Cooperative Interactions of p53 Mutation, Telomere Dysfunction, and Chronic Liver Damage in Hepatocellular Carcinoma Progression

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Abstract

Hepatocellular carcinoma is among the most common and lethal cancers in humans. Hepatocellular carcinoma is commonly associated with physical or functional inactivation of the p53 tumor suppressor, high levels of chromosomal instability, and disease conditions causing chronic cycles of hepatocyte death and regeneration. Mounting evidence has implicated regeneration-induced telomere erosion as a potential mechanism fueling genome instability. In mouse models of hepatocellular carcinoma, telomere dysfunction has been shown to enhance initiation of hepatic neoplasias yet constrain full malignant progression of these neoplasms possibly due to activation of a p53-dependent checkpoint and/or intolerable levels of genomic instability. Here, in a hepatocellular carcinoma–prone model brought about through toxin-induced hepatocyte injury and regeneration, we sought to determine the cooperative interactions of germ line p53 mutation and telomere dysfunction [produced by telomerase reverse transcriptase (mTERT) gene knockout]. In the setting of intact telomeres, p53 mutation had no effect on hepatocarcinogenesis, whereas in the setting of telomere dysfunction, p53 mutation enabled advanced hepatocellular carcinoma disease. Notably, there was no evidence of deletion or mutation of the wild-type p53 allele in the late generation mTert−/−p53+/− mice, suggesting that reduced levels of p53 potently enable hepatocellular carcinoma progression in the setting of telomere dysfunction. Thus, this study supports a model that, in the face of chronic liver damage, attenuated p53 function and telomere-induced chromosomal instability play critical and cooperative roles in the progression of hepatocellular carcinoma. (Cancer Res 2006; 66(9): 4766-73)

Introduction

Hepatocellular carcinoma is among the most prevalent and lethal cancers worldwide with death, occurring in 75% of documented cases (1). The magnitude of the hepatocellular carcinoma problem has attracted sustained basic, clinical, and epidemiologic investigations directed towards the dissection of disease pathology and molecular circuitry, construction of refined animal models, and elucidation of environmental and host risk factors (2). To date, these efforts have generated a compendium of signature mutations, including p53, IGF2R, RB1, p16INK4a, and PTEN (3), and have also highlighted our limited understanding of the molecular mechanisms driving the genesis and progression of this disease. Hepatocellular carcinoma development is associated with diverse factors and conditions, including chronic hepatitis B and C viral infection, chronic alcohol intake, aflatoxin B1 exposure from food, and cirrhosis (4, 5). Across the many etiologies, it has been postulated that hepatocarcinogenesis results in part from repeated rounds of hepatocyte destruction and regeneration, which produces a procarcinogenic cirrhotic microenvironment in the liver. Another not mutually exclusive theory suggests that continuous hepatocyte renewal promotes telomere shortening, which ultimately leads to chromosomal instability. Indeed, telomere attrition has been documented in hepatocytes of chronic hyperproliferative liver disease and end-stage liver cirrhosis (6–10), and a correlation between telomere shortening and increased chromosomal instability has been established in human hepatoma (11). In previous work using the mouse telomerase RNA component (mTerc) knockout, we addressed the role of telomeres in hepatocarcinogenesis and showed that telomere dysfunction enhanced initiation of hepatic neoplasias yet impaired full malignant progression of these lesions (12). Similarly, Rudolph et al. have shown that hepatocellular carcinoma in a Hepatitis B virus surface antigen transgenic model is suppressed in telomerase-deficient mice with short telomeres (13). Collectively, these results support the view that telomere-induced genomic instability can initiate hepatic neoplasias, but this mechanism alone is insufficient to drive progression to advanced hepatocellular carcinomas. These observations imply the need for additional procarcinogenic genetic events and/or the presence of robust telomere checkpoints serving to cull early-stage premalignant cells.

