THE CODON 47 POLYMORPHISM IN p53 IS FUNCTIONALLY SIGNIFICANT

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Running title: Serine 47 polymorphism impairs p53 function

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In addition to a common polymorphism at codon 72, the p53 tumor suppressor gene also contains a rare single nucleotide polymorphism at amino acid 47. Wild type p53 encodes proline at this residue, but in less than 5% of African Americans this amino acid is serine. Notably, phosphorylation of the adjacent serine 46 by the proline-directed kinase p38 MAPK is known to greatly enhance p53’s ability to induce apoptosis. Here we show that the serine 47 polymorphic variant, which replaces the proline residue necessary for recognition by proline-directed kinases, is a markedly poorer substrate for phosphorylation on serine 46 by p38 MAPK. Consistent with this finding, we show that the serine 47 variant has up to five-fold decreased ability to induce apoptosis compared to wild type p53. Mechanistically we find that this variant has decreased ability to transactivate two p53-target genes, p53AIP1 and PUMA, but not other p53-response genes; this is the first time that phosphorylation of serine 46 has been implicated in transactivation of PUMA by p53. Down-regulation of PUMA in cells with wild type p53 using short interfering RNAs reduces apoptosis in these cells to a level comparable to that in cells containing the serine 47 variant. The combined data indicate that, like the codon 72 polymorphism, the codon 47 polymorphism of p53 is functionally significant, and may play a role in cancer risk, progression, and the efficacy of therapy.

We recently reported that a common polymorphism in p53 at codon 72 is functionally significant. Codon 72 of p53 can encode either arginine (R72) or proline (P72). We found that the R72 form of p53 has up to 15-fold increased apoptotic ability compared to the P72 form, in both inducible cell lines and in cells with endogenous p53 homozygous for each variant (1). At least part of the increased apoptotic potential of R72 is due to enhanced mitochondrial localization of this protein, where we have found that p53 can interact directly with the pro-apoptotic protein BAK, displacing Mcl-1 and allowing BAK oligomerization (2). These functional studies on the codon 72 polymorphic variants of p53 have led to a number of studies testing the impact of this polymorphism on the risk and progression of human cancer. Several of these reports indicate that the lesser apoptotic P72 form is associated with increased risk of cancer (3-5). Another report indicated that individuals in families predisposed to colon cancer who are homozygous for the P72 allele have an earlier age of onset for cancer, and tend to have increased tumor number, compared to individuals homozygous for R72 (6). Recently a polymorphism in the gene encoding MDM2, which negatively regulates p53, was shown to lead to attenuated p53 function and increased cancer risk (7). The combined data support the premise that identification of functionally significant polymorphisms in the p53 tumor suppressor gene will have an impact on our understanding of genetic determinants of cancer risk and progression.

In the present report we provide data that another polymorphism in p53, at codon 47, is also functionally significant. Codon 47 encodes proline in wild type p53, but in a small subset of individuals it can encode serine (CCG -> TCG). A single study, performed over ten years ago, was done on the serine 47 (S47) polymorphic variant. In that study, the S47 polymorphism was found in less than 5% of African Americans, and not at all in Caucasians (8). A preliminary functional analysis failed to reveal differences between S47 and wild type p53; importantly however, the
ability to induce apoptosis was not assessed (8). Additionally, it was not known at that time that phosphorylation of the adjacent residue, serine 46, was critical for p53’s ability to induce apoptosis.

One of the kinases that directly catalyzes serine 46 phosphorylation is the proline-directed kinase p38 MAPK. The importance of serine 46 phosphorylation to apoptosis induction by p53 is epitomized by the findings that mutation of serine 46 to alanine, incubation with specific chemical inhibitors of p38 MAPK, or overexpression of proteins that inhibit p38 MAPK, have all been shown to markedly inhibit p53-dependent apoptosis (9-14). Significantly, the serine 47 polymorphism in p53 replaces the proline residue necessary for phosphorylation of serine 46 by proline-directed kinases like p38 MAPK. This raises the possibility that the S47 variant has decreased phosphorylation on serine 46, and impaired apoptotic ability.

In this report we present data indicating that the serine 47 polymorphic variant is a poorer substrate for phosphorylation by p38 MAPK. Consistent with this we show that in multiple clones of stably transfected cells containing equivalent levels of p53, the S47 variant has up to 5-fold decreased ability to induce programmed cell death, compared to wild type p53. This decreased ability to induce programmed cell death is accompanied by a decreased ability to transactivate p53AIP1 and the pro-apoptotic p53 target gene PUMA, but not other p53 target genes. PUMA (p53-upregulated mediator of apoptosis) is known to be a critical mediator of p53-dependent apoptosis. Down-regulation of PUMA using short interfering RNAs reduces the ability of wild type p53 to induce apoptosis, to levels roughly equivalent to the S47 variant. The combined data indicate that the codon 47 polymorphism in p53 is functionally significant, and suggest that the influence of this polymorphism on altered cancer risk in African Americans should be assessed.

