biology are depicted in this review article.

Keywords: liver cancer ; hepatocellular carcinoma ; intrahepatic cholangiocarcinoma ; telomere shortening ; TERT promoter mutation ; telomerase

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Liver cancer is one of the most common cancer types worldwide and the fourth leading cause of cancer-related death. Liver carcinoma is distinguished by a high heterogeneity in pathogenesis, histopathology and biological behavior. Dysregulated signaling pathways and various gene mutations are frequent in hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA), which represent the two most common types of liver tumors. Both tumor types are characterized by telomere shortening and reactivation of telomerase during carcinogenesis. Continuous cell proliferation, e.g., by oncogenic mutations, can cause extensive telomere shortening in the absence of sufficient telomerase activity, leading to dysfunctional telomeres and genome instability by breakage–fusion–bridge cycles, which induce senescence or apoptosis as a tumor suppressor mechanism. Telomerase reactivation is required to stabilize telomere functionality and for tumor cell survival, representing a genetic risk factor for the development of liver cirrhosis and liver carcinoma. Therefore, telomeres and telomerase could be useful targets in hepatocarcinogenesis. Here, we review similarities and differences between HCC and iCCA in telomere biology.

1. Introduction

Liver cancer is predicted to be the sixth most common tumor disease worldwide and the fourth leading cause of cancer-related death [1]. Liver cancer presents with a high heterogeneity in pathogenesis, histopathology and biological behavior. The heterogeneous disease in terms of etiologies reflects the poor prognosis of patients with liver cancer. The two most common types of liver cancer are hepatocellular carcinoma (HCC) (75–85% of cases) and intrahepatic cholangiocarcinoma (iCCA) (10–15% of cases) [1]. Most liver carcinomas are diagnosed at advanced stages despite the surveillance program of patients with liver cirrhosis to diagnose early liver tumors. To date, therapy options are limited to the multikinase inhibitors sorafenib and lenvatinib as first-line treatment options and regorafenib and cabozantinib as second-line treatment options for liver cancer patients [2][3][4]. A recent clinical trial revealed significantly longer overall and progression-free survival in patients with unresectable hepatocellular carcinoma, who received atezolizumab, a programmed death ligand 1 (PD-L1) inhibitor, combined with bevacizumab, a monoclonal antibody targeting the vascular endothelial growth factor (VEGF), in comparison to sorafenib only [5]. These findings point to new treatment options in patients with unresectable hepatocellular carcinoma and support the development of new therapy options.

The major risk factors in hepatocarcinogenesis are chronic viral infections with hepatitis B virus (HBV) and hepatitis C virus (HCV), heavy alcohol consumption, obesity, type 2 diabetes, smoking and long-term exposure to aflatoxin B [6]. Most liver tumors arise on the basis of chronic liver diseases and often result in liver fibrosis and cirrhosis formation, which itself represents a risk factor for tumor development. In western countries, HCC is mainly related to HCV, high alcohol consumption and non-alcoholic steatohepatitis (NASH) and connected with cirrhosis formation [7][8], whereas in Asia, most

HCC patients are related to HBV infection; in addition, HCC can also develop in normal liver without fibrosis/cirrhosis or liver with limited fibrosis formation [9]. Interestingly, in Japan, chronic HCV infection is more common than HBV, and HCV infection accounts for the majority of HCC [10]. The burden of hepatocellular carcinoma is continuously growing due to increased rates of obesity, type 2 diabetes and nonalcoholic fatty liver disease (NAFLD), especially in low-risk HCC areas and thereby replaces viral- and alcohol-related chronic liver diseases [11]. Due to the diversity of risk factors, a high heterogeneity in liver tumors is reported. The molecular heterogeneity in terms of various gene mutations in liver cancer requires the identification of molecular targets for designing individualized therapies. Recent studies have described various sub-classification of HCC and iCCA tumor types [12][13][14]. Individual studies underline the importance of specific treatment options based on the tumor subtypes as a key to achieving a better overall survival of liver cancer patients [15][16][17]. The identification of dysregulated molecular pathways in premalignant lesions is required for an early disease detection in hepatocarcinogenesis [18]. A detailed description of molecular targets in the diversity of liver cancer subtypes would be beneficial for targeted therapies.

Telomere shortening and reactivation of telomerase, two common hallmarks of carcinogenesis, are described in a broad range of human cancers, including liver cancer [19][20][21]. Telomere shortening and reactivation of telomerase, through TERT promoter mutations, for example, represent genetic risk factors for the development of liver cirrhosis and liver cancer [22]. Therefore, disruptions in telomere biology could be a useful target for the treatment of liver cancer. In the following, we will present the current knowledge of telomere biology in HCC and iCCA.

