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

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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
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/6×129/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.

Fig. 1. Tumor incidence and survival in 41 p53+/+, 217 p53+/– and 72 p53–/– mice. All mice were of mixed inbred C57BL/6×129/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.

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.
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 overexpressing mutant p53 (Lavigueur 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 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)
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 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+/− 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...
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 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 childhood retinoblastomas and pituitary tumors from 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 childhood retinoblastomas and pituitary tumors from...
p53 dosage reduction and tumor formation

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

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<th>Average CGH abnormalities per tumor</th>
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<tr>
<td></td>
<td>p53+/– (no LOH)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1.8 (n = 5)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>0.0 (n = 2)</td>
</tr>
<tr>
<td>Soft tissue sarcoma</td>
<td>1.0 (n = 1)</td>
</tr>
<tr>
<td>All tumors</td>
<td>1.0 (n = 8)</td>
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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.
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; Saylors 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 as 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%) × 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’-CGTCTGACGGACCACATCCT-3’) and exon 11 primer (5’-AGGATGGTGTCAGCGCTCCTG-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’-GATAATACGACTCACTATAGGG-3’), exon 5 primer (5’-GTCGCCAGCTTGGAGGTTC-3’), exon 8 primer (5’-CTTCTTGATGCGCGG-3’), exon 9 primer (5’-CTCTGGACAGCGGAGGTTG-3’), and exon 10 primer (5’-GTCCTCGAGGATTGTGTCTCAGCCCTG-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 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 32P-labeled cDNA fragments generated by a commercial oligo labeling procedure (High Prime Oligo labeling 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 minced, and homogenized in lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris–HCl, pH 7.5, 7.5 μg/ml aprotinin, 20 μg/ml leupeptin and 5 μg/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–agarose coated with a mixture of anti-p53 monoclonal antibodies (p53 Ab1 and Ab5; Oncogene Science, Inc., and pAb 4 and Ab7, Neogenov, 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 (Lavigueur 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 washed further with TBST and incubated with (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 reconstituted system (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 and then washed according to the described protocols and stained with appropriate antibodies (rabbit anti-sheep peroxidase-conjugated antibody) at 1:2000 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 left side 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 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 (Doneddat 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**

EMSA’s 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, 15 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 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 to the labeled DNA solution and loaded onto 5% polyacrylamide gel containing 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 C57BL 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/2x SSC], denatured at 70°C for 5 min, at 2× SSC and air-dried for 20 min. The probe was then hybridized on metaphase spreads and then air dried. The hybridization mixture was overlaid with 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–labeled (red fluorescence) at 2 μg/ml (to visualize bound digoxigenin-labeled probes with red fluorescence). Samples were counterstained with 0.1 μM 4,6-diamino-2-phenylinode (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/control hybridizations.
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References

p53 dosage reduction and tumor formation


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