Materials and Methods

Human studies, genotyping analysis - All human subjects were collected with the approval of the Fox Chase Cancer Center Institutional Review Board. DNA was isolated from peripheral blood collected from 200 African Americans from the Biosample Repository at Fox Chase Cancer Center. PCR amplification of a 241 bp fragment of p53 exon 4 was carried out in a 25 µl reaction containing 20 ng genomic DNA and 0.2 µM primers (forward-5’CAC CCA TCT ACA GTC C and reverse-5’ ACC GTA GCT GCC CTG GTA G) with JumpStart RedTaq ReadyMix (Sigma). The reverse primer was biotinylated to facilitate single-strand DNA template preparation for pyrosequencing. Primers were synthesized and HPLC-purified by Thermo Hybaid Interactiva Division (Ulm, Germany). Cycle conditions were as follows: 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec. Amplicon size and purity was verified on a 2% agarose gel containing 0.5 µg/ml ethidium bromide (Life Technologies; Rockville, MD). Single-stranded DNA template preparation was performed in a 96-well plate. The biotinylated strand was immobilized onto streptavidin-coated Sepharose beads (Amersham Pharmacia Biotech) and processed using the PSQ 96 Sample Preparation Kit (Pyrosequencing AB) according to manufacturer’s instructions. The template was incubated with a sequencing primer specific for codon 47 (5’GGA TGA TTT GAT GCT GTC) and automatically analyzed on a PSQ 96 Instrument (Pyrosequencing AB) using the PSQ 96 SNP Reagent Kit (Pyrosequencing AB). Genotyping and quality assessment of the raw data was performed using PSQ 96 SNP Software (Pyrosequencing AB).

Plasmid Construction - The human p53 cDNA construct encoding temperature sensitive p53 (valine 138, ref. 15) and the GST-p53 (1-92) construct (1) were modified by site-directed mutagenesis using Stratagene’s Quick-Change Kit. The A46 p53 mutant was made by introducing alanine at codon 46 using primers:

5’-GATTTGATGCTGCCCAGACGATATTG-3’
and
5’-CAATATCGTCCGGGGCCAGCATCAAATC-3’.

The S47 p53 polymorphic variant was made using primers:

5’-GATTTGATGCTGGCCCGGGACGATATTG-3’
and
5’-CAATATCGTCCGGGGCCAGCATCAAATC-3’.

The serine 33 to alanine GST-p53 (1-92) was mutated using primers:

5’-AACAACGTTCTGGCCGCCCTTGGCGTCC-3’
and
5’-
GGACGGCAAGGGGCCAGAACGTTGTT-3'. These mutations were confirmed by DNA sequencing. All p53 constructs have proline at codon 72.

Cell culture, p53 induction - H1299 and Saos2 cell lines containing temperature-sensitive p53 were generated by stable transfection with CMV promoter-driven human p53 cDNA containing the temperature-sensitive Valine 138 mutation. These H1299 and Saos2 cells were maintained at 39°C in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and streptomycin, and 400 µg/mL (Saos2) or 800µg/mL (H1299) G418 in a 5% CO₂ humidified atmosphere. For temperature shift and p53 induction, cells were plated at 2x10⁶ cells per 100-mm plate and either shifted to 32°C (active p53) or maintained at 39°C (mutant conformation p53). For experiments with the p38 kinase inhibitor, cells were incubated with SB-203580 (Upstate Biotechnology) to a final concentration of 40 uM, or vehicle alone (DMSO) for 30 minutes prior to temperature shift or irradiation. Cells were irradiated with 7.5 J/m² of UV with a Spectroline X series UV lamp, and output was measured with a traceable UV light meter (Fisher Scientific).

Western and Northern analysis - Western analysis was performed as described (1). Antisera for p53 (Ab-6, Calbiochem), phosphorylated p53 (Cell Signalling), PUMA (Oncogene Sciences), MDM2 (Oncogene Sciences) and p21/waf1 (Oncogene Sciences) were used at 1:1000 dilution, and antisera for cleaved PARP (p85PARP, Promega) was used at 1:400 dilution. For analysis of RNA, cells were harvested 24 hrs after temperature shift and total RNA was prepared using Trizol (Invitrogen) or via cesium chloride gradients, as described (16). Polyadenylated RNA was purified from cesium gradient total RNA using Poly A Quick columns, as per the manufacturer (Stratagene). Eight micrograms of total RNA, or two micrograms polyA RNA were resolved by denaturing agarose gel electrophoresis, transferred onto a nylon membrane, hybridized with probes and exposed to an X-ray film as described, as described (16).

