Telomere dysfunction provokes regional amplification and deletion in cancer genomes

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Summary

Telomere dysfunction and associated fusion-breakage in the mouse encourages epithelial carcinogenesis and a more humanized genomic profile that includes nonreciprocal translocations (NRTs). Here, array comparative genomic hybridization was used to determine the pathogenic significance of NRTs and to determine whether telomere dysfunction also drives amplifications and deletions of cancer-relevant loci. Compared to tumors arising in mice with intact telomeres, tumors with telomere dysfunction possessed higher levels of genomic instability and showed numerous amplifications and deletions in regions syntenic to human cancer hotspots. These observations suggest that telomere-based crisis provides a mechanism of chromosomal instability, including regional amplifications and deletions, that drives carcinogenesis. This model provides a platform for discovery of genes responsible for the major cancers affecting aged humans.

Introduction

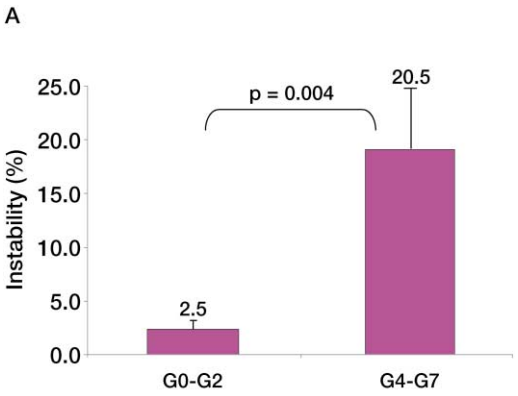
Chromosomal structural aberrations are a hallmark feature of human epithelial cancers and the end product of compromised genome stability mechanisms. Perhaps the most prominent and pathogenetically relevant aberrations are those that result in the regional amplification and deletion of oncogene and tumor suppressor gene loci. Consequently, significant effort has been devoted to elucidating the mechanisms that generate these "chromosomal numerical aberrations" (CNAs), which clearly drive would-be cancer cells toward a threshold of altered gene functions required for malignant transformation. This threshold has been estimated to be higher in epithelial cancers relative to neoplasms originating in mesenchymal or hematopoietic lineages (Armitage and Doll, 1954). Several possible mechanisms have been proposed and experimentally validated, and include defects in the mitotic checkpoint machinery (Elledge, 1996; Nojima, 1997; Wassmann and Benezra, 2001), increased oxidative stress (Bohr et al., 1998; Olinski et al., 1998; Bohr and Dianov, 1999; Karanjawala et al., 2002), and diminished nonhomologous end-joining (Karanjawala et al., 1999; Shen et al., 2000; O'Driscoll et al., 2001; Sharpless et al., 2001; Karanjawala et al., 2002), among others (DePinho, 2000; Lengauer, 2001). The analysis of human cultured cells in crisis (Counter et al., 1992), evolving human colorectal cancers (Hastie et al., 1990; Chadeneau et al., 1995; Rudolph et al., 2001), engineered telomeric double strand breaks in mouse embryonic stem cells (Lo et al., 2002), and tumors arising in mice with telomere dysfunction (Chin et al., 1999; Artandi et al., 2000) has supported the view that telomere dysfunction can serve as a potent driving force in the production of complex chromosomal rearrangements and aneuploidy.

Unlike humans, the laboratory mouse, *Mus musculus*, possesses long telomeres and shows ready activation of telomerase in the setting of neoplasia (Kipling and Cooke, 1990; Broccoli et al., 1996); thus, telomere-based crisis does not appear to take place in mouse cancer cells (Prowse and Greider, 1995). In addition, there are significant species differences in tumor

SIGNIFICANCE

Dissection of the molecular mechanisms underlying cancer-associated chromosomal structural aberrations, such as amplifications and deletions, remains an important focus in the cancer field. The data of this report lend support to the thesis that telomere dysfunction and resultant fusion-bridge-break cycles represent one mechanism that drives the numerous regional amplifications and deletions across the cancer genome. That these chromosomal aberrations are clonal and recurrent in independent tumors suggests pathogenetic relevance to epithelial carcinogenesis. Importantly, many of these amplifications and deletions target regions syntenic to cancer hotspots in human epithelial cancers, providing a system for the discovery of genes responsible for the most common human cancers.

