

Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation

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Tumor suppressor genes are generally viewed as being recessive at the cellular level, so that mutation or loss of both tumor suppressor alleles is a prerequisite for tumor formation. The tumor suppressor gene, p53, is mutated in ~50% of human sporadic cancers and in an inherited cancer predisposition (Li–Fraumeni syndrome). We have analyzed the status of the wild-type p53 allele in tumors taken from p53-deficient heterozygous (p53 +/-) mice. These mice inherit a single null p53 allele and develop tumors much earlier than those mice with two functional copies of wild-type p53. We present evidence that a high proportion of the tumors from the p53 +/- mice retain an intact, functional, wild-type p53 allele. Unlike p53 +/- tumors which lose their wild-type allele, the tumors which retain an intact p53 allele express p53 protein that induces apoptosis following γ -irradiation, activates p21^{WAF1/CIP1} and Mdm2 expression, represses PCNA expression (a negatively regulated target of wild-type p53), shows high levels of binding to oligonucleotides containing a wild-type p53 response element and prevents chromosomal instability as measured by comparative genomic hybridization. These results indicate that loss of both p53 alleles is not a prerequisite for tumor formation and that mere reduction in p53 levels may be sufficient to promote tumorigenesis.

Keywords: genomic instability/loss of heterozygosity/mouse/p53/tumor suppressor

Introduction

In 1971, Alfred Knudson formulated the ‘two-hit’ hypothesis to provide a genetic explanation for the incidence patterns of inherited and sporadic childhood retinoblastomas (Knudson, 1971). This hypothesis in its current form stipulates that individuals inheriting one defective copy of a tumor suppressor gene are predisposed to tumors because of the higher likelihood of the remaining wild-type allele incurring a mutation (Knudson, 1985). Sporadically arising tumors in non-predisposed individuals occur less frequently because both wild-type alleles of the tumor

suppressor must incur *de novo* lesions. Mutation or inactivation of both copies of the tumor suppressor gene is considered a prerequisite for tumor formation. Thus, at the cellular level, tumor suppressor genes are recessive. Since then, it has generally been accepted that tumor suppressor genes, including the retinoblastoma (Rb) gene, conform to the two-hit rule in those inherited and sporadic cancers exhibiting mutations in these genes (Cavenee *et al.*, 1985; Jacks *et al.*, 1992).

Inherited mutations in another tumor suppressor gene, p53, have been implicated in the Li–Fraumeni syndrome, a familial cancer predisposition (Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Malkin, 1994). Approximately 50% of Li–Fraumeni family members who inherit a mutated p53 allele develop cancer by the age of 30. Analysis of the tumors from Li–Fraumeni patients reveals that in many cases there is apparent loss of heterozygosity (LOH) at the p53 locus, consistent with the predictions of the two-hit hypothesis (Malkin *et al.*, 1990; Iavarone *et al.*, 1992; Srivastava *et al.*, 1992).

Almost half of all sporadic human tumors examined to date have evidence of p53 gene mutations. p53 is the most frequently mutated gene in human cancers (Greenblatt *et al.*, 1994). Vogelstein and colleagues, in their examination of colon carcinomas and other cancers, found that the most frequent pattern of p53 mutation was a point mutation in one allele and complete loss of the second allele (Baker *et al.*, 1989; Nigro *et al.*, 1989). This p53 mutational pattern has been confirmed in scores of subsequent studies (Greenblatt *et al.*, 1994). However, a subset of tumors with p53 mutations were observed with a point mutation or deletion in one allele while the wild-type allele remained intact (Nigro *et al.*, 1989; Mulligan *et al.*, 1990; Davidoff *et al.*, 1991; Mazars *et al.*, 1992). It was suggested that the ability of the tumors with a point mutation to grow in the presence of the wild-type allele was due to the dominant-negative activity of the mutant form of p53 (Fearon and Vogelstein, 1990). In addition, some p53 mutant proteins may also possess a dominant gain-of-function activity which can increase genomic instability and promote cancer formation in the presence of a wild-type allele (Gualberto *et al.*, 1998). However, the mere reduction of p53 gene dosage in these tumors was not considered to be a factor which might in itself contribute to tumorigenesis.

To address the role of p53 in tumorigenesis in an animal tumor model, we have generated p53-deficient mice through embryonic stem (ES) cell technology (Donehower *et al.*, 1992). Null (p53^{-/-}) mice are developmentally viable yet develop tumors very rapidly; all succumb to tumors by 10 months of age (Figure 1). Heterozygous (p53^{+/-}) mice also develop tumors, though at a later stage in their life span. By 2 years of age, >95% of heterozygous mice have died or developed tumors, in

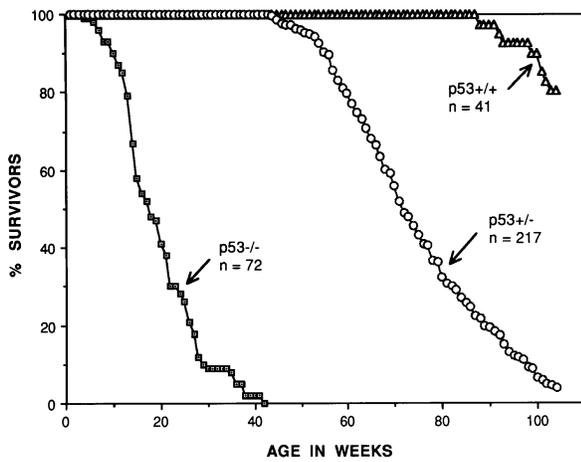


Fig. 1. Tumor incidence and survival in 41 p53^{+/+}, 217 p53^{+/-} and 72 p53^{-/-} mice. All mice were of mixed inbred C57BL/6 \times 129/Sv background. The percentages of tumor-free survivors are plotted as a function of age in weeks. Animals were monitored for tumors, morbidity or spontaneous death over a period of 2 years (104 weeks). Moribund or tumor-bearing mice were sacrificed and necropsied. Growths or enlarged tissues were subjected to histopathological analysis to confirm their status as tumors.

contrast to an 80% survival to 2 years in their wild-type (p53^{+/+}) littermates (Figure 1). While the p53^{-/-} mice primarily develop lymphomas, the p53^{+/-} mice exhibit a wider array of tumors, including soft tissue sarcomas, osteosarcomas and carcinomas of various types (Harvey *et al.*, 1993a; Jacks *et al.*, 1994; Purdie *et al.*, 1994; Donehower, 1996). The tumor spectrum of the p53^{+/-} mice resembles to some extent that of the Li-Fraumeni families, leading to the proposal that the p53 heterozygotes might make a useful animal model for Li-Fraumeni syndrome (Donehower, 1996).

Here, we have analyzed the fate of the wild-type allele in p53^{+/-} tumors. The two-hit model would predict that the remaining wild-type p53 allele would be structurally or functionally inactivated. Surprisingly, we found that over half of the p53^{+/-} tumors appear to retain an intact wild-type p53 allele. We show several fundamental differences between those tumors which retain wild-type p53 and those that lose it during tumor progression. The fact that tumorigenesis is still accelerated in the presence of a single wild-type p53 gene suggests that the mere reduction of p53 gene dosage may be sufficient for cancer formation. These findings are consistent with the failure to find p53 LOH in a subset of sporadic and inherited human tumors with p53 mutations.