The effect of dysfunctional telomeres in carcinogenesis has been shown to be dependent upon cell type and p53 status. In general, in the setting of intact p53, telomere dysfunction has been shown to inhibit progression to advanced malignancy in a variety of models (14–18). In contrast, the combination of telomere dysfunction and p53 mutation causes acceleration in tumor onset and shift towards epithelial cancers relative to telomere-intact p53 mutant controls. For purposes of the current study, it is worth emphasizing the tissue-specific effect of telomere dysfunction in processes of carcinogenesis. That is, in late-generation mTerc−/−p53+/− mice, there is an increase in epithelial cancers (primarily breast, colon, and skin) as opposed to all epithelial cancer types, and there is a minimal effect on the occurrence of mesenchymal or hematopoietic malignancies (19, 20). Of relevance to the current study, hepatocellular carcinomas were not observed in the late-generation mTerc−/− p53 mutant mice, which could relate to the presence...
of a p53-independent telomere checkpoint and/or insufficient hepatocyte turnover. With regard to the latter, it is reasonable to consider that hepatocyte turnover would engender telomere-induced bridge-fusion-breakage cycles and accumulation of cancer-promoting genomic alterations. Thus, the context-dependent actions of telomere dysfunction and DNA damage checkpoints in cancer development underscore the need for systematic direct assessment of the singular and combinatorial effect of telomere dysfunction and p53 mutation against the backdrop of physiologic and pathophysiologic cell renewal across different tissues.

In human hepatocarcinogenesis, p53 inactivation is a common event across the diversity of etiologic factors. Specifically, the hepatitis B virus HBx protein has been shown to bind and inactivate p53 in vitro (21), and the hepatitis C virus NS5A protein binds and sequesters p53 to the perinuclear membrane (22). Moreover, allelic deletions of p53 and mutations have been detected in hepatocellular carcinoma in the setting of HBV or HCV infection (23). The fungal toxin aflatoxin B1 acts as a mutagen, and exposure correlates with increased rate of p53 mutations in codon 249 in aflatoxin-associated hepatocellular carcinoma cases (24–26). Finally, oxidative stress can serve as a mutational mechanism in hepatocellular carcinoma, generating reactive oxygen species and free radicals, which can somatically alter key oncogenes and tumor suppressor genes, including p53. In fact, a strong correlation between p53 mutations and generation of oxyradicals has been forged in hepatocellular carcinoma cases associated with iron overload, which causes increased oxidative stress (27).

Considering the complex interactions of p53 and telomeres in normal and neoplastic processes and the commonality of p53 mutation and telomere erosion in human hepatocellular carcinomas, we sought to provide genetic evidence for a cooperative interaction among p53 compromise, telomere dysfunction, and chronic liver damage in hepatocellular carcinoma progression. To this end, we developed an hepatocellular carcinoma–prone mouse model that involves chronic intermittent exposure to carbon tetrachloride (CCl4) to recapitulate certain key aspects of human chronic liver disease associated with hepatocellular carcinoma, such as development of cirrhosis, liver inflammation, hepatocyte necrosis and regeneration, and induction of oxidative stress (28).

Materials and Methods

Generation of the mTERT allele. The mTert−/− allele was generated by deletion of exon 1 that contains the 5′ portion of the open reading frame (Fig. 1A and B). A 5′ fragment of the mTERT genomic DNA was cloned into the pPNT-Neo targeting vector using EcoRI-XbaI sites. This 5′ mTERT genomic fragment along with the Neo cassette were subsequently ligated into the pDT-scrambler vector using EcoRI-Xhol sites. The 3′ fragment of the mTERT genomic DNA was also cloned into the pDT-scrambler using BglII-Xhol sites. The 5′ and 3′ fragments of mTERT genomic DNA were subsequently cloned (using the EcoRI sites at the 5′ and 3′ ends) into the pTK vector (which has the herpes simplex virus TK gene). Embryonic stem cells were electroporated with the targeting vector, and selection of positive clones was done as described previously (29). To verify telomerase deficiency, telomeric repeat amplification protocol (TRAP) assays were done according to standard protocols as previously described using E13.5 mouse embryonic fibroblasts (MEFs; ref. 29).