spectrum associated with advancing age: aged humans develop predominantly epithelial cancers, while mice sustain a high incidence of lymphomas and soft tissue sarcoma throughout life (DePinho, 2000). As such, these cross-species differences provided an ideal experimental framework in which to engineer telomere-based crisis in the mouse in order to assess the impact of crisis on the cancer process in vivo. In p53 mutant mice lacking the telomerase RNA component (mTerc), telomere dysfunction and advancing age has been shown to result in a marked shift in tumor spectrum toward one more typical of aged humans: epithelial cancers of the colon, breast, and skin, among others (Artandi et al., 2000). Significantly, these murine cancers also present with a more classical human carcinoma cytogenetic profile, most notably marked aneuploidy and numerous complex unbalanced translocations (UBTs) (Artandi et al., 2000). In this study, the presence of amplifications and deletions, another hallmark and perhaps more pathogenically relevant feature of the cancer genome, was not assessed. Along these lines, it is worth noting that previous work in cultured cells has established that chromosome damage and DSBs can provide a nidus for amplification or deletion at the site of breakage (Windle et al., 1991; Kimmel et al., 1992; Pipiras et al., 1998). As UBTs result from the production and repair of double-stranded DNA breaks (DSBs), we utilized array-comparative genomic hybridization (array-CGH) to determine whether the bridge-fusion-breakage process that accompanies telomere dysfunction was associated with clonal and regional alterations in gene/loci dosage and whether such numerical aberrations involve regions with potential relevance to the transformation process.



B

Genotype	Tumor	Instability (%) G0-G2	G4-G7
mTerc$^{-/-}$,*p53*$^{+/-}$	Osteosarcoma	2.8	17.2
mTerc$^{-/-}$,*p53*$^{+/-}$	Lymphoma	4.9	29
mTerc$^{-/-}$,*p53*$^{+/-}$	Breast	3.2	23
mTerc$^{-/-}$,*Wnt*$^{+}$	Breast	0.4	9.2

Figure 1. Late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ cancer genomes exhibit significantly greater levels of genomic instability than early generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ tumors

A: Histogram illustrates the frequency of BACs exhibiting copy number changes as determined by array-CGH. G0–G2 represents tumors from *mTerc*$^{+/+}$,*p53*$^{+/-}$ and early generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice. G4–G7 represents tumors from late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice.
B: Table illustrates the frequency of BACs exhibiting copy number changes as determined by array-CGH for individual tumor types.

Results and Discussion

Array-CGH assays were conducted on a collection of mammary carcinomas, squamous cell carcinomas of the skin (SCC), and colorectal cancers derived from late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice, as well as on tumors derived from early generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice of the same cohort. These studies utilized arrays of 931 mouse BACs at 3 Mb coverage of the mouse genome. Late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ cancer genomes showed extensive focal alterations involving 20.5% (SEM = 3.1) of the arrayed BACs, which contrasts with more modest CNA levels (2.5%, SEM = 1.3) detected in tumors arising in early generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice from the same cohort ($p = 0.004$). Since epithelial carcinomas are rare in mice with intact telomere function, it was not possible to perform a precise comparison of the level of telomere-induced genomic changes in the same cancer type. However, an examination of late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ lymphomas and osteosarcoma genomes revealed a significantly greater level of chromosomal instability than *mTerc*$^{+/+}$,*p53*$^{+/-}$ lymphomas and osteosarcoma genomes (Figure 1B), indicating that telomere dysfunction can provoke a marked increase in chromosomal alterations independent of tumor type. More directly, we also examined a small collection of mammary carcinomas arising in late generation *mTerc*$^{-/-}$ MMTV-*Wnt1* transgenic mice and found a higher level of chromosomal instability (9.2%, SEM = 3.9) relative to mammary carcinomas arising in *mTerc*$^{+/+}$ MMTV-*Wnt1* transgenic mice (0.4%, SEM = 0.1; $p = 0.05$) These array-CGH results are in line with previous spectral karyotype analyses showing a marked increase in aneuploidy and NRTs in tumors derived from late generation mTerc$^{-/-}$ mice with telomere dysfunction (Artandi et al., 2000).

