Multiple Stress Signals Activate Mutant p53 In Vivo

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Abstract

p53 levels are tightly regulated in normal cells, and thus, the wild-type p53 protein is nearly undetectable until stimulated through a variety of stresses. In response to stress, p53 is released from its negative regulators, mainly murine double minute 2 (Mdm2), allowing p53 to be stabilized to activate cell-cycle arrest, senescence, and apoptosis programs. Many of the upstream signals that regulate wild-type p53 are known; however, limited information for the regulation of mutant p53 exists. Previously, we showed that wild-type and mutant p53R172H are regulated in a similar manner in the absence of Mdm2 or p16. In addition, this stabilization of mutant p53 is responsible for the gain-of-function metastatic phenotype observed in the mouse. In this report, we examined the role of oncogenes, DNA damage, and reactive oxygen species, signals that stabilize wild-type p53, on the stabilization of mutant p53 in vivo and the consequences of this expression on tumor formation and survival. These factors stabilized mutant p53 protein which oftentimes contributed to exacerbated tumor phenotypes. These findings, coupled with the fact that patients carry p53 mutations without stabilization of p53, suggest that personalized therapeutic schemes may be needed for individual patients depending on their p53 status. Cancer Res; 71(23); 7168–75. ©2011 AACR.

Introduction

The p53 pathway is impaired in most human cancers. More than 50% of human tumors carry p53 mutations, and most of these are missense mutations that result not only in loss of tumor-suppressing activities but also acquisition of oncogenic activities, defined as gain of function. Specifically, mutant p53 enhances proliferation and survival in cells and tumorigenesis in mice when compared with cells or mice that are deficient for p53 (1).

Wild-type p53 normally exists in a latent state but becomes stabilized and activated in response to various genotoxic and cellular stress signals, allowing for transcriptional modulation of multiple genes that play important roles in controlling cell-cycle progression, senescence, and apoptosis (2). The regulation of wild-type p53 is mediated mainly by protein turnover. Primarily, its stability is regulated by murine double minute 2 (Mdm2), an E3 ubiquitin ligase that targets p53 for proteolytic degradation (3–7). Recently, several other ubiquitin ligases have been identified that also regulate p53 stability and include COP1 (8), Pirh2 (9), ARF-BP1/Mule (10), and Trim24 (11), although their roles in vivo are less clear.

The importance of DNA damage in p53 signaling has been extensively studied. In particular, exposure to chemotherapeutic drugs results in stabilization of wild-type p53 through posttranslational modifications in the amino terminus (2). These modifications disrupt the ability of Mdm2 to interact with p53 (12). The generation of reactive oxygen species (ROS), cytokines, and γ-radiation also play roles as potent activators of p53 (13). Mechanistically, ROS activates p53 through direct damage to DNA. In addition, ROS contributes to cross-talk and activates other signaling pathways such as p38, c-jun-NH2-kinase (JNK), or NF-xB signaling resulting in synergy of p53 activation through phosphorylation, stabilization, and activation (14).