The mTert−/− allele was generated on a mixed background (containing FVB and Sv129). This mTert null allele was backcrossed onto the C57Bl6 background to generate genetically homogeneous mTert mutant mice and to generate average shorter telomeres, which is typical of the C57Bl6 strain (30). Speed congenic methodology was used to expedite backcrossing, in which the N2 offspring and beyond were PCR tested for homozygosity of 18 C57Bl6 alleles: MIt markers D1-318, D2-168, D4-72, D5-161, D6-19, D8-94, D9-306, D10-40, D11-320, D12-189, D13-76, D14-7, D14-55, D17-123, D17-216, and D19-78. The mTert−/− male offspring homozygous for the highest number of C57Bl6 markers were chosen for further backcrossing at each generation. N5 mTert−/− males that were homozygous for the above C57Bl6 markers were then crossed to The Jackson Laboratory, Bar Harbor, ME) to obtain mTert−/− and mTert−/+ p53−/− mice (N6/G0 mTERT−/− or mTERT−/+ with and without a p53 null allele). Interbreeding of G0 mTert−/− and G0 mTert−/+ p53−/− mice resulted in G1 mTert−/− and G1 mTert−/+ p53−/− mice. Subsequent intercrossing of these mice resulted in G2 mice and so on (for the breeding scheme, see Fig. 2A).

For mTert genotyping, tail DNA was obtained using the Puregene DNA isolation kit and used for genotyping. The primers used were as follows (Fig. 1B): P1, 5′-ATTACCCGAGGTGTGCGCGT-3′; P2, 5′-GAGAAGAAGAGGTCCGCCAGTTG-3′; P3, 5′-CGACCGGACGTCGGCAGGGGGG-3′. The PCR conditions were as follows: 95°C for 15 minutes (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) × 28, 72°C for 6 minutes.

CCl4 liver injury protocol and tissue analysis. Mice were subjected to CCl4 administration (10 µL/g body weight of a 10% solution in olive oil) by ip. injection thrice a week for 4 months beginning at 6 weeks of age. Mice were monitored regularly following the course of the CCl4 administration and were treated and sacrificed in accord with approved protocols as per institutional guidelines. The livers were removed by gross inspection of the entire liver to look for the presence of surface nodules. These surface nodules were recorded and fixed in formalin, embedded in paraffin, and subsequently all were analyzed histologically (by H&E and reticulin staining of tissue sections) to enable definitive classification of hepatocellular carcinoma versus hyperplastic nodules. Hyperplastic nodules are composed of regenerating hepatocytes that lack cytologic or architectural features of malignant transformation. Initiation foci are microscopic lesions of regenerating hepatocytes with atypical cytologic (increased nuclear density, pleomorphism, and clear cell changes) or architectural (increased trabecular thickness and disarray), features indicative of an early transition to malignant transformation. For the quantification of initiation foci, the entire "normal liver" (i.e., without gross lesions) was sectioned, and all of these sections were examined histologically. The number of initiation foci per total number of high power fields of liver tissue was recorded. Finally, hepatocellular carcinomas are mass lesions with unequivocal cytologic (nuclear crowding and pleomorphism) and architectural (perinuclear thickenings) features of malignant transformation. For immunohistochemistry, unstained sections were cut from the paraffin-embedded livers and stained with various antibodies according to standard protocols (Cell Signaling Technology, Beverly, MA; Immunohistochemistry Protocol). The p53 antibody (CM5 antibody, Novocastra, Benton Lane, United Kingdom) was used at 1:400 dilution, and the mTERT antibody (Ab-2, Calbiochem, San Diego, CA) was used at 1:5,000 dilution and incubated overnight at 4°C. Antigen unmasking was achieved using a pressure cooker. The secondary antibody was applied at 10 µg/mL (Vector, Burlingame, CA) for 1 hour at room temperature and was followed by EnVision+, Peroxidase (DAKO, Carpinteria, CA).