32 CNAs were found to be recurrent in at least one of the three tumor types, with recurrence defined as present in at least 25% of the tumors examined in this study. The most prominent and consistent change was a 5- to 6-fold amplification of distal chromosome 6, observed in 8 of 15 samples representing all 3 tumor types. The involvement of distal chromosome 6 corresponds well with previous spectral karyotype analyses showing UBTs involving distal chromosome 6 in some late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ breast tumors and the absence of rearrangements in this region in breast tumors derived from *mTerc*$^{+/+}$ MMTV-*Wnt1 p53* mutant mice (Artandi et al., 2000). Amplification of this region has also been observed in viral oncogene-induced skin cancers arising in late generation *mTerc*$^{-/-}$ mice (D. Argilla et al., submitted). Together, these findings raise the possibility that this locus encodes an oncogene relevant to the pathogenesis of many epithelial cancers or an adaptive factor that enables tumorigenesis in the face of telomere dysfunction.

To characterize the locus on distal chromosome 6 at higher resolution, the mouse genome sequence database was used to identify closely spaced markers spanning the region of amplification identified by array-CGH. Using these markers, slot blot DNA analysis of the same set of *mTerc*$^{-/-}$,*p53*$^{+/-}$ tumors delimited the minimal region of involvement to a 250 kb region

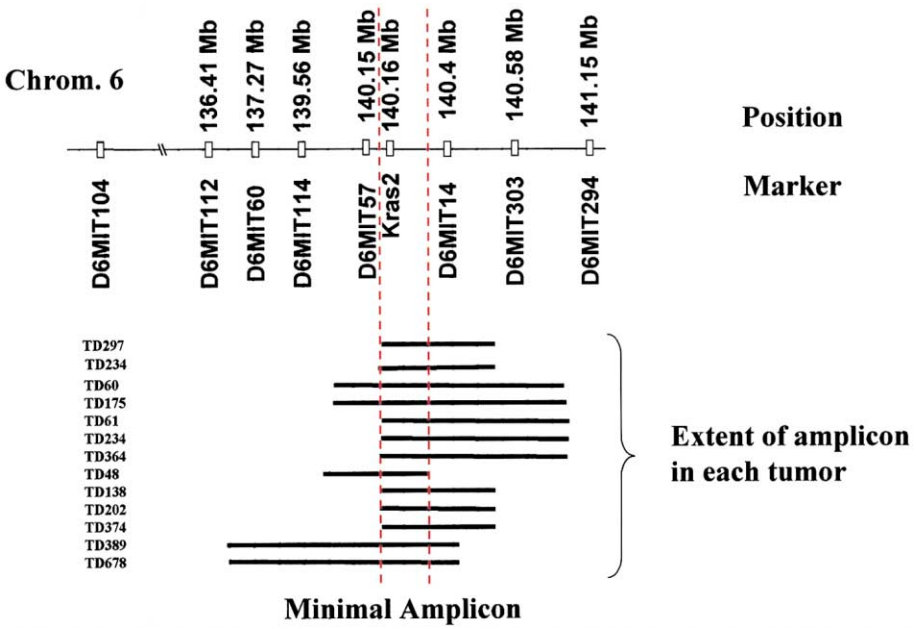


Figure 2. Identification of the minimal amplicon on mouse chromosome 6

The extent of the region of amplification at distal chromosome 6 is indicated by the black bars for 13 *mTerc*$^{-/-}$,*p53*$^{+/-}$ tumors that contained amplifications in this region. The name of the marker, and its position on chromosome 6 (defined by mouse genome database), are indicated in the upper part of the figure.

located between 140.15 and 140.40 Mb on chromosome 6 (Figure 2). This region includes six known or predicted genes with no obvious links to DNA repair responses, recombination, alternative lengthening of telomeres (ALT), or other telomere-relevant pathways (note that *Rad52* falls outside of the minimal amplicon boundary). *K-Ras* is contained within the amplicon and this gene, while clearly important in the pathogenesis of a broad spectrum of epithelial cancers, is not known to be involved in modulating the cellular response to telomere dysfunction. It is conceivable that *K-Ras* might regulate the expression or activity of genes involved in this adaptive response, since K-, H-, and N-Ras have been shown to enhance the expression of a number of DNA processing enzymes (Zuber et al., 2000). It is noteworthy that amplification of distal chromosome 6, including *K-ras*, is not observed in MMTV-*Wnt1* late generation mTerc$^{-/-}$ breast cancers. In these tumors, Wnt1 acts as a dominant oncogene, and activation of the Wnt signaling pathway was recently shown to be sufficient to replace receptor-tyrosine-kinase/Ras signaling in C. elegans vulval development (Gleason et al., 2002). This genetic evidence provides a rational explanation for the lack of *K-ras* amplifications in the Wnt1-driven tumors and supports the hypothesis that *K-ras* is the target of the distal chromosome 6 amplification in the late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ breast adenocarcinomas.

