p53 Selective and Nonselective Replication of an E1B-deleted Adenovirus in Hepatocellular Carcinoma


ABSTRACT

An E1B gene-attenuated adenovirus (dl1520) has been proposed to have a selective cytolytic activity in cancer cells with a mutation or deletion in the p53 tumor suppressor gene (p53-null), a defect present in almost half of human hepatocellular carcinomas (HCCs). In this study, the in vitro and in vivo antitumor activity of dl1520 was investigated focusing on two human HCC cell lines, a p53-wild type (p53-wt) cell line and a p53-null cell line. dl1520 was tested for in vitro cytopathic effects and viral replication in the human HCC cell lines Hep3B (p53-null) and HepG2 (p53-wt). The in vivo antitumor effects of dl1520 were investigated in tumors grown s.c. in a severe combined immunodeficient mouse model. In addition, the combination of dl1520 infection with systemic chemotherapy was assessed in these tumor xenografts. At low multiplicities of infection, dl1520 had an apparent p53-dependent in vitro viral growth in HCC cell lines. At higher multiplicities of infection, dl1520 viral replication was independent of the p53 status of the target cells. In vivo, dl1520 significantly retarded the growth of the p53-null Hep3B xenografts, an effect augmented by the addition of cisplatin. However, complete tumor regressions were rare, and most tumors eventually grew progressively. dl1520 had no effect on the in vivo growth of the p53-wt HepG2 cells, with or without cisplatin treatment. The E1B-deleted adenoviral vector dl1520 has an apparent p53-dependent effect in HCC cell lines. However, this effect is lost at higher viral doses and only induces partial tumor regressions without tumor cures in a human HCC xenograft model.

INTRODUCTION

HCC is the most common cause of cancer death worldwide (1). Surgical resection or liver transplantation provide long-term disease-free remissions, but only a minority of patients are candidates for these treatments (2). Furthermore, recurrence rates for those who undergo resection approach 70% (3). Therefore, the great majority of HCC patients will ultimately die of their disease. Homozygous mutations or deletions of p53, a tumor suppressor gene that encodes a nuclear phosphoprotein that is a key regulator of the cell cycle (5), have been reported in 30–50% of HCCs (4). Mutant p53 correlates with tumor dedifferentiation, metastatic potential, and other poor outcomes (4). Mutant p53 correlates with nuclear phosphoprotein that is a key regulator of the cell cycle (5), suggesting that the E1B-deleted adenovirus may have selectivity for tumors in which the functional p53 is required for normal cell growth. This hypothesis has been recently challenged by Hall et al. (11), who have suggested that functional p53 is required for dl1520 replication. Based on these conflicting reports, we tested the effects of dl1520 in several cell lines to determine whether this vector has a selective tumor cytotoxic effect capable of treating p53-null liver cancers. Our data suggest a viral dose-dependent effect rather than a p53-selective effect of dl1520 in p53-wt and p53-null HCC cell lines.

MATERIALS AND METHODS

Mice and Cell Lines. Male 6–9-week-old CB17 Beige SCID mice were bred and housed in a controlled pathogen environment at the Experimental Radiation Oncology Animal Facility at the UCLA. All animal studies were conducted in accordance with the UCLA Animal Care Policy as prescribed by the Chancellor’s Animal Research Committee. Hep3B and HepG2 are well-characterized human HCC cell lines (13), whereas HeLa and C33A are human cervical carcinoma cell lines. Hep3B, HepG2, and HeLa were obtained from American Type Culture Collection (Manassas, VA). C33A was provided by Dr. Arnold Berk (UCLA). All four cell lines were maintained as monolayers and serially passaged in culture in RPMI 1640 containing 10% FCS (Gemini Products, Calabasas, CA) and 1% (v/v) penicillin, streptomycin, and fungizone (Gemini; complete media) and incubated at 37°C in 5% CO2. Human embryonal kidney 293 cells, provided by Dr. Kohnosuke Mitani (UCLA), were passaged similarly in DMEM. Tumors for in vivo studies were maintained through serial passage in SCID mice as described previously (14).