Preparation of GST fusion proteins, p38 MAPK and HIPK2 kinase assays - GST fusion proteins were generated exactly as described (1, 2). The GST-p53 proteins were concentrated using Microcon columns as per the manufacturer (Millipore). For the in vitro p38 MAPK kinase reactions, the indicated amounts of purified GST-p53 (wt, S47 or A46) or myelin basic protein (MBP, Sigma) were mixed with 10µL ADBI buffer (20mM Tris, pH 7.4, 20mM β-glycerol phosphate, 5mM EGTA, 1mM Na3VO4, 1mM DTT and 20mM MgCl2), 0.6 mM cold ATP, 20 µCi [γ-32P] ATP (NEN), and 100 ng active p38β2 (Upstate Biotechnology). After a 15 min incubation at 30°C, the reaction was stopped by adding Lithium Dodecyl Sulfate and boiling for 10 min. The samples were loaded on a NuPage Novex 10% Bis-Tris gel (Invitrogen), and the gel was dried and exposed to X-ray film. For the HIPK2 kinase assays, the cDNA encoding flag-tagged HIPK2 or a kinase-dead version of this enzyme (kindly provided by Ettore Appella, National Institutes of Health) were transfected into H1299 cells using FuGene (Roche). After 24 hours cells were harvested and HIPK2 was immunoprecipitated from 400 µg of lysate with anti-flag antibody (M2, Sigma). These immunoprecipitates were washed and incubated as described (17) with 20 ug of GST-p53 (1-92) or GST-S47.

Apoptosis assays, siRNA studies - Temperature-sensitive H1299 clones were seeded onto 6-well plates at a density of 50,000 cells per well. Saos2 cells were plated at 1 x 10⁶ cells/100-mm plate. Cells were shifted to 32°C and harvested at the times indicated after temperature shift. Control cells were maintained at 39°C. TUNEL and multi-caspase assays were conducted using the Guava Personal Cytometer (Guava Technologies) using the Guava TUNEL and multi-caspase detection kits, using protocols provided by the manufacturer (Guava Technologies). For the siRNA studies, equal number of H1299 cells with temperature sensitive wt p53 were seeded onto a 10-cm plate; after 24 hours 25 µL of siRNA for PUMA (20 uM BBC3 SmartPool oligos, Dharmacon) or control RNA (20 uM, Dharmacon) were transfected using Oligofectamine as per the manufacturer (Invitrogen). After 24 hours of temperature shift cells were harvested and subjected to Western analysis as described above.
Results

A previous study by Harris and colleagues first described the existence of a coding region polymorphism in the p53 gene at codon 47. This codon encodes proline in wt p53, but these researchers found it encodes serine in less than 5% of African Americans (8). Codon 47 occurs in exon 4 of p53, where we have shown another functionally significant polymorphism, at codon 72, also exists. Before testing the hypothesis that the S47 and wild type p53 proteins might have altered apoptotic function, it was necessary to determine the frequency with which this polymorphism was linked to either codon 72 variant (P72 or R72). Toward this end, we analyzed both the codon 47 and the codon 72 polymorphisms in genomic DNA isolated from 200 African Americans from the Fox Chase Cancer Center Biosample Repository. We identified four DNA samples that were heterozygous for the S47 variant, for an allele frequency of 1% (4/400 alleles); this is somewhat less than the previous study, although that study had considerably smaller sample size. Sequence analysis revealed that in all four cases the S47 allele occurred in cis with the proline 72 polymorphism (P72). This suggests that the S47 and P72 polymorphisms may be linked. Therefore, for this study we focused on the S47 allele in cis with P72 (which we designate S47), and we compare it to the P72 protein, which we designate as wild type (wt) p53.

