Differential Impact of Telomere Dysfunction on Initiation and Progression of Hepatocellular Carcinoma

Paraskevi A. Farazi, Jonathan Glickman, Shan Jiang, Alice Yu, Karl Lenhard Rudolph, and Ronald A. DePinho

ABSTRACT

Telomere maintenance and telomerase reactivation are near universal features of human hepatocellular carcinoma (HCC), yet the shorter telomeres and highly abnormal cytogenetic profiles of HCC suggest that telomere erosion and dysfunction may be operative during the formative stages of tumorigenesis. Previous studies have established that the cancer-enhancing or suppressing impact of telomere dysfunction is highly dependent on several parameters including cell type, tumor stage, and p53 status. Here, to understand better the pathogenetic role of telomere dysfunction in the initiation and progression in human HCC, we have used three mechanistically distinct liver cancer-prone model systems (urokine plasminogen activator transgenic mice, carbon tetrachloride exposure, and diethylnitrosamine treatment) in the context of successive generations of telomerase-deficient mice null for the telomerase RNA component, mTERC. Across all of the HCC model systems, telomere dysfunction suppressed both the incidence and growth of HCC lesions, a trend that mirrored the level of intratumoral proliferative arrest and apoptosis. On the histological level, telomere dysfunction was associated with a significant increase in the number of early stage neoplastic lesions and a reciprocal decline in the occurrence of high-grade malignancies. These genetic data in the mouse indicate that telomere dysfunction exerts an opposing role in the initiation versus progression of HCC and provide a framework for understanding the intimate link among chronic liver disease, chromosomal instability, and increased HCC in humans.

INTRODUCTION

HCC is among the most prevalent and lethal cancers in humans. Worldwide, the high incidence of HCC has been linked to diverse etiologies including chronic hepatitis B and C viral infection, and alcohol exposure (1). The precise mechanisms through which these factors drive hepatocarcinogenesis are not well understood, although the unifying pathophysiological correlate of chronic continual hepatocyte destruction and renewal is thought to allow for accumulation of cancer-relevant changes over time. Along these lines, it is notable that the HCC genome is characterized by extensive chromosomal structural aberrations including complex nonreciprocal translocations, and recurrent amplifications and deletions. This cytogenetic profile has pointed to a chromosomal instability mechanism functioning to drive hepatocytes toward a threshold of genetic lesions needed for malignant transformation (2). Indeed, it was this cytogenetic profile, emerging after a prolonged premalignant phase of cell turnover, which fueled speculation of a telomere-dependent chromosomal instability process (3).

Telomere shortening is a recognized feature of chronic hyperproliferative liver disease (4–6) that has been shown to affect hepatocytes (7), and studies in the telomerase knockout mouse have established a link between telomere attrition and chromosomal instability of the type typical of human HCC described above (8, 9). Mounting evidence supports the view that telomere-based chromosomal instability mechanisms are operative during early stages of carcinogenesis in humans (10). In advanced malignancy, high telomerase activity has been detected in ~90% of cases, correlating well with increasing steady-state human telomerase reverse transcriptase mRNA levels (11). Furthermore, elevated telomerase activity has been shown to correlate well with the potential for HCC recurrence after surgical resection, pointing to its use as a possible predictive marker for HCC recurrence (12). Although such correlations are consistent with a role for telomere dysfunction in early carcinogenesis and subsequent telomerase activation in promoting more advanced stages of malignant progression, this view is confounded by the detection of telomerase activity in chronic liver diseased tissues as well as in early neoplastic lesions (13–15). However, this early stage telomerase activation has been called into question in light of the presence of telomerase-positive infiltrating lymphocytes and resident sinusoidal cells in these nonmalignant tissues (16). Regardless of the timing of telomerase activation in the evolution of HCC, the shorter telomeres encountered in HCC suggests that the telomerase holoenzyme may not be assembled and active at the telomere during the early stages of these cancers. As these cancers progress, telomerase activation may function to restore a level of chromosomal stability required for cancer cell viability, a view that is reinforced by loss of tumorigenic potential of telomerase-positive hepatoma cell lines on enforced expression of a dominant negative mutant form of human telomerase reverse transcriptase (17). These complex patterns of telomere attrition and telomerase activation point to the need to assess more directly how telomere dynamics contribute to the initiation and/or progression phases of HCC.