Loss of heterozygosity and mutations analysis. DNA was isolated using the Puregene DNA isolation kit (Genta Systems, Plymouth, MN). For the mutation analyses, 10 ng of DNA was used for nested PCR amplification with primers that amplify exons 5 to 9 of p53. Primer sequences were located in the intron sequences flanking either side of each exon. One microliter of the product from the first PCR reaction was subsequently used for the nested PCR. The PCR products were run on 2% agarose gels, and the purified DNA fragments were sequenced and analyzed with Qiagen’s SeqWrite services. For loss of heterozygosity (LOH) analyses, the DNA was used for PCR amplification using the standard genotyping protocol as previously published (31). Briefly, 100 ng of genomic DNA was used for the PCR reaction with the following conditions: 94°C for 3 minutes, (94°C for

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1 minute, 62°C for 1 minute, and 72°C for 1 minute) × 30 cycles, 72°C for 6 minutes. The reactions were done using Qiagen Taq. The PCR products were run on 2% agarose gels.

**Statistical analysis.** All statistical analyses were done using the GraphPad InStat 3 and GraphPad Prism 4 software. The time-dependent incidence of tumors was determined using the Kaplan-Meier survival curve and log-rank test. Fisher's exact test was used for a comparison of the overall incidence of hepatocellular carcinomas, and the Mann-Whitney test was used for the comparison of hepatocellular carcinoma size among the various genotypes of mice.

**Results**

Robust mTERT expression in the regenerating and malignant liver. In the CCl4 model, we observed robust anti-mTERT signal in 20% to 90% of hepatocytes during the regeneration phase after CCl4 exposure (in p53+/+ or p53−/− mice; data not shown). In addition, mTERT overexpression was evident in the majority of CCl4-induced hepatocellular carcinomas (7 of 11 p53+/+ and 9 of 11 p53−/− mice) but not detectable in the adjacent quiescent normal liver (Supplementary Fig. S1, i and ii, respectively). As hepatocellular carcinoma development is preceded by hyperplastic lesions and initiation foci (12), we also assayed mTERT expression as a function of disease progression. mTERT overexpression was detected in one of three hyperplastic nodules and five of five initiation foci (Supplementary Fig. S1, iii and iv). TERT expression was increased in the hepatocellular carcinomas relative to the early-stage lesions; furthermore, ~81% of tumor cells showed robust signal compared with 10% to 40% of signal-positive cells in initiation foci. This pattern suggests that TERT expression may become activated in neoplastic lesions, and the biological pressures in tumor formation may favor increasingly elevated mTERT levels. These mTERT expression patterns prompted assessment of the effect of mTERT elimination and resultant telomere dysfunction on hepatocellular carcinoma development.

**Generation and characterization of the mTert-deficient mouse.** Standard methods (29) were used to generate a null mTert allele in the germline. This allele contains a 169-bp exon 1 deletion, which contains the start codon “ATG” and encodes the RNA/DNA binding domain of mTert (Fig. 1A and B). Several lines of evidence confirmed generation of a null mTert allele. First, first-generation (G1) mTert−/− MEF cultures possessed no detectable telomerase activity via the highly sensitive TRAP assay, compared with activity in mTert+/+ and mTert+/− control cultures (Fig. 1C). Second, robust mTERT expression detected in the CCl4-induced regenerating hepatocytes of mTert+/− mice was absent in mTert−/− regenerating hepatocytes (Fig. 1D). Third, successive generational intercrosses yielded mice with progressively shorter telomeres and, by the third/fourth generations (G3/G4), showed...
classic cytogenetic and constitutional signs of telomere dysfunction as reported for mice null for the telomerase RNA component, mTerc (29, 32). Specifically, late generation mTert−/− mice showed decreased fecundity with reduced litter size, testicular atrophy, and gastrointestinal hallmarks of anaphase bridging, apoptosis, and p53 induction (Fig. 2B-D; data not shown). Thus, the preliminary phenotypic characterization of the mTert−/− mouse indicates a close phenocopy of the mTerc−/− model (29, 32) and mirrors the previous findings of another mTert null strain generated by Harrington et al. (33).

