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Germ Cell Tumors of the Testis Overexpress Wild-Type p53

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Several recent studies have suggested that testicular germ cell tumors express high levels of wild-type p53 protein. To clarify and confirm this unexpected result, we have investigated seminomatous and nonseminomatous germ cell tumors at the genomic, mRNA, and protein levels. Thirty-five tumors were examined for p53 overexpression using antibodies directed against the p53 (PAb1801, PAb240, and CM1), mdm2 (IF2), and p21$^{Waf1/Cip1}$ (EA10) proteins. Thirty-two tumors were screened for p53 mutations by single-strand conformation polymorphism analysis. Eighteen tumors were screened with a functional assay that tests the transcriptional competence of human p53 protein expressed in yeast. On frozen sections, 100, 65, 35, 73, and 0% of tumors reacted with the CM1, PAb240, PAb1801, IF2, and EA10 antibodies, respectively. No p53 mutations were detected by single-strand conformation polymorphism or by functional assay. The fact that many tumors overexpress wild-type p53 but not mdm2 rules out mdm2 overexpression as a general explanation for the presence of wild-type p53 in these tumors. The absence of p21 overexpression suggests that p53 may be unable to activate transcription of critical target genes, which may explain why the presence of wild-type p53 is tolerated in this tumor type, although the mechanism for this transcriptional inactivity remains to be established. (Am J Pathol 1996, 149:1221–1228)

The p53 protein is a transcription factor that induces cell cycle arrest and apoptosis in response to DNA damage.[1,2] It is inactivated in a large variety of human tumors by mutations in the DNA-binding domain that reduce its affinity for DNA.[3,4] Several studies have shown that tumors with p53 mutations are more aggressive than those without p53 mutations.[4]

Mutant p53 protein accumulates to high levels in many tumors because of metabolic stabilization,[5] which renders it immunohistochemically detectable using many different anti-p53 antibodies.[6–8] Testicular germ cell tumors are unusual in that they overexpress p53 protein[9–14] but apparently do not contain p53 mutations.[12,13,15–18] Overexpression of wild-type p53 in tumors is sufficiently unusual for it to warrant additional investigation. In particular, it is important to rule out the possibility that rare or unusual mutations may have been systematically missed by standard DNA sequence- and structure-based techniques.

We recently developed a conceptually different approach to the detection of p53 mutations, functional analysis of p53 protein expressed in yeast.[19] The basis of this approach is that tumorigenic p53 mutants are unable to bind DNA and activate transcription. The functional assay provides a powerful independent way to test for the presence of p53 mutations. Accordingly, we have examined a series of testicular germ cell tumors using conventional and functional techniques.

We show that testicular germ cell tumors consistently overexpress p53 protein that is wild type by both single-strand conformation polymorphism (SSCP) and functional criteria. We further demonstrate that, although some tumors overexpress the p53 inhibitor protein mdm2, this cannot be a general explanation for the retention of wild-type p53 be-

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cause many tumors overexpress p53 but not mdm2. Finally, we show that p21, a p53 target gene implicated in p53-dependent cell cycle arrest,[20] is not overexpressed in these tumors, which may partly explain why the presence of wild-type p53 is tolerated in this tumor type.

Materials and Methods

Patients

Thirty-five patients with seminomatous and nonseminomatous germ cell tumors of the testis were investigated. No treatment was given before tumor resection. The age of the patients ranged from 2 to 62 (mean, 33) years. Frozen sections of six testes removed for the following conditions were used as negative controls: hormonal deprivation in prostate cancer (four cases), orchitis (one case), and penile cancer (one case).

Morphology

Tumors were classified according to the World Health Organization International Histological Classification of tumors. Intratubular germ cell neoplasia, unclassified type, was diagnosed on morphological criteria[21] and, in doubtful cases, by staining with an anti-placental alkaline phosphatase antibody.[22]

Immunohistology

All tumors were studied using frozen tissues. Sections were examined using the avidin-biotin (ABC) technique.[23] The methodology used has been previously detailed.[24] Counterstaining of nuclei with hematoxylin was omitted when necessary to obtain unequivocal results.

