Adenoviral Expression of p53 Represses Telomerase Activity through Down-Regulation of Human Telomerase Reverse Transcriptase Transcription

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Abstract

Telomerase activation is a critical step in cellular immortality and oncogenesis. The activity of telomerase is known to be correlated with cell proliferation, but its regulation by cell cycle regulators is not well understood. In the present study, we examined the effects of p53 on telomerase activity. Wild-type p53 was introduced into SiHa cells via a recombinant adenoviral vector, Ad5CMV-p53, and change in telomerase activity was examined by quantitative telomerase assay. Telomerase activity in the Ad5CMV-p53-infected cells was significantly repressed 36 h after infection following down-regulation of human telomerase catalytic subunit [human telomerase reverse transcriptase (hTERT)] mRNA expression, whereas no change in telomerase activity was observed in the cells infected with control vector Ad5CMV-β-gal. Interestingly, repression of telomerase activity was an early event that preceded cell growth inhibition or apoptosis induced by p53 overexpression, suggesting that p53 directly regulates telomerase activity. Transient expression assays using hTERT-promoter reporter constructs revealed that overexpression of p53 significantly repressed promoter activity of hTERT. 5′-Truncation of the promoter sequences revealed that the proximal core promoter region containing multiple binding sites for transcription factor Sp1 was responsible for p53-mediated transcriptional repression. Mutations in these binding sites for Sp1 led to failure of p53 to repress transcription. These findings suggest that p53 repressed telomerase activity through down-regulation of hTERT transcription and that interaction of p53 with Sp1 or other transcription factors may be involved in this regulation.

Introduction

Telomeres are the distal ends of human chromosomes composed of tandem repeats of the sequence TTAGGG (1). Possible functions of telomeres include prevention of chromosome degradation, end-to-end fusions, rearrangements, and chromosome loss (2). Human telomeres in somatic cells undergo progressive shortening with cell division through replication-dependent sequence loss at DNA termini (3). The shortening of telomeres results in chromosomal instability, and critically short telomeres induce cell cycle arrest, termed replicative senescence. Telomerase is a specialized ribonucleoprotein polymerase containing an integral RNA with a short template element that directs the synthesis of telomeric repeats at chromosome ends (1). Activation of telomerase is thus thought to be essential for stabilizing telomere length and overcoming replicative senescence, which may lead to cellular immortality (4).

A number of studies using newly developed telomeric repeat amplification protocol assays have demonstrated that telomerase activity is observed in most malignant tumors but is usually repressed in normal somatic tissues, suggesting that telomerase activation may be a critical step in cellular immortality and carcinogenesis (5, 6). However, subsequent studies have revealed that telomerase is activated not only in tumors but also in some types of normal somatic cells, such as hematopoietic stem cells, keratinocytes in basal layers of the epidermis, and uterine endometrial cells, all of which have high regenerative and proliferative capacity (7–9), and that telomerase activity is up-regulated on cell proliferation and cell cycle exit into G0 or cell differentiation accompanies down-regulation of telomerase activity (10–12). These findings suggest that the factors that regulate the cell cycle may play roles in telomerase regulation. However, little information on such factors has been obtained.

Recent studies have identified three major subunits comprising the human telomerase complex. The RNA component of human telomerase (hTR)2 provides the template for telomere repeat synthesis (13). Telomerase-associated protein (TP1) has also been cloned as a component of telomerase, but its function remains unclear (14, 15). The most important component responsible for the enzymatic activity of telomerase is hTERT (16, 17). Studies have found that hTERT is expressed in most malignant tumors but not in normal tissues, and the expression of

Received 10/20/99; revised 1/24/00; accepted 2/1/00.

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2 The abbreviations used are: hTR, human telomerase RNA; hTERT, human telomerase reverse transcriptase; β-gal, β-galactosidase; LUC, luciferase; MOI, multiplicity of infection; RT-PCR, reverse transcription-PCR; HPV, human papillomaviruses.
hTERT is closely associated with telomerase activity, whereas two other factors are constitutively expressed in both tumors and normal tissues and are not directly associated with the enzymatic activity of telomerase (18–20). Introduction of hTERT cDNA into normal cells confers telomerase activity in these cells (21, 22). hTERT-expressing normal cell clones have an extended life span without any change in karyotype (23). These findings suggest that hTERT is a critical determinant of telomerase activity.

