Human p53 knock-in (hupki) mice do not differ in liver tumor response from their counterparts with murine p53

Maike Jaworski1, Stephan Hailfinger1, Albrecht Buchmann, Manfred Hergenhahn1, Monica Hollstein1, Carina Itrich2 and Michael Schwarz*

Department of Toxicology, Institute of Pharmacology and Toxicology, University of Tübingen, Wilhelmstrasse 56, 72074 Tübingen, Germany, 1Department of Genetic Alterations in Carcinogenesis and 2Central Unit Biostatistics, German Cancer Research Center, PO Box 101949, 69009 Heidelberg, Germany

*To whom correspondence should be addressed. Tel: +49 7071 29 77398; Fax: +49 7071 29 2273; Email: michael.schwarz@uni-tuebingen.de

Mouse models are important tools in toxicologic research. Differences between species in pathways contributing to tumor development, however, raise the question in how far mouse models are valid for human risk assessment. One striking difference relates to the frequency of spontaneous liver cancer which is high in certain mouse strains but rather low in humans. Similarly, mutation frequencies in cancer genes are characteristically different, i.e. P53 mutations are frequent in human but very rare in murine liver tumors, whereas Ras genes are often mutated in mouse liver tumors but hardly ever in human liver cancers. Since P53 has been shown to control oncogenic Ras in human cells, we hypothesized that this function of the tumor suppressor could differ in mouse hepatocytes. To test this hypothesis, we used hupki (human p53 knock-in) mice which carry a partly humanized P53 sequence (P53KI). In this study, we report the results of the first hepatocarcinogenesis experiment with this strain of mice. Mice of the genotypes P53KI/KI, P53WT/KI and P53WT/WT were treated with N-nitrosodiethylamine at 2 weeks of age and killed 35 weeks later. The frequency of liver tumors and glucose-6-phosphatase-altered liver lesions was almost identical in all three P53 genotypes and ~40–50% of liver tumors showed activating mutations in codon 61 of the Ho-Ras gene independent of genotype. Moreover, only very few P53-positive lesions were observed but without nuclear localization of the protein, suggesting the absence of P53 mutations. These data suggest that the hupki allele behaves like its murine ortholog in mouse hepatocarcinogenesis.

Introduction

Primary hepatocellular carcinomas (HCCs) are one of the ten most frequent human cancers worldwide (1). Risk factors like hepatitis B and C virus and aflatoxins as given in southern Africa and southern China make HCC the most frequent cause of cancer death in these countries. Most populations from industrialized countries, however, show a comparatively low incidence of HCC, indicating that development of ‘spontaneous’ HCC is a rather rare event in humans. By contrast, certain strains of laboratory mice, e.g. C3H, show a very high incidence of spontaneous liver tumors which can reach up to 100% at the end of the animals’ lives (2). These opposing findings are paralleled by differences at the molecular level. One of the most striking differences lies in the mutation frequencies of genes in two pathways, namely the P53 and the mitogen activated protein (MAP) kinase signaling pathway.

The transcription factor P53 is a well-known tumor suppressor protein that induces cell growth arrest, apoptosis and senescence in response to various types of stress. In unstressed cells, P53 is maintained at low levels by the action of the ubiquitin E3 ligase MDM2 which earmarks P53 for degradation in the proteasome (3). MDM2 can also bind to the transactivation domain of P53 thus reducing its transcriptional activity (4). DNA damage or oncogenic stress, e.g. caused by mutated CTNNB1 (β-catenin), c-MYC or RAS lead to a stabilization of the P53 protein (5–7) and transcriptional activation of a battery of ‘caretaker’ genes (8).

RAS-mediated signal transduction pathways are involved in the regulation of cell growth, apoptosis and differentiation, which constitute fundamental processes that are deregulated in tumors carrying mutated Ras genes (9–11). The most common mutations in Ras affect the GTPase activity of the small monomeric G-protein resulting in the constitutively active molecule which may, amongst others, permanently stimulate cell-cycle progression via activation of MAP kinase and other effector pathways (9). The tumorigenic transformation of cells by Ras oncogenes can be prevented by P53-controlled mechanisms: inappropriate stimulation of cell-cycle progression leads to an E2F-mediated upregulation of the tumor suppressor protein p14/19ARF which can bind MDM2 and inactivate its ability to initiate P53 degradation (7,12–14). Thus, in human cells, P53 controls oncogenic RAS and activation of RAS in tumor cells is often accompanied by ablation of P53-dependent responses.