Results

Tumorigenesis and wild-type allele status

In order to form a clearer understanding of tumor progression in p53^{+/-} mice, we monitored tumor formation in 217 p53^{+/-} animals and 41 control p53^{+/+} animals over a period of 2 years. The genetic background of these mice was mixed inbred C57BL/6 (87.5%) \times 129/Sv (12.5%). The tumor incidence of these mice is shown in Figure 1 and compared with historical tumor incidences for p53^{-/-} mice of similar background. Over 95% of p53^{+/-} mice develop tumors (primarily soft tissue sarcomas, osteosarcomas and lymphomas) or die prematurely by 2 years of

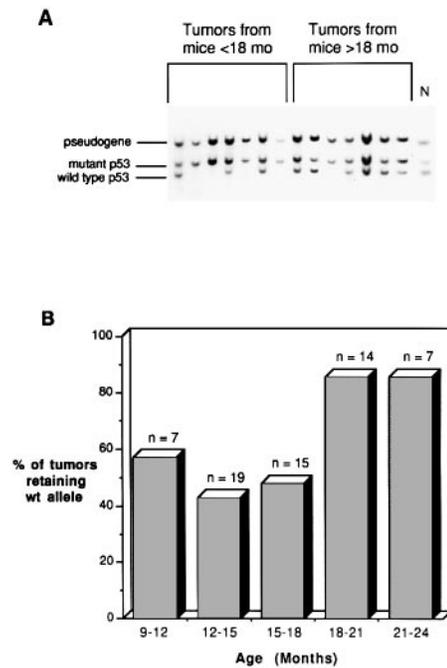


Fig. 2. Loss of p53 heterozygosity (LOH) in tumors from p53^{+/-} mice. (A) LOH in p53^{+/-} tumors assessed by Southern blot hybridization. High molecular weight DNA from p53^{+/-} tumors was isolated, cleaved with restriction endonuclease *Bam*HI and subjected to agarose gel electrophoresis followed by Southern blot hybridization with a ³²P-labeled p53 exon 2-6 cDNA probe. The wild-type p53 gene, the mutant allele and the p53 pseudogene present in the mouse genome are indicated. Note that more of the tumors from the mice <18 months of age appear to exhibit loss of the wild-type allele. (B) LOH frequency is age dependent. Compilation of the results from p53 genotype analysis of 62 p53^{+/-} tumors shows a statistically significant difference in LOH frequency depending on the age when the tumor was taken.

age, in contrast to the p53^{+/+} control mice which show an incidence of only 20% deaths or tumors by this age. The incidences reported here are comparable with those reported in previous studies by us and others (Harvey *et al.*, 1993; Jacks *et al.*, 1994; Purdie *et al.*, 1994).

We analyzed the status of the wild-type allele in the p53^{+/-} tumors by Southern blot hybridization analysis. We found that approximately half of the tumors in p53^{+/-} mice under 18 months of age showed retention of the wild-type allele (Figure 2A). Retention or loss of the wild-type allele occurred with similar frequencies among all the major tumor types. However, p53 allele retention increased to >85% in tumors from mice older than 18 months of age (Figure 2B). When the frequency of wild-type allele retention is compared in animals <18 months of age versus animals >18 months of age by *t*-test, the difference between these two groups is significant ($P = 0.025$).

The increased frequency of retention of the wild-type allele in tumors from older mice suggested that perhaps these tumors were arising later because the wild-type allele was structurally intact and its continued presence might be retarding tumor development. To test this hypothesis, we sequenced the entire coding sequence of p53 cDNAs from two tumors with retention of the wild-type allele. In both tumors, the sequence of the p53 transcript was wild-type, suggesting that the p53 gene did remain structurally intact.

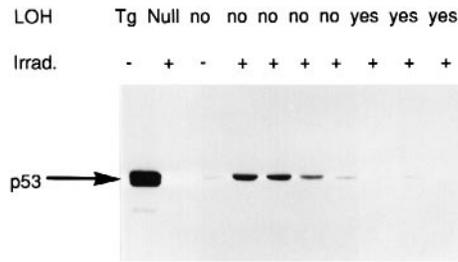


Fig. 3. p53 protein in p53^{+/-} tumors. Tumor-bearing p53^{+/-} mice were irradiated with 20 Gy ionizing radiation and tumors were harvested 2–3 h later. Lysates from each tumor were prepared and immunoprecipitated with a cocktail of monoclonal antibodies specific for mouse p53. The immunoprecipitates were subjected to SDS-PAGE and Western blotting, prior to incubation with sheep polyclonal antibody specific for p53. This was followed by incubation with a peroxidase-conjugated anti-sheep secondary antibody. p53, which is indicated by the arrow, was detected using chemiluminescence. In the first lane, Tg is the positive control, a lysate from a tumor taken from a mutant p53-overexpressing transgenic mouse. The negative control (Null) was a lysate from an irradiated p53^{-/-} tumor. The remainder of the lanes are from p53^{+/-} tumors with (yes) or without (no) p53 LOH.

p53 protein expression

Although the p53 gene remained structurally intact in some p53^{+/-} tumors, it might be down-regulated in expression at the transcriptional or post-transcriptional level. We tested this possibility by performing immunoprecipitation–Western blot analyses for p53 protein expression in tumor lysates from p53^{+/-} tumors which retained or lost their wild-type allele as assayed by Southern blot analysis. Since steady-state levels of wild-type p53 protein are normally modest, even in tumors, we optimized for detection of the protein by γ -irradiation (20 Gy) of the tumor-bearing mouse 2–3 h prior to sacrifice. p53 protein levels have been shown to be greatly increased following ionizing radiation (Kuerbitz *et al.*, 1992; Lu and Lane, 1993). Tumor lysates were immunoprecipitated with p53 monoclonal antibodies followed by Western blot analysis with a sheep polyclonal antibody. The results of these assays showed that p53 protein could be detected in p53^{+/-} tumors which retained the wild-type allele, but was essentially undetectable in p53^{+/-} tumors with LOH (Figure 3). Positive control [from a transgenic p53 mouse tumor overexpressing mutant p53 (Lavigneur *et al.*, 1989)] and negative control (from a p53^{-/-} mouse tumor) lysates show the expected high level and absence of p53 expression, respectively. These results suggest that the p53 protein is expressed at significant levels in the p53^{+/-} tumors which retain their wild-type p53 allele. Moreover, the fact that p53 protein levels are relatively modest compared with levels in the tumor lysate with a mutant version of p53 (see Figure 3, lane 1) is consistent with a wild-type configuration, since wild-type p53 has a much shorter half-life in tumor cells than most mutant forms of p53 (Lane, 1994).

p53 functional tests

Even if structurally intact wild-type p53 protein is expressed in the p53^{+/-} tumors with no LOH, it is important to demonstrate that the protein is functioning normally. One possibility is that p53 is intact but inactivated by overexpression of Mdm2, a cellular oncogene protein which can bind to p53 (Momand *et al.*, 1992).

mdm2 gene amplification has been observed in one-third of human sarcomas (Oliner *et al.*, 1992). We examined 25 p53^{+/-} tumors (19 of 25 tumors retained the wild-type allele) for evidence of *mdm2* amplification and found that all tumors were apparently diploid for the *mdm2* gene, suggesting that Mdm2 is not inactivating p53 function. RT-PCR analysis and Northern blot analysis of *mdm2* transcripts in a subset of these tumors also revealed no obvious increase in Mdm2 RNA expression (data not shown).