Two independent tumor suppressors, p16INK4a and p19ARF (p14ARF in humans) also impact the p53 signaling pathway through different mechanisms. p16INK4a loss, as occurs in some tumors, allows cyclin D/CDK4 kinase activity to phosphorylate retinoblastoma (Rb) and results in its dissociation from E2F. Released E2F then transcriptionally activates p19ARF which binds Mdm2 and thereby affects p53 stabilization (15, 16). In cancers, p19ARF is also upregulated following oncogene activation (17–19). Mutant Ras or increased levels of c-Myc, for example, stimulate the ARF–Mdm2 complex formation (18, 20–22) which in turn causes a sequestration of Mdm2 and subsequent stabilization of p53 (23). Although the signaling pathways targeting wild-type p53 are well documented, the regulatory signals that control mutant p53 levels are less well understood. Recently, we and others showed that mutant p53 is inherently unstable in normal tissues and that some of the factors that regulate wild-type
p53 are also responsible for the stabilization of mutant p53 protein (24–26). Loss of Mdm2, for example, stabilizes mutant p53 in many normal tissues (26). In these experiments, stabilization of mutant p53 led to gain-of-function phenotypes manifested as increased tumor incidence and metastasis. Disruption of the Rb pathway via p16INK4A deletion also stabilized mutant p53 in vivo (26). We have now explored whether other signals that stabilize wild-type p53 likewise affect mutant p53 stabilization. Because p53 is mutated in the majority of human tumors and expression of mutant p53 is often associated with poor outcomes, we examined a variety of cellular stimuli that may potentially stabilize mutant p53 in vivo and may thus lead to an enhanced tumor phenotype. We found that activation of oncogenes stabilized mutant p53 resulting in more potent tumor phenotypes than mice that only harbored the p53 mutation; however, these types of stimuli did not impact overall survival. On the other hand, when we examined the effect of doxorubicin, a chemotherapeutic DNA-damaging agent, on mutant p53 stability, we observed stabilization of mutant p53 as well as decreased survival compared with the untreated p53 homozygous mutant mice. Furthermore, we analyzed the effect of a ROS scavenger on mutant p53 protein stabilization and observed a gain-of-function phenotype. In most cases, stabilization of mutant p53 led to worse phenotypes than the absence of p53. These results led us to propose that multiple cellular stress pathways that regulate wild-type p53 also act to increase mutant p53 levels yielding gain-of-function phenotypes.

Materials and Methods

Generation of Eμ–myc and K-rasL141 cohorts

p53515A+/+, p53515A−/−, K-rasL141, and p53515A+/515A mice were maintained on more than a 95% C57BL/6 background (24, 27, 28). Eμ–myc mice (29) were crossed to p53515A+/515A to generate p53515A−/− Eμ–myc. The background of Eμ–myc, Eμ–myc p53515A/515A, or Eμ–myc p53515A−/− mice were 75% C57BL/6 and 25% 129Sv. Tails from Eμ–myc, Eμ–myc p53515A/515A, or Eμ–myc p53515A−/− mice were genotyped using primers previously described (30). K-rasL141 mice (27) were crossed to p53515A+/515A to generate p53515A+/515A K-rasL141 mice. p53515A+/515A K-rasL141 mice were further crossed with p53515A+/515A to generate p53515A+/515A K-rasL141 mice. To determine mouse genotypes, PCR analysis was conducted on tail DNA using published primer sets for the p53515A, p53515A+/515A, and K-rasL141 alleles (24, 27, 28, 31). The animal studies were conducted according to the MD Anderson and IACUC guidelines.

Statistics

Survival curves were plotted by the Kaplan–Meier method by GraphPad Prism to assess statistical differences. A factor was considered statistically significant if it had a 2-sided P value less than 0.05.

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was conducted as previously described (32). Protein expression was analyzed using rabbit α-p53 (CM5) antibodies (Vector Laboratories) at a 1:200 dilution for 2 hours at 37°C, and visualized using ABC and DAB kits from (Vector Laboratories). Slides were counterstained with Nuclear Fast Red. Hematoxylin and eosin staining was used for pathologic analysis of tumors.

Western blotting

Protein lysates were prepared from either the tissues or tumors of mice. Fifty microgram of total proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (GE Bioscience). After blocking with 5% skim milk in PBS–0.1% Tween 20 (PBS-T) for 1 hour at room temperature, membranes were incubated with α-p53 (CM5; Vector Laboratories, 1:1,000 dilution) antibodies at 4°C overnight. Membranes were then washed with PBS-T and incubated with anti-rabbit horseradish peroxidase–conjugated secondary antibody and visualized with ECL plus (GE Bioscience). Anti-β-actin or vinculin (Sigma; 1:5,000) antibodies were used as a loading control. All antibodies were diluted in blocking buffer.