The study of hepatocarcinogenesis has been aided by several distinct mouse model systems. In the Alb-uPA transgenic mouse model, the underlying mechanism of hepatocarcinogenesis is thought to be related to hepatocellular destruction and DNA rearrangements that occur in the ensuing regenerative nodules, which have spontaneously deleted the hepatotoxic transgene (18). Alb-uPA mice start developing HCCs after 10 months of age and reach up to 75% penetrance by 18 months depending on the line examined. In the CCL4 model, massive necrosis and regeneration induced by CCl4 may allow for the accumulation of cancer-relevant genetic lesions in continuously dividing liver cells (19). Other possible mechanisms may include DNA damage of liver cells by CCl4 (20). A reasonable speculation is that the continual necrosis and regeneration induced by CCl4 may allow for the accumulation of cancer-relevant genetic lesions in continuously dividing liver cells (19). Other possible mechanisms may include DNA damage of liver cells by CCl4 (20).
hepatocytes. Finally, the third model of hepatocarcinogenesis uses the chemical carcinogen, DEN, which causes ethylation of the DNA, cell toxicity, and limited cell proliferation. DEN-treated mice develop HCCs with high penetrance in a dose- and strain-dependent manner (21, 22).

In this study, we sought to dissect additionally how telomerase deficiency and telomere dysfunction influence the initiation versus the progression of hepatocarcinogenesis in these mechanistically distinct model systems. Previous studies have demonstrated that telomere dysfunction brought about by successive generational matings of mice null for the essential RNA component of telomerase (mTerc) can suppress or enhance cancer initiation depending on cell type and/or integrity of the p53-dependent telomere checkpoint responses (23–26). In several studies, the feeble progression phenotype of these initiated neoplasms is consistent with the concept that ongoing telomere dysfunction constrains malignant progression (27, 28) and that initiated neoplasms is consistent with the concept that ongoing telomere dysfunction constrains malignant progression (27, 28) and that telomerase reactivation restores genomic stability to a level permissive for cancer cell viability and full malignant progression including metastases (29). The complex impact of telomere dysfunction on carcinogenesis, particularly in light of the modulating effects of cell type and initiating genetic lesions, necessitates a systematic and direct analysis of how telomere dysfunction influences the development of different tumor types in vivo.

MATERIALS AND METHODS

**Mice.** mTerc+/− mice of mixed background (C57Bl/6Sv129; Ref. 23) were crossed to the uPA transgenic mice (uPA+) uPA+/−, uPA+/− mice were then crossed to mTerc+/− mice to obtain uPA+ G1 mTerc+/− mice. Additional successive generational matings were conducted (see Fig. 1) to obtain uPA+ third and fourth generation mTerc−/− mice. This mating scheme was used to derive the experimental cohorts (25 G0 and 6 G3/G4 mTerc+/− mice 11–16 months old; 22 G0 and 11 G3/G4 mTerc−/− mice 17–24 months old). For the CCl4 and DEN experiments mTerc+/− mice were intercrossed to obtain mTerc−/− mice, and successive generational matings were conducted to give rise to G5 and G6 mice. In the DEN protocol, 15-day old male mice were injected DEN with a dose of 10 mg per kg body weight by a single i.p. injection (11 G0 and 9 G6). Mice were sacrificed and analyzed between 8 and 12 months of age. In the CCl4 protocol, 10 μg/gram body weight of a 10% solution of CCl4 in olive oil was injected i.p. 3 times a week over a period of 12 months. Mice were sacrificed and analyzed at monthly intervals after the end of the treatment (6–12 months of age).