One test of p53 function is to demonstrate radiation-induced apoptosis in these tumors. Wild-type p53 has been shown to be a potent inducer of apoptosis in tumor cells following γ -irradiation (Lowe *et al.*, 1993, 1994). To perform this assay, we monitored radiation-induced apoptosis in p53^{+/-} tumors. We performed a DNA fragmentation assay on total DNA isolated from the tumors before and after radiation. In tumor cells exhibiting high levels of apoptosis, ethidium bromide staining of the gels revealed a ladder of DNA bands which were multiples of ~170 bp, due to activation of cell endonucleases which cleave DNA in internucleosome regions (Kerr and Harmon, 1991). Prior to radiation, the tumors exhibited varying levels of apoptosis. After radiation, however, only the p53^{+/-} tumors which retained their wild-type allele showed significant increases in levels of apoptotic DNA fragments (Figure 4A). Tumors which lost the wild-type allele invariably showed no significant increases in apoptotic bands following radiation. The degree of apoptosis induction was quantified by comparing the relative amounts of DNA in the high and low molecular weight regions of the gel for each sample (Figure 4B). With a single exception, p53^{+/-} no LOH tumors showed significant increases in apoptosis following radiation, while p53^{+/-} LOH tumors in all cases did not.

A second p53 functional test focused on the transcriptional activity of the p53 target genes p21^{WAF1/CIP1} and *mdm2* in the p53^{+/-} tumors following treatment with ionizing radiation. Both of these genes are transcriptionally activated in a p53-dependent manner following radiation (Barak *et al.*, 1993; El-Deiry *et al.*, 1993, 1994; Perry *et al.*, 1993). Northern blot hybridization analysis of irradiated p53^{+/-} tumor mRNAs demonstrated that the p53^{+/-} no LOH tumors showed 2.5-fold higher levels of p21 and Mdm2 mRNA on average compared with p53^{+/-} LOH tumors (Figure 5). These differences in mRNA levels were statistically significant for p21 and were near statistical significance for Mdm2. Unirradiated p53^{+/-} no LOH and LOH tumors were approximately equivalent in p21 and Mdm2 mRNA levels to irradiated p53^{+/-} LOH tumors, indicating that the higher p21 and Mdm2 mRNA levels in the irradiated p53^{+/-} no LOH tumors were p53-dependent. The modest, though significant, p21 induction levels in the no LOH tumors are also observed in p53^{+/-} normal tissue, where radiation-induced p21 induction levels are only one-eighth as robust as in the p53^{+/+} normal tissue (S.Venkatachalam and L.Donehower, unpublished data).

A third test of p53 function involved the examination of a target gene known to be transcriptionally down-regulated by p53. Proliferating cell nuclear antigen (PCNA) has been shown to be repressed directly by wild-type p53 (Mercer *et al.*, 1991; Subler *et al.*, 1992). Thus,

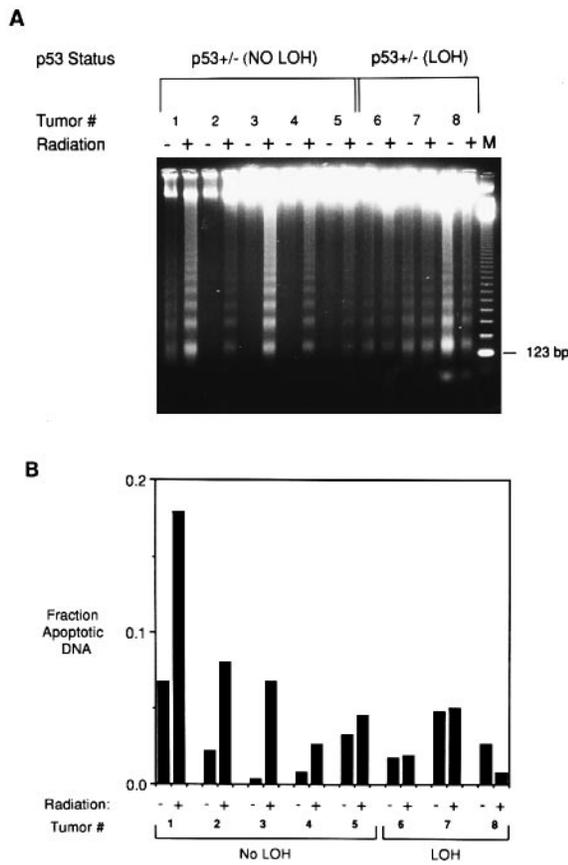


Fig. 4. Radiation-induced apoptosis in p53+/- tumors. **(A)** Apoptotic DNA fractions from p53+/- tumors before and after radiation. Total DNA was isolated from p53+/- tumors before and 24 h after 20 Gy ionizing radiation treatment. The DNA samples were subjected to agarose gel electrophoresis and stained with ethidium bromide. Apoptotic DNA is represented by the low molecular weight bands which form a ladder on the gel. Note the increased intensity of the apoptotic bands in the p53+/- no LOH samples after irradiation. **(B)** Quantitation of apoptotic DNA fractions relative to total DNA in pre- and post-radiation-treated p53+/- tumors. The ethidium bromide-stained gel shown in (A) was blotted to nylon and hybridized to ³²P-labeled mouse genomic DNA. The resulting blots were quantitated for radioactivity using a Molecular Dynamics PhosphorImager. The radioactivity signal in the low molecular weight region of each lane was divided by the signal from the entire lane to derive a value for the percentage of the apoptotic DNA fraction which is represented as a bar on the graph.

if p53 is active in the p53+/- no LOH tumors, PCNA levels should be lower in these tumors compared with LOH tumors. To investigate this possibility, we performed Western blot analysis of lysates from p53+/- tumors irradiated with 20 Gy ionizing radiation 24 h prior to harvest. The results show that p53+/- no LOH tumors tend to have lower levels of PCNA than the p53+/- LOH tumors (Figure 6A). Note that some of the p53+/- no LOH tumors have low but detectable levels of PCNA. This is likely to be due to the long half-life of PCNA (24 h) and the high variability of PCNA levels in the tumors prior to irradiation. However, three of seven p53+/- no LOH tumors had undetectable levels of PCNA, while none of eight p53+/- LOH tumors had undetectable PCNA. Quantitation of the PCNA Western blots by densitometry of seven p53+/- no LOH tumors and eight p53+/- LOH tumors demonstrated an average 3- to 4-fold

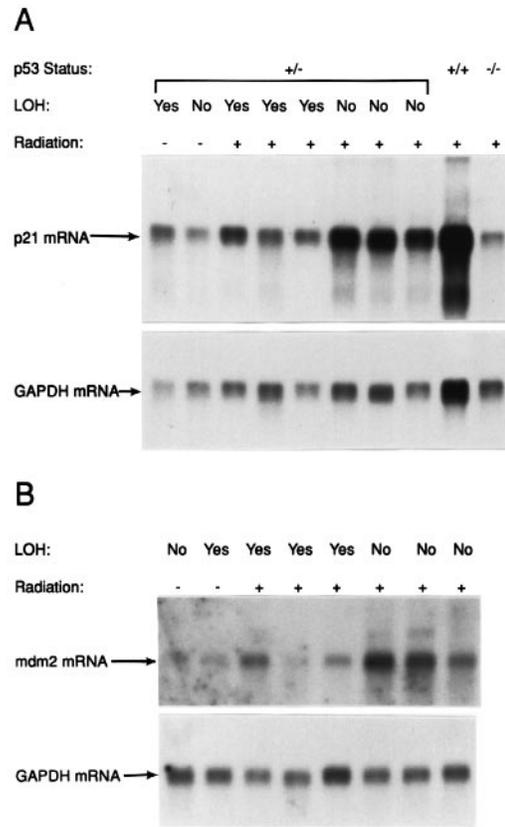


Fig. 5. Comparison of p21^{WAF1/CIP1} and Mdm2 mRNA levels in irradiated p53+/- tumors. Tumors from p53+/- mice were harvested 3 h after 20 Gy ionizing radiation. mRNA was purified from each tumor and subjected to denaturing agarose gel electrophoresis and Northern blot hybridization with p21 and Mdm2 probes. **(A)** Northern blot analysis of p21 mRNA expression in irradiated p53+/- tumors (lanes 3-8), irradiated p53+/+ and p53-/- tumors (lanes 9 and 10) and unirradiated p53+/- no LOH and LOH tumors (lanes 1 and 2). The p21 message is indicated, and subsequent hybridization to a GAPDH probe (to control for variations in loading) is shown in the lower part of the figure. Also indicated at the top of the figure are p53 status, LOH status of p53+/- tumors and radiation status. **(B)** Northern blot analysis of Mdm2 mRNA expression in irradiated and unirradiated p53+/- tumors. The Mdm2 and GAPDH messages are indicated, and the LOH status and radiation status for each lane is shown.

reduction in PCNA levels in the presence of an intact p53 allele (Figure 6B). This difference was significant by *t*-test (*P* = 0.03).