Hepatocellular carcinoma development. The evolution of murine hepatic neoplasms into hepatocellular carcinoma is defined by a series of histopathologic changes that begins with hyperplastic nodules followed by initiation foci and culminating in the appearance of fully malignant hepatocellular carcinoma (see Materials and Methods and ref. 12). To assess the singular or combined effect of telomere and p53 status on cancer initiation, we quantified the number of early neoplastic lesions in mice well before the known onset of hepatocellular carcinoma in this chronic liver injury model. As shown in Fig. 3, the telomere-intact G0 p53+/+ and G0 p53+/− mice developed hyperplastic and initiation lesions with similar incidence. In contrast to these G0 cohorts, the G3/G4 p53+/+ mice exhibited a marked reduction in the incidence of early neoplastic lesions (P < 0.01), and this suppression was markedly, albeit only partially, alleviated in G3/G4 p53+/− mice (P = 0.6785 compared with G0 p53+/+ mice). Thus, in this model, telomere dysfunction was associated with a marked suppression of cancer initiation, and, notably, the effect of p53 mutation on cancer initiation was observed exclusively in the setting of telomere dysfunction.

The lack of an effect of p53 mutation in mice with intact telomeres was somewhat unanticipated because germ line p53 mutation exerts a strong effect on tumor kinetics in many diverse cancer models (31). These findings prompted us to speculate that
a major role of p53 compromise in hepatocellular carcinoma pathogenesis might be to enable the survival of hepatocytes experiencing telomere dysfunction. Finally, on a separate point, it is worth noting that these above results with the mTert knockout model differ from our previous studies with the mTerc−/− model wherein telomere dysfunction was found to enhance initiation foci even in the setting of intact p53 (12). Although these contrasting outcomes raise the intriguing prospect of additional procarcinogenic Tel activities beyond its orthodox telomere maintenance functions (34–37), we stress that the mTerc and mTert studies were performed on different genetic backgrounds. Nevertheless, these observations are intriguing in the light of the robust mTERT expression in the G0 hepatic neoplasms.

Next, following a full course of CCl4 treatment, we monitored hepatocellular carcinoma incidence as a function of age, telomere integrity, and p53 status. In these serial studies, we observed suppression of hepatocellular carcinoma in G3/G4 p53+/− compared with G0 p53+/− mice (P = 0.0036). Indeed, the two hepatocellular carcinomas in G3/G4 p53+/− mice occurred at an advanced age (32 weeks of age; Fig. 4A). In contrast, hepatocellular carcinomas arose readily in the G3/G4 p53−/− mice, which exhibited tumor kinetics that were more comparable with that of the G0 p53−/− mice (Fig. 4A). Thus, p53 heterozygosity functions to partially relieve the telomere-induced suppression and enables progression to fully malignant hepatocellular carcinoma. In end point studies, when the overall incidence of hepatocellular carcinomas was examined across all genotypes, there was a trend towards accelerated progression relative to G0 p53+/− mice (Fig. 4B). Although the end point data did not achieve statistical significance in tumor incidence, it is worth noting that the G3/G4 p53+/− hepatocellular carcinomas in the aged mice were of limited size relative to the other genotypes (Fig. 4C). We also quantified tumor multiplicity in these mice and observed a statistically insignificant reduction in G3/G4 p53+/− mice (average of 1.5 hepatocellular carcinomas per mouse) compared with G0 p53+/− mice (average of 3.8 hepatocellular carcinomas per mouse; P = 0.5035) and G0 p53−/− mice (average of 3.3 hepatocellular carcinomas per mouse; P = 0.0997). The two aged G3/G4 p53+/− mice that developed hepatocellular carcinoma only had one hepatocellular carcinoma each (the low number of hepatocellular carcinomas in this group and the fact that the SD is 0 do not allow for statistical comparisons with the other groups).