Slides were reviewed independently by two pathologists (L. Guillou and P. Chaubert). A tumor was scored as positive if unequivocal nuclear staining was observed, regardless of the number of positive nuclei and the intensity of nuclear staining. Staining varied in its intensity and distribution from case to case and from one area to another in a given tumor. Therefore, areas rich in stained nuclei were selected and the percentage of positively stained nuclei was assessed in 10 contiguous high-power fields (high-power field surface area = 0.174 mm^2).

Antibodies

All of the antibodies used recognize both wild-type and mutant p53 protein in tissue sections. Although PAb240 does not recognize wild-type p53 in protein extracts, it recognizes all forms of p53 after denaturation.[25]

PAb1801 (monoclonal; Cambridge Research Biochemicals, Northwick, UK),[26] which recognizes an epitope between p53 amino acids 46 and 55,[8] was diluted 1/4000.

PAb240 (monoclonal; Oncogene Science, Manhasset, NY),[25] which recognizes an epitope between p53 amino acids 213 and 217,[27] was diluted 1/400.

CM1 (polyclonal; Novocastra Laboratories, Newcastle upon Tyne, UK),[28] which recognizes predominantly epitopes at the p53 amino terminus,[8] was diluted 1/2000.

IF2 (monoclonal; Oncogene Science, Uniondale, NY), which recognizes an amino-terminal epitope of the mdm2 protein, was diluted 1/10.

EA10 (monoclonal; Oncogene Science), which is directed against the $p21^{Waf1/Cip1}$ protein, was diluted 1/50.

Detection of p53 Mutations

For each sample, the ratio of tumor to normal tissue was assessed by light microscopy of 1000 cells on hematoxylin and eosin (H&E)-stained sections (see Table 1). Samples containing almost exclusively neoplastic tissue were tested directly for p53 mutations. Samples containing a mixture of normal and tumor tissue (cases 5, 8, 9, 12, 18, 19, and 35) were microdissected to remove the normal tissue before testing for p53 mutations.

SSCP Analysis

Genomic DNA was extracted from frozen biopsies by digestion with proteinase K, extraction with phenol/chloroform, and precipitation with ethanol.[29] Exons 5 to 9 of the p53 gene were amplified separately by polymerase chain reaction using the primers and conditions previously described.[7] SSCP was performed as previously described[30] using 30% (exon 5) or 40% (exons 6 to 9) MDE gels (AT Biochem, Malvern, PA), and DNA was visualized by silver staining. Two testes removed for nonmalignant conditions were processed identically and used as negative controls.

p53 Functional Assay

Frozen samples of 18 tumors were tested by functional assay.[19] Total RNA was purified from 6 tumors using acidified phenol (Trizol, BRL, Gaithersburg, MD) and reverse transcribed with Superscript II (BRL) and random hexamers (Pharmacia, Uppsala, Sweden). mRNA was purified from 12 tumors using

Table 1. *p53 and mdm2 Immunostaining and p53 Functional Analysis in Testicular Germ Cell Tumors (Frozen Tissue)*