In addition to amplification of distal chromosome 6, array-CGH analyses of the *mTerc*$^{-/-}$,*p53*$^{+/-}$ carcinomas revealed numerous recurrent amplifications or deletions affecting previously recognized cytogenetic hotspots in human cancers (Tables 1–3 and Figure 3). Significantly, these CNAs were not detected in numerous tumor types derived from *mTerc*$^{+/+}$,*p53*$^{+/-}$ mice, and no recurrent loci were identified in *mTerc*$^{+/+}$,*p53*$^{+/-}$ tumors (data not shown). In the late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ tumors, a number of CNAs were noteworthy when compared to the database of CNAs derived from human tumors. For each recurrent CNA in the mouse, the largest single occurrence was mapped onto the human genome using the NCBI mouse-human homology database, and the corresponding human chromosomal region analyzed for the presence of known cancer hotspots and/or breakpoints. In Tables 1–3, the neighboring genes define the extent of the human chromosome that is homologous to the CNA observed in the mouse.

In the *mTerc*$^{-/-}$,*p53*$^{+/-}$ breast adenocarcinomas, eight recurrent CNAs were identified. The chromosome 11 amplicon, present in four of seven breast tumors, corresponds to human

Table 1. Recurrent CNAs in *mTerc*$^{-/-}$,*p53*$^{+/-}$ breast cancers (n = 7)

Mouse BAC[1]	Chromosome	Location (cM)	Frequency of amplification	Frequency of deletion	Human synteny	Neighboring genes	Breast tumor number
D1MIT369	1	73	3	0	1q31	*PTPRC, MYOG*	2,6,7
D5MIT132	5	29	0	3	4p15	*CD38*	1,3,4
D6MIT295	6	73.6	3	0	12p12	*KRAS2, BCAT1, BHLHB3*	1,2,5
D6MIT15	6	74	3	0	12p12	*KRAS2, BCAT1, BHLHB3*	1,2,5
D7MIT46	7	69	3	0	11p15	*LSP1, IGF2*	1,2,6
D11MIT362	11	75	4	0	17q23-25	*GRB2, MAFG*	1,2,3,5
D13MIT312	13	44	0	3	5q13-21	*CRTL1, PCSK1*	2,3,4
D17MIT87	17	33.8	0	4	19p13; 16pter	*CLPP; SULT1A2*	1,2,3,5

[1]Mouse BAC indicates the minimal common region altered in all recurrences of each CNA identified.

Table 2. Recurrent CNAs in *mTerc*$^{-/-}$,*p53*$^{+/-}$ SCCs (n = 4)

Mouse BAC[1]	Chromosome	Location (cM)	Frequency of amplification	Frequency of deletion	Human synteny	Neighboring genes	SCC tumor number
D5MIT155	5	53	3	0	4q13-20	*GKP2, SCYB9*	2,3,4
D6MIT295	6	73.6	3	0	12p12	*KRAS2, BCAT1, BHLHB3*	2,3,4
D8MIT45	8	40	4	0	16q12-13; 18q23	*ADCY7, CBLN1*	1,2,3,4
D10MIT218	10	29	3	0	6q21	*ROS1, AIM1*	1,3,4
D11MIT362	11	75	3	0	17q23-25	*GRB2, MAFFG*	2,3,4
D13MIT279	13	36	4	0	9q22	*FANCC, PTCH*	1,2,3,4

[1]Mouse BAC indicates the minimal common region altered in all recurrences of each CNA identified.