A final test of p53 function in the p53+/- no LOH tumors relied on the ability of wild-type p53 protein to bind specifically to oligonucleotides containing a p53 response element using a standard electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from p53+/- tumors irradiated (20 Gy) 3 h prior to sacrifice. These extracts were incubated with a short oligonucleotide fragment containing either a wild-type consensus p53 response element or a mutated p53 response element sequence previously shown to be incapable of binding wild-type p53. To some of the lysates containing the wild-type p53 response element we added the p53-specific monoclonal antibody, pAb421. The complexes were visualized on a non-denaturing gel, and typical results are shown in Figure 7. Note that the positive control extracts (from irradiated p53+/+ mouse embryonic fibroblasts)

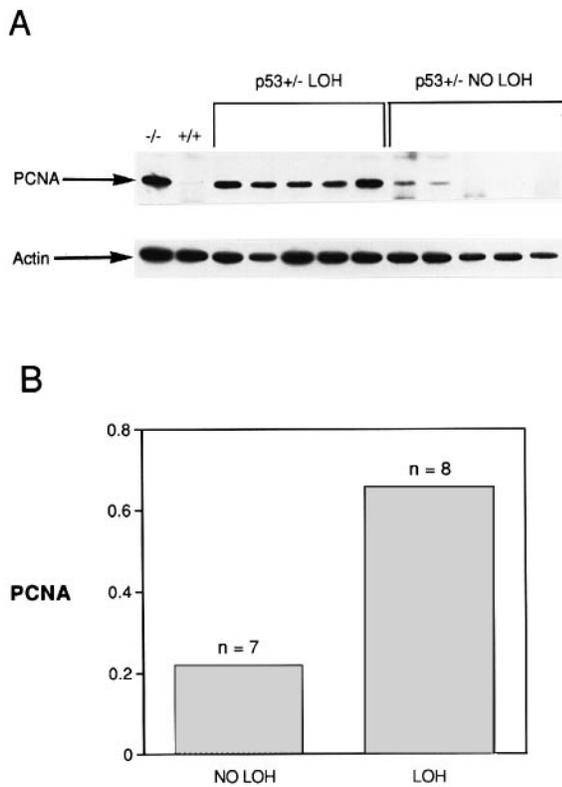


Fig. 6. Comparison of PCNA protein levels in irradiated p53^{+/-} tumors. Tumors from p53^{+/-} mice were harvested 24 h after 20 Gy ionizing radiation. Lysates from p53^{+/-} LOH and no LOH tumors were subjected to SDS-PAGE followed by immunoblotting. PCNA protein was visualized by chemiluminescence protocols as described in Figure 3. **(A)** Western blot analysis of PCNA levels in tumors from p53-deficient and wild-type mice. The first two lanes are controls from irradiated p53^{+/+} and p53^{-/-} tumors. The LOH status of the other 10 p53^{+/-} tumors is indicated in the figure. The bottom panel serves as a loading control showing relative levels of actin in each lysate. **(B)** Graph showing average PCNA levels for seven p53^{+/-} no LOH tumors and eight p53^{+/-} LOH tumors. PCNA levels were quantitated by densitometry analysis of PCNA bands on film, and PCNA levels for each lane were then normalized to the band intensities of actin in that lane.

and the p53^{+/-} no LOH extracts show high levels of binding to wild-type p53 response elements, and not to mutated response elements. In contrast, the p53^{+/-} LOH extracts show very low or undetectable binding to the wild-type p53 response elements. In addition, the p53^{+/-} no LOH extracts show much higher levels of supershifted complexes following incubation with the p53-specific monoclonal antibody compared with the p53^{+/-} LOH extracts. The residual supershift activity observed with the p53^{+/-} LOH extracts is likely to be due to small amounts of contaminating cells in the tumor which did not lose the wild-type allele. These results clearly demonstrate specific wild-type p53 activity in the p53^{+/-} no LOH tumors.

Chromosomal instability

Loss of p53 has been shown to be highly correlated with chromosomal instability *in vitro* and *in vivo* in mouse and human systems (Bischoff *et al.*, 1990; Livingstone *et al.*, 1992; Bouffler *et al.*, 1995; Donehower *et al.*, 1995; Gualberto *et al.*, 1998). To examine the effects of p53 loss or retention on genomic integrity at the chromosomal

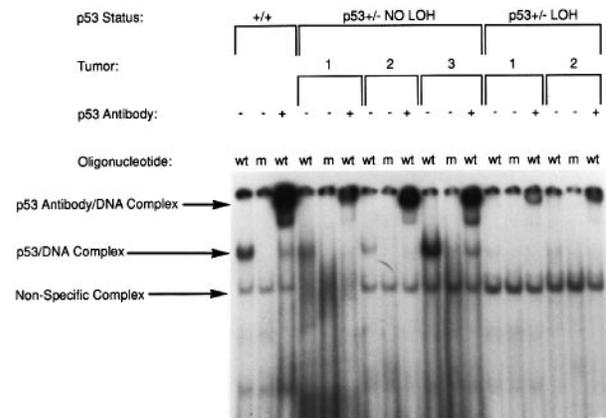


Fig. 7. Specific p53 DNA-binding activity in p53^{+/-} tumors with and without LOH. Nuclear extracts were prepared from p53^{+/-} tumors isolated from mice sacrificed 2–3 h after γ -irradiation. Nuclear extracts (50 μ g) were incubated with short oligonucleotide sequences containing a p53 response element which specifically interacts with wild-type p53 or a mutated version of the response element which does not bind p53. In some cases, a p53-specific monoclonal antibody (pAb421) was incubated with the oligonucleotide. The reaction mixes were electrophoresed on a 5% polyacrylamide gel and complexes visualized following autoradiography. For each tumor, three reactions are shown, including incubation of the extract with wild-type oligos (wt), mutant oligos (m) and wild-type oligos with pAb421 (wt + Ab). The results for three p53^{+/-} no LOH tumors and two p53^{+/-} LOH tumors are shown. The positive control (p53^{+/+}) is from irradiated p53^{+/+} mouse embryo fibroblast extracts.

level and to identify candidate loci which cooperate with p53 loss in tumor progression, we performed comparative genomic hybridization (CGH) analyses on tumor DNAs from p53^{-/-} and p53^{+/-} mice. CGH detects chromosomal regions of increased and decreased DNA copy number throughout the genome of tumor cells (Kallioniemi *et al.*, 1992). The tumor DNAs analyzed were from 24 lymphomas, seven osteosarcomas and four soft tissue sarcomas. The results of the CGH analyses for these 35 tumor DNAs are shown in Table I and Figure 8. The tumors which lacked wild-type p53 (p53^{-/-}) or lost wild-type p53 during tumor progression (p53^{+/-} LOH) showed significantly more regions of copy number variation than p53^{+/-} tumors which retained a wild-type p53 allele, as measured by DNA copy number losses and gains involving whole or partial chromosomes. Summary data on total copy number changes are compared for the lymphomas, osteosarcomas, soft tissue sarcomas and for all of the tumors (Table II). For each tumor type, tumors missing (p53^{-/-}) or losing p53 (p53^{+/-} LOH) consistently showed 5-fold more CGH chromosomal abnormalities than tumors retaining wild-type p53. For the lymphoma, osteosarcoma and all tumor categories, these differences are statistically significant (as measured by *t*-test).