2. Telomere Shortening in Liver Cirrhosis and Hepatocellular Carcinoma

Chronic liver disease is associated with chronic liver inflammation, which can lead to cell death and compensatory cell regeneration. The liver is characterized by a high regenerative reserve [23], which decreases in the context of chronic liver disease, consequently leading to telomere shortening and limiting the regenerative reserve of the liver. The frequent appearance of senescent hepatocytes in liver cirrhosis is the result of the loss of telomeric repeats and the extensive proliferation [24][25][26][27]. These senescent hepatocytes exhibit markers like $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ and are positive for senescence-associated β-galactosidase staining. A disruption of the p53-signaling pathway overcomes the senescence checkpoint and leads to further cell division of hepatocytes with already-shortened telomeres until the telomeres become critically short. At this point, the cells enter the crisis checkpoint, which is characterized by massive cell death [27][28].

In the liver, telomerase activity is downregulated during early embryonic development, and telomerase activity is absent in the adult liver. The healthy liver is a slowly proliferating organ, and most hepatocytes are in a quiescent stage (only one out of 20,000 cells (0.005%) is in the cell cycle [29]). Thus, telomerase activity seems not to be essential for hepatocyte function in a healthy liver. However, telomere shortening occurs in the absence of sufficient telomerase activity in hepatocytes under conditions of chronic liver diseases or upon injury [25]. Importantly, low levels of telomerase activity were observed under regenerative conditions, indicating the potential physiological activation of telomerase in adult hepatocytes [30][31]. So far, two distinct mechanisms were made responsible for telomere shortening upon increased proliferative signals, either due to oncogene activation (e.g., *Ras* mutations or *c-Myc* amplification) or expression of viral oncogenic proteins: (i) in most of the cases, absence of sufficient telomerase reactivation [32][33] and (ii) in some cases, germline mutations within the coding region of telomerase, which impair the enzymatic activity of telomerase in proliferating hepatocytes (see Section 5) [34][35].

Reactivation of telomerase activity has been shown in more than 80% of HCCs, which suggests that telomerase activation is a rate-limiting process for liver cancer formation [36][37]. The reactivation of telomerase correlates with the upregulation of both essential components TERT and TERC, respectively. It has been reported that the re-expression of TERT and activation of telomerase occurs at early premalignant stages in regenerative nodules and cirrhotic livers [38][39]. Importantly, telomerase activity was detected both in HCC and in iCCA to a similar extent (80–85%) [40]. Thus, it is important to emphasize at this point that the majority of both HCCs and iCCAs are similarly telomerase-positive, highlighting the necessity of telomerase activity for telomere functionality and tumor progression.

Irrespective of the mechanism, insufficient telomerase activity leads to accelerated telomere shortening in proliferating liver cells and as a result to genomic instability by breakage–fusion–bridge cycles (see review by Meena et al. [41]). In cells with intact DNA-damage response (DDR) checkpoints, telomere shortening leads to senescence/apoptosis and functions as a tumor suppressor mechanism. On the other hand, in cells lacking functional DDR, telomere shortening promotes genome instability and tumor formation.

Consequently, telomere shortening is an important risk factor for tumor initiation in liver carcinogenesis. The risk of tumor formation drastically increases at the cirrhosis stage, which is characterized by increased hepatocyte senescence, and upon further cell division at the crisis checkpoint by apoptosis of hepatocytes. Several studies have shown that telomere

shortening is more pronounced in liver carcinoma compared to the surrounding liver tissue (see review by Satyanarayana et al. [42]). Furthermore, the progressive shortening of telomeres and the inactivation of cell cycle checkpoints in premalignant lesions led to the identification of a preneoplastic-sequence in human hepatocarcinogenesis, suggesting that small cell changes (SCC) are more advanced precursor lesions compared to large cell changes (LCC) [43]. In addition, telomere shortening was more pronounced in HCCs with a high degree of aneuploidy compared to diploid HCCs [44][45]. In fact, several studies provide evidence for a role of telomere shortening in the induction of chromosomal instability and increased risk for tumor formation [46][47][48][49]. The importance of telomere shortening and dysfunctional telomeres in HCC initiation was shown in transgenic mouse models (see Section 3).