**Laparotomies.** For the gross analysis of tumors, we performed laparotomies on live mice (ages 11–24 months) anesthetized with avertin by i.p. injection. The number and size of nodules observed with naked eye was recorded, and the mice were allowed to recover and were monitored weekly.

![Breeding Colonies](Breeding_Colonies.png)

Fig. 1. Breeding scheme used to obtain the experimental cohort of the uPA mice. The matings that were conducted are shown on the left, whereas the experimental cohorts obtained from these matings are shown on the right.

![Experimental Cohorts](Experimental_Cohorts.png)

**Histological Analysis.** Mice were sacrificed according to institute guidelines, and their livers were fixed in formalin and paraffin embedded. Subsequently, tissue sections were obtained and stained with H&E to enable classification of the lesions and quantification of the mitoses in the tumors. In addition, sections were stained with reticulin to aid in distinguishing an HCC nodule from a hyperplastic nodule in cases that were hard to classify based on H&E. Immunohistochemical Staining. TUNEL assays were performed on no-bake sections according to the manufacturer’s instructions (ApopTag detection kit; Intergen) and PCNA staining was performed with the PCNA (antibody-1) monoclonal antibody from oncogene at a 1:150 dilution using the M.O.M. immunodetection kit according to manufacturer’s instructions (Vector Labo-

**Fig. 2. Inhibition of hepatocarcinogenesis in telomere-dysfunctional mice in the uPA model.** A, the average number of nodules observed during laparotomies is plotted for the different generations of mice (G0 versus G3/G4) and ages (11–16 months and 17–24 months). B, the graph shows the average size of nodules observed grossly in uPA mice of various generations and ages. C, the graph shows the size of nodules during sequential laparotomies of the individual mice. White bars represent the size of the nodules of the first time the mice were examined, and gray bars represent the size of the nodules at a second, later examination. The numbers underneath the bars represent individual mice. All Ps were obtained using the Mann-Whitney statistical test.

**Fig. 3. Telomere dysfunction impairs hepatocarcinogenesis in mice treated with liver carcinogens (DEN and CCl4).** A, the graph shows the number of gross nodules observed in 8–15-month-old DEN-treated mice of various generations. B, the average size of the observed nodules for the various DEN-treated generations is shown on this graph. C, the number of nodules observed grossly in 6–12-month-old mice of various generations treated with CCl4. D, the size of the gross nodules observed in the CCl4-treated mice.
RESULTS AND DISCUSSION

Telomere Dysfunction Impairs Liver Tumorigenesis and Tumor Growth. In the Alb-uPA regeneration model, the Alb-uPA transgene was brought through successive generational intercrosses of mTerc−/− mice to produce transgenic and nontransgenic mice pos-

Statistical Analysis. All of the statistical analyses were performed using the GraphPad InStat program. Comparisons of the incidence of lesions were based on Fisher’s exact test. Comparisons of the number of lesions, mitoses, PCNA+, and TUNEL+ cells were based on the Mann-Whitney test.

Fig. 4. Enhanced initiation, yet impaired tumor progression in uPA late generation mTerc−/− mice. A, histological analysis of the lesions identified in the uPA transgenic mice. The top panel shows H&E stains, and the bottom panel shows sections of the same lesion stained with reticulin. The name of the lesion represented by each set of stains is shown on the top part of the figure. The insets show the normal thickness of the trabeculae as indicated by an arrow (hyperplastic nodule), thickening of the trabeculae (initiation focus), and loss of reticulin staining (HCC). B, the graph indicates the percentage incidence of the lesions described in A in the different generations and ages studied. P values were obtained using Fisher’s exact test. C, the average number of initiation foci per hpf in mice of both generations and ages examined is shown in this graph. D, the average number of hyperplastic and HCC nodules in mice of both generations and ages examined is depicted in this graph. All P values were once again obtained using the Mann-Whitney test.