The patterns of chromosomal CGH gains and losses were non-random in nature (Figure 8). In the lymphomas, frequent (at least 25% of tumors) copy number gains were observed in chromosomes 4, 11, 14 and 15. In many cases, entire chromosomes showed copy number gains but, in some cases (e.g. centromere-proximal regions of chromosome 14 in lymphomas), subchromosomal regions were involved. Frequent copy number gains in chromosome 15 (eight of 23 tumors) are consistent with previous observations of trisomy 15 in murine lymphomas (Spira

Table I. CGH copy number gains and losses in p53-deficient tumors

Tumor genotype	Tumor	Tumor type	Modal karyotype	
p53+/- (no LOH)	31	lymphoma	normal	
	37	lymphoma	dec4(A-C); dec5(A-D)	
	354	lymphoma	inc8E	
	470	lymphoma	dec10; inc15	
	967	lymphoma	inc1(C-E); inc11(B-D); inc14(A5-B); inc18B	
	192	osteosarcoma	normal	
	382	osteosarcoma	normal	
	136	soft tissue sarcoma	incY	
	p53+/- (LOH)	23	lymphoma	inc3; inc5; inc11; inc12; dec13(B-D); inc17
		70	lymphoma	inc11A; inc16C
191		lymphoma	dec3; inc5(D-G); inc17; dec18	
347		lymphoma	inc3(A-F); inc6(F-G); inc11A; dec13B2; dec14A	
371		lymphoma	dec2(C-H); inc4(A-E); inc8; inc11; dec12; dec13; inc14(B-C); inc15	
960		lymphoma	dec7; dec10; inc14A; dec16A; dec17(A-D); dec18(A-D)	
1033		lymphoma	inc2(A-B); dec2C; dec12; inc15	
216		osteosarcoma	dec8(C-E); dec11(B-E); dec12(B-F); inc15; inc17B	
272		osteosarcoma	dec8; inc11(C-E); dec12	
346		osteosarcoma	dec7(C-F); dec8(B-E); dec9(B-F); dec12(C-F); dec13(B-D); inc14(A-B); inc17A; dec19D	
637		osteosarcoma	dec9; dec12; inc14(A-B)	
1371		osteosarcoma	inc1; inc3; dec7(C-F); dec10(B-D); dec11(B-E); dec12(C-F); inc14A; inc18A; inc19A	
43		soft tissue sarcoma	inc3; dec8(B-E); inc14A; inc15; inc16; inc17A; incY	
148		soft tissue sarcoma	inc14A; dec16; inc17A; dec18(D-E)	
p53-/-		121	lymphoma	dec3; inc4; dec6; dec10; dec13; inc14; inc15; dec16; inc17; dec18; decX; incY
		247	lymphoma	dec6; dec9(B-F)
	278	lymphoma	inc4(D-E); dec13; dec16A; incY	
	301	lymphoma	dec10; inc15	
	341	lymphoma	dec1(A-G); inc5; inc11; inc14; inc15	
	375	lymphoma	dec3; inc4; dec5; dec6; dec9; dec13; inc14; dec15; dec16; dec19	
	417	lymphoma	inc11(C-E); inc14(A-B); dec17E; dec19(C-D)	
	448	lymphoma	inc4; inc5; inc11; inc14; dec17(C-D)	
	509	lymphoma	inc2(A-B); inc3; inc6; dec9(E-F); inc10; inc11A; inc15; dec17E; dec18E	
	510	lymphoma	inc4; inc5; inc14A; dec16(A-B4); dec18(B-E)	
	538	lymphoma	inc6; inc14(A-B); incY	
	908	lymphoma	dec3; inc4; dec6; dec9; inc14; inc15; dec17(A-C)	
	904	soft tissue sarcoma	inc3; dec9(B-F); dec11(A-B); decX; incY	

Tumor DNAs were analyzed by comparative genomic hybridization. Chromosomal abnormalities are indicated 'dec' for DNA copy number decreases and 'inc' for DNA copy number increases, followed by the mouse chromosome number affected. Subchromosomal regions are indicated in parentheses by uppercase letters. Each letter represents a band visible by DAPI staining. A is the band closest to the centromere.

et al., 1979). Frequent copy number losses in the lymphomas were observed on chromosome 13. Among the p53+/- osteosarcomas, five of five p53+/- LOH tumors exhibited copy number losses of chromosome 12, suggesting that inactivation of one or more loci on this chromosome has an important contributory effect on osteosarcoma progression. Surprisingly, only two of 14 p53+/- (LOH) tumors showed copy number decreases in chromosome 11, the site of the p53 gene. This suggests that the deletion of the remaining p53 allele in most of these tumors was on a scale too small to be detected by CGH. The 5-fold average reduction in chromosomal instability in p53+/- tumors without LOH is consistent with the presence of a functional wild-type p53 allele.

Discussion

We have shown that mice with a single null p53 allele have a much higher tumor incidence than mice with two functional copies of p53 and often retain the remaining wild-type allele during tumorigenesis. Moreover, the retained wild-type p53 allele appears structurally and functionally intact. The evidence for intact p53 structure and function rests primarily on seven observations: (i) sequencing of p53 transcripts in two p53+/- no LOH tumors reveals a structurally intact coding region;

(ii) intact p53 protein is observed in the p53+/- no LOH tumors; (iii) radiation increases apoptosis in p53+/- no LOH tumors but not in p53+/- LOH tumors; (iv) p21^{WAF1/CIP1} and *mdm2* are transcriptionally up-regulated following radiation in p53+/- no LOH tumors, but not in p53+/- LOH tumors; (v) PCNA expression, a target of p53-mediated transcriptional repression, is significantly reduced in p53+/- no LOH tumors compared with p53+/- LOH tumors following radiation; (vi) extracts from p53+/- no LOH tumors show much higher levels of binding activity to a p53 response element than do extracts from p53+/- LOH tumors; and (vii) chromosomal abnormalities are 5-fold less frequent on average in p53+/- no LOH tumors than in p53+/- LOH tumors and p53-/- tumors. Taken together, we believe that these data provide a strong argument that loss of both p53 alleles is not a prerequisite for accelerated tumor formation.