Treatment of mice

Two-day-old homozygous pups were treated with 2 Gray (Gy) of γ-radiation. For doxorubicin treatment, pups were injected with either PBS or 2 μg of doxorubicin suspended in PBS per gram of body weight at days 5 and 6. Five hours after the last injection, pups were sacrificed. Protein expression in tissue was measured by Western blot analysis as described earlier. For ROS experiments, parent mice were treated with the ROS scavenger N-acetyl-cysteine (NAC) for 2 weeks before mating through drinking water and continued while pups were nursing. At day 5, homozygous mutant pups were irradiated with 2 Gy. Four hours after irradiation, mice were injected with either PBS or 2 μg of NAC in PBS per gram of body weight. One hour later, pups were sacrificed and protein expression was examined.

Measurement of intracellular ROS

The dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Molecular Probes) was used to detect intracellular ROS. The fluorescence intensity of intracellular DCFDA is a linear indicator of ROS in stained cells (33). To compare intracellular ROS in thymocytes from p53515A+/515A mice, freshly isolated thymocytes were incubated with 10 μmol/L DCFDA in the culture medium (RPMI-1640) for 30 minutes at 37°C. The cells were harvested and the DCFDA fluorescence profiles determined by flow cytometric analysis with a Coulter Epics software program, version 4.02.
Results

K-ras activation stabilized the p53R172H mutant and exacerbated tumor progression

Wild-type p53 is stabilized and activated in response to hyperproliferative signals and thus protects cells from aberrant growth signals. Tissue culture stabilizes wild-type and mutant p53 and is, thus, not an optimal system to study these effects (24). Therefore, we examined the effect of oncogenes on mutant p53 protein stabilization in vivo by taking advantage of 2 tumor prone mouse models that are driven by hyperproliferative signals. The first is the mouse carrying an oncogenic allele of K-rasLA1 (27). Following a spontaneous recombination event in the Ras allele, the recombined cells express activated Ras and thus drive tumor progression. The second is the Eμ-myc transgenic mouse model that expresses high levels of c-Myc in B cells and rapidly develops B-cell lymphomas (29). Myc overexpression elevates p53 activity (30, 35). These were crossed to a p53 mutant mouse model inheriting the p53515A missense mutation, encoding the mutant p53R172H protein, and to p53-null mice for comparison of stabilization of mutant protein and tumor phenotypes. We generated homozygous p53 mutant mice that carry the K-rasLA1 allele to avoid wild-type p53 activation by K-ras. The latent K-rasLA1 allele is spontaneously recombined and expresses an active ras protein harboring a glycine to aspartic acid alteration at amino acid 12 (27). We examined whether activated K-ras expression in the lungs affected p53 stability in 4-week-old mice, prior to overt tumor development. By immunohistochemistry, we observed that mutant p53 is visible only in hyperplastic lesions in lungs from young K-rasLA1 p53515A/515A but not in the lungs from young K-rasLA1 mice with wild-type p53 (Fig. 1A). The level of p53 protein expression in different genotypes was also analyzed by Western blotting. p53 levels were higher in the lungs from K-rasLA1 p53515A/515A mice than wild-type, K-rasLA1, or p53515A/515A mice at 4 weeks of age (Fig. 1B). The absence of wild-type p53 staining may be due to the fact that cells activating wild-type p53 initiate senescence or apoptotic programs, quickly rendering p53 undetectable. Stabilization of mutant p53 was also examined in tumors from K-rasLA1 p53515A/515A mice. Immunohistochemistry revealed that mutant p53 was expressed in all tumors (Fig. 1C), which contrasts to the 75% detection rate in tumors that spontaneously arise in p53515A/515A mice (ref. 26; and this study). Thus, activation of K-ras stabilizes mutant p53 in vivo.

Despite stabilization of p53R172H, the survival of K-rasLA1 p53515A/515A and K-rasLA1 p53+/− was similar (Fig. 1D). However, the K-rasLA1 p53515A/515A mice developed more advanced and metastatic lung adenocarcinomas than K-rasLA1 p53−/− mice (Table 1). This fact is highlighted by the observation that 55.4% of the tumors from K-rasLA1 p53515A/515A mice were adenocarcinomas, whereas only 27.8% of K-rasLA1 p53−/− mice developed adenocarcinomas. Taken altogether, these data suggest the shift to a more aggressive tumor phenotype is the result of stabilized p53R172H gain-of-function activities following oncogenic K-ras stimulation.