Inactivation of P53 by mutation is frequently observed in human HCCs (15–17) whereas RAS and its downstream effector B-RAF are only very rarely mutated in human HCCs (2,18). Liver tumors from mice, however, are hardly ever mutated in P53 (2,19) but rather harbor activating mutations in Ha-Ras (20,21) and B-Raf (22). This evidence could indicate that murine P53 may not control the activated RAS oncoprotein in mouse liver as opposed to its human ortholog, suggesting that the P53 protein may differ in function between mice and men. Although P53 shows high homology between the two species both in the overall amino acid sequence (74%) and the DNA binding domain (91%), the homology is less in the N-terminal and C-terminal regions and the polyproline domains (PPDs). Mouse P53 lacks, for example, residues that constitute important phosphorylation sites in its human ortholog, e.g. serine 37 and 46, amino acids which are relevant for

Abbreviations: DEN, N-nitrosodiethylamine; G6Pase, glucose-6-phosphatase; GS, glutamine synthetase; HCC, hepatocellular carcinoma; hupki, human p53 knock-in; PPD, polyproline domain.

These authors contributed equally to this work.
the modulation of P53 stress response in cells, and proline 72 with a role in apoptotic responses (23,24).

The aim of our present work was to investigate whether structural differences between human and mouse P53 proteins are critical determinants during hepatocarcinogenesis. A potent tool to address this question is the transgenic *hupki* (human p53 knock-in) mouse, in which parts of the murine P53 gene are replaced by the respective human sequences (exons 4–9) containing serine 46, proline 72, and threonines 76 and 86 (25). The Jackson Laboratory Repository designation for the knock-in allele is Trp53<sup>mHoi</sup>/m; it will be abbreviated as P53<sup>K1</sup> in the following, as opposed to the wild-type allele (P53<sup>+</sup>). The humanized *hupki* P53 seems to carry out all normal functions of the mouse P53 investigated to this point (25–27). We now analyzed the frequency of liver tumors induced by a single injection of N-nitrosodiethylamine (DEN) in C3H mice carrying the P53<sup>K1</sup> allele homozygously or heterozygously in comparison with C3H mice with the wild-type murine P53 (P53<sup>3/3</sup>). C3H mice were chosen because of their high susceptibility to liver tumor formation (28). In addition, the ploidy status of hepatocytes in normal liver and *Ha-Ras* mutation frequencies in liver tumors of mice of the different genotypes were analyzed.

**Materials and methods**

**Breeding of C3H P53<sup>K1/K1</sup>, P53<sup>K1/+</sup> and P53<sup>+/+</sup> mice**

C57BL P53<sup>K1/+</sup> mice were introduced to our laboratory (University of Tübingen) via embryo transfer. These mice were backcrossed into C3H for six generations prior to the study. C3H P53<sup>K1/+</sup> (F6) were mated and the male offspring used in the experiment. P53- genotyping was performed by duplex PCR using the following primer pairs: GECE7F (5'-GCCCTACATCGTGGCCTTTGAC-3') and GCEX7R (5'-GGCCAGTGGTCAGGCGAAGAGGTC-3') specific for human P53; P42A (5'-ACTCCATGCGGCCCTGGTAC-3') and P42B (5'-AGTTGACAGCAAAGACAA-GACAAT-3') specific for mouse P53 (25,29).

**Induction of liver tumors**

An animal study was carried out according to the German guidelines for animal care and treatment. A single dose of N-nitrosodiethylamine (10 μg/g body wt) was injected (i.p.) to 15 male C57BL mice of each genotype (P53<sup>K1/+</sup>, P53<sup>K1/K1</sup> and P53<sup>+/+</sup>) at 2 weeks of age. After weaning, the mice were housed individually in macrolon cages and kept on a standard diet without further treatment. All mice were killed 35 weeks after carcinogen treatment and livers were removed and weighed. Normal appearing liver tissue was excised for isolation of nuclei; larger tumors were isolated and immediately frozen in liquid nitrogen and stored at −1830°C.