17q24-25—a region of amplification reported previously in 10% to 67% of human breast tumors (Orsetti et al., 1999; Larramendy et al., 2000). The focal deletion on chromosome 5, detected in three of seven breast tumors, bears synteni to human 4p14-15, which sustains deletions in 16% to 39% of human breast cancer cases (Orsetti et al., 1999; Richard et al., 2000). Of particular interest is the chromosome 1 amplification, observed in three of seven breast tumors, and which corresponds to human 1q31, a region amplified in 26% to 89% of human breast carcinomas (Loveday et al., 1999; Larramendy et al., 2000). In a recent human breast cancer study correlating cytogenetics and telomerase activity, 25% of the tumors were found to lack telomerase activity, and this parameter correlated with amplification of 1q31 (Loveday et al., 1999). Also detected in multiple *mTerc*$^{-/-}$,*p53*$^{+/-}$ breast tumors was an amplification corresponding to human 11p15 and deletions syntenic to human 5q13-21 and 19p13. Deletion or loss of heterozygosity at 5q13-21 or 19p13 has been reported in ovarian carcinomas (Aman et al., 1993; Jenkins et al., 1993; Kiechle-Schwarz et al., 1995; Wang et al., 2001), and 5q13 is frequently deleted in myeloid neoplasias (Fairman et al., 1996; Gogineni et al., 1996; Castro et al., 1998, 2000). These data suggest several areas of common amplification and deletion between telomerase deficient murine and human spontaneous breast tumors.

In the skin carcinomas, six recurrent CNAs (defined as present in three of four samples) were identified (Table 2 and Figure 1A). Two of these, the amplifications on distal chromosome 6 (*K-Ras* locus) and distal chromosome 11, were also present in the mouse breast cancers. Of the remaining four that were specific to SCCs, three CNAs were syntenic to cancer hotspots at human chromosomes 4q, 9q, and 16q that were reported previously in human SCC by conventional cytogenetic analyses (Atkin and Fox, 1992; Worsham et al., 1993). In light of the proposed connection between breakage and amplification or deletion (Windle et al., 1991; Kimmel et al., 1992), it is noteworthy that these human cancer hotspots correspond to breakpoints in human SCC translocations (Atkin and Fox, 1992; Worsham et al., 1993).

In four late generation *mTerc*$^{-/-}$*p53*$^{+/-}$ colon cancers examined, 13 recurrent CNAs (present in two of four samples) were identified, and four bear synteni to loci (11p15, 3p21, 8q22, and 9q31) known to experience copy number changes in human colon carcinomas (Table 3 and Figure 1A). Amplification of chromosome 7 in a region syntenic to human 11p15 was identified in three of four mouse colon cancers. A number of genes encoding mucins are found in this region of 11p15, including *MUC2*, which is overexpressed in metastatic colon cancers (Jang et al., 2002). Amplification of chromosome 9 with synteni to human 3p21 was observed in three of four mouse colon tumors studied. Breakpoints in human 3p21 have been identified in metastatic colon cancers (Gagos et al., 1995), and overexpression of the *CDCP1* gene, which is located at 3p21 also has been observed in colorectal cancers (Scherl-Mostageer et al., 2001). The mouse chromosome 15 deletion in two of four mouse colon tumors corresponds to a deletion at human 8q22 which appears to predispose to cancer and provide an initiating event in a subset of human colon adenocarcinomas (Shabtai et al., 1988). In addition to known hotspots for colon cancers, amplification or dele-

Table 3. Recurrent CNAs in *mTerc*$^{-/-}$,*p53*$^{+/-}$ colon cancers (n = 4)

Mouse BAC[1]	Chromosome	Location (cM)	Frequency of amplification	Frequency of deletion	Human synteny	Neighboring genes	Colon tumor number
D1MIT227	1	81.6	3	0	1q25	*SOAT1, XPR1*	1,2,4
D4MIT55	4	19.6	3	0	9q33-34	*TGFBR1*	1,2,4
D4MIT242	4	24.5	0	3	9q31	*Thioredoxin1, KLF4*	1,2,4
D6MIT294	6	73.4	2	0	12p11-p12	*KRAS2, BCAT1, BHLHB3*	1,2
D7MIT280	7	51.5	3	0	11p15.1-15.5	*HRAS, RRAS2, MRV1*	1,2,4
D8MIT92	8	73.2	3	0	10p12	*NRP (VEGF165R)*	1,2,4
D9MIT93	9	27.0	3	0	11q23	*APOA1; N-CAM*	1,3,4
D9MIT280	9	67.0	3	0	3p21.3-21.2	*TGFBR2, CDCP1*	1,3,4
D15MIT251	15	13.6	0	2	8q22-23	*SYNDECAN2, FZH6*	1,2
D16MIT143	16	10.8	0	3	22q11	*BID*	1,2,4
D17MIT113	17	6.5	4	0	6q25-27	*MEKK4*	1,2,3,4
D18MIT2	18	37.0	3	0	18p11.2	*MC2R, ACTHR*	1,2,3
DXMIT56	X	5.7	3	0	Xp11	*PCTK1, UBE1X*	1,3,4

[1]Mouse BAC indicates the minimal common region altered in all recurrences of each CNA identified.