c-Myc stabilized p53R172H mutant protein but did not accelerate tumor progression

To expand the generality of these findings to other oncogenes, we next assayed how overexpression of c-Myc influenced the stability of p53R172H in vivo. We generated heterozygous p53 mutant mice harboring the Eμ-myc transgene due to prenatal lethality of homozygous mutant p53 mice with Eμ-myc (30). Western blotting and IHC analysis of Eμ-myc p53515A/515A lymphomas showed that all Eμ-myc p53515A/515A splenic lymphomas overexpressed p53 as compared with wild-type spleens (Fig. 2A and B). On the other hand, Eμ-myc p53−/− lymphomas, which delete the one wild-type p53 allele, failed to express p53 (Fig. 2B; ref. 35). To explore whether the expressed
p53 in lymphomas from Eμ-myc p53<sup>15A/−</sup> is wild-type or mutant protein, we analyzed the p53 loci for LOH. Lymphomas from the Eμ-myc p53<sup>15A/−</sup> mice retained a p53 allele, whereas Eμ-myc p53<sup>−/−</sup> mice lost their single wild-type p53 allele (Fig. 2C). Sequencing revealed that 100% (15 of 15) Eμ-myc p53<sup>15A/−</sup> lymphomas lost the wild-type p53 allele (Fig. 2C). Thus, increased expression of c-Myc stabilized p53R172H in vivo.

We also monitored the survival of Eμ-myc p53<sup>15A/−</sup> and Eμ-myc p53<sup>−/−</sup> mice. The Eμ-myc p53<sup>15A/−</sup> mice succumbed to lymphomas at a similar rate as the Eμ-myc p53<sup>−/−</sup> mice showing mean survival of 40 days and 38 days, respectively (Fig. 2D). Thus, while the mutant p53 allele was retained and the protein was stable, no alteration in tumor development was observed.

**Mutant p53 stabilized by p16<sup>Ink4a</sup> loss does not affect overall survival**

Given the fact that oncogenic activation resulted in stabilization of mutant p53, we next examined how loss of a second tumor suppressor, p16<sup>Ink4a</sup>, affected mutant p53 stability and tumor progression. Previously, we showed that mutant protein is stabilized in a variety of tissues from young mice in the absence of p16<sup>Ink4a</sup> (26). To this end, we analyzed the effect of stabilized mutant protein in a cohort of p16<sup>Ink4a</sup>−/− p53<sup>15A/−</sup> mice with stable p53R172H. Western blotting and IHC analysis revealed that mutant p53 protein was detectible in all (11 of 11) p16<sup>Ink4a</sup>−/− p53<sup>15A/−</sup> tumors at varying levels (Fig. 3A and B). Thus, p16<sup>Ink4a</sup> loss also resulted in stabilization of p53R172H in all tumors. Although the survival of p53 homozygous mutant and p53<sup>−/−</sup> mice in a p16<sup>Ink4a</sup>-null background was not statistically significant (Fig. 3C), loss of p16 enhanced the mutant p53 gain-of-function phenotype. We observed that 5 of 17 (29.4%) of the p16<sup>Ink4a</sup>−/− p53<sup>15A/−</sup> mice developed metastatic sarcomas in contrast to the lack of metastatic disease in the p16<sup>Ink4a</sup>−/− and p53<sup>15A/−</sup> mice (26).

**ROS stabilized mutant p53 protein in vivo**

It is widely appreciated that the p53 pathway is activated in response to gains (oncogenes) or losses (tumor suppressors) of genetic material. However, exogenous factors have also been implicated in activation of wild-type p53. One factor, oxidative stress, is caused by ROS and is known to stabilize and activate wild-type p53. High levels of ROS are often observed in solid tumors and are elevated following radiation treatment in cancer patients (36). To test the role of ROS in stabilization of mutant p53 in vivo, we treated pups with γ-radiation to first induce ROS and then treated with the ROS scavenger NAC. We first measured ROS induction in the thymus of p53<sup>15A/−</sup>/C0 mice following irradiation. As shown in Fig. 4A, thymi from p53<sup>15A/−</sup>/C0 mice had 3-fold higher levels of ROS after irradiation. We next irradiated additional pups to examine the levels of p53R172H. Four hours after treatment with ionizing radiation, the pups were then intraperitoneally injected with NAC or PBS. Protein expression was analyzed one hour after NAC or PBS treatment. For this experiment, we treated 2 mice for each condition. Mutant p53 protein was increased in the spleen and thymus after irradiation; however, mutant p53 stabilization was dampened following treatment with NAC in both the thymus and spleen (Fig. 4B). This result indicates that increased ROS levels resulting from γ-radiation also stabilize mutant p53.