p38 MAPK is a proline-directed kinase, and requires a proline residue adjacent to the target phosphorylation site for efficient recognition of that site; notably, the S47 polymorphism replaces this proline with serine (Fig. 1A). In order to test the hypothesis that the S47 polymorphism alters serine 46 phosphorylation by p38 MAPK, we created GST fusion proteins representing amino acids 1-92 of wt p53 (wt) and the S47 variant (S47). As a control for these studies we generated a p53 construct in which serine 46 was mutated to alanine (A46). Because serine 33 is also phosphorylated by p38 MAPK (Fig. 1A), initially these constructs were generated with an alanine substitution at amino acid 33; this replacement has no effect on phosphorylation of serine 46 (17 and our unpublished results). Increasing amounts of GST-p53 and GST-S47 were incubated with a constant amount of purified active p38 MAPK (Upstate Biotechnology) and γ32P-ATP, using kinase conditions previously described (9). Phosphorylated p53 was then resolved by SDS-PAGE and autoradiography. As depicted in Figure 1, wt p53 was efficiently phosphorylated by p38 MAPK. In contrast, the S47 variant protein was phosphorylated markedly less well (Fig. 1B). As expected, neither GST alone nor the A46 mutant was detectably phosphorylated under these conditions (Fig. 1B). Coomassie staining of the input proteins confirmed equal loading and purity of the wt, S47 and A46 substrates (Fig. 1C). Notably, decreased phosphorylation of the S47 variant by p38 MAPK was also evident using GST fusion proteins that retained serine at amino acid 33, except that the background level of phosphorylation was higher (Supplementary Fig. 1).

The Michaelis Menton model was fit to the averaged data from three independent p38 MAPK kinase experiments performed using S47 and wt p53 as substrates. This modeling revealed a three-fold decrease in the K_m, and an eight-fold decrease in the V_max, for S47 compared to wt p53 (Fig. 1D). These data suggest that the S47 protein binds reasonably well to p38 MAPK, but that the ability of this enzyme to phosphorylate serine 46 is markedly reduced. To control against the possibility that the S47 protein was denatured or misfolded, we tested the ability of another kinase, the homeo-domain interacting protein kinase-2 (HIPK2) to phosphorylate S47 and wt p53. Like p38 MAPK, HIPK2 also phosphorylates serine 46, but it is not reported to be a proline-directed kinase (17, 19). As shown in Figure 1E, following transfection into p53 null cells, immunoprecipitated FLAG-tagged HIPK2 was able to phosphorylate S47 and wt p53 proteins identically. In contrast, a kinase dead version of HIPK2 (K221R, ref. 17) did not detectably phosphorylate either protein (Fig. 1E). These data indicate that the S47 variant of p53 is impaired for phosphorylation on serine 46 by the proline-directed kinase p38 MAPK.

We next determined whether the S47 polymorphism alters p53 apoptotic function in vivo. We generated stably-transfected cell lines containing inducible versions of wild type p53 (wt) and the S47 variant, as well as the A46 mutant. These p53 variants were generated using
the temperature sensitive version of p53 encoding valine at amino acid 138. This temperature-sensitive form p53 is well characterized; the p53 protein is denatured and inactive when cells are cultured at 39 degrees. Temperature shift of cells to 32 degrees results in wild type p53 conformation and apoptosis induction (15, 20). Twenty independent clones for each variant (wt, S47 and A46) were generated in the human lung adenocarcinoma cell line H1299 (p53-/-), which was chosen because it contains high levels of active p38 MAPK, as determined by western blotting using antisera specific for the active enzyme (Supplementary Fig. 2). Two clones for each variant were selected for further analysis based upon western analysis indicating comparable levels of p53 protein (Fig. 2A, inset western). These six clones were analyzed for apoptosis induction following temperature shift using TUNEL assay (terminal dUTP nick end labeling); additionally, three of these clones (wt-clone 4, S47-clone 8 and A46-clone 8) were further analyzed using an assay that measures multi-caspase activation. As depicted in Figure 2, TUNEL analysis following temperature shift indicated that the S47 variant consistently had between 2 to 5-fold decreased ability to induce apoptosis, relative to wt p53, while the A46 variant was almost completely compromised for apoptosis induction (Fig. 2A). Western analysis of the level and activity of p53 in our H1299 clones indicated that these approached those reached physiologically, in MCF7 cells treated with the DNA damaging agent doxorubicin (Figure 2B). Consistent with our TUNEL analyses, analysis of multi-caspase activation indicated that the S47 variant had significantly decreased ability to activate caspases, relative to wt p53 (p<0.001). The ability of A46 to induce apoptosis was more compromised than S47 (Fig. 2C), possibly due to residual phosphorylation of S47 by p38 MAPK or HIPK2.

To confirm these findings in another cell background, we generated stably-transfected wt and S47 cell lines in the p53-null human osteosarcoma cell line Saos2. Again, clones were selected that contained equivalent levels of p53 (Fig. 2D, inset western). For this comparison we also included a Saos2 cell line containing an inducible version of the arginine 72 (R72) polymorphic variant; in this way, all three polymorphic variants of p53 could be compared for apoptosis induction (S47, P72 and R72). As shown in Figure 2D, TUNEL analysis following temperature shift in these cell lines indicated there was an over 2-fold decrease in apoptosis in the S47 cell line compared to wild type p53 (P72, p = 0.01), and a 7-fold decreased ability when compared to R72 (p<0.001).