3. Mouse Models of Telomere Dysfunction in Hepatocarcinogenesis

To understand the severe situation of dysfunctional telomeres and telomere shortening during chronic liver disease, transgenic mouse models were used to analyze the functions of telomeres. To this end, it is important to note that, firstly, there is a substantial difference in the regulation of telomerase between mouse and human liver. Telomerase activity is detectable in resting mouse liver but not in resting human liver [50][51][52][53]. The limiting component, restricting telomerase activity in human tissues, is the catalytic subunit of the telomerase TERT [54]. Concordantly, there is a marked difference in *TERT* mRNA levels in human and mouse livers [52][53][54]. In fact, in vivo experimental evidence supports the idea that the species-specific differential regulation is based on different promoter organization [53][55]. Secondly, the average telomere length in laboratory mice is about five times longer than that of human telomeres, to some extent due to constitutive telomerase activity in mouse cells [56][57].

The telomerase knockout mouse (*Terc*−/−) lacking the RNA component of the telomerase enzyme was used to analyze telomere shortening in liver regeneration, chronic liver disease, and hepatocarcinogenesis [58][59][60]. Mice are characterized by the existence of longer telomeres compared to humans [56]. Mice of different backgrounds differ a lot in telomere length [61]. For this reason, the *Terc*−/− mouse has to be crossed until the third to the sixth generation, depending on the used background strain to generate mice with critically short telomere lengths [58][60][62]. In an experimental model of liver regeneration involving the removal of two-thirds of the liver by partial hepatectomy of G3 *Terc*−/− and *Terc*+/+ mice, telomere shortening was observed to be a heterogeneous event at the cellular level, which led to the inhibition of a subpopulation of cells with critically short telomeres to enter the cell cycle and prevent those cells from participating in liver regeneration [58]. By comparing the mean telomere fluorescence intensities measured by FISH analysis, Satyanarayana and colleagues [58] observed no significant differences in *TERC*+/+ mice between BrdU-positive cells (952.53 ± 144.19) and BrdU-negative cells (957.44 ± 130.57) but saw a dramatic reduction of the mean telomere fluorescence intensity of BrdU-negative cells (364.94 ± 116.45) in comparison to BrdU-positive cells (509.65 ± 101.30) in G3 *TERC*−/− mice. In a mouse model of experimentally induced acute liver damage in which *Terc*−/− mice were subjected to genetic, surgical and chemical impairment of the liver, dysfunctional telomeres were associated with defective liver regeneration and accelerated formation of liver cirrhosis, which could be partly rescued by adenoviral delivery of the telomerase RNA [24]. In an approach comprising three different cancer-prone model systems—1) treatment with CCl_4 (carbon tetrachloride), (2) treatment with DEN (diethylnitrosamine) and (3) a genetic model (urokinase plasminogen activator transgenic mice) in *Terc*−/− mice—it could be shown that telomere dysfunction has a differential impact on tumor initiation and tumor progression. In all three model systems, dysfunctional telomeres were associated with higher amounts of tumor initiation and a decline in tumor progression [63]. In mouse models of chronic liver damage achieved by crossing HBsAg-expressing mice (the mice express the hepatitis B surface antigen under the liver-specific albumin promoter [64]) with *Terc*−/− mice, contrary effects of telomere shortening were shown between the beneficial effect on suppression of tumor growth and the negative effect on organismal survival [59]. In another mouse model of chronic liver disease, HBsAg mice were crossed with *Terc*−/− and *Trp53* cKO mice. We generated mice with critically short telomeres by an intercross of *Terc*−/− and *Terc*+/− to generate siblings with loss of telomerase function in one group and telomerase expression in the other group. This study yielded the evidence for telomerase to be a critical component in the progression of *Trp53*-deficient hepatocellular carcinoma with short telomeres in the setting of chronic liver damage [60]. In addition, it was also shown that telomerase limits the accumulation of telomere dysfunction and the generation of aneuploidy by the activation of TRP53-independent checkpoints which suppress carcinogenesis [60]. Increased rates of chromosomal aberrations could be also shown in a DEN-induced liver cancer mouse model with dysfunctional telomeres. Telomerase knockout mice (*Terc*−/−) with chronic telomere dysfunction as well as a model of transient telomere dysfunction by inducing a dominant-negative variant of the TRF2 (telomeric repeat-binding factor 2) protein exhibited higher levels of chromosomal aberrations. In summary, the model of transient telomere dysfunction promotes chromosomal instability and liver carcinogenesis in telomerase-competent mice [65]. RAP1 (Ras-proximate-1 or Ras-related protein 1), like TRF2, is a component of the shelterin complex, which caps the telomere end for the protection of chromosome ends [66]. A recent

publication suggested an important role of RAP1 in the protection of liver damage and liver carcinogenesis. DEN-induced *Rap1−/−* female mice were more prone to liver damage and hepatocellular carcinoma [67]. These models reflect the complexity and opposing roles of dysfunctional telomeres in hepatocarcinogenesis.