**Enzyme and immunohistochemistry**

Sections (10 μm thick) were prepared from frozen liver tissue with a cryostat. Sections of G6Pase-altered liver lesions were quantified using a computer-assisted digitizer system as previously described (31).

P53 was stained immunohistochemically in sections (5 μm thick) from paraffin-embedded liver blocks as previously described (32). In brief, the sections were deparaffinized, rehydrated and incubated with a polyclonal, pan-specific rabbit anti-P53 antibody (NCL-p53-CM1; Novocasta Laboratories, Newcastle, UK; 1:1500 dilution). Following incubation with a secondary goat anti-rabbit-IgG antibody coupled with biotin (Biospa, Milano, Italy; 1:200 dilution), the sections were incubated with streptavidin alkaline phosphatase conjugates (Biospa, Milano, Italy) for 30 min. After washing the sections in phosphate-buffered saline, the bound antibody was visualized using the alkaline phosphatase substrate FastRed™ (Kem-En-Tech, Copenhagen, Denmark). For glutamine synthetase (GS) staining, sections were incubated with a monoclonal anti-GS antibody (BD Biosciences, Heidelberg, Germany; 1:500 dilution) after blocking the endogenous peroxidase activity. After incubation with the HRP-coupled anti-mouse-IgG secondary antibody (Sigma-Aldrich, Taufkirchen, Germany; 1:20 dilution) sections were stained with aminoethylcarbazole/H<sub>2</sub>O<sub>2</sub> solution. Nuclei were counterstained with hematoxylin.

**Isolation of nuclei from mouse liver and FACS analysis**

For the isolation of liver nuclei a standard protocol was used (33). About 100–280 mg normal tissue of 17 mouse livers were removed and transferred immediately into cold 0.25 M TKM solution (0.25 M sucrose, 50 mM Tris–HCl, pH 7.5, 25 mM KCl and 5 mM MgCl₂). All subsequent operations were performed at a temperature near 0°C. The tissue was weighed and homogenized in a potter with a motor-driven Teflon pestle (Elvehjem Tissue Grinder, clearance 0.025 cm) in two volumes of ice-cold 0.25 M sucrose in TKM. The homogenate was filtered through four layers of gauze, the liquid fraction mixed with two volumes of 2.3 M sucrose in TKM. The preparation was underlaid by one volume of 2.3 M sucrose in TKM and centrifuged for 30 min at 126 000 × g, at 4°C (Beckman Coulter, Fullerton, USA, Optima™ TL Ultracentrifuge with a TL-A 100.4 rotor, 55 000 × g, r.p.m.). The supernatant was discarded, the nuclear pellet taken up in 1 ml TKM buffer and the nuclei concentration determined.

From each sample an aliquot of ~100 000 nuclei was centrifuged and the nuclei were resuspended in 15 μl PI solution [0.1% (w/v) sodium citrate; 0.1% (w/v) Triton X-100; 50 μg/ml propidium iodide]. After 5 min of incubation, the DNA content of nuclei was measured by FACS analysis (FACScalibur, Becton Dickinson, Franklin Lakes, USA). From each sample 10 000 events were counted; triplicate measurements were made from each animal and averaged. Data were analyzed with CellQuest 3.1f software.

**Statistical analyses**

Statistical analyses were carried out using the software package R, version 2.0.1 (34). Comparisons of relative liver weight, area fraction of G6Pase-altered lesions in liver and ploidy status between the three groups (P53<sup>K1/K1</sup>, P53<sup>3/3</sup> and P53<sup>+/+</sup>) were performed by Kruskal–Wallis rank sum tests. Frequencies of *Ha-Ras* mutations in the three groups (P53<sup>K1/K1</sup>, P53<sup>3/3</sup> and P53<sup>+/+</sup>) were compared by Fisher’s exact test for count data.