We extended these analyses to include a time course of apoptosis in the inducible wt, S47 and A46 cell lines following exposure to gamma radiation (clones wt-4, S47-8 and A46-8 from Figure 2, which were analyzed for the remainder of this study). In this experiment, apoptosis was measured as the appearance of the 85 kDa caspase cleavage product of Poly (ADP) ribose polymerase (PARP), using an antibody specific for this caspase cleavage product (p85 PARP). Decreased abundance of p85 PARP following induction of S47 compared to wt p53 could be seen at all time points, although at later time points the difference was more marked (2-fold difference at 6 hours, and 10-fold difference at 24 hours, see Fig. 3A). Similar findings were made following treatment with doxorubicin as the source of DNA damage; additionally, similar fold-differences were seen in the absence of radiation (not shown). We next examined the phosphorylation pattern of p53 in these inducible cell lines following temperature shift, using antisera specific for p53 phosphorylated at serines 15, 46 or 392. These analyses revealed no difference in the phosphorylation pattern on serines 15 or 392 between the wt, S47 and A46 proteins (Fig. 3B). As expected there was a marked decrease in reactivity of the S47 variant for the serine 46 phospho-specific antibody, although we cannot rule out the possibility that the proline-to-serine change in the S47 variant interferes with recognition by this antibody. Interestingly, despite clear differences in apoptosis induction, we saw no evidence of a gross difference in the transactivation potential of these proteins. The ability of the S47 and A46 variants to transactivate the p53-response genes MDM2 and p21/waf1 was indistinguishable from wt p53 in a time course following radiation (MDM2, see Fig. 3A) or following temperature shift (p21 and MDM2, Fig. 3B).

The combined data support the hypothesis that the S47 variant has impaired apoptotic ability...
relative to wt p53, due in part to decreased phosphorylation on serine 46 by p38 MAPK. To solidify these findings, we made use of a well-characterized inhibitor of p38 MAPK, SB-203580 (9). Specifically, we temperature-shifted our inducible H1299 clones in the presence of SB-203580 or dilution vehicle, and apoptosis induction and the phosphorylation pattern of p53 were assessed. As depicted in Figure 4, western analysis using an antibody specific for phosphorylated serine 46 confirmed that the p38 MAPK inhibitor effectively inhibited phosphorylation on this residue. Prior to temperature shift wt p53 was already phosphorylated on serine 46 (Fig. 4A, lane 1, S-46), and following temperature shift this phosphorylation of p53 increased 3 fold (lane 4, S-46). In contrast, temperature shift in the presence of SB-203580 reduced serine 46 phosphorylation 2-fold (lane 7, S-46). As expected, this decrease in serine 46 phosphorylation was accompanied by a marked decrease in apoptosis (compare p85 PARP, lanes 4 and 7). Notably, incubation with SB-203580 brought the levels of the p85 PARP caspase cleavage product and TUNEL positive cells to levels comparable to the untreated S47 variant (Figs. 4A and 4B, respectively), indicating that the different apoptotic potential of the S47 and wt p53 proteins reflects altered serine 46 phosphorylation. SB-203580 also marginally inhibited apoptosis induced by the S47 variant, possibly due to residual phosphorylation of serine 46, or to phosphorylation of serine 33 by p38 MAPK, which can also play a role in apoptosis induction (9). Consistent with these data, we found that the SB-203580 inhibitor also effectively inhibited phosphorylation of serine 46, and apoptosis (p85 PARP appearance), in MCF7 cells (wt p53) treated with ultra-violet light (Fig. 4C).

It has been reported previously that serine 46 phosphorylation is not required for the ability of p53 to transactivate the majority of target genes. However, at least one, the pro-apoptotic target gene p53AIP1, requires this phosphorylation event for efficient transactivation (10). In line with this, we analyzed poly-adenylated RNA isolated from our inducible H1299 cell lines, and found that there was a modest induction of p53AIP1 in cells containing wt p53, but there was no evidence for induction in lines containing the S47 or A46 variants (Fig. 5A). Unfortunately we have been unable to detect p53AIP1 protein using commercially available antibodies, possibly because the level of p53AIP1 in the cell types employed in this study (H1299, Saos2 and MCF7) is extremely low following p53 induction. The low level of p53AIP1 in these cells prompted us to broaden this analysis to include other p53-target genes. Northern analysis of total RNA for the level of other p53 response genes revealed no difference in the ability of S47, A46 and wt p53 proteins to transactivate the p53-induced genes Killer/DR5, Gadd45, p21/waf1, Mdm2 or bax, or to repress the genes encoding survivin or cyclin B1, either in a time course (not shown) or following 24 hours of temperature shift (Fig. 5B). Interestingly, however, there was a consistent decrease in the ability of the S47 and A46 variants to transactivate the p53 response gene PUMA. Western analysis confirmed that the S47 variant had a decreased ability to induce PUMA (2-fold decrease compared to wt p53 at 8 hours, 7-fold decrease at 24 hours, see Fig. 5C), but not the p53 target gene MDM2. Decreased PUMA levels correlated with decreased apoptosis, as measured by the appearance of p85 PARP (Fig. 5C). These data suggest that PUMA transactivation is sensitive to serine 46 phosphorylation. In support of this premise, we found that the p38 MAPK inhibitor SB-203580 efficiently inhibited PUMA transactivation in MCF7 cells following treatment with ultra-violet light (Supplementary Fig. 3).