Assessment of p53 Status. The p53 status of Hep3B, HepG2, and HeLa tumor cell lines, which is already well documented (15–17), was reconfirmed using a Human p53 Amplifier Panel (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. DNA from cultured cells was subjected to the PCR and found to be as reported: HepG2 and HeLa were found to be positive for all exons examined (2–3, 5–11); whereas Hep3B was deleted in exons 8–11. The human cervical carcinoma C33A contains an inactivating mutation of the p53 gene at codon 273 (18).

Viruses. dl1520 and Ad5 (wt Ad5) were kind gifts of Dr. Arnold Berk (UCLA). dl1520 is a E1B double mutant human group C chimera (Ad2 and Ad5) in which a deletion of nucleotides 2496–3323 and a C to T transition at position 2022 render the E1B M5,50,000 gene product functionally inactive (9). AdV-Lac (generously provided by Dr. Michael Barry, University of Texas–Southwestern, Dallas, TX) is a replication-incompetent, E1 region-deleted type-5 AdV that contains the firefly Photinus pyralis luciferase reporter gene to and inactivating the p53 gene product (7, 8), thereby enabling the virus to overcome restrictions imposed on viral replication by the host cell cycle. In hosts with tumors carrying p53 mutations, a wt AdV will have a replication advantage in the p53-mutated tumor cells but will have difficulty replicating in p53-wt normal cells (due to the p53 block). If the AdV is modified to lack expression of the E1B M5,50,000 protein, it will maintain its ability to replicate in p53-null tumor cells while having further difficulty replicating in p53-wt normal cells (the effect of p53 will not be blocked by the AdV E1B gene product). Barker and Berk (9) constructed one such virus (dl1520), and its in vitro and in vivo effects on p53-wt and p53-null cell lines have been analyzed by Bischoff et al. (10) and Heise et al. (11). Results from these studies suggest that dl1520 replicates more efficiently in cells lacking a functional p53 gene. When tested in vivo, this virus was able to eradicate p53-null tumors, supporting a selective growth advantage in cells lacking functional p53. This hypothesis has been recently challenged by Hall et al. (12), who have suggested that functional p53 is required for dl1520 replication. Based on these conflicting reports, we tested the effects of dl1520 in several cell lines to determine whether this vector has a selective tumor cytotoxic effect capable of treating p53-null liver cancers. Our data suggest a viral dose-dependent effect rather than a p53-selective effect of dl1520 in p53-wt and p53-null HCC cell lines.

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4 The abbreviations used are: HCC, hepatocellular carcinoma; AdV, adenovirus; SCID, severe combined immunodeficient; MOI, multiplicity of infection; CPE, cytopathic effect; pfu, plaque-forming unit; wt, wild type; UCLA, University of California Los Angeles; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
under the transcriptional control of the cytomegalovirus promoter (14, 19). Working stocks of virus were grown on 293 human embryonal kidney cells, purified on a CsCl gradient, and titered on 293 cells (19).

**In Vitro CPE Assays.** Cultured cells were added to 34-mm wells at a concentration of 3 x 10^4 cells/well. Cultures were transduced 16 h later in AdV infection media (RPMI 1640 + 2% FCS) with dl1520, AdV-Luc, or wt AdV for 2 h at various MOIs. Cells were then washed with PBS, incubated at 37°C, and observed daily for a CPE by the wt AdV at a MOI of 0.1 (which served as a positive control for viral replication). Once this effect was demonstrated (usually between days 4 and 9, depending on the individual cell culture), the cells were fixed and stained with 10% formalin/crystal violet solution.

**Colorimetric Cell Viability Assay.** A colorimetric assay using tetrazolium and MTT (Sigma, St. Louis, MO) was used to assess cell viability after viral infection. Cells were infected at various MOIs of wt AdV or dl1520 for 2 h at 37°C. Uninfected cells served as controls. After infection, cells were washed with PBS and placed, in triplicate, in a 96-well plate at a concentration of 1 x 10^5 cells/well in 200 μl of complete growth media. Eight days later, 25 μl of MTT (4 mg/ml) were added to each well and incubated at 37°C for 4 h. Plates were centrifuged at 500 x g for 5 min, and the medium was aspirated with care so as not to disturb the formazen crystals at the bottom of the well. DMSO (100 μl) was added to each well to solubilize the crystals. Plates were read at A570 nm on a scanning multwell spectrophotometer (Bio-Rad, Hercules, CA). Results are reported as the percentage of absorbance of uninfected control cells.