Patient	Histology	Tumor cell ratio*	p53/PAb240†	p53/CM1†	mdm2/IF2†	p53 functional assay‡
1	Embryonal carcinoma/teratoma	40	−/−	35/5	−/−	Not done
2	Embryonal carcinoma	35	−	25	−	14 ± 4 SD§
3	Embryonal carcinoma/teratoma	−	1/1	30/5	30/5	Not done
4	Seminoma	40	20	40	−	8§
5	Seminoma	30 (m)	70	40	70	Not done
6	Seminoma	53	80	70	80	4§
7	Seminoma	40	100	55	45	3§
8	Seminoma	38 (m)	70	60	−	Not done
9	Embryonal carcinoma	49 (m)	−	80	15	Not done
10	Seminoma	85	−	25	−	3§
11	ITGCN	−	+	+	−	Not done
12	Seminoma/ITGCN	68 (m)	20/+	90/+	2/+	Not done
13	Seminoma	−	90	70	10	Not done
14	Yolk-sac tumor	54	−	5	−	Not done
15	Yolk-sac tumor/teratoma	48	−/−	70/70	1/1	3§
16	Seminoma	80	−	10	15	4§
17	Yolk-sac tumor	35	−	10	5	Not done
18	Embryonal carcinoma/ITGCN	76 (m)	40/+	80/+	40/+	5‖
19	Embryonal carcinoma/teratoma	87 (m)	−/−	80/80	10/10	Not done
20	Embryonal carcinoma	67	−	80	15	4§
21	Teratoma	79	−	20	−	Not done
22	Seminoma	53	1	70	−	Not done
23	Seminoma	57	65	30	10	5§
24	Embryonal carcinoma/teratoma/ ITGCN	58/82	−/−/+	70/80/+	40/−/−	6§
25	Yolk-sac tumor/teratoma/ITGCN	42	5/5/+	50/20/+	15/−/−	4§
26	Embryonal carcinoma	57	1	90	40	4§
27	Seminoma	86	60	90	20	3‖
28	Seminoma	56	100	1	15	10‖
29	Embryonal carcinoma/ITGCN	78	20/+	35/+	10/−	Not done
30	Seminoma	72	10	20	10	13‖
31	Seminoma/ITGCN	64	100/+	10/+	5/+	Not done
32	Seminoma/yolk-sac/ITGCN	42/64	90/95/+	65/80/+	5/−/−	7‖
33	Seminoma	52	5	65	−	Not done
34	Yolk-sac tumor	96	20	60	30	5‖
35	Embryonal carcinoma	44 (m)	50/	35	5	Not done

The proportion of positive cells is not specified in intratubular germ cell neoplasia (ITGCN).

*Ratio (percentage) of tumor to normal tissue in the sample submitted to molecular studies. m, microdissected material; −, cases not investigated for p53 mutations by SSCP.

†Percentage of positive tumor cell nuclei. When more than one value is given, each value refers to a separate histological component of the tumor.

‡Percentage of red-stained yeast colonies.

§Cases tested in yeast using mRNA.

‖Cases tested in yeast using total RNA.

oligo-dT coupled to magnetic beads (Dynabeads mRNA direct kit, Dynal, Oslo, Norway), and reverse transcribed with Superscript II (BRL) and a specific primer (CGG GAG GTA GAC). cDNA was amplified using *Pfu* polymerase with primers P3 and P4[19] and transformed into the yIG397 yeast strain as previously described.[19]

Results

Detection of p53 Protein

Invasive tumors were present in all cases except for one (case 11) with only intratubular germ cell neoplasia (Table 1). All 34 cases were positive with CM1, whereas only twenty-two and twelve cases reacted with PAb240 (Figure 1A) and PAb1801, respectively. Nonseminomatous tumors showed generally stronger staining than seminomas. Features of intratubular germ cell neoplasia, including focal intratubular seminoma, were present in eight cases. In all of these cases, a significant proportion of atypical nuclei reacted with both CM1 and PAb240 (Figure 2, A and B). In every case, the adjacent invasive tumor also reacted with at least one anti-p53 antibody. Nine samples also contained seminiferous tubules with persistent spermatogenesis adjacent to foci of intratubular germ cell neoplasia, and morphologically normal spermatocytes showed nuclear reactivity with CM1 in one (case 29) of these cases. CM1, PAb240, and PAb1801 did not stain any component of nontumor control testes.