ratories). Positively stained cells were labeled with the 3,3′-diaminobenzidine reagent (Vector Laboratories).
sessing progressively shorter telomeres. Consistent with previous work (30), third and fourth generation mTerc−/− (designated G3 and G4 mTerc−/−) mice exhibited signs of telomere dysfunction across many organ systems (including impaired liver regeneration). To monitor the impact of advancing age and telomere functional status on the rate of liver tumor formation, exploratory laparotomies were performed at ages 11–16 months and 17–24 months in the various experimental cohorts (Fig. 1). Gross examination of Alb-uPA mTerc+/+ and +/- livers revealed numerous surface nodules that increased in both number and size with advancing age (Fig. 2, A and B). By comparison, younger Alb-uPA G3/4 mTerc−/− mice exhibited marked reduction in nodule multiplicity and average size (Fig. 2, A and B). These differences in number and size of nodules were less dramatic in the older age group; however, serial laparotomies of individual mice readily confirmed the constraining effects of telomere dysfunction on nodule growth as a function of advancing age (Fig. 2C).

Similar tumor phenotype patterns were established in the comparisons of wild-type and late-generation mTerc−/− mice subjected to the CCl4 and DEN protocols (see “Materials and Methods”). In both models, telomere dysfunction was associated with a significant decrease in the number and size of the surface liver nodules (Fig. 3, A and B: DEN model; Fig. 3, C and D: CCl4 model). These preliminary findings suggested that, regardless of the mechanisms of hepatocarcinogenesis, telomere dysfunction impairs the emergence and growth of grossly visible liver neoplasms.

Telomere Dysfunction Impacts Differentially on Liver Neoplasm Initiation and Progression. The above gross examinations were complemented by serial histological studies to assess more precisely the initiation, evolution, and malignant nature of the liver tumors. To this end, we determined the occurrence of three types of neoplastic lesions: hyperplastic nodules, initiation foci, and HCC. “Hyperplastic nodules” are discrete nodular foci of regenerating hepatocytes that do not exhibit cytological or architectural features of malignant transformation. “Initiation foci” are microscopic lesions of regenerating hepatocytes that possess atypical cytologic (increased nuclear density, pleomorphism, and clear cell changes) or architectural (increased trabecular thickness and disarray) features suggestive of an early transition to malignant transformation. HCCs in that this stain can reveal the thickened trabeculae that are diagnostic for HCC. Initiation foci often reside within larger hyperplastic nodules, consistent with the view that hyperplastic nodules can give rise to initiation foci, which, in turn, carry the potential to evolve into HCC (Fig. 4A). This progression analysis is in agreement with those reported previously in

![Graph](image-url)

**Fig. 5.** Inhibition of tumor progression in the DEN and CCl4 models. A, the size of the HCC nodules is depicted in this graph for G0 and G6 mTerc−/− mice treated with DEN. B, the ratio of initiation foci:HCC was quantified for G0 and G6 mTerc−/− mice. C, the number of HCCs identified after histological analysis in G0 and G5 CCl4-treated mice is represented in this graph.

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the mouse and, furthermore, mirrors the sequence of events observed in human hepatocarcinogenesis (2, 18, 21, 31).