**Results**

In this study, we determined the tumor response in livers of mice after a single injection (i.p.) of the liver carcinogen N-nitrosodiethylamine. Mice of differing P53 genotype were used (15 per group): mice carrying the mouse P53 wild-type allele (P53<sup>+/+</sup>), mice heterozygous for the humanized P53<sup>KI</sup>-KI allele (hupki) allele (P53<sup>K1/+</sup>) and mice homozygous for the P53<sup>KI/KI</sup> allele. Animals were killed 35 weeks after treatment, except for one animal in the P53<sup>KI/KI</sup> group which was killed at 19 weeks of the study because it had developed overt signs of malady. At the end of the study, all animals had enlarged, sponge-like livers harboring numerous tumors visible at the surface of the livers. Mice of all three groups showed increased liver weights and liver/body weight ratios caused by the high liver tumor burden, without significant differences between groups (Table I). In historical controls, untreated wild-type C3H mice showed a liver/body weight ratio of ~4–5%, a value that was almost doubled in DEN-treated mice of the present study.

The neoplastic response in liver was determined by enzyme-histochemical staining of frozen sections for G6Pase, which is, under most circumstances, decreased in activity in...
hepatocellular preneoplastic foci, adenomas and carcinomas, and is frequently employed as a marker for the identification of these lesions (35). In this study the majority of liver lesions were of a G6Pase-negative phenotype but in addition, lesions were observed which showed increases rather than decreases in G6Pase activity, often with inhomogeneous staining patterns. The fraction of G6Pase-altered tissue in liver was quantitatively evaluated and was found to range between ~60 and 70% without significant difference between groups (Table I). Therefore, if P53 has an effect on the development of liver tumors in mice, this effect does not differ between the endogenous mouse protein and its humanized P53 ortholog.

The role of P53 in maintaining genomic integrity is well established. Mammalian cells show a P53-dependent growth arrest when they reach tetraploidy (36), and a failure of the P53 pathway can lead to aneuploidy (37,38). P53 may thus play a role in the control of cellular ploidy and differences may exist in this activity between the murine and the human protein. We therefore analyzed the ploidy distribution of nuclei isolated from non-tumorous liver tissue of DEN-treated P53+/+, P53hupki+/- and P53hupki +/- mice. Nuclei were isolated and their DNA content was measured by FACS analysis after staining with propidium iodide. Diploid nuclei formed the majority (~45%) closely followed by tetraploid (~33%) and octaploid nuclei (~7%), without significant differences between mice from the three groups (Table II).

Since hepatocytes are often binuclear or polynuclear, liver sections were stained with hematoxylin and scored microscopically for binuclear cells. About 6% of hepatocytes were found to be binuclear (without correction for stereology) without apparent differences between genotypes.

We have previously screened liver tumors from mice of the present study for the presence of mutation in B-Raf (22). In the course of that study, Ha-Ras mutations were analyzed in parallel. Since the emphasis of the study was on B-Raf and since no significant differences in the mutation frequencies between p53 genotypes were detectable, we did not discriminate in our previous publication between genotypes. In Table III we now present Ha-Ras codon 61 mutation patterns of liver tumors grouped by genotype of the animals. Point mutations in Ha-Ras codon 61 were detected in ~40–50% of tumors without significant differences in frequencies and patterns between mice of the three genotypes.

Mutation of P53 is often associated with nuclear accumulation of the protein. Thus, immunohistochemical P53 staining is a common and fast method to indirectly score for the presence of P53 mutations in tumor tissues. In this study, we analyzed 42 liver sections stained for P53, 14 of each genotype (P53+/+, P53+/KI and P53hupki+/-), for P53 accumulation in tumor transections. Altogether 347 tumor transections could be identified, but only 4 of these showed a positive P53 staining. Nuclear staining, often indicative of P53 mutation, was not observed in these lesions (Figure 1), of which two were from P53+/+ mice and the other two were from P53hupki+/- mice. P53-positive

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**Table II.** Distribution of nuclear ploidy of hepatocytes from hupki (P53hupki+) as compared with P53-wild-type (P53+/+) and P53 heterozygous (P53+/KI) mice

<table>
<thead>
<tr>
<th>P53 genotype</th>
<th>+/+</th>
<th>+/KI</th>
<th>KI/KI</th>
<th>P-valuesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals analyzed</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Ploidy status (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>45.92 ± 0.35</td>
<td>44.25 ± 0.49</td>
<td>44.62 ± 0.25</td>
<td>0.8164</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>32.86 ± 0.25</td>
<td>33.22 ± 0.21</td>
<td>32.24 ± 0.25</td>
<td>0.5479</td>
</tr>
<tr>
<td>Octaploid</td>
<td>7.06 ± 0.18</td>
<td>7.06 ± 0.18</td>
<td>6.78 ± 0.1</td>
<td>0.9983</td>
</tr>
<tr>
<td>Other ploidy status</td>
<td>14.16 ± 0.24</td>
<td>15.47 ± 0.33</td>
<td>16.36 ± 0.18</td>
<td>0.4694</td>
</tr>
</tbody>
</table>

Data represent mean ± standard error.