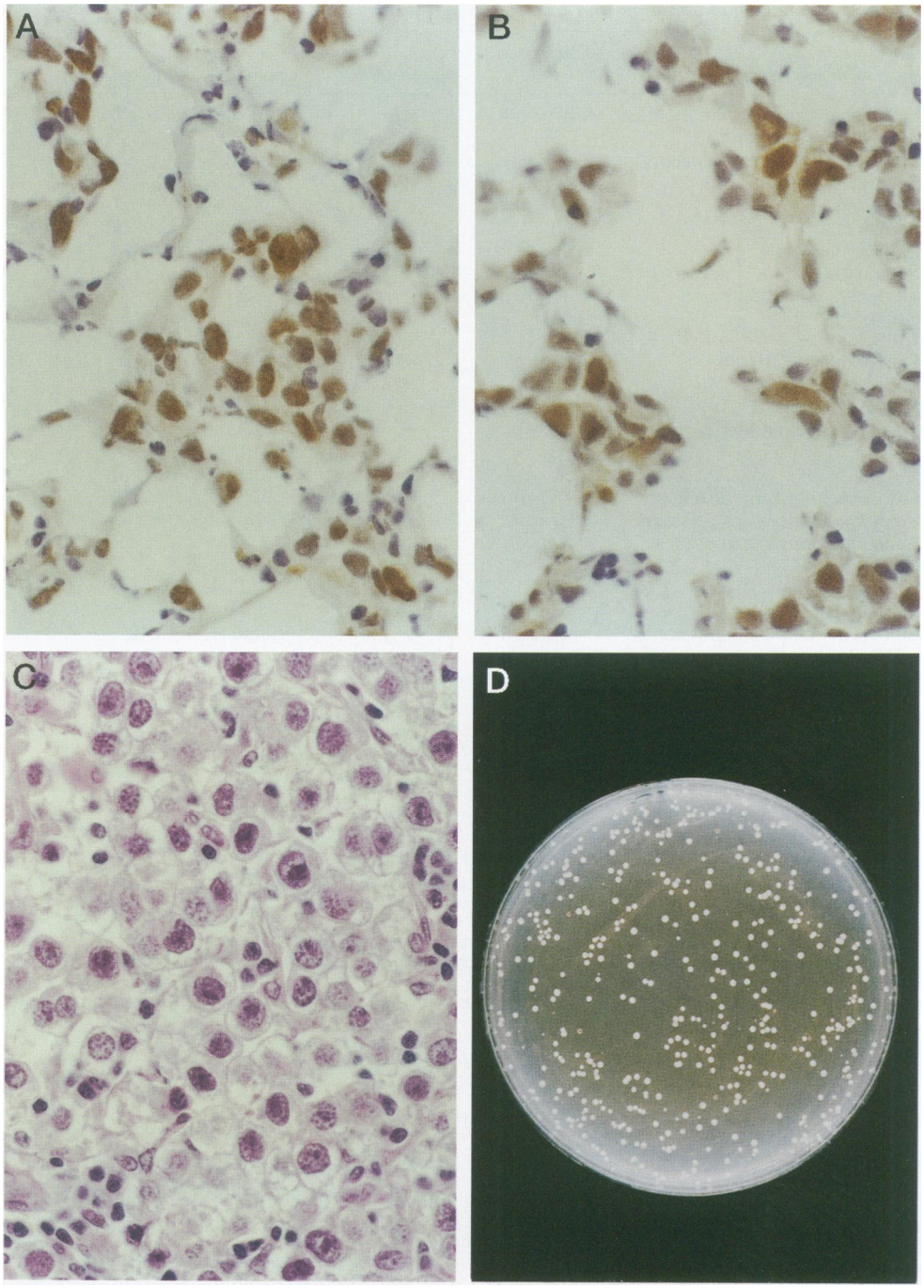

Figure 1. *Immunoperoxidase staining and p53 functional analysis of a seminoma (case 6) Magnification, ×410.* **A:** *Frozen tissue section stained with the anti-p53 monoclonal antibody PAb240 with hematoxylin nuclear counterstaining.* **B:** *Frozen tissue section stained with the anti-mdm2 monoclonal antibody IF2 (hematoxylin nuclear counterstaining).* **C:** *Paraffin-embedded tissue sections stained with H&E.* **D:** *Results of the p53 functional assay in yeast. On frozen sections, a large proportion of tumor cells showed strong nuclear positivity with both anti-p53 and anti-mdm2 antibodies. The p53 functional analysis (4% red colonies) showed that the p53 protein was wild type.*

Detection of mdm2 and p21$^{Waf1/Cip1}$ Proteins

Results obtained with the IF2 antibody are given in Table 1. Among tumors positive for both p53 and mdm2 (Figure 1, A and B), there was no correlation in a given tumor between the intensity of p53 and mdm2 staining or the percentage of cells positive for the two proteins. No reactivity was observed with the anti-p21$^{Waf1/Cip1}$ antibody in germ cell tumors, although positive nuclei were seen in irradiated colonic mucosa used as a positive control.