Histological analyses at 11–16 months of age revealed that, relative to Alb-uPA G0 mTerc+/+ or +/− mice, the Alb-uPA G3/4 mTerc−/− mice showed a higher penetrance of hyperplastic nodules (0% in G0 versus 33% in G3/G4) and initiation foci (4% in G0 versus 67% in G3/G4 mice) yet a lower incidence of HCC (68% in G0 versus 17% in G3/G4; Fig. 4B). The incidence of hyperplastic nodules and initiation foci was measured for mice that were HCC-free to assess the specific impact of telomere dysfunction in the process of initiation before progression has occurred. These trends were strengthened additionally by a determination of the average number of lesions in individual mice aged 11–16 months. As shown in Fig. 4, C and D, relative to intact telomere controls, the Alb-uPA late-generation mTerc−/− mice showed a higher average number of initiation foci and hyperplastic nodules per mouse (0.005 versus 0.027 initiation foci per hpf, P = 0.0115; 0 versus 0.5 hyperplastic nodules per mouse; note Ps cannot be calculated because the value in the G0 group is 0) but show a decrease in the average number of HCC nodules (1.5 versus 0.17 HCC nodules per mouse; P = 0.0471). Furthermore, it appears that the initiated lesions in young G3/G4 mice (6-fold enhancement in initiation in G3/G4 mice compared with G0 controls; see Fig. 4C) do not all progress to form HCCs, as in the older age group the difference of HCCs among the generations is not statistically different (2.364 HCCs in G0 mice and 1.818 HCCs in G3/G4 mice; Fig. 4D). This argues against a delayed progression of HCCs in telomere dysfunctional mice, as the number of HCCs observed in older late-generation telomerase-deficient mice does not parallel the enhanced levels of initiation at a younger age. Therefore, consistent with our previous observations (27), telomere dysfunction appears to enhance the initiation of liver neoplasms, yet impair their progression into highly malignant tumors. As noted above, advancing age muted the impact of telomere dysfunction on processes of tumor initiation and progression (Fig. 4, B–D, 17–24 months).

Similar histological analyses were performed for the DEN and CCl4 models of hepatocarcinogenesis. In the DEN-treated mice, the size of the HCC lesions was reduced significantly in G6 mTerc−/− mice relative to G0 mTerc+/+ and +/− controls (Fig. 5A). Furthermore, the ratio of initiation foci to HCC lesions increased dramatically in G6 mTerc−/− mice compared with G0 mTerc mice (Fig. 5B; Table 1). In the CCl4-treated mice, we observed a similar pattern of increased initiation foci and decreased HCC nodules in G5 mTerc−/− mice compared with G0 controls (Fig. 5C; Table 2). It was not possible to determine the initiation foci:HCC ratio, because only 1 of 9 G5 mTerc−/− mice developed HCC. In summary, three mechanistically distinct models of hepatocarcinogenesis reveal consistent trends with advancing telomere dysfunction, and these trends strongly support the view that telomere dysfunction serves to promote the initiation of hepatocarcinogenesis, yet impairs tumor progression as evidenced by decreased size and number of HCCs in the late-generation mTerc−/− mice.

**Cellular Consequences of Telomere Dysfunction in Murine HCCs.** The telomere checkpoint exhibits many features similar to those elicited by DNA damage (reviewed in Ref. 32). The specific cell biological response to telomere dysfunction varies greatly across different cell types ranging from growth arrest and senescence in fibroblasts (26) to robust apoptosis in germ cells (33). In the setting of intact p53 function, these cellular mechanisms have been shown to inhibit tumor progression in mouse models of intestinal carcinoma (27) and skin carcinogenesis (28).

To ascertain the nature of the telomere checkpoint response underlying inhibition of liver tumor growth, we measured intratumoral....

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**Fig. 6. Telomere dysfunction impairs proliferation and triggers apoptosis within tumors of telomere-compromised mice.** A, the graph shows the number of mitoses per hpf quantified in tumors from G0 and G3/G4 uPA mice. The P was obtained using the Mann-Whitney test. B, representative pictures of tumors from the two experimental cohorts stained with PCNA. C, representative pictures of tumors from the two experimental cohorts stained for apoptosis. Cells that exhibit brown nuclear staining are positive for apoptosis. D, the table shows the average number of PCNA+ cells, as well as the percentage of TUNEL+ cells within a tumor for the various experimental cohorts. The Ps were obtained using the Mann-Whitney test. E, the number of mitoses within tumors of the various generations of DEN-treated mice was quantified and the average numbers are plotted in this graph.
proliferation and apoptosis in relation to telomere status in the Alb-uPA model. Relative to G0 controls, Alb-uPA G3/4 mTerc−/− tumors showed a marked reduction in mitotic figures (Fig. 6A) as well as a reduction in PCNA-stained nuclei (1.294 in G0 versus 0.012 PCNA+ cells/hpf in G3/G4; Fig. 6, B and D). Late-generation mTerc−/− tumors also exhibited a significant increase in apoptosis relative to tumors arising in mice with functional telomeres (14% versus 2.3% TUNEL+ cells; Fig. 6, C and D). These results demonstrate that impaired proliferation and increased apoptosis are among the mechanisms contributing to inhibition of tumor growth in the hepatocytes possessing telomere dysfunction.