Recently, abrogation of p53 function by the introduction of mutant p53 has been reported to induce cellular immortality and telomerase reactivation in mammary epithelial cells (24). It has also been reported that HPV-16 oncoprotein E6, which is known to bind p53 and to promote its degradation, up-regulates telomerase activity (25, 26). These findings suggest the possible involvement of p53 in telomerase regulation. In the present study, we use adenovirus-mediated gene transfer to examine the effect of p53 on telomerase activity in cervical cancer cells.

Materials and Methods

Cell Lines. SiHa, ME180, TSU-PR1, and DU145 cells were obtained from the American Type Culture Collection (Manassas, VA). Ishikawa cells were kindly provided by Dr. M. Nishida (Department of Obstetrics and Gynecology, Tsukuba University, Tsukuba, Japan). These cells were grown in DMEM with 10% FCS in the presence of 5% CO2 at 37°C.

Recombinant Adenovirus and Infection. The recombinant p53 adenoviral vector Ad5CMV-p53 contains the cytomegalo-virus promoter, wild-type p53 cDNA, and a SV40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified adenovirus Ad5 (27). The adenovirus containing the β-gal gene, AdICMV-LacZ, was used to determine transduction efficiencies or was used as a control. Viral stocks were propagated in 293 cells, which were derived from primary embryonal kidney cells transformed by introducing sheared fragments of Ad5 DNA. This cell line contains E1 and is thus permissive for the replication of the E1 replication-deficient adenovirus. Virus was purified from this cell to obtain viral stock. Cells were harvested at various time points after infection, pelleted, resuspended in PBS, and lysed by three cycles of freezing and thawing. Cells were harvested at various time points after infection, pelleted, resuspended in PBS, and lysed by three cycles of freezing and thawing. Cell debris was removed by subjecting the lysed cells to CsCl gradient centrifugation. Concentrated virus was dialyzed and stored at −80°C. Infection was carried out by adding the virus to high-glucose DMEM and to the cell monolayers. The cells were infected at 37°C for 60 min with constant agitation. Medium was added, and the cells were incubated at 37°C for the desired length of time. The viral titers were determined by plaque assays.

Stretch PCR Assay. For quantitative analysis of telomerase activity, stretch PCR assays were performed using the Telochaser system according to the manufacturer’s protocol (Toyobo, Tokyo, Japan). The PCR products were electrophoresed on a 7% polyacrylamide gel and visualized with SYBR Green I Nucleic Acid Gel Stain (FMC BioProducts, Rockland, ME). The band intensity of the telomerase ladders was analyzed by NIH Image picture analyzing software.

RNA-PCR Analysis. Analysis of Bel-2 mRNA expression was performed by RT-PCR amplification using the primer pair 5′-ACTTGTGCGCCCGATAGGGGACCAGC-3′ (forward primer) and 5′-CGACTTCGCGAGATGTCAGCCAG-3′ (reverse primer) as described previously (28). hTERT mRNA was amplified using the primer pair 5′-CGGAAGATGTGCTGGAGCAA-3′ (LT5) and 5′-GGATGAAGCGGATCTGGA-3′ (LT6; Ref. 17). hTR was amplified using the primer pair 5′-TCTAACCTCAAAGTGAGGCGTGC-3′ (F3b) and 5′-GTGTCAGCTAGAATGACCGTGAAG-3′ (R3c; Ref. 17). Total RNA was isolated from the tissues using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. cDNA was synthesized from 1 μg of RNA using the RNA PCR kit version 2 (TaKaRa, Otsu, Japan) with random primers. To amplify the cDNA, 2-μl aliquots of the reverse-transcribed cDNA were subjected to 28 cycles of PCR in 50 μl of 1× buffer [10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, and 50 mM KCl] containing 200 μM each of dATP, dCTP, dGTP, and dTTP; 2.5 units of Taq DNA polymerase (TaKaRa); and 0.2 μM of specific primers. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s. PCR products were electrophoresed in 7% polyacrylamide gel and stained with SYBR green (FMC BioProducts). The efficiency of cDNA synthesis from each sample was estimated by PCR with glyceraldehyde-3-phosphate dehydrogenase-specific primers as described previously (17).