Although the comparison of tumor multiplicity across G0 p53+/−, G0 p53−/−, and G3/G4 p53+/− mice did not yield statistical significance, there was still a 2.5- and 2.2-fold reduction in tumor multiplicity in G3/G4 p53−/− mice compared with G0 p53−/− mice and G0 p53+/− mice, respectively. On the other hand, it is worth noting that one G0 p53+/− mouse developed 19 tumors, thus skewing the average number of hepatocellular carcinomas. In considering all of these findings, whereas it is clear that p53 heterozygosity greatly alleviates the block on progression, our data do not exclude the possibility that there remains some degree of impaired progression in G3/G4 p53+/− tumors.

The effect of telomere dysfunction and p53 mutation on hepatocellular carcinoma progression was assessed further on the level of histopathologic grade: well differentiated (grade 1), moderately differentiated (grade 2), and poorly differentiated (grades 3 and 4). This is the same classification system used in the clinical assessment of human hepatocellular carcinomas (38). Here, we defined “aggressive tumors” as those designated grade 2 to 4 and/or measuring >0.5 cm in size. Using these criteria, we observed a complete suppression of advanced disease in G3/G4 p53+/− (Fig. 4B; P = 0.0083 relative to G0 p53+/−). In contrast, G3/G4 p53−/− mice developed aggressive hepatocellular carcinomas with a trend towards accelerated progression relative to G0 p53−/− controls (Fig. 4B; P = 0.1). The average size of hepatocellular carcinomas was comparable in G3/G4 p53−/− to that of G0 p53−/− mice (Fig. 4C). The overall incidence of aggressive hepatocellular carcinomas was similar in G3/G4 p53+/− (29%), G0 p53+/− (22%), and p53−/− mice (17%; Fig. 4D). In summary, these results show that germ line p53 heterozygosity can significantly attenuate telomere-induced tumor suppression, enabling increased frequency of advanced hepatocellular carcinomas in this model.

Retention of the wild-type p53 allele in hepatocellular carcinomas of p53+/− mice. We next examined the status of the wild-type p53 allele in G0 p53+/− and G3/G4 p53+/− tumors by LOH analysis. All tumors analyzed had ≥70% tumor cells (the other 30% was composed of nonparenchymal liver cells), which were viable, contained minimal fibrous tissue, and were devoid of necrotic cells. These hepatocellular carcinomas retained a near equimolar ratio of wild-type and knockdout alleles of p53, with only one sample suggestive of a minor reduction in the wild-type allele (Supplementary Fig. S2). As a positive control, a lymphoma from a p53−/− mouse shows LOH relative to normal tissue (last two lanes). The retention of the wild-type allele was evidenced further by sporadic anti-p53 immunoreactivity in these hepatocellular carcinomas (data not shown). Mutational analysis of the tumors failed to detect mutations in p53 exons 5 to 9 (data not shown), which were readily detected in an analogous CCl4-induced model of cholangiocarcinoma (PAF and RAD, see accompanying submission). Finally, it is worth stressing that these findings stand in sharp contrast to many other tumor types arising in late-generation mTerc−/− p53+/− mice, in which virtually all tumors sustain loss of the wild-type p53 allele (19). Thus, in this hepatocellular carcinoma model, retention of the wild-type p53 allele may be explained in a number of ways: (a) p53 haploinsufficiency is sufficient to drive hepatocarcinogenesis; (b) other p53 pathway

![Figure 3](cancerres.aacrjournals.org) p53 heterozygosity alleviates the telomere-induced suppression of cancer initiation. The incidence of hyperplastic nodules and initiation foci is shown in mice that were hepatocellular carcinoma (HCC)-free.