**In Vitro Viral Replication Assay.** Cells were plated in 6-well plates at a concentration of 4 x 10^3 cells/well. Sixteen h later, the cellular monolayers were washed with PBS and infected with virus at a MOI of 0.05 mg/ml of AdV infection media at 37°C for 90 min. After removal of the unadsorbed virus, the monolayers were washed once with PBS and incubated at 37°C for different periods of time in reduced serum (5%) growth media. Plates corresponding to individual time points were subsequently frozen at −80°C. Lysates were prepared by three cycles of freezing and thawing. Serial dilutions were subsequently titered on 293 human embryonic kidney cells.

**In Vivo Tumor Model.** Progressively growing tumors were passaged in vivo as described previously (14). When tumors reached an average diameter of 4–6 mm, mice were randomly allocated to various treatment groups. Intratumoral dosing regiments varied with each particular study, ranging from a total dose of 1 x 10^5 to 6 x 10^5 pfu/tumor, applied over 3–5 consecutive days. In most experiments, those doses at 2 x 10^5 pfu/tumor of either dl1520, AdV-LUC, or wt AdV were injected in 100 μl of PBS vehicle (equally distributed in all four quadrants of the tumor). For studies combining virus and chemotherapy, 2 x 10^5 pfu of dl1520 were injected intratumorally for 3 consecutive days, followed by i.p. administration of cisplatin ([cis-diaminodichloroplatinum(II); 4 mg/kg]) every other day over the ensuing 6 days. Tumor growth was monitored twice weekly using a caliper. Tumor volume was estimated using the following formula: volume = 0.5 x l x w^2.

**Statistical Analysis.** Student's t test or the rank-sum test (if failing the Kolmogorov-Smirnov test for normality) was performed to interpret the significance between final tumor volumes of animals treated with dl1520 and those treated with other therapies. Two-sided P values reflect individual comparisons.

**RESULTS**

**In Vitro Effects of dl1520 on p53-wt and p53-null HCC Cell Lines.** The CPE of dl1520 on the HCC cell lines HepG2 (p53-wt) and Hep3B (p53-null) was examined. The p53-wt cell line Hela and the p53-mutated human cervical carcinoma (C33A) were used as negative and positive controls, respectively. The effect of dl1520 was compared to a wt AdV (expected to lyse all cell lines) and a replication-incompetent E1-deleted AdV (AdV-Luc, which was expected to be nonreplicative and nonlytic in all cell lines except 293 cells). Results of a crystal violet CPE assay are presented in Fig. 1. The wt AdV completely lysed all cell types even at a MOI of 0.1, corresponding to 1 viral particle for every 10 tumor cells, suggestive of active viral replication and a CPE leading to complete cell lysis. The E1-deleted AdV-Luc vector only displayed a CPE on the permissive 293 cells that express E1 gene products in trans. dl1520 had different effects in the tested cell lines. It lysed 293 cells, consistent with the presence of the E1 gene products including the E1B M protein, enabling effective viral replication. It lysed the two p53-null lines, the cervical carcinoma C33A and the HCC line Hep3B, at a MOI of 0.01–0.1, suggestive of active viral replication. This effect was not noted in the p53-wt cell lines Hela and HepG2 at a MOI of 0.1. However, the HepG2 culture was completely lysed at a MOI of 1. In an MTT assay, dl1520 also displayed a higher CPE on the two p53-null cell lines tested (Fig. 2). To achieve a similar decrease in cell colonies, dl1520 had to be added at a MOI of at least 1 to the p53-wt cell lines Hela and HepG2. Taken together, the observed effects of low viral multiplicity of dl1520 on the HCC cell lines correspond to 1–2-log difference in cytopathic efficacy for the p53-null Hep3B in both the qualitative crystal violet CPE assay and the more quantitative MTT chromogenic assay. This differential effect decreases or disappears when a higher MOI is used.