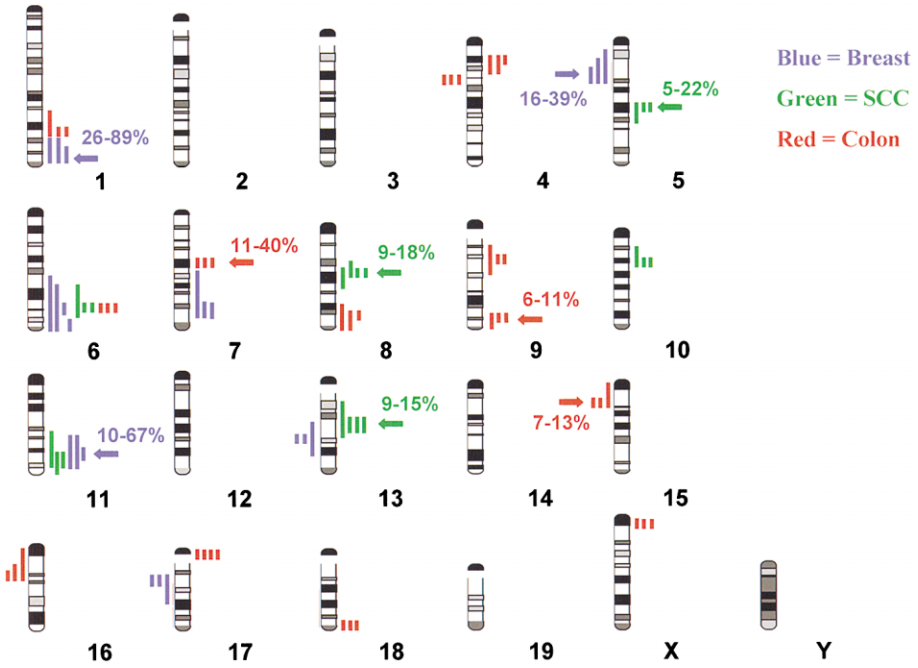


Figure 3. Chromosomal alterations in tumors of *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice are homologous to human cancer hotspots

Chromosomal locations of CNAs detected by array-CGH in breast (blue), skin (green), and colon (red) carcinomas in *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice. Gains are indicated by lines to the right of the chromosome ideograms, and losses are indicated by lines to the left. Numbers and arrows indicate the frequency with which the homologous region of a particular CNA was affected in the corresponding human tumor.

tion of mouse chromosomes in regions that contain potential cancer-relevant genes was also observed. The gut-enriched Kruppel-like factor (*KLF4*) is located in a region of mouse chromosome 4 syntenic to human chromosome 9q31 and deleted in three of four colon tumors. Expression of *KLF4* inhibits proliferation in the gut, suggesting that loss of this gene might promote colon cancer (Shie et al., 2000; Chen et al., 2001; Dang et al., 2001). The recent finding of the related KLF6 protein as a prostate cancer tumor suppressor in humans adds significance to our observations (Narla et al., 2001). As was the case for breast tumors, these data show that the increased epithelial carcinogenesis induced in skin and colon by telomere instability is driven by a spectrum of amplifications and deletions reminiscent of those seen in the corresponding human carcinomas.

These array-CGH data demonstrate clonal selection of CNAs within a tumor cell population, recurrence of these CNAs in independently derived tumors of similar and different cancer types, and correspondence to known cytogenetic hotspots in human cancers. On this basis, we conclude that telomere dysfunction engenders regional CNAs that drive epithelial carcinogenesis in this mouse model. The identification of chromosomal alterations in the mouse that mirror alterations identified in human SCC, breast cancer, and colon cancer is striking and lends further support to the hypothesis that telomere-based crisis and associated fusion-breakage not only drives age-dependent epithelial carcinogenesis, but is also a major force underlying the complex chromosomal changes present in such cancer genomes in human carcinoma. That CNAs result from telomere dysfunction and contribute to tumorigenesis is supported by a markedly reduced number of rearrangements (Artandi et al., 2000; Lu et al., 2001) and CNAs (Hodgson et al., 2001) in tumors arising in cancer-prone mice with intact telomere function (Lu et al., 2001), including early generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice from the same cohort as the late generation *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice studied herein. Additional support derives from the lower incidence of CNAs in two viral oncogenesis models, despite telomere dysfunction, presumably related in part to the capacity of these viral oncoproteins to directly commandeer many cancer-relevant pathways (D. Argilla et al., submitted). Direct comparison of the extent of genomic instability in breast tumors derived from MMTV-*Wnt1*, *mTerc*$^{-/-}$ (9.2%, SEM = 3.9) and *mTerc*$^{-/-}$,*p53*$^{+/-}$ (20.5%, SEM = 3.1) mice supports the notion that the presence of a dominantly acting oncogene reduces the necessity for telomere-dysfunction induced genomic instability in oncogenesis. With data reported here, we conclude that telomere dysfunction and its associated fusion-breakage process is a major mechanism driving the accumulation of CNAs that alter the dose of cancer-relevant genes. As the already considerable power of array-CGH will further increase with more dense coverage of the genome, this mouse model of epithelial carcinogenesis should provide a robust platform for discovery of genes responsible for the major cancers affecting the aging human population.