<table>
<thead>
<tr>
<th># Mice with Hyperplastic Nodules/ Total # HCC-Free Mice</th>
<th># Mice with Initiation Foci/ Total # HCC-Free Mice</th>
</tr>
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<tbody>
<tr>
<td>G0 p53+/+</td>
<td>8/15 (53%)</td>
</tr>
<tr>
<td>G0 p53+/−</td>
<td>10/20 (50%)</td>
</tr>
<tr>
<td>G3/G4 p53−/−</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>G0 p53+/−</td>
<td>7/15 (47%)</td>
</tr>
<tr>
<td>G0 p53+/−</td>
<td>1/20 (5%)</td>
</tr>
<tr>
<td>G0 p53−/−</td>
<td>8/20 (40%)</td>
</tr>
<tr>
<td>G3/G4 p53−/−</td>
<td>3/10 (30%)</td>
</tr>
</tbody>
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*p = 0.0113, **p = 0.0111

Note: All genotypes were compared to the G0 p53+/− mice. Except where noted all other comparisons to the G0 pp33+/+ did not give statistically significant results. Mice were examined 2-30 weeks post end of treatment.

[Unpublished observations.](cancerres.aacrjournals.org)
components are compromised in these tumors; or (c) p53 is functionally inactivated via mechanisms other than direct genetic alteration.

**Discussion**

In this work, we address the role of p53 in hepatocellular carcinoma development in the setting of intact and dysfunctional telomeres and further assess the role of p53 as a transducer of the telomere-induced DNA damage response. In this chronic liver injury model, there were no differences in tumor kinetics of G0 p53+/+ versus G0 p53+/-/C0 mice. In sharp contrast, the status of p53 had a major effect on hepatocellular carcinoma progression in the setting of telomere dysfunction and suggests that a major role of p53 in hepatocarcinogenesis is to attenuate the telomere checkpoint response. Furthermore, the retention of p53 heterozygosity in these tumors suggests that p53 haploinsufficiency can compromise the telomere checkpoint to a level that permits the development of hepatocellular carcinoma in the setting of telomere dysfunction and chronic liver injury. Interestingly, in the analysis of human hepatocellular carcinomas associated with HBV and/or HCV (where chronic liver disease and hence telomere shortening occur before hepatocarcinogenesis), loss of only one allele with retention of an intact p53 allele has been observed (39). Finally, we note that hepatocellular carcinoma multiplicity was modestly reduced in G3/G4 p53+/-/C0 mice compared with G0 p53+/+ and G0 p53+/-/C0 mice, an observation that raises one of several possible mechanisms, including partial retention of p53 function encoded by the remaining intact allele, p53-independent telomere checkpoint mechanisms, mTERT-dependent functions in

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**Figure 4.** Acceleration of hepatocarcinogenesis and tumor progression in G3/G4 p53+/- compared with G3/G4 p53+/+ mice. A, the time-dependent incidence of all hepatocellular carcinomas (HCC) is compared in G0 and G3/G4 mice with intact p53 alleles (left) and in G0 and G3/G4 mice that are also p53 heterozygous (right). B, the time-dependent incidence of aggressive hepatocellular carcinomas (size > 0.5 cm and differentiation grade ≥ 2) is compared in G0 and G3/G4 mice with intact p53 alleles (left) and in G0 and G3/G4 mice that are also p53 heterozygous (right). All HCCs, small, large, well, moderately, and poorly differentiated hepatocellular carcinomas were included in the analysis. C, the size of all hepatocellular carcinomas observed in the various genotypes of mice is compared (all ages are included). D, the incidence of all hepatocellular carcinomas and aggressive hepatocellular carcinomas is indicated in the various genotypes of mice (all ages are included). Note: We would like to point out that we elected to use p53+/- and not p53+/+ mice in our studies, due to competing lymphomas, sarcomas, and the development of cholangiocarcinomas with high penetrance (another type of liver cancer observed in p53+/- mice treated with CCl4; reported by Farazi et al. in another recent submission) in p53+/- mice, all of which impair analysis of hepatocellular carcinoma.
hepatocellular carcinoma progression, and/or intolerable levels of genomic instability driven by telomere dysfunction. Our present study cannot distinguish among these mechanisms, which are also not mutually exclusive.