In the DEN model, we observed a similar decrease in the number of intratumoral mitoses in G6 mTerc−/− samples compared with G0 controls (Fig. 6E). The limited number of HCCs in the CCL4 model (1 HCC identified in 9 G5 mice examined) did not permit statistical analysis, although the lone G5 mTerc−/− HCC showed decreased PCNA staining, lower number of intratumoral mitoses, and increased TUNEL staining compared with the average of G0 tumors (data not shown). Together, these analyses demonstrate that impaired proliferation and/or increased apoptosis accompany the impaired growth and progression of liver tumors in the setting of telomere dysfunction.

**Concluding Remarks.** The progression paradigm of human HCC provided a framework for interpreting the gross, histological, and molecular profiles of three mechanistically distinct murine models of hepatocarcinogenesis. In each case, telomere dysfunction was shown to increase the frequency of initiated neoplasms yet constrain their subsequent growth and progression into HCC. This differential tumor stage-specific impact of telomere dysfunction is very similar to that described in evolving intestinal neoplasms in mice, and correlates well with the progression analyses of human colorectal cancers (27) and pancreatic adenocarcinomas (34). At the same time, it is important to note that the significant muting effects of telomere dysfunction on murine lymphomagenesis, even in the setting of p53/ATM deficiency (8, 35), underscores the complex interplay of the cellular context, telomere checkpoint response, and chromosomal instability on the processes of cancer initiation.

In these liver studies, short dysfunctional telomeres appear to serve an “oncogenic” role, presumably by promoting chromosomal instability across the population of regenerating hepatocytes. Such instability mechanisms have been shown to be the result of telomere erosion and associated bridge-fusion-breakage cycles that, in turn, produce regional amplifications and deletions at the site of breakage. In the studies described here, we speculate that this telomere-based mechanism could promote oncogene amplification and tumor suppressor gene inactivation events, which would then be selected during the initial stages of liver carcinogenesis. Because limited information exists on the genes/loci driving hepatocarcinogenesis in humans (2), such a model may provide an opportunity for the identification and validation of these HCC loci.

In the models described here, we observed a robust telomere checkpoint response that we propose functions to constrain the growth and progression of HCC in the mouse. In many different cell types and tissues, the adverse cellular consequences of the telomere checkpoint response can be attenuated significantly by loss of p53 function (25). Along these lines, it is worth noting that there exists a high mutation/loss of heterozygosity rate of p53 in human HCCs. Recurrent loss of chromosome 17p, where p53 resides, has been reported frequently in human HCCs (reviewed in Ref. 2). In addition, various groups have reported mutations of p53 at codon 249 in human HCCs associated with dietary aflatoxin intake and hepatitis B virus infection (36–38). It is well established that p53 deficiency plays many roles in cancer biology, ranging from enhanced tumor cell survival to activation of angiogenesis (39). Given the frequency of p53 inactivation in HCC and its role in the DNA damage checkpoint, it is possible that one outcome of p53 inactivation in this cancer would be compromise of the telomere checkpoint response thereby expanding the pool of genomically unstable cells from which a procancer genotype may emerge. Finally, the consistent correlation of telomerase activation in the progression to HCC in humans, coupled with the impaired HCC potential of late-generation mTerc−/− hepatocytes, anticipates that the restoration of telomere function (by telomerase or alternative lengthening of telomeres mechanisms) plays a permissive role in the malignant progression of these initiated neoplasms by restoring genomic stability to a level compatible with cell viability. Thus, the models described here could provide an experimental framework in which to dissect the adaptive responses to telomere dysfunction, the contributions of p53 loss of function, and the reactivation of telomerase in the evolution of HCC.

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**REFERENCES**


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