In order to confirm the significance of decreased PUMA transactivation to the reduced apoptotic potential of the S47 polymorphic variant, we used transfection of short interfering RNA oligonucleotides specific for the PUMA transcript to reduce its level in cells with inducible wt p53. Apoptosis in these cells was measured as the appearance of the caspase cleavage product p85 PARP. As depicted in Figure 6, western analysis of cells treated with siRNA specific for PUMA (SmartPool RNA, Dharmacon), or control scrambled RNA, indicated that the PUMA siRNA efficiently reduced the levels of this transcript, while the control siRNA had no effect. Exposure to siRNA for PUMA, or control oligos, had no effect on the ability of p53 to transactivate MDM2 (Fig. 6). Significantly, reduction of PUMA in cells with wt p53 reduced the level of apoptosis in these cells to a level equivalent to cells containing the inducible S47 variant (Fig. 6). These data
support the premise that impaired transactivation of PUMA underlies at least part of the apoptotic defect of the S47 variant of p53.

**Discussion**

Our data indicate that the S47 variant of p53 has decreased ability to serve as a substrate for phosphorylation by p38 MAPK, as well as 2 to 5-fold decreased ability to induce apoptosis in vivo. Underlying this apoptotic defect we have found that the S47 variant exhibits decreased ability to transactivate the p53 response genes p53AIP1 and PUMA. In contrast, we have seen no differences in the ability of S47 to bind to DNA, induce G1 arrest, or to localize to mitochondria, compared to wt p53 (X. Li and M. Murphy, unpublished observations). These data are the first to suggest that the serine 47 polymorphism is functionally significant, and to implicate serine 46 phosphorylation in the transactivation of the p53 target gene PUMA. While the changes in PUMA transactivation by S47 appear to range only from 2- to 7-fold, transactivation of PUMA is known to be particularly critical for apoptosis induction by p53 (21-23). For example, even the heterozygous PUMA knock-out mouse (Puma +/-), with 2-3 fold reduction in PUMA levels, has a marked defect in apoptosis of irradiated thymocytes, compared to wild type mice (23).

Our data indicate that there are two polymorphisms in p53 that are functionally significant, at codons 47 and 72. It remains to be determined if the S47 polymorphism has an impact on cancer risk. In support of this possibility, missense mutations of amino acid 47 (proline to leucine) are reported in the p53 mutation data base (24).

It remains to be determined why functionally significant polymorphic variants in a tumor suppressor gene that decrease its function would persist in the human population. It is of note that both of the polymorphic variants of p53 that are associated with decreased apoptotic potential (P72 and S47) are more prevalent in populations whose origin is near the equator (Africa). This suggests that the selection for these variants may have occurred as a response to high exposure to ultraviolet light. We speculate that the decreased apoptotic potential of p53 may have been selected for in environments of high UV exposure in order to enable cells to more efficiently repair DNA, and perhaps also to accumulate the pigmentation necessary to inhibit excessive fixation of vitamin D, which can be toxic in such environments. In contrast, the higher apoptotic potential of the R72 variant, which is associated with skin of lighter pigmentation (25), would be selected for in environments where UV exposure was limited, and the increased absorption of UV rays afforded by lighter pigmentation would enhance vitamin D fixation. These hypotheses remain to be tested. The combined data suggest that a careful epidemiological analysis of the impact of the S47 polymorphism on cancer risk, progression, and the efficacy of chemotherapy and radiation therapy is warranted. Additionally, it will be important to determine if the ethnic bias of this polymorphism explains some of the differences in cancer incidence between Caucasian and African American populations.
References