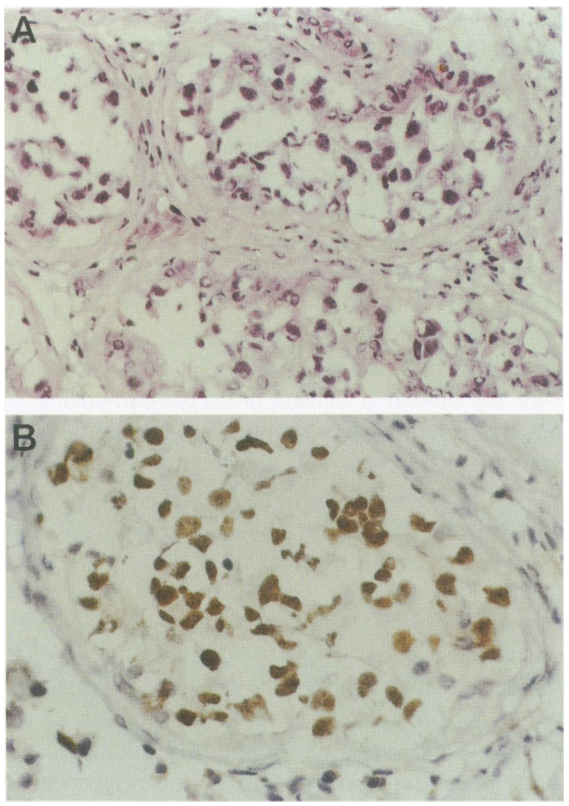

Figure 2. A: *Frozen tissue section of an intratubular seminoma stained with H&E (case 31) Magnification, ×200.* B: *The enlarged and atypical germ cell nuclei showed strong reactivity with the anti-p53 antibody PAb240. Magnification, ×330.*

SSCP Analysis of Genomic DNA

Frozen tissue was examined for p53 mutations by SSCP. Two cases (3 and 13) with <30% tumor content were not investigated. Case 11, showing only features of intratubular germ cell neoplasia, was also discarded. In none of the remaining thirty-two cases, nor in the two control testes, were p53 mutations detected by SSCP (data not shown).

Yeast p53 Functional Assay

In this assay, human p53 is expressed in a yeast strain containing the ADE2 gene controlled by a p53-responsive promoter.[19] Yeast colonies containing wild-type p53 express ADE2 and form white colonies, whereas colonies containing mutant p53 fail to express ADE2 and form red colonies. The basis of the assay is that tumorigenic p53 mutants are transcriptionally inactive. Frozen tissue from 18 p53-overexpressing tumors was tested (Table 1 and Figure 1D). In 6 cases, total RNA was used; in the remaining 12 cases, mRNA was used. All except 1 tumor gave normal percentages of red colonies, indicating that the p53 protein was wild type in these tumors. The intrinsic background in the assay is higher with total RNA (<20% red colonies) than mRNA (<10% red colonies; A. Estreicher and R. Iggo, unpublished data). Case 2 gave 14 ± 4% red colonies, a value above the background for samples tested using mRNA. Six plasmids were rescued from red colonies to determine the reason for the elevated value in this case. Three of these plasmids contained a partial retention of intron 9 (I9+). The I9+ splice variant is occasionally seen in normal tissue examined by functional assay, where its presence is not indicative of mutation.[31] When the alternatively spliced clones are excluded from the analysis, the value for case 2 falls within the normal range.

Discussion

Previous studies have shown that p53 protein is overexpressed in many germ cell tumors.[9–14] Bartkova et al[9] observed positive staining in 84% of formalin-fixed cases (tumors with ≤5% stained nuclei were considered positive in their study). In the present series, 100, 65, and 35% of cases were positive with CM1, PAb240, and PAb1801 on frozen material, respectively. PAb1801 was much less sensitive than PAb240 and CM1, consistent with previous studies on testicular and ovarian germ cell tumors.[32,33]

Overexpression of p53 protein has repeatedly been observed in intratubular germ cell neoplasia.[9,34] In the series of Bartkova et al,[9] 59% of formalin-fixed cases were positive, whereas nontumor testes were consistently negative. In our series, 100% of foci of intratubular germ cell neoplasia reacted with at least two anti-p53 antibodies on frozen sections.