Experimental procedures

Generation of mice and tumor analysis

Double heterozygous mice (*mTerc*$^{+/-}$,*p53*$^{+/-}$) were generated from matings between multiple pairs of *mTerc*$^{+/-}$ mice and *p53*$^{+/-}$ mice (Jackson Labs). Both strains are of mixed genetic background, primarily 129SV and C57Bl/6. These mice were intercrossed to generate G1 *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice; G1 *mTerc*$^{-/-}$,*p53*$^{+/-}$ intercrosses yielded G2 *mTerc*$^{-/-}$,*p53*$^{+/-}$ mice and so on until G8. We used randomized cousin mating schemes to maintain genetic heterogeneity and prevent the generation of substrains (Artandi et al., 2000).

DNA isolation

Genomic DNA was isolated from tumors or normal tissue using the PUREGENE DNA isolation kit (Gentra Systems) according to manufacturer's protocol. 5 μg of gDNA was digested with EcoRI, extracted with phenol:chloroform, ethanol precipitated, and resuspended in sterile water prior to labeling.

Labeling and hybridization to arrays

Array-CGH experiments were performed with SpectralChip arrays (Spectral Genomics) according to the manufacturer's protocol. These arrays cover

the mouse genome at a density of approximately 3 Mb and comprise 976 mapped mouse BACs from the RPCI11 BAC library. 1 μg of tumor and normal genomic DNA was labeled with Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia Biotech). Fluor-reversal experiments were performed in all cases. Hybridizations were performed in sealed chambers for 20 hr at 60°C. After hybridization, slides were rinsed in 2× SSC, 0.5% SDS at room temperature, washed for 40 min in 2× SSC, 0.5% SDS at 65°C, washed for 10 min in 0.2× SSC at 65°C, and washed for 1 min in 0.2× SSC at 2°C. Slides were then air dried by centrifugation prior to imaging.

Image and data analysis

16-bit TIF images were collected using an Axon 4000B scanner, and processed initially using GenePix Pro. Subsequently, custom software was utilized to exclude spots that demonstrated low signal/noise ratios or poor spot morphology. Data were normalized to the ratio of medians of Cy3 and Cy5, and the normalized values converted to log base 2 ratios to weight gains and losses equally. Dye swap experiments were merged to calculate the mean and standard deviation for each BAC. The threshold for defining a CNA was based on the distribution of log ratios in self-self hybridizations. Regions were scored as significantly increased/decreased if the CGH ratio was 4 standard deviations above/below the mean (± 0.35, log base 2).

Slot blot analysis of genomic DNA

DNA concentrations were determined by UV absorbance spectroscopy, diluted to 5 μg/ml in 0.4N NaOH, 10 mM EDTA (pH 8.0) and boiled for 5 min before blotting. Slot blots (Schleicher & Schuell, Inc.) were prepared according to the manufacturer's recommendations on Hybond-N+ membranes (Amersham Pharmacia Biotech) and hybridized with 32-P random prime labeled probes for the indicated markers or genes. Membranes were washed at 65°C in 2× SSC, 0.1% SDS for 15 min., 0.5× SSC, 0.1% SDS for 15 min., 0.25× SSC, 0.1% SDS for 15 min. Signal intensities were determined on a Fujifilm FLA-2000 phosphorimager using ImageGauge 3.3 software. Background subtractions were performed and values were normalized relative to markers for which no CNAs were observed in the tumors studied.

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