Our observations suggest that p53 may be an exception to the current 'two-hit' model for inherited tumor suppressor genes. In the two-hit model, loss of function or mutational activation of both relevant tumor suppressor alleles is a prerequisite for tumor formation (Knudson, 1971, 1985; Cavenee *et al.*, 1985). The two-hit model is highly consistent with the observation that the remaining wild-type allele is invariably lost or mutated in inherited childhood retinoblastomas and pituitary tumors from

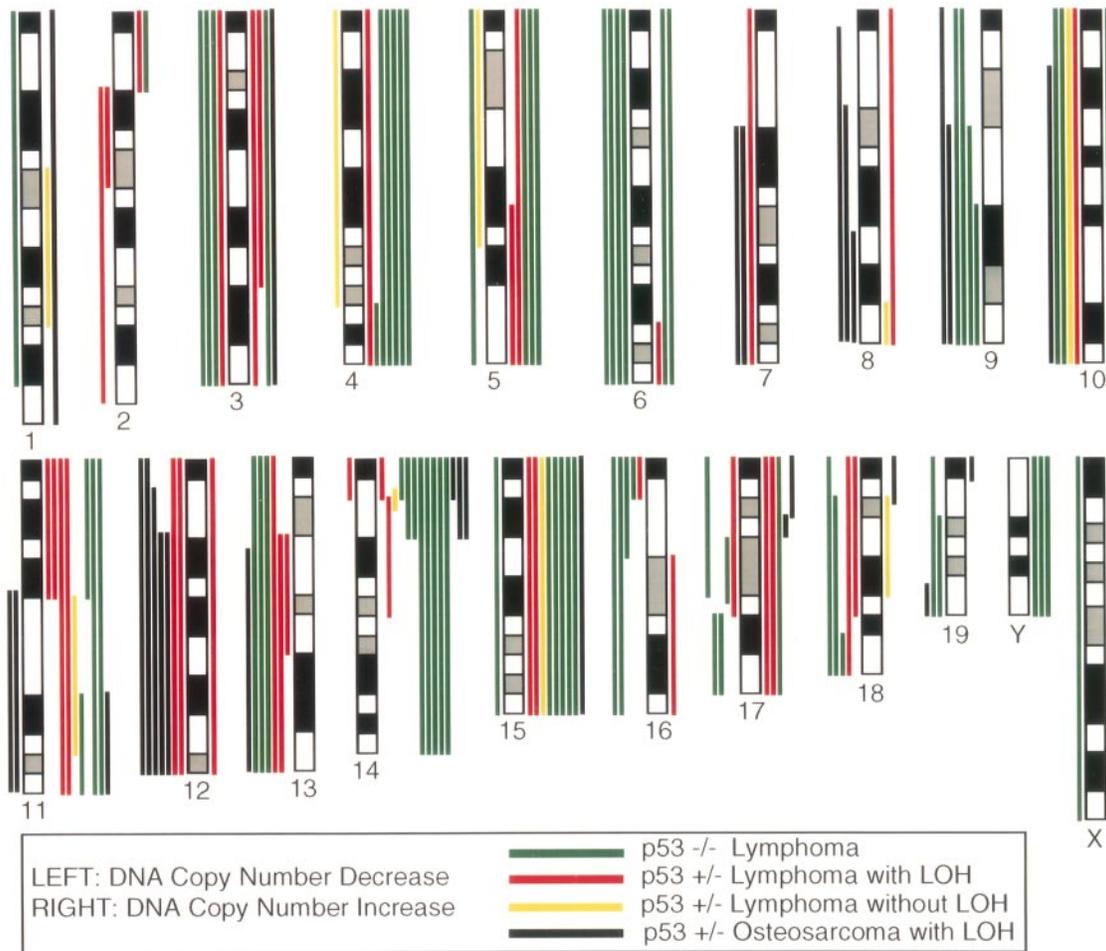


Fig. 8. Chromosomal localization of DNA sequence copy number changes in 24 lymphomas and seven osteosarcomas from p53-deficient mice. Individual bars along each chromosome represent the regions of copy number gains and losses for a single tumor. Bars representing DNA copy number gains are on the right of the chromosome, while bars to the left of a chromosome indicate DNA copy number losses. Each bar color represents a particular tumor type and p53 status as indicated in the box. The chromosomal band locations indicate the observed DAPI staining patterns.

Table II. Comparison of CGH abnormalities in p53-deficient mouse tumors

	Average CGH abnormalities per tumor		
	p53+/- (no LOH)	p53+/- (LOH)	p53-/-
Lymphoma	1.8 (n = 5)	5.0 (n = 7)	5.7 (n = 12)
Osteosarcoma	0.0 (n = 2)	5.6 (n = 5)	ND
Soft tissue sarcoma	1.0 (n = 1)	5.5 (n = 2)	5.0 (n = 1)
All tumors	1.0 (n = 8)	5.3 (n = 14)	5.6 (n = 13)

The data from Table I were compiled and the average number of CGH abnormalities for each tumor type and each of three p53 genotypes was calculated. Numbers in parentheses indicate the number of tumors examined for each category. ND, not done.

Rb+/- mice (Figure 9). However, in our revised model for p53, inheritance of only one intact wild-type p53 allele is sufficient to promote early tumors. Given that p53+/- animals do not usually develop tumors before 9 months of age, other cooperating oncogenic lesions are postulated to be required for tumor formation. Such oncogenic lesions may arise more readily in cells with half the normal p53 dosage. During tumor progression, the remaining wild-type p53 allele may or may not be lost (Figure 9). If it is

lost early enough, the clone with the loss is likely to progress faster and become the dominant clone in the tumor at the time of analysis; hence the observation that a higher fraction of the early arising tumors exhibit p53 LOH. Since chromosomal instability correlated with p53 LOH, this may play a role in the accelerated appearance of the p53+/- LOH tumors.

How could p53 display this apparent gene dosage effect when other tumor suppressors do not? We and others have noted intermediate dosage effects in p53+/- cells *in vitro* with respect to a number of biological properties, including cell proliferation and induction of apoptosis (Harvey *et al.*, 1993b; Clarke *et al.*, 1994). One possible reason is that p53 appears to function primarily as a tetramer (Friedman *et al.*, 1993). If the level of total p53 protein is halved in a cell due to allele loss, then the effective concentration of tetramers may be more than halved, depending on the affinity of the p53 monomers for each other. Thus, the level of functional p53 may actually be reduced by >50% in a heterozygous state. Supporting evidence for this high dependence of function on concentration has been provided by Oren and colleagues (Gottlieb *et al.*, 1997), who showed that mice heterozygous for p53 are only marginally more effective in transcriptionally activating a marker

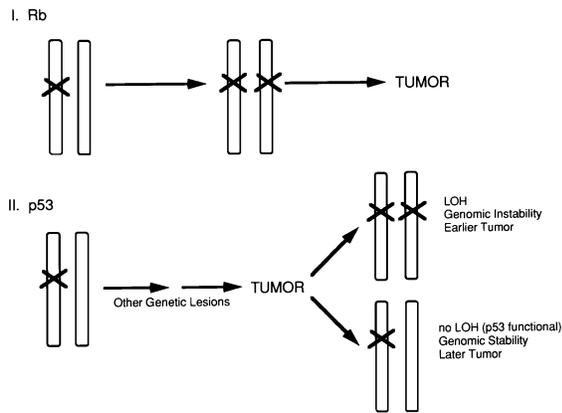


Fig. 9. Models for the effects of inherited tumor suppressor gene mutations on tumorigenesis. The upper model shows a simplified version of the classic 'two-hit' hypothesis which fits the inheritance of a defective Rb allele. In this model, loss of function in both tumor suppressor alleles is a prerequisite for tumor formation. The lower model is a modified version of the two-hit model which hypothesizes that the inheritance of a single defective p53 allele promotes cancer formation. This model presumes that a reduction in p53 gene dosage is sufficient to provide a cellular environment more conducive to the subsequent development of further oncogenic lesions and the initiation of a tumor. During tumor progression, the remaining p53 allele may be lost early. These cells would have a further growth advantage and exhibit increased genomic instability. Such cells would soon become the dominant clone in the growing tumor mass and would be classified as p53^{+/-} LOH. Those tumors which retain a functional wild-type p53 allele would show decreased growth rates, exhibit fewer chromosomal abnormalities and arise later in the mouse life span.

gene with a p53 response element than p53^{-/-} mice and are much reduced in this activity compared with corresponding wild-type mice.