4. Telomere Shortening in Cholangiocarcinoma

Intrahepatic cholangiocarcinoma (iCCA) is the second most common malignant liver tumor which arises from the biliary tract and is characterized by a very poor prognosis with rising incidence and mortality in recent years [68][69]. The main risk factors described for HCC are also reported for iCCA. Additional risk factors are primary sclerosing cholangitis (PSC), hepatobiliary flukes, biliary duct cysts and hepatolithiasis. A study by Verma and colleagues analyzed telomere shortening during aging in normal liver with no history of liver disease. Interestingly, they observed that the cholangiocytes exhibited the longest telomeres compared to all other analyzed intrahepatic lineages [70]. Similar to $CD4^+$ and $CD8^+$ lymphocytes, no significant telomere shortening was observed in cholangiocytes and hepatocytes of individuals without liver disease during aging. The authors only observed an age-related telomere shortening in Kupffer cells and stellate cells [70]. On the other hand, a consistent telomere shortening was reported during the development of biliary tract carcinoma, starting early in carcinogenesis in the inflamed biliary tract, metaplasia, dysplasia and carcinoma [71]. In contrast, the normal and the inflamed epithelium of the biliary tract showed a uniform telomere length [71]. Within cholangiocarcinoma, a frequent intratumoral heterogeneity of telomere length is reported [71]. As indicated above, telomere shortening in hepatocytes triggers cellular senescence in the context of intact DDR checkpoints. Similarly, an investigation of telomere shortening and senescence in the pathogenesis of primary biliary cirrhosis (PBC) showed telomere shortening. Moreover, DNA damage accumulation was detectable in biliary epithelial cells in the damaged small bile ducts and bile ductules in PBC in comparison to normal-looking bile ducts and bile ductules in PBC, chronic viral hepatitis and normal livers [72]. Of note, the accumulation of DNA damage foci correlated with increased expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$, which characterize biliary cellular senescence [72].

5. Loss of Function Mutations in Telomerase Components

Germline loss-of-function mutations in the telomerase components were found in a variety of human diseases, including dyskeratosis congenita, aplastic anemia, familial idiopathic fibrosis and acute myeloid leukemia [73][74][75][76][77][78][79][80]. These mutations provoked an impaired tissue regeneration due to telomere dysfunction and stem/progenitor cell exhaustion. Similar mutations were also reported in a subset of liver cancer samples. The authors analyzed *TERT* and *TERC* mutations in buccal mucosa tissue and peripheral blood of patients with liver cirrhosis and compared them with healthy non-cirrhotic controls. An increased number of telomerase mutations were found in the group with liver cirrhosis. The study by Calado et al. reported nine patients with a mutation in the *TERT* gene and one patient with a mutation in the *TERC* gene among 134 patients with liver cirrhosis. Similarly, Hartmann et al. reported mutations in the *TERT* and *TERC* genes in 16 out of 521 patients. The Calado study reported a significantly higher allele frequency for the gene variants in the *TERT* and *TERC* genes in patients with cirrhosis (allele frequency 0.037) compared to controls (0.008; $p = 0.0011$). A similar result was shown by the Hartmann study, which stated an increased incidence of telomerase mutations detected in cirrhosis patients (allele frequency 0.017) compared to non-cirrhotic controls (0.003, $p = 0.0007$). The mutations in telomerase components led to decreased telomerase activity in comparison to wildtype telomerase enzyme activity. Consequently, patients with these mutations showed shorter telomeres in peripheral white blood cells . Rare *TERT* mutations were also reported in patients with nonalcoholic fatty liver disease (NAFLD). Here, an enrichment of *TERT* mutations could be found in NAFLD-associated HCC [81]. Functional evaluation of these mutations exposed reduced protein synthesis from some of the mutations compared to the TERT wild-type protein. It is speculated that these *TERT* mutations could also impair the DNA-binding function of TERT. In summary, these results indicate that *TERT* mutations result in impaired telomerase activity, accelerated telomere shortening and impaired regeneration in chronic liver disease. These findings are supported by the above-mentioned studies indicating low/absent telomerase activity in resting liver and telomerase activation in the regenerating liver . Taken together, these studies show that telomerase activity acts as a protective mechanism in chronic diseases to prevent telomere shortening during accelerated cell proliferation, whereas *TERT* mutations result in telomere shortening and may promote hepatocarcinogenesis by dysfunctional telomeres.

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