**Doxorubicin stabilized mutant p53 protein in vivo**

Although we have shown that both ionizing radiation and ROS result in stabilization of mutant p53 in vivo, other DNA-damaging agents are also often used in clinical settings. The impact of these chemotherapeutic agents on the stabilization of mutant p53 is currently unknown. Doxorubicin is used as a treatment strategy for multiple tumor types. It induces DNA damage resulting in wild-type p53 activation. To determine whether doxorubicin treatment resulted in the stabilization of mutant p53 in vivo, we injected doxorubicin...
in loss of wild-type p53. A, Western blot analysis of p53 in lymphomas from Eμ-myc p53+/-/+ mice. Wild-type (WT) and irradiated (IR) spleens were used for comparison of c-Myc levels. All spleens were lysed by tissue homogenization in NP-40 lysis buffer, followed by centrifugation to remove insoluble proteins and debris. β-Actin serves as a loading control. B, immunohistochemistry of Eμ-myc p53+/-/+ and Eμ-myc p53-/-/+ lymphomas using a p53 antibody. C, PCR analysis of the p53 locus in lymphomas from Eμ-myc p53+/-/+ and Eμ-myc p53-/-/+ mice. The PCR primers were designed to amplify p53 from exon 5 to intron 6. The asterisk denoted the p53 allele results into 5- and 6-day-old pups. Western blot analysis revealed that p53 levels increased after doxorubicin treatment not only in the thymi of homozygous mutant mice but also in wild-type and p53 heterozygous mice (Fig. 5A). Homozygous p53 mutant pups treated with doxorubicin once a day for 2 consecutive days starting at day 5 were monitored for tumor formation and survival. Doxorubicin-treated mutant mice showed no difference in survival as compared with doxorubicin-treated p53-null mice (Fig. 5B). Interestingly, treated p53 homozygous mutant mice died significantly earlier due to increased tumor burden than untreated p53 mutant mice, suggesting that timing or mechanisms by which the mutant p53 protein is stabilized may impact tumor onset.

Stabilization of p53R172H by γ-radiation alters survival

Previously, we observed that γ-irradiation of 4-week-old p53 homozygous mutant mice resulted in a longer half-life of the mutant protein over a 15-hour time period than the wild-type protein (26). To further explore the effects of mutant p53 stabilization, we treated pups with a low dose of radiation as
p53-/- pups treated with γ-radiation show significant differences in survival as compared with untreated mice (37). Irradiated p53+/+ pups died significantly sooner than irradiated p53-/- mice and nontreated control mice (Fig. 5C; \( P = 0.036, P < 0.0001 \), respectively). Importantly, 100% of the tumors from irradiated mice expressed stable mutant p53 (Fig. 5D). This result is contrasted by the fact that only 75% of spontaneous tumors from p53+/+ mice expressed stable mutant p53 (ref. 26, and this study). Together, these results suggest that radiation-induced stability of mutant p53 results in a deleterious gain-of-function phenotype manifested by development of multiple tumors and decreased survival as compared with irradiated p53-/- mice.

Discussion

Mutant p53, like wild-type p53, is inherently unstable (26). However, p53R172H becomes stabilized in the absence of Mdm2 and p16\(^{ink4a}\) and also has an extended half-life as compared with wild-type p53 in response to DNA damage signals (26). These data indicate that mutant p53 may be stabilized by mechanisms that also stabilize wild-type p53, implying that chemotherapeutic strategies that aim to activate wild-type p53 will also stabilize mutant p53. Because p53 is mutated in more than 50% of tumors and expression of mutant p53 is associated with poor outcomes, we asked whether other factors, such as those that contribute to human tumor formation (oncogenic activation) and clinical therapeutics (irradiated and doxorubicin), stabilize mutant p53.