p53 overexpression in tumors is commonly associated with mutation of the p53 gene, yet we identified no p53 mutations in the present study using two independent techniques. The absence of mutations cannot be attributed to tumor heterogeneity as DNA was extracted from the same frozen tissue block as was used for staining. Nor can it be attributed to the presence of an excess of normal tissue in the blocks, as only samples containing at least 30% tumor were screened for mutations. The functional assay detects mutations in a larger region of the p53 gene (amino acids 67 to 346) than is commonly tested by SSCP, denaturing gradient gel electrophoresis, or sequencing, and it can also detect splicing defects. RNA from 18 tumors was tested and showed that the encoded p53 protein was functionally normal in all but 1 case. In that case, a rare splice variant was

detected: partial retention of intron. This splice variant is occasionally seen with the functional assay in normal tissue, where its presence is not indicative of mutation.[31] Our findings parallel those published by six different teams that used conventional screening techniques,[12,13,15–18] but they conflict with those of Wei et al,[35] who found missense mutations in 4 of 17 (23.5%) seminomas from Chinese patients.

Although accumulation of wild-type protein has been observed sporadically in a wide variety of tumors,[36–44] it remains a surprising event, given that deliberate overexpression of wild-type p53 in transformed cells generally triggers cell cycle arrest or apoptosis.[1,2] Failure of wild-type protein to interfere with tumor cell growth thus suggests either a defect in activation of p53, for example, by regulatory kinases, or the presence of inhibitors of p53 activity. There is very little direct *in vivo* evidence for the former possibility. In contrast, several p53 inhibitors are known: the mdm2 protein,[45] SV40 large T antigen,[46,47] human papillomavirus E6 protein,[47] and adenovirus E1B protein.[48] In the absence of viral infection, mdm2 overexpression is thus the simplest explanation for the fact that wild-type p53 overexpression is tolerated by germ cell tumors. To test this possibility, we screened tumors for immunohistochemical evidence of mdm2 protein expression (Table 1). mdm2 protein was detected in 25 cases (73%). Importantly, we detected p53 protein in 9 tumors that failed to overexpress mdm2 protein. Thus mdm2 overexpression could be responsible for maintaining p53 in an inactive state in some of the tumors, but unless the IF2 antibody is insensitive, this cannot be the general explanation for the presence of wild-type p53 in germ cell tumors. Similar conclusions were drawn by Riou et al,[12] who studied mdm2 at the DNA and RNA levels and found evidence of mdm2 amplification or overexpression in only 12% of cases. Two other studies failed to detect mdm2 protein expression or gene amplification in any testicular germ cell tumor examined.[13,16]

An additional possibility is that, although p53 is active, the cell is refractory to the effects of p53 target genes. A precedent for this is that, although p53 can activate production of the cell cycle inhibitor protein p21 in c-*myc*-overexpressing cells, the cdk/cyclin complexes in these cells are resistant to its effects.[49] To examine this possibility, germ cell tumors were examined for p21 expression using the EA10 monoclonal antibody. The antibody readily detected p21 on positive control sections of irradiated colon, but no staining was seen in germ cell tumors. Such entirely negative staining results should be treated with caution, but they certainly do not support the hypothesis that overexpressed p21 is bypassed in germ cell tumors. Instead, they suggest that p53 is unable to activate p21 transcription, a phenomenon that could arise, for example, from changes in chromatin structure at the p21 promoter.

Our results show that in rare situations cells can be resistant to the growth-inhibitory effects of wild-type p53. Interestingly, early embryos undergo rapid cell division despite the presence of high levels of wild-type p53 protein,[50] showing that physiological mechanisms to overcome the effects of p53 must exist. The p53 overexpression in testicular tumors may be due to a similar process, particularly as nonseminomatous tumors often contain a variety of components resembling developmental tissues. A similar phenomenon may explain the overexpression of wild-type p53 in hepatoblastomas, an unusual childhood tumor containing developmental tissues.[41] In addition, testis is one of the few adult tissues in which positive staining for p53 has been observed in morphologically normal cells in mice.[51] Malignant cells arising in such naturally resistant populations could readily give rise to tumors expressing high levels of wild-type p53. Although resistance to wild-type p53 appears uncommon in tumors, understanding the underlying mechanism has great practical importance.

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