Plasmid Construction. The structures of hTERT promoter-LUC plasmids are shown in Fig. 4A. Various lengths of DNA fragments upstream of the initiating ATG codon of the hTERT gene were PCR-amplified and inserted into LUC reporter vector pGLO3-Basic, a promoterless and enhancerless vector (Promega, Madison, WI), in a sense orientation relative to the LUC coding sequence at the MluI and BglII sites. A mutant reporter plasmid (pGL3-181Sp1 MT) containing mutations in five Sp1 binding sites at −109, −88, −56, −36, and −7 was prepared using PCR-based site-specific mutagenesis. As a positive control plasmid, pGL3-Control (Promega) was used, in which the LUC gene is driven by the SV40 LT enhancer/promoter. The names of reporter constructs with serial deletions of the 3.3 kb of hTERT promoter were assigned according to the 5′-end nucleotide numbers of inserted promoter sequences (29). p53 expression vector (pC53-SN3) was kindly provided by Dr. Bert Vogelstein (30).

LUC Assay. Transfection of the plasmids was performed using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) for SiHa cells according to the protocols recommended by the manufacturer or using the calcium phosphate precipitation method for the other cell types (31). LUC assays were performed using the Dual-Luciferase Reporter Assay System (Promega), in which Renilla LUC plasmids were cotransfected as control plasmids to standardize transcription efficiency. All experiments were performed at least three times for each plasmid, and the mean relative LUC activities were determined.

Results

Introduction and Expression of Exogenous p53 in SiHa Cells. We introduced the wild-type p53 gene into SiHa cells via the recombinant adenoviral vector Ad5CMV-p53. To determine the adenoviral transduction efficiency, SiHa cells were infected for 2 days with Ad5CMV-LacZ, an adenovirus that expresses the β-gal gene. The transduction efficiency was as-
pressed by scoring 500 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside-positive cells and then determining the percentage of β-gal-positive blue-stained cells. As shown in Fig. 1A, there was a linear correlation between the number of infected cells and the number of adenovirus particles used for infection. The cells inoculated with a single dose of Ad5CMV-LacZ at a MOI of 12.5 or greater exhibited almost 100% blue-stained cells.

Next, to determine the expression of p53 proteins in the Ad5CMV-p53-infected SiHa cells, Western blot analyses were performed using the mouse antihuman p53 monoclonal antibody DO7. At 24 h after infection, a p53 band recognized by DO7 was observed in cellular extracts isolated from infected SiHa cells (Fig. 1B), especially at a MOI of 25 or greater. Samples isolated from noninfected SiHa cells exhibited very low levels of p53 protein. The time course of p53 expression in SiHa cells is shown in Fig. 1C. Cell extracts were prepared at various time points after infection. p53 protein expression peaked 3 days after infection and declined thereafter, but detectable expression was observed even at day 7.

**Overexpression of p53 Represses Telomerase Activity with Down-Regulation of hTERT.** To examine the effects of p53 overexpression on telomerase activity, SiHa cells were infected with Ad5CMV-LacZ or Ad5CMV-p53 at various MOIs, and cell extracts were isolated 72 h after infection and examined for telomerase activity by the quantitative stretch PCR assay. As shown in Fig. 2A, infection by Ad5CMV-p53 decreased telomerase activity at a MOI of 25 and significantly eliminated it at a MOI of 50, whereas infection by Ad5CMV-LacZ had no effect on telomerase activity. Time course experiments were also performed in which repression of telomerase activity became obvious 48 h after infection, and complete loss of telomerase activity was observed 72 h after infection (Fig. 2B). In contrast, no significant change in telomerase activity was detected in cells infected with Ad5CMV-LacZ as negative controls. We extended analyses on the onset of telomerase repression, monitoring telomerase activity every 6 h, and we found that repression occurred 36 h after the infection (Fig. 2C). Expression of hTERT and hTR was next examined by RT-PCR analyses. A significant decrease in hTERT mRNA expression was observed 12 h after infection with Ad5CMV-p53, whereas expression of hTR was not altered during the time course of observation (Fig. 2D). Thus, overexpression of p53 down-regulated hTERT mRNA expression, followed by telomerase repression. A recent study reported that antiapoptotic factor Bcl-2 activates telomerase (32). Because the expression of Bcl-2 is known to be regulated by p53 in some cell types (33), the change in Bcl-2 expression after infection by Ad5CMV-p53 was examined by RT-PCR analyses, but no significant change in expression was detected during the time course of observation, suggesting that deregulation of Bcl-2 is not involved in telomerase repression by p53.