Two potent oncogenes, K-ras and c-Myc, were used to determine their impact on mutant p53 stability in vivo.
mouse models. Expression of each oncogene resulted in the stabilization of mutant p53 but did not dramatically shorten life span compared with p53-null mice. Significantly, the K-ras\textsuperscript{LA1} p53\textsuperscript{515A/515A} displayed a more aggressive tumor phenotype than the K-ras\textsuperscript{LA1} p53\textsuperscript{+/−} mice; 55% and 28% adenocarcinoms, respectively. These results confirm that the gain-of-function ability of p53R172H is associated with stabilized mutant protein and indicate that p53 mutations are more detrimental than p53 loss. However, this does not occur in E6–Myc p53\textsuperscript{515A/515A} mice, suggesting either the timing of stabilization or tissue specific differences impact manifestation of the gain-of-function phenotypes.

Many chemotherapeutic strategies aim to activate the p53 tumor suppressor signaling pathway. Here, we tested whether different DNA-damaging agents such as doxorubicin, γ-radiation, and ROS affect mutant p53 protein levels in vivo. Doxorubicin and γ-radiation are standard therapeutic agents used to treat various cancers as they induce a powerful DNA-damaging response which elicits a p53 response. Our findings show that these therapeutic strategies stabilize p53 expression regardless of mutation status. As a result, γ-radiation yielded a gain-of-function phenotype by decreasing survival of mutant mice. Given that irradiated p53\textsuperscript{515A/515A} mice die sooner than irradiated p53\textsuperscript{+/−} mice, it is tempting to speculate that harmful outcomes may occur in human patients who harbor mutated p53 following therapeutic treatment regimens. Furthermore, the median survival for doxorubicin-treated p53\textsuperscript{515A/515A} and p53\textsuperscript{+/−} mice was 99 and 126 days, respectively. Given the potential clinical importance of these preliminary findings, it will be imperative going forward to compare the outcome and survival of patients harboring wild-type or mutant p53.

In addition to chemotherapeutic treatment, oxidative stress, mainly caused by ROS, is an important factor leading to the activation of the p53 pathway (38, 39). Accumulating data suggest that tumors treated with radiation contain high amount of ROS (40). Studies in p53\textsuperscript{+/−} mice have shown that the antioxidant function of p53 may directly contribute to the prevention of tumor development (41). This may have important implications for p53 in the regulation of redox-sensitive survival pathways. Our results showed that decreasing the level of ROS resulted in decreased mutant p53 protein expression. This result indicates the gain-of-function phenotype resulting from stabilized mutant p53 may be overcome by inhibiting DNA damage caused by ROS and suggests that management of ROS levels in patients with mutant p53 may be warranted.

The survival of mutant mice was clearly affected in response to certain stresses but not others. Irradiated mutant mice showed a significant decreased latency in tumor formation as compared with p53\textsuperscript{+/−} mice. Interestingly, however, the survival of p53 mutant mice that are also p16\textsuperscript{−/−} or have activated Ras was not different from p53\textsuperscript{+/−} mice in these respective backgrounds, even though there were changes in tumor spectrum and metastatic potential. Therefore, other cooperating events, timing of insult, or tissue specificity may all contribute to outcome.

In conclusion, our data indicate that p53R172H is regulated by many of the same signals that regulate wild-type p53. The importance of this study cannot be overemphasized, especially with regards to tumor treatment. The molecular mechanisms of mutant p53 stabilization present a fundamental conundrum in therapeutic intervention, not only for patients with Li-Fraumeni syndrome but also for cancer patients with spontaneous p53 mutations. These data suggest that direct knowledge of the p53 status of a patient may be critical in preventing unintended consequences when determining therapeutic strategies. This study also emphasizes the need for individually tailored treatment for cancer patients depending upon their p53 mutation status.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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