How do these observations impact on the role of p53 mutation in human inherited and sporadic tumors? There are observations in Li-Fraumeni tumors that are consistent with our revised version of the model. One recent comprehensive study of tumors from Li-Fraumeni patients reveals that over half of all tumors show retention of the wild-type p53 allele by restriction fragment length polymorphism (RFLP) mapping (Varley *et al.*, 1997). This result could be explained by point mutations in the presumptive wild-type allele, but it is also consistent with retention of wild-type p53.

These results may also have implications for the role of p53 mutation in sporadically arising tumors. Since the most frequent pattern of p53 lesions has been a p53 point mutation accompanied by complete loss of the remaining allele, the two-hit model may apply well to most human sporadic tumors. However, numerous examples have been noted where one allele has a point mutation and the other appears to remain intact, or one allele has been deleted while the other is intact (Nigro *et al.*, 1989; Mulligan *et al.*, 1990; Davidoff *et al.*, 1991; Saylor *et al.*, 1991; Frankel *et al.*, 1992; Mazars *et al.*, 1992). These exceptions to the two-hit rule have been explained as possibly a result of p53 mutations outside the usual sequenced regions (exons 5–8), or a result of mutations in other closely linked tumor suppressors, or due to a non-specific allelic deletion (Nigro *et al.*, 1989). At least one group, however, has entertained the possibility that 50% dosage of p53 confers a selective growth advantage to the cell (Coles *et al.*, 1992). We propose that while a point mutation of

a single p53 allele may indeed provide a growth advantage through a dominant-negative or gain-of-function effect, this is likely to be in conjunction with a gene dosage effect resulting from the reduction of total wild-type p53 protein in the cell.

Materials and methods

Mice

The p53-deficient mice were generated by gene targeting methods as previously described (Donehower *et al.*, 1992). The mice used in this study were of mixed C57BL/6 (87.5%) \times 129/Sv (12.5%) genetic background. p53 gene status of each individual mouse was determined by standard Southern blot hybridization analysis of tail DNA as previously described (Donehower *et al.*, 1992). Tumors were harvested from the p53-deficient mice, samples taken for histopathological identification and the remainder frozen at -70°C for subsequent DNA, RNA and protein analyses.

Southern blot and DNA sequencing analyses

Analysis of wild-type p53 allele loss or retention in the p53^{+/-} tumors was performed as for genotyping. Briefly, 5–10 μg of BamHI-cleaved genomic DNA was subjected to agarose gel electrophoresis (0.7%), blotted to nylon and hybridized to a murine p53 cDNA probe (exons 2–6). Autoradiographs were performed and the blots were examined on a Molecular Dynamics PhosphorImager to compare the signal ratio of wild-type p53 bands (migrating at 5.0 kb) with the mutant allele bands (migrating at 6.5 kb). Wild-type/mutant ratios of 60–100% were considered 'no LOH', ratios of 30–60% were considered 'partial LOH' and ratios of 0–30% were considered 'LOH'. The vast majority of tumors fell into the 'LOH' or 'no LOH' categories. Assessment of *mdm2* copy number by Southern blot analysis was essentially as described previously (Jones *et al.*, 1995).

Total RNA was also purified from small pieces of representative p53^{+/-} (no LOH) tumors using the Clontech Micro-Scale Total RNA Separator Kit according to the manufacturer's specifications. RT-PCR with murine p53-specific primer sets was used to amplify the complete coding region of the p53 gene in two of the tumors. The Perkin Elmer RNA PCR kit was employed for these amplifications. Amplification primers used were: exon 1 primer (5'-CAGTTCATTGGGACCATCCT-3') and exon 11 primer (5'-AGGATTGTGTCTCAGCCCTG-3'). Amplified PCR fragments were cloned into the PKS BlueScript II vector (Stratagene) and sequenced with a US Biochemical kit according to the manufacturer's specifications. Sequencing primers were: universal T3 primer (5'-AATTAACCCTCACTAAAGGG-3'), universal T7 primer (5'-GTAATACCGACTCACTATAGGGC-3'), exon 5 primer (5'-GTC-CCAGCTGGAGGTGTG-3'), exon 8 primer (5'-CTTCTGTACGGCGG-TCTCTC-3') and exon 10 primer (5'-CTCCGGAACATCTCGAAGC-3'). Three independent cDNAs were sequenced for each tumor to greatly reduce the likelihood of errors derived from the RT-PCR procedure.

Messenger RNA analyses

Northern blot analysis was performed essentially as described earlier (Sambrook *et al.*, 1988). Poly(A) RNA was isolated from ~500 mg of tumor tissue using the Fast Track 2.0 kit (Invitrogen), and 2 μg of mRNA was separated on a 1.2% agarose-formaldehyde gel. The separated mRNA samples were transferred to nitrocellulose membranes, pre-hybridized, hybridized and washed as described (Sambrook *et al.*, 1988). The p21, Mdm2 and GAPDH mRNAs were detected by sequential hybridization of the membranes with the respective ^{32}P -labeled cDNA fragments generated by a commercial oligo labeling procedure (High Prime Oligo labelling kit, Boehringer Mannheim). Following hybridization and washing, the membranes were exposed to X-OMAT AR5 film (Kodak) for 24–48 h and the bands were quantitated by densitometric scanning.

Protein analyses

Prior to immunoprecipitation and immunoblot analyses, tumor segments were thawed, minced and homogenized in lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl, pH 7.5, 40 $\mu\text{g}/\text{ml}$ aprotinin, 20 $\mu\text{g}/\text{ml}$ leupeptin and 5 $\mu\text{g}/\text{ml}$ pepstatin) using a Polytron homogenization device. Lysates were incubated at 4°C for 30 min and centrifuged at 14 000 r.p.m. in an Eppendorf microcentrifuge for 20 min. The supernatant was removed and stored at -80°C .

Immunoprecipitations for p53 protein were performed according to

established protocols (Sambrook *et al.*, 1988) using 2.5 mg of total protein from the tumor lysates. Protein concentrations were measured with the Bio-Rad protein assay kit. Briefly, the protein samples were pre-cleared for 60 min at 4°C with 10 µl packed volume of protein A-agarose. The lysates were then centrifuged and the supernatant was incubated with 10 µl packed volume of protein G-agarose. The lysates were then centrifuged and the supernatant was incubated with 10 µl packed volume of protein G plus protein A-agarose coated with a mixture of anti-p53 monoclonal antibodies (p53 Ab1 and Ab5, Oncogene Science, Inc, and p53 Ab4 and Ab7, Neomarkers, Inc.) for 3 h at 4°C on a rotating wheel. Samples were then centrifuged at 14 000 r.p.m. in a microcentrifuge and the pellets were washed sequentially with NET gel buffer (50 mM Tris-HCl pH 7.5, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 40 µg/ml aprotinin, 20 µg/ml leupeptin and 5 µg/ml pepstatin) containing increasing amounts of NaCl (150 and 500 mM) followed by a final wash with 10 mM Tris-HCl pH 7.5, 0.1% NP-40. The protein A-agarose beads were then resuspended in 1× loading buffer [2% SDS, 10% glycerol, 50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol (DTT) and 0.05% bromophenol blue], heated at 95°C for 5 min, electrophoresed in a 10% SDS-polyacrylamide gel, and transferred to immobilon-P membrane (Millipore) using an electroblotter. For the positive control lane, we used 100 µg of lysate from a tumor with a mutant p53 transgene (Lavigneur *et al.*, 1989), skipping the immunoprecipitation step.