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Figure Legends

Figure 1. The S47 polymorphic variant of p53 is impaired for phosphorylation by p38 MAP kinase.
A. Amino acid sequence of residues 30-57 of human wt p53, with the p38 MAPK phosphorylation sites underlined, and the site of the serine 47 polymorphism marked with an asterisk.
B. (Left panel) Kinase assay using purified p38 MAPK (Upstate Biotechnology) and the indicated concentration of purified GST-p53 and GST-S47 (both containing amino acids 1-92, with serine 33 replaced by alanine). (Right panel) p38 MAPK assay using myelin basic protein (MBP) as a positive control, and as a negative control, GST alone and GST (1-92) with serines 33 and 46 replaced with alanine (A46), 10 ug of substrate per reaction.
C. Coomassie stained gel verifying the loading and purity of GST fusion proteins used in A; Bovine serum albumin (BSA) is included as a standard.
D. The Michaelis Menton model was fit to the averaged data from three independent experiments performed in A, using GraphPad Prism v. 3.0a (GraphPad Software, San Diego CA). The Km for wt p53 and S47 are indicated; the Vmax for p38 MAPK for wt p53 is estimated to be eight-fold greater than for S47. Rate is indicated on the y axis as the densitometry units of phosphorylation per minute per microgram of enzyme.
E. Flag-tagged HIPK2, or a kinase-dead version of this enzyme, were immunoprecipitated with anti-flag antibody following transient transfection of H1299 cells and incubated with myelin basic protein (MBP), GST alone, GST-p53 or GST-S47 (the latter two encode amino acids 1-92 of human p53, with alanine at amino acid 33).

Figure 2. The S47 variant has impaired ability to induce apoptosis, relative to wt p53.
A. TUNEL assay results from individual clones of cells stably transfected with inducible versions of p53, as indicated. Western analysis for total p53 in each clone is indicated in the inset. Values of TUNEL positive cells are given at 39 degrees (mutant p53) and 32 degrees (wild type p53 conformation) following twenty-four hours of temperature shift. Values given are the mean +/- standard error of measurement from three independent experiments. p values reflect comparison of indicated clones to clone wt-4.
B. Western analysis of the level of p53 in the inducible H1299 clones, versus MCF7 cells treated with a DNA damaging agent. 10 ug of total lysate is loaded for the H1299 clones, compared to 50 ug of lysate for MCF7 treated with doxorubicin (Dox) for eight hours. The increased levels of p53 response genes p21 and MDM2 in MCF7 lysates indicates that the level of activity of p53 in the inducible clones is comparable to that reached physiologically, in MCF7 cells treated with DNA damage. The higher molecular weight species of MDM2 is that derived from the upstream housekeeping promoter (26).
C. Multi-caspase activity assay in clones wt-4, S47-8 and AP-8 from A, following twenty-four hours of temperature shift. Levels of caspase activity in uninduced samples (39 degrees, mutant conformation p53) were set to 1, and the fold increase is depicted. The values shown are the averaged results from three independent experiments, with standard error of measurement. The p value indicated is relative to clone wt-4 at 32 degrees.
D. TUNEL analysis of clones of Saos2 cells stably transfected with each p53 polymorphic variant (R72, P72 and S47), analyzed by western analysis for p53 level (see inset) and for TUNEL positive cells after twenty-four hours of temperature shift. Values are expressed as the mean +/- standard error of measurement. p values are given for each cell line (R72 and S47) compared to wt p53 (P72) at 32 degrees.

Figure 3. The S47 variant has reduced apoptotic ability, but apparently normal transactivation of MDM2 and p21/waf1.
A. Western analysis of apoptosis induction (appearance of the caspase-cleaved form of poly (ADP) ribose polymerase, p85-PARP) in cells harvested at the indicated timepoints after temperature shift. Just prior to temperature shift, cells were exposed to 6 Gy of radiation. The clones depicted are the same as
those in Figure 2B. 39 degrees is mutant (inactive) p53, and 32 degrees is wild type conformation and activity. Levels of β-actin are included as a loading control.

B. Western analysis for p53 level, apoptosis (p85-PARP) and phosphorylated serines 46, 15 and 392, using phosho-specific antisera (Cell Signaling), as well as induction of the p53 target genes MDM2 and p21/waf1. Cells were temperature shifted for 24 hours. Levels of β-actin are included as a loading control.

Figure 4. The p38 MAPK inhibitor SB-203580 inhibits phosphorylation of p53 serine 46 and abrogates p53-dependent apoptosis.

A. Western analysis for the proteins indicated of H1299 clones containing the indicated inducible proteins (wt, S47 and A46) treated with the p38 inhibitor SB-203580 or vehicle alone (dimethyl sulfoxide, DMSO) 30 minutes prior to temperature-shift to 32 degrees to induce wild type p53 conformation. The data depicted are representative of three independent experiments, and the values depicted below the S-46 lane are the values for that lane in three independent experiments, plus standard deviations. Levels of β-actin are included as a loading control.