*aKruskal–Wallis rank sum test.

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**Table III.** Frequencies and patterns of Ha-ras mutations in liver tumours from hupki (P53hupki+) as compared with P53-wild-type (P53+/+) and P53 heterozygous (P53+/KI) mice

<table>
<thead>
<tr>
<th>P53 genotype</th>
<th>+/+</th>
<th>+/KI</th>
<th>KI/KI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ha-Ras mutations (codon 61)</td>
<td>15/29 (51.7%)</td>
<td>11/26 (42.3%)</td>
<td>13/27 (48.1%)</td>
</tr>
<tr>
<td>CAA→CGA (Gln→Arg)</td>
<td>6 (20.7%)</td>
<td>7 (26.9%)</td>
<td>7 (25.9%)</td>
</tr>
<tr>
<td>CAA→CTA (Gln→Leu)</td>
<td>2 (6.9%)</td>
<td>0 (0%)</td>
<td>2 (7.4%)</td>
</tr>
<tr>
<td>CAA→AAA (Gln→Lys)</td>
<td>7 (24.1%)</td>
<td>4 (15.4%)</td>
<td>4 (14.8%)</td>
</tr>
</tbody>
</table>

Liver tumors from mice of the three groups did not differ significantly in their mutation frequencies. Data are taken from ref. (22) and grouped according to p53 genotype. Base substitutions are indicated in boldface. *P = 0.82, Fisher’s exact test.

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**Fig. 1.** P53 and glutamine synthetase (GS) colocalize in P53-positive mouse liver lesions. Both proteins were stained on frozen sections by immunohistochemistry. Note the cytoplasmic localization of P53. Horizontal bars equal 0.1 mm.
lesions stained also positive for glutamine synthetase, which is indicative for activation of β-catenin by mutation of the gene (39,40) (Figure 1).

Discussion

The results of our present study demonstrate that carcinogen-treated hupki mice, which harbor the human P53 sequence from exons 4–9 replacing the corresponding murine exons 4–9, show no differences in their neoplastic response in liver as compared with mice harboring the respective murine allele. Several studies have pinpointed the fact that P53 plays a central role in tumorigenesis both in humans and in experimental systems. For example, the gene dosage of P53 affects malignant development in murine skin and P53 null mice are highly susceptible to radiation-induced tumorigenesis (41,42). It is also well known that heterozygous P53 null mice show an overall increase in tumor burden when compared with wild-type mice (43). Interestingly, however, hepatocarcinogenesis was not enhanced on treatment of heterozygous P53 null mice with a chemical carcinogen (43–48). These results may be interpreted in the sense that murine P53 plays an important role in the control of malignant development in some organs of mice but not in their liver, quite in contrast to human liver where the frequent inactivation of P53 in HCCs points toward an important control function of the suppressor protein during malignant transformation. However, recent data from mouse models of the Li–Fraumeni syndrome show that point mutant P53 mice develop carcinomas in a number of tissue sites, including the liver, reopening the debate on effects of P53 mutation on murine hepatocarcinogenesis (49).

The humanized P53 protein contains several important phosphorylation sites which are possibly regulated differentially in mice and men. The P53 proteins share a high homology between species and, although the post-transcriptional modifications of the mouse protein are not as well understood as those of the human counterpart, there is evidence that there are a number of species-specific sequence differences especially in the replaced PPD which possibly lead to a differential post-translational regulation of P53’s function.