These studies show that the role of p53 in hepatocarcinogenesis is complex and depends on the context of the cellular environment. The carcinogenic protocol used in our studies involves CCl₄ exposure, which translates into the induction of inflammation, oxidative stress, hepatocyte necrosis, and regeneration. One of the features of this process is the release of cytokines and growth factors that promote regeneration after toxic chemical exposure. In the setting of long telomeres, the cells are exposed to damage exerted by the chemical and the resulting induction of oxidative stress. The fact that carcinogenesis occurs to a similar extent in p53⁺/⁺ and p53⁻/⁻ mice suggests that loss of one allele of p53 does not promote extensive proliferation or severely impair the cell’s ability to repair any damage and induce apoptosis. This is consistent with studies done in cultured hepatocytes, whereby complete loss of p53 confers little survival advantage and has no effect on apoptosis in an environment characterized by abundance of survival factors (40). In the setting of chronic liver disease, the cytokines and growth factors released to promote regeneration resemble the scenario of abundant survival factors. Moreover, there was overexpression of telomerase in the regenerating livers and the tumors of these mice, suggesting that activation of telomerase may also provide an additional growth advantage. Thus, in the setting of a growth-promoting environment, p53 heterozygosity does not significantly affect liver tumorigenesis in the mouse (Fig. 5, left).

In the setting of telomere dysfunction, p53 heterozygosity affected liver tumorigenesis by alleviating the tumor suppressive effect of telomere dysfunction and promoting tumor progression. Chronic liver disease occurs in the same way as was described above for mice with intact telomeres. However, a major difference is that the cells now experience additional damage from shortened telomeres, which induce genomic instability. Thus, in the setting of telomere dysfunction, one allele of p53 may not be sufficient to combat the additional damaging effects of telomere attrition and may allow cells to escape growth control and ultimately result in tumor progression. In addition, tumors from telomere dysfunctional mice lack mTERT expression, which may also contribute to the accumulation of even more DNA damage. In fact, it has recently been shown that suppression of hTERT in fibroblasts lead to impaired DNA damage response and defective DNA repair (41). Along similar lines, our findings are consistent with the view that mTERT may also contribute to tumor progression.

In conclusion, a tumor progression phenotype associated with p53 haploinsufficiency became evident only in cells possessing telomere dysfunction (Fig. 5, right). Our previous (12) and current study provide a picture of how telomere dynamics may function to drive hepatocarcinogenesis. Telomere shortening (that occurs during the chronic phase of liver disease) results in genomic instability, which drives hepatocellular carcinoma progression only in the face of telomere checkpoint compromise (i.e., p53 inactivation or other p53-independent mechanisms). In this progression model, increasing expression of mTERT would be predicted to provide additional key activities required for efficient hepatocellular carcinoma progression. Considering that we built our current model using the mTert⁻/⁻ (and not the mTerc⁻/⁻) mice, it is conceivable that future studies can use this model to address the effect of somatic activation of telomerase (via viral transduction of mTert in vivo) on inhibition of cirrhosis and/or inhibition/promotion of hepatocellular carcinoma development. Such a model would better mirror key features of the human situation, such as undetectable to low levels of hepatocyte TERT/ telomerase expression in normal physiologic conditions, and reactivation of telomerase during carcinogenesis.

**Figure 5.** p53 haploinsufficiency in mice with telomere dysfunction. The schematic shows the hypothesis formulated by the data of this work with regard to the role of p53 in hepatocarcinogenesis (in the setting of intact versus dysfunctional telomeres). A detailed explanation of this hypothesis can be found in Discussion.
In conclusion, these observations suggest that the shortened telomeres, high levels of genomic instability, and frequent functional/physical inactivation of p53 in human hepatocellular carcinomas are not mere bystanders but rather play critical pathogenetic roles in the formation of this important cancer type.

References


Role of Telomeres and p53 in Hepatocarcinogenesis

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