**dl1520 Reproductive Cycle in p53-wt and p53-null HCC.** The adenoviral reproductive cycle in target cells was studied using a one-step growth curve assay (Fig. 3). In this assay, the infectious process is initiated at high viral concentrations (MOI, 5), which ensures a rapid, nearly synchronous infection in all target cells. To eliminate unadsorbed virus, cultures were washed after AdV infection. Then, at various times after the initiation of infection, culture samples were tested for infective virus levels on a plaque assay in 293 cells as described in “Materials and Methods.” The wt AdV shows a viral replication curve in the three tested cell lines. This viral growth curve consists of an initial adsorption period, an eclipse period when no infective viral particles can be recovered from the culture, and a virus release or burst period, when the virions are released from the infected cells and can be titrated in a plaque assay. The E1-deleted replication-defective vector AdV-Luc demonstrates a nonreplicative viral cycle in all three cell lines. After viral adsorption, AdV-Luc viral particles cannot reassemble and generate new infective virions due to the absence of the E1 gene products. The viral reproductive cycle of dl1520 was tested on one p53-null cell line (Hep3B) and two p53-wt cell lines (HeLa and HepG2). The viral growth curve of dl1520 on Hep3B cells was nearly equivalent to that of the wt AdV, suggesting that the absence of E1B genes in dl1520 did not hamper its ability to replicate in this p53-null HCC cell line. As originally described by Barker and Berk (9), dl1520 infection of the cervical carcinoma line HeLa generates a 2-log lower growth curve compared to the growth curve of wt AdV in this cell line, suggesting a somewhat defective growth of dl1520 in HeLa cells. However, dl1520 generated an effective replicative growth curve in the other p53-wt cell line. As observed in the CPE assays (Figs. 1 and 2), infection of HepG2 with dl1520 at a MOI greater than 0.1 generates a viral growth cycle with similar kinetics to wt AdV in this p53-wt HCC cell line. In conclusion, at a sufficiently high MOI, dl1520 replicates at a similar rate in p53-null and p53-wt HCC cell lines.

**In Vivo Antitumor Activity of dl1520 in Human HCC Xenografts.** The antitumor effects of repeated intratumoral injections with dl1520 were studied in two human HCC xenografts: (a) Hep3B (p53-null); and (b) HepG2 (p53-wt). dl1520 was administered at doses ranging from 1–6 x 10^5 pfu/injection to mice with tumors with mean diameters of 4–6 mm, whereas randomly allocated control mice received injections with PBS. Intratumoral injection of dl1520 into the p53-wt HepG2 cells did not alter tumor growth in a total of eight studies using 34 mice with HepG2 tumors (Fig. 4a). No growth retardation was observed in PBS-treated tumors. In contrast, dl1520 resulted in significant tumor growth retardation in nine studies in which 39 mice with Hep3B tumors (p53-null) were treated with dl1520; again, no responses were seen in the PBS-treated mice (Fig.
However, complete tumor regression was rare (observed only in one animal), and tumors ultimately grew progressively, despite repeated dl1520 treatment (data not shown).

The in vivo effect of dl1520 was compared to the wt AdV and the E1-deleted AdV-Luc (Fig. 5). Significant tumor growth inhibition of Hep3B was observed with either dl1520 (P = 0.01) or wt AdV (P = 0.02) treatment, whereas the tumor growth curve was only slightly diminished by administration of AdV-Luc (P = 0.4). In HepG2 cells, wt AdV significantly altered the tumor growth when compared with saline-injected controls (data not shown), whereas dl1520 repeatedly showed no effects, ruling out the possibility that the lack of in vivo effect of dl1520 in this tumor is an inherent defect of in vivo AdV replication. In conclusion, dl1520 significantly retards the growth of a p53-null HCC xenograft but has no effect on the growth of a p53-wt HCC xenograft.

dl1520 and Chemotherapy Have Additive Antitumor Effects. The combination of dl1520 with the chemotherapeutic agent cisplatin, which has moderate efficacy for human HCC, was investigated. When compared to saline-injected controls, single-agent therapy with either intratumoral dl1520 (P = 0.003) or systemic chemotherapy (P = 0.007) displayed equivalent growth retardation. Combined treatment with dl1520 followed by cisplatin demonstrated markedly improved antitumor effects when compared to treatment with either dl1520 or cisplatin alone (Fig. 6a). Four of five tumors in the combined therapy group initially regressed, whereas the fifth tumor stabilized. Although some tumors in this group displayed a transient complete response, eventual tumor outgrowth occurred in all five tumors. In contrast, the addition of dl1520 to cisplatin treatment of HepG2 tumors did not improve the antitumor effect of cisplatin alone (Fig. 6b).
DISCUSSION