For p53 protein detection, the immobilon-P membrane was blocked with 5% milk powder (Carnation) in TBST buffer (10 mM Tris pH 7.5, 150 mM NaCl and 0.05% Tween) overnight and then incubated with anti-p53 polyclonal antibody (anti-p53 Ab7, Calbiochem) at 1:3000 dilution (2% milk powder in TBST) for 3 h at room temperature. The membrane was incubated further with the secondary antibody reagent (rabbit anti-sheep peroxidase-conjugated antibody) at 1:2000 dilution for 2 h at room temperature. Following each antibody incubation, the membrane was washed thoroughly with TBST buffer. The p53 protein bands were visualized using the enhanced chemiluminescence substrate reaction (Pierce) according to the manufacturer's instructions using X-OMAT AR5 film (Kodak).

For PCNA detection, similar amounts (30 µg) of protein lysates from tumors were electrophoresed on a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and blocked overnight with 5% milk powder in Tris-buffered saline. The membranes were incubated with anti-PCNA (Calbiochem) and anti-actin (Boehringer Mannheim) monoclonal antibodies at a 1:1000 dilution for 2 h followed by secondary antibody reagent (goat anti-mouse-peroxidase conjugate) at 1:3000 dilution for an additional 2 h. The specific protein bands were detected with enhanced chemiluminescence substrate as described earlier. Quantitation of PCNA and actin band intensities was performed at several different exposures using a Molecular Dynamics Densitometer. The numbers obtained for each PCNA band (after subtracting background) were divided by the corresponding actin value for that lane to obtain relative PCNA levels for each tumor.

Apoptosis assays

p53[±] mice were monitored for tumors. When an overt tumor was observed on the surface of an animal, the animal was anesthetized by intraperitoneal injection with 0.1 ml of Combination Anesthetic III (37.5 mg/ml ketamine, 1.9 mg/ml xylazine, 0.37 mg/ml acepromazine). A small biopsy specimen of the tumor was then surgically removed from the tumor. The animal was then whole-body irradiated with 20 Gy ionizing radiation. Twenty four hours after irradiation, the mouse was sacrificed and the tumor immediately removed and frozen at -80°C. Total DNA was prepared from the tumor segments by mincing a piece of tumor tissue with a razor followed by overnight incubation of the minced tissue in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM EDTA, 100 mM sodium chloride, 1% SDS, 5 mM DTT and 100 µg/ml proteinase K) at 55°C. The lysate was then extracted twice with phenol-chloroform and precipitated in two volumes of 100% ethanol. The DNA was then centrifuged in an Eppendorf microcentrifuge at 14 000 r.p.m. for 30 min. The precipitated DNA was then resuspended in 100–400 µl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE). DNA concentrations were determined by spectrophotometry.

For DNA fragmentation assays, ~10 µg of total DNA from each tumor sample was subjected to electrophoresis on a 1.5% agarose gel. Following electrophoresis, DNA was visualized by staining in ~1 µg/ml ethidium bromide and exposure to UV light. The DNA gels were photographed, then blotted to nylon according to published procedures. To generate a probe for the apoptotic DNA, total mouse genomic DNA was prepared by *AluI* and *HaeIII* cleavage of high molecular weight mouse DNA. The cleaved DNA was extracted with phenol-chloroform,

ethanol precipitated and resuspended in TE. Total mouse DNA (100–500 ng) was ³²P labeled with the High Prime oligo labeling kit (Boehringer Mannheim). The probe was hybridized to the filter as previously described (Donehower *et al.*, 1992). Following washing of the blot and autoradiography, the blot was examined on the Molecular Dynamics PhosphorImager. The fraction of apoptotic DNA in each lane was measured by dividing the counts in the low molecular weight portion of the gel (<1700 bp) by the total number of counts in the lane.

Electrophoretic mobility shift assays

EMSAs were performed essentially as described earlier, with minor modifications (Funk *et al.*, 1992). Tumor tissues from irradiated mice were homogenized in lysis buffer [20 mM HEPES (pH 7.6), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 15 µg/ml aprotinin] and pelleted at 3000 r.p.m. at 4°C. Tissue pellets were resuspended in nuclear extraction buffer (lysis buffer with 500 mM NaCl), incubated at 4°C for 1 h on a rocking platform and centrifuged at 13 000 r.p.m. for 10 min. The supernatant was aliquoted and stored at -80°C until use. The p53 consensus and mutant double-stranded oligonucleotides (Santa Cruz Biotechnology) were end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase.

For EMSA, 10 µl of nuclear extract (25 or 50 µg of protein) was mixed with an equal volume of binding buffer (40 mM HEPES, 3 mM MgCl₂, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 15 µg/ml aprotinin, 1 µg of sonicated salmon sperm DNA) containing 0.1–0.3 ng of ³²P-labeled probe (100 000 c.p.m.) and incubated for 30 min at room temperature. After incubation, 0.8 µl of 0.05% bromophenol blue was added and loaded onto 5% acrylamide gels containing 89 mM Tris, 89 mM boric acid, 2 mM EDTA and electrophoresed at 150 V for 2–3 h at room temperature. For antibody supershift analysis, 2 µl of anti-p53 antibody (Ab-1, Oncogene Science) was added to the reaction and incubated for an additional 20 min before electrophoresis.

Comparative genomic hybridization

Metaphase chromosome spreads were prepared from C57BL normal mouse fibroblasts (graciously provided by Maria Pallavicini) as previously described (Kallioniemi *et al.*, 1992). The newly prepared slides were kept at room temperature for 2–3 weeks and then stored at -20°C in nitrogen for 2 months before using. Genomic DNAs from the tumors and normal tissue were isolated and labeled with biotin and digoxigenin, respectively, by nick translation (Kallioniemi *et al.*, 1992, 1994). Then 120 ng of each of the labeled probes with the optimal size of 600–1000 bp and 40 µg of unlabeled mouse C₆t-1 DNA (Gibco-BRL) were co-precipitated with ethanol and redissolved in 10 µl of hybridization buffer [50% (v/v) formamide/10% (w/v) dextran sulfate/2× SSC], denatured at 70°C for 5 min, dehydrated in a sequence of ethanol solutions and then air dried. The hybridization mixture was applied to slides and hybridized for 4 days at 37°C. After hybridization, the slides were washed according to the described protocols and stained with avidin-fluorescein isothiocyanate (FITC) (to visualize bound biotinylated probes with green fluorescence) at 5 µg/ml and anti-digoxigenin-rhodamine at 2 µg/ml (to visualize bound digoxigenin-labeled probes with red fluorescence). Samples were counterstained with 0.1 µM 4,6'-diamino-2-phenylindole (DAPI) in an anti-fade solution.

Image analysis

The hybridization reactions were analyzed with a Quantitative Image Processing System (QUIPS) which is based on a Zeiss Axioplan fluorescence microscope as described elsewhere (Kallioniemi *et al.*, 1992; Piper *et al.*, 1995). Separate images of the DAPI, fluorescein (green) and rhodamine (red) were obtained. Morphological boundaries of the chromosomes were determined by thresholding, and the fluorescence intensities from the genomic probes were normalized so that the overall green-red ratio for the entire metaphase was set at 1.0. The chromosomes were identified interactively by the analyst using the banding pattern from a contrast-enhanced DAPI image. Background fluorescence in the vicinity of a chromosome in the green and red images was determined and subtracted from the intensities of the corresponding chromosome image. Green and red fluorescence intensity profiles were determined by integrating fluorescence across the width of the chromosome at ~0.1 µm steps along the medial axis. Fluorescence intensity ratio profiles were then calculated. Three to five metaphases from each hybridization were analyzed. Regions of significant deviation in ratio were determined from inspection of the ratio profiles and the digital images. Criteria were established based on the noise in the profiles and the range of deviations seen in normal/normal control hybridizations.

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