B. Quantitation of apoptosis using TUNEL assay on the inducible H1299 clones indicated, following 24 hours of temperature shift; cells were pre-treated for 30 minutes prior to temperature shift with p38 MAPK kinase inhibitor (SB-203580) or vehicle alone (DMSO). Apoptosis was quantitated on a Guava Personal Cell Analysis machine (Guava Technologies).

C. Western analysis of asynchronously growing MCF7 breast carcinoma cells treated with ultra-violet light (7.5 J/m2) and harvested after the times indicated. Cells were pre-treated for 30 minutes with SB-203580 to a final concentration of 40 μM, or vehicle alone (DMSO), before harvesting and western analysis using antisera to the proteins indicated. The values depicted below the p85-PARP lane are the values averaged from three independent experiments, plus standard deviations.

Figure 5. The S47 variant has impaired ability to transactivate the p53-response genes p53AIP1 and PUMA.

A. Northern analysis of 2 μg of polyadenylated RNA isolated from the inducible H1299 clones indicated probed with cDNA specific for p53AIP1 and gapdh (glyceraldehyde 3 phosphate dehydrogenase). Temperature shift is for 24 hours.

B. Northern analysis of total RNA isolated from the H1299 clones indicated, following 24 hours of temperature shift. Results are representative of 3 independent experiments. Densitometry indicates a consistent 3 to 4-fold decrease in PUMA level in S47 and A46, relative to wt p53. Gapdh is included as a control for loading and integrity.

C. Western analysis of a time course of p53 induction in the H1299 clones indicated for p53 level, apoptosis (p85-PARP) and PUMA level. Levels of β-actin are included as a loading control.

Figure 6. Decreased ability to induce PUMA underlies in part the decreased apoptotic potential of the S47 variant.

H1299 cells containing the inducible versions of p53 indicated were temperature shifted for 24 hours; 24 hours prior to temperature shift cells were transfected with siRNA specific for PUMA, or control scrambled oligonucleotide. Western analysis was performed using the antisera indicated, and levels of β-actin are included as a loading control. The values depicted below the p85-PARP lane are the average values for that lane in three independent experiments, plus standard deviations.
Li et al., Fig. 1

A

30-NVLSPLP SQAMDDLMSPDIEQWT ED-57

* P→S, P47S polymorphism

p38MAPK sites

B

GST-p53(wt)

GST-p53(S47)

C

D

E

MBP GST GST-p53 wt S47 HIPK2

MBP GST GST-p53 wt S47 HIPK2(KD)
Li et al., Fig. 2

A

![Graph showing cell death percentages](image)

B

![Graph showing protein expression](image)

C

![Graph showing caspase activity](image)

D

![Graph showing cell death percentages](image)
Li et al., Fig. 3

A

H1299 treated with IR

0 3 6 12 24 hrs

wt S47 A46 wt S47 A46 wt S47 A46

p53
MDM2
p85-PARP
actin

B

H1299

39 32

wt S47 A46 wt S47 A46

p53
p85-PARP
S-46
S-15
S-392
p21
MDM2
actin
Li et al., Fig. 6

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Fig. 1. The S47 variant is a poorer substrate for phosphorylation of active p38 MAPK kinase.
A, B. One microgram of the indicated GST fusion proteins (amino acids 1-92 of p53, without the serine to alanine 33 replacement) were used as substrates for phosphorylation by active, purified p38 MAPK kinase. Myelin basic protein (MBP) is used as a positive control, and GST is used as a negative control. Coomassie staining of input substrates is included in B.

Fig. 2. The levels of active p38 MAPK in H1299 cells are constitutively high, and are not significantly increased by UV radiation. H1229 cells were treated with UV (20 J/m²) and harvested 1 hr after UV treatment. The whole cell lysates were subjected to Western analysis using antibodies detecting total p38 MAPK (N-20, Santa Cruz) and activated p38 MAPK (D-8, Santa Cruz).

Fig. 3. Western analysis of PUMA levels in MCF-7 cells treated with 7.5 J/m² of UV and harvested at the indicated timepoints. 30 minutes prior to irradiation, cells were treated with the p38 inhibitor SB-203580 or vehicle alone (DMSO).
**Li et al., Supplementary Fig. 1**

A

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B

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**Li et al., Supplementary Fig. 2**

UV

- +

total p38

active p38

**Li et al., Supplementary Fig. 3**

MCF7 treated with UV

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The codon 47 polymorphism in p53 is functionally significant
Xiaoxian Li, Patrick Dumont, Anthony Della Pietra, Cory Shetler and Maureen E. Murphy

J. Biol. Chem. published online April 25, 2005

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