AdVs are DNA viruses that cause limited pathology in humans, mainly conjunctivitis and flu-like syndromes. Their life cycle consists of: (a) an adsorption and viral entry into the host cells, when the viral capsid interacts with the Coxsackie and AdV receptor on the target cell membrane (20); (b) an eclipse period, when viral particles are nondetectable in the infected cells because they disintegrate and use the host’s cell replication machinery to synthesize their own DNA, mRNA, and proteins; and (c) the assembly and burst period, when multiple virions assemble in the cytoplasm and eventually lyse the host cell to infect neighboring cells. As vehicles for gene therapy strategies, AdVs are rendered replication incompetent by disruption of the E1 region, which encodes early proteins required for the synthesis of new viral DNA. The cDNA of the gene of interest to be used in gene therapy strategies is placed in the empty AdV E1 region. This results in viral particles capable of infecting a single host cell, where they produce mRNA of the inserted gene, but the eclipse period stops before the production of new viral particles because the E1 gene products are not synthesized. To allow viral particle assembly, the 293 cell line was constructed (21). This cell line, derived from a human embryonal kidney cancer line, was stably transfected with the AdV E1 gene. Therefore, when E1-deleted AdV vectors infect 293 cells, they will generate infective viral particles because all viral products will be synthesized in the host cells.

AdV E1 consists of two regions, E1A and E1B. dl1520 was originally constructed by Barker and Berk (9) to study the function of the E1B genes. They demonstrated that the E1B gene region has a critical function in AdV replication because it encodes proteins that dysregulate normal cell growth to the advantage of viral replication. One of its products, the M₅₅,000 protein, binds and inactivates the p53 gene product in the infected cells. If the E1B gene is not expressed by the AdV, replication does not proceed because the p53 protein detects a malfunction in the cell’s replication cycle and drives its machinery to cell cycle arrest or apoptosis. However, when the E1B region is expressed by the infecting AdV particles, it will bind to p53 and inactivate it. This allows the virus to use the host cell replication machinery to generate multiple viral particles and ultimately lyse the cell (7, 8, 17).

Fig. 3. Viral replication in cultured human HCC cells Hep3B (p53-null) and HepG2 (p53-wt). HeLa cervical carcinoma cells serve as a p53-wt, non-HCC comparison. The viral growth curves illustrated were generated after infection with dl1520, wt AdV, or the replication-incompetent AdV-Luc at a MOI of 5. After a 90-min incubation, cells were washed with PBS, incubated at 37°C in 5% complete media, and frozen at various time points. After three freeze/thaw cycles, the viral content of the lysates was titered on a 293 plaque assay. Data were consistent in two replicate studies.

Fig. 4. In vivo growth of human HCC after treatment with dl1520. The 4–6-mm HepG2 (p53-wt; a) and Hep3B (p53-null; b) tumors in C17/Beige SCID mice were treated intratumorally with 2 × 10⁷ pfu of dl1520 for 5 consecutive days (as indicated by arrows; days 21–25 for Hep3B and days 14–18 for HepG2) in 100 µl of PBS. Control mice received an equivalent volume of intratumoral PBS. Significant tumor growth inhibition was evident by day 22 after initiation of therapy for Hep3B cells (P < 0.0001), but not for HepG2 cells (P = 0.8 at day 20 after initiation). Data are presented as the mean tumor volume ± SE.
presented as the mean tumor volume compared with p53 replacement gene therapy, the curative, despite repeated virus administration. Also, when directly propagated in SCID mice, may exert p53-dependent growth inhibition in human HCC xenografts.

We tested the activity of vectors have been shown to selectively target the liver following any route of administration (14, 19). Our results using cervical carcinoma and glioblastoma cell lines suggest that d1520 replicates 100 times more efficiently in cells lacking functional p53. However, when testing was extended to additional cell lines, this selective growth was not supported. d1520 demonstrated a CPE in 7 of 10 p33-wt cell lines (including HepG2) tested (11). Furthermore, there is evidence that, in certain situations, p53-wt plays a necessary role in mediating cellular destruction to allow a productive AdV infection (12).