**Repression of Telomerase Activity Does Not Accompany Growth Inhibition or Apoptosis of SiHa Cells.** Recent studies have demonstrated a close correlation between telomerase activity and cell proliferation (7–12). To examine whether repression of telomerase activity was secondary to growth inhibition induced by p53, cell growth was monitored after infection by Ad5CMV-p53. The growth rate of SiHa cells infected with Ad5CMV-p53 was comparable to that of control cells infected with Ad5CMV-LacZ, suggesting that telomerase repression by p53 did not result in growth inhibition or apoptosis of SiHa cells.
Fig. 2 Repression of telomerase activity by p53. A, stretch PCR assays to quantify relative telomerase activity in SiHa cells infected with Ad5CMV-p53 or Ad5CMV-LacZ at different MOIs. IC, internal controls to verify the efficacy of PCR amplification. P, positive control samples (C33A cell extracts). N, negative control samples (lysis buffer only). B, time course of telomerase activity in SiHa cells infected with Ad5CMV-p53 or Ad5CMV-LacZ at a MOI of 50. C, time course of telomerase activity between 24 and 42 h after infection. D, expression of hTERT mRNA, hTR, and Bcl-2 mRNA as determined by RT-PCR assays at various time points after infection.
with Ad5CMV-LacZ, and no significant growth inhibition was observed for 42 h after infection (Fig. 3A).

p53 is known to induce apoptosis. To examine whether apoptotic changes induced by p53 play a causative role in telomerase repression, the cell cycle profile was examined using flow cytometric analyses. Between 24 and 42 h after infection, no significant change in cell cycle profile was observed, and the sub-G0-G1 fraction, a characteristic marker of apoptosis, was not detected, suggesting that apoptosis was not induced during this period of observation (Fig. 3B). When cells infected with Ad5CMV-p53 were fixed and subjected to a terminal deoxynucleotidyl transferase-mediated nick end labeling assay, no increase in stained cells was detected after infection (data not shown). Taken together, these findings indicate that overexpression of p53 in SiHa cells represses telomerase without altering cellular growth properties or inducing apoptosis.