Residue 72 is an important site for apoptosis and response to chemotherapy in human cells (50,51). In humans, it is polymorphic, encoding arginine or proline, whereby the arginine variant (the more common form in Northern European populations) correlates with an enhanced response to apoptotic stimuli. Comparing this sequence with mice is not straightforward since it lies in one of the most divergent parts of the protein [discussed in (52)], but at the corresponding position in mouse P53 (codon 69) there is an alanine. In the hupki protein, this part of the sequence is human with an arginine at this site, but our data indicate that this does not induce a difference with respect to control of Ras-mutated cells by P53 since the prevalence of Ha-Ras mutated tumors was very similar in mice of all three P53 genotypes.

Serine 46 in human cells and in hupki cells (M.H., unpublished data) is phosphorylated by a yet unidentified kinase after massive DNA damage (24). Substitution of serine 46 blocks the expression of P53AIP1 (P53 regulated apoptosis inducing protein 1) which is an important mediator of P53-dependent apoptosis (53). The murine protein lacks this phosphorylation site. Serine 46 might also provide a link of P53 to cell-cycle control since, based on sequence homologies, it could be a target for cyclin-dependent and MAP kinases in human cells (24). Constitutive activation of MAP kinase signaling in Ras-mutated cells carrying the humanized KI alleles could therefore have a lead, by feedback inhibition, to fewer Ras-mutated tumors, an effect that was not detectable in our study.

Murine threonines 76 and 86 in the PPD are expected sites of phosphorylation by MAP kinases (54,55). Increased phosphorylation was detected after stimulation of cells with TPA (12-O-tetradecanoyl-phorbol-13-acetate) (54,56). There is no equivalent to this site in the human sequence, but again our data show that the lack of this phosphorylation site affects neither the tumor response in liver nor Ha-Ras mutation prevalence of tumors.

Liver lesions of the present study stained very infrequently positive for P53 (~1%). Accumulation of P53 is a hallmark of mutation of the gene. Nuclear P53 accumulation, however, was not observed in the positively stained lesions. Mutation within the DNA binding domain of P53 often results in loss of the protein’s ability to act as a transcription factor. As a result Mdm2, one of its target genes, is no longer transcribed which eliminates the feedback loop that negatively controls the intracellular concentration of P53 (57). We therefore conclude that mutation of P53 was infrequent in the mouse liver tumors, irrespective of whether the mice harbored the murine or the humanized P53 alleles. We also found that the rare P53-positive lesions we detected were simultaneously positive for glutamine synthetase which is absent from most normal hepatocytes except those in a small ring of cells directly located around the central veins (58). Glutamine synthetase is overexpressed in hepatoma cells with an activated version of β-catenin and may serve as marker indicating mutation of the gene (39,40). β-Catenin and P53 may in fact interact: activation of β-catenin signaling provoked by LiCl treatment of cells or by mutation of the gene leads to stabilization of P53, cell-cycle arrest, and induction of senescence in endothelial cells (59) and mouse fibroblasts (60). We therefore suggest that the accumulation of P53 protein in glutamine synthetase-positive liver lesions of this study may be caused by the presence of an activated form of β-catenin in cells of these lesions.

The results of our present study demonstrate that hupki mice behave like P53-wild-type mice with respect to hepatocarcinogenesis induced by our DEN treatment protocol. There was no difference in tumor response, the Ha-ras mutation prevalence of tumors was similar and there was no indication of P53 mutations in liver tumors from hupki mice, in analogy to what has been demonstrated in mice with an unmodified P53 locus (19). These findings do not support the hypothesis that P53 harboring the human polyproline and DNA binding domains has a growth control function that is lacking in normal murine P53 of mouse hepatocytes. We cannot exclude the possibility that the segments of murine P53 that are not replaced in the hupki model could have had an influence on the outcome of this study (Figure 2). Another possibility is that the hupki allele may have novel properties but nevertheless behaves like the mouse protein in the genetic environment of the mouse, either because other human-specific proteins that interact with P53 are missing or mouse-specific proteins block the control function mediated by the human allele. The reason why the mutation prevalences in Ras and in P53 in liver tumors from mice and men are so different remains to be unveiled.
Fig. 2. Comparison of mouse wildtype, hupki and human P53 protein sequences. Gray boxes indicate sequences of mouse, white boxes of human origin. TAD, transactivation domain; PPD, polyproline domain, DBD, specific DNA binding domain, OM/MF, oligomerization/multifunctional domain. Important residues mentioned in the text are indicated.

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Conflict of Interest Statement: None declared.

References