There is no effective systemic treatment for HCC (1–3). Because most human HCC tumors have mutations or deletions in the p53 gene (4), we tested the activity of d1520 in this disease. Moreover, AdV vectors have been shown to selectively target the liver following any route of administration (14, 19). Our in vivo data suggest that d1520 may exert p53-dependent growth inhibition in human HCC xenografts propagated in SCID mice. In vivo inhibition of tumor growth was not curative, despite repeated virus administration. Also, when directly compared with p53 replacement gene therapy, the in vivo effect of d1520 was fairly comparable and was certainly no better. In our immunodeficient mice, which are unable to mount antibody or cellular immune responses to repeated viral exposure, tumor escape after d1520 injection suggests an acquired resistance to AdV infection, a change in killing susceptibility of tumor cells, or a lack of adequate viral distribution in the tumors. These possibilities were not studied by us. In vivo efficacy of d1520 treatment could be enhanced with concomitant cisplatin therapy; however, it was not a goal of this study to optimize this combined therapy.

Bischoff et al. (10) investigated the effects of d1520 infection on p53-null cancer cells. Theoretically, d1520 would replicate in these cells because p53 is not present to prevent viral replication. This would generate a replication-competent environment in which the E1B-deleted AdV could produce multiple viral particles that would lyse the p53-null target cells. However, if these viral particles infected p53-wt cells, viral replication would not proceed. Therefore, these investigators (10) used d1520 to infect a panel of p53-wt and p53-null cell lines both in vitro and in vivo. Their results using cervical carcinoma and glioblastoma cell lines suggest that d1520 replicates 100 times more efficiently in cells lacking functional p53. However, when testing was extended to additional cell lines, this selective growth was not supported. d1520 demonstrated a CPE in 7 of 10 p33-wt cell lines (including HepG2) tested (11). Furthermore, there is evidence that, in certain situations, p53-wt plays a necessary role in mediating cellular destruction to allow a productive AdV infection (12).

Unpublished observations.
A wide range of dl1520 viral doses could not be tested in vivo because most mice died after an intratumoral injection of 5 × 10⁹ pfu. Therefore, we used in vitro CPE testing and viral growth curve assays over a wide MOI range. In these studies, the apparent p53 selectivity was lost, suggesting that other factors play a role in dl1520 biology (11, 17). In the original description of dl1520, Barker and Berk (9) demonstrated that this vector replicated in p53-wt HeLa cells in a viral dose-dependent manner, generating viral growth curves superimposable to ours. Further testing of dl1520 in HeLa cells by Goodrum and Ornelles (17) suggests that the E1B M₉ 55,000 protein functions directly in overcoming the growth restrictions imposed on viral replication by the cell cycle in a mechanism that is p53-independent. In their extensive studies, HeLa cells were synchronized to S phase or G₁. Compared to cells infected in G₁, cells in S phase infected with dl1520 had a greater number of infectious centers, produced higher levels of virus progeny, and demonstrated a near wt CPE. Therefore, dl1520 has acquired a dependence on the cell cycle for virus progeny production and replicates in cells in S phase. Taken together with our data, when sufficient dl1520 viral particles are delivered and sufficient target cells are in S phase, dl1520 may replicate regardless of the p53 status. The presence of contaminating wt replication-competent virus would be an alternative explanation for the loss of p53 selectivity in vitro. Although it is certainly possible that our dl1520 may be contaminated with replication-competent virus, the similarity of our viral growth curves and the ones from Barker and Berk (9) and Goodrum and Ornelles (17) tested in the same cell line (HeLa) argue against their presence.

Our in vivo data cannot be translated directly to a clinical setting. We used immunodeficient mice that have a greatly diminished ability to block viral replication. This may allow for greater viral replication and corresponding tumor lysis in this animal model. The great majority of adult humans have antiadenoviral-neutralizing antibodies, which might limit the effectiveness of this treatment approach. Furthermore, AdVs infect human cells more easily than murine cells (21). Therefore, dl1520 would have a selective advantage in lysing human cancers in our SCID mouse model without generating active infection in the mouse. In conclusion, treatment of p53-null HCC with the E1B-deleted dl1520 vector generates permissive viral replication sufficient to lyse p53-null cells in vivo. However, this response is a function of viral dose and schedule and is not entirely dependent on the p53 status of the tumor.

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