**p53 Represses Transcriptional Activity of hTERT Promoter.**

The finding that p53 down-regulates hTERT mRNA suggests that hTERT expression is controlled by p53 at the transcriptional level. In a previous study, we cloned 3.3 kb of the 5’-flanking sequences of the hTERT gene (29). A computer-assisted homology search revealed two potential p53 binding motifs at −1877 (AGGCTGGTCT) and −1240 (AGGCCTGTTC) in the 5’-flanking sequences (Fig. 4A). To examine whether p53 regulates the promoter activity of hTERT, LUC assays were performed using SiHa cells in which p53 expression vectors were cotransfected with 3.3 kb hTERT-promoter LUC reporters (pGL3–3328). As a result, overexpression of p53 significantly repressed the promoter activity of hTERT (Fig. 4B). Similar inhibition of telomerase activity was observed for other types of cells, such as ME180 and Ishikawa cells (derived from uterine...
Fig. 4 Repression of the transcriptional activity of hTERT by p53. A, LUC reporter plasmids used in the assays. The 181-bp core promoter region essential for transcriptional activation is shown in the black box in which binding sites for Myc/Max and Sp1 are shown. The transcription start site and the ATG initiation codon are shown as +1 and +78, respectively. Putative p53 consensus binding motifs are shown as blank boxes. Mutations introduced into Sp1 binding sites in pGL3–181Sp1 MT are shown as crossed-out boxes. B, transcriptional activity of the 3.3-kb promoter was examined by LUC assays using a variety of cell lines. p53 expression vectors (pC53-SN3) or blank vectors were cotransfected with pGL3–3328 reporter plasmids into various types of cells, and LUC assays were performed. Relative LUC activities, which were determined by comparison with control activities in samples transfected with blank vectors, are shown. Bars, SD. C, identification of the elements responsible for p53-mediated transcriptional repression. p53 expression vectors or blank vectors were cotransfected with each deletion or substitution mutant reporter plasmid, and LUC assays were performed. Bars, SD.
The Proximal Core Promoter of the hTERT Gene that Binds Cellular Factor Sp1 Is Responsible for p53-mediated Transcriptional Repression. To identify the elements responsible for the down-regulation of hTERT, serial deletion mutants of reporter plasmids were prepared (Fig. 4A), and LUC assays were performed. p53 overexpression repressed transcrip-
tional activity not only in wild-type pGL3–3328 but also in its deletion mutants (Fig. 4C). Interestingly, transcriptional activity in pGL3–1375 and pGL3–1175, which lack one or two potential p53 binding motifs, respectively, was also repressed by p53 overexpression. Repression was further observed in mutants with more extended deletions, and the proximal 181-bp region (pGL3–181) could respond to p53 overexpression. Our previous study demonstrated that this region functions as a core promoter essential for transactivation of hTERT in cancer cells (29). This core promoter contains five GC-boxes and an E-box that bind transcription factor Sp1 and Myc/Max, respectively (Ref. 29; Fig. 4A). Myc proteins have been found to be critical factors for activation of hTERT transcription (34, 35). However, deletion of the E-box (pGL3–150) did not affect the repressive effect of p53, indicating that the remaining 150-bp region containing five GC-boxes was responsible for this repression. Interestingly, even the proximal 32-bp region (pGL3–32) containing one Sp1 site responded to p53 overexpression. In contrast, p53 overexpression failed to repress transcription of mutant p-181 (pGL3–181Sp1 MT), in which all Sp1 sites had been eliminated by substitution mutations.

Discussion

In the present study, we demonstrated that p53 represses telomerase activity in SiHa cells through down-regulation of hTERT transcription. In our assays, we used SiHa cells in which HPV-16 DNA is integrated in the genome. To evaluate the effects of p53 overexpression, it might be better to use cell lines in which wild-type p53 functions have been disrupted. We therefore initially used the C33A cervical cancer cell line, in which both alleles of the p53 gene are mutated and normal p53 function has been lost. However, adenovirus-mediated introduction of p53 easily induced apoptosis in this cell type, and most cells died with even minimal titers of viral infection, making it difficult to evaluate the effects of p53 on telomerase activity. In contrast, SiHa cells were more resistant to p53-induced apoptosis and remained alive for at least several days after introduction of the p53 gene, allowing significant expression of exogenous p53. In addition, HPV-16 E6 protein expressed in SiHa cells is known to bind endogenous p53 and to promote its degradation, suggesting that normal function of p53 is at least partly disrupted in this cell type. These findings prompted us to use SiHa cells for p53 gene transfer.

Telomerase is known to be a regulated enzyme, and its activity is critically controlled in association with cell proliferation. p53 is a cell cycle regulator and induces G1 arrest of the cell cycle by activating p21 transcription on DNA damage signals (36). It also induces apoptosis through transcriptional activation of Bax or p53-induced genes (37, 38). We therefore examined whether telomerase repression was due to growth inhibition or apoptosis induced by p53. However, under our assay conditions, no significant growth inhibition or apoptosis was observed until 42 h after introduction of the p53 gene. These findings suggest that repression of telomerase activity is not simply an effect of growth inhibition or apoptosis of the cells.

Telomerase repression was observed 24 h after down-regulation of hTERT mRNA expression. This is consistent with the observation that the half-life of hTERT is approximately 24 h (12) and suggests that telomerase repression is achieved at the transcriptional level of hTERT. The early onset of hTERT down-regulation (12 h after infection) indicates that p53 directly regulates hTERT transcription. We have, in fact, identified putative p53 binding motifs in the hTERT promoter. Transient expression assays using hTERT promoter-reporter plasmids demonstrated that p53 significantly represses the transcriptional activity of hTERT. However, these motifs did not play causative roles in this down-regulation. Serial deletion assays revealed that the 181-bp core promoter region that contains an E-box and five GC-boxes responded to p53 overexpression. However, deletion of the E-box did not alter the effect of p53, indicating that the remaining 150-bp region containing five Sp1 sites was responsible for transcriptional repression. Interestingly, even the 32-bp minimal promoter region containing one Sp1 site could respond to p53 overexpression. Abrogation of Sp1 bindings by substitution mutations (pGL3–181Sp1 MT) resulted in a marked loss of transcriptional activity (Fig. 4C), indicating that Sp1 is a critical transactivator possibly involved in the basal transcription of hTERT. Interestingly, p53 overexpression failed to repress transcription of pGL3–181Sp1 MT. These findings suggest that Sp1 plays a role in p53-mediated transcriptional repression. Recent studies have demonstrated that p53 negatively regulates gene transcription from GC-rich promoters containing Sp1 binding sites, such as the insulin-like growth factor I receptor gene (39). This negative regulation is performed via protein-protein interaction of p53 with Sp1. Although such an interaction was not confirmed in this study, it might be one of the mechanisms by which p53 represses hTERT transcription.

Interaction of p53 with transcriptional coactivator p300 has recently been demonstrated. p300 is known to bind specific transcription factors such as the cAMP-responsive element-binding protein, the Stat family, c-Myc, c-jun, and nuclear factor κB, all of which are essential transactivators for various genes, through protein-protein interaction (reviewed in Ref. 40). p300 also binds to basal transcription factors such as transcription factor IID. Thus, p300 functions as a bridging factor that connects enhancer-binding proteins with basal transcription factors to facilitate transcription of target genes (40). It has been suggested that p300 is a limited cellular factor and that overexpression of p53 sequesters p300 (41), which may disturb the interaction of p300 with specific transcription factors, resulting in decreased transcription of target genes. This may be an alternative mechanism by which p53 represses transcription of hTERT. Further analyses are needed to clarify the molecular mechanisms by which p53 represses hTERT transcription via the proximal promoter region.

Our findings suggest a novel function of p53, although the biological significance of this function remains unclear. It is possible that p53 controls telomerase activity in normal cells...
and that its deregulation by p53 gene mutation, which is frequently observed in a wide variety of tumors, may contribute to increased telomerase activity in tumors. An inhibitory effect on telomerase may thus be a novel function of the antitumor activity of p53. Gene therapies targeting telomerase have been developed recently. It has been suggested that transient inhibition of telomerase activity by antisense hTR strategies leads to a rapid decrease in cell viability and tumorigenicity (42). This effect did not require a lag period during which telomeres shorten and reach a critical length as the threshold for chromosomal instability. Telomerase may thus play roles in cell proliferation other than the maintenance of telomere length, suggesting that repression of telomerase is a promising strategy for cell growth control. These findings indicate a novel aspect of gene mutation, which is frequently observed in a wide variety of tumors, may contribute to increased telomerase activity in tumors. An inhibitory effect on telomerase may thus be a novel function of the antitumor activity of p53. Gene therapies targeting telomerase have been developed recently. It has been suggested that transient inhibition of telomerase activity by antisense hTR strategies leads to a rapid decrease in cell viability and tumorigenicity (42). This effect did not require a lag period during which telomeres shorten and reach a critical length as the threshold for chromosomal instability. Telomerase may thus play roles in cell proliferation other than the maintenance of telomere length, suggesting that repression of telomerase is a promising strategy for cell growth control. These findings indicate a novel aspect of gene mutation, which is frequently observed in a wide variety of tumors, may contribute to increased telomerase activity in tumors. An inhibitory effect on telomerase may thus be a novel function of the antitumor activity of p53. Gene therapies targeting telomerase have been developed recently. It has been suggested that transient inhibition of telomerase activity by antisense hTR strategies leads to a rapid decrease in cell viability and tumorigenicity (42).

Acknowledgments

We thank Dr. Masuo Yutsudo (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and Atsushi Mizokami (Department of Urology, University of Occupational and Environmental Health, Kitakyushu, Japan) for critical discussion and advice. We also thank Dr. Bert Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MD) for providing the p53 expression vector.

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