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A Single Nucleotide Polymorphism in the *MDM2* Promoter Attenuates the p53 Tumor Suppressor Pathway and Accelerates Tumor Formation in Humans

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Summary

The tumor suppressor p53 gene is mutated in minimally half of all cancers. It is therefore reasonable to assume that naturally occurring polymorphic genetic variants in the p53 stress response pathway might determine an individual's susceptibility to cancer. A central node in the p53 pathway is the MDM2 protein, a direct negative regulator of p53. In this report, a single nucleotide polymorphism (SNP309) is found in the *MDM2* promoter and is shown to increase the affinity of the transcriptional activator Sp1, resulting in higher levels of MDM2 RNA and protein and the subsequent attenuation of the p53 pathway. In humans, SNP309 is shown to associate with accelerated tumor formation in both hereditary and sporadic cancers. A model is proposed whereby SNP309 serves as a rate-limiting event in carcinogenesis.

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Introduction

The tumor suppressor protein, p53, is activated upon cellular stresses such as DNA damage and oncogene activation and initiates a transcriptional program which leads to DNA repair, cell cycle arrest, and in some cases, apoptosis (Jin and Levine, 2001). The p53 stress response pathway has been shown to be crucial for the prevention of tumor formation. For example, both mice and humans harboring a germline inactivating mutation in one allele of the p53 gene develop tumors very early in life and at dramatically high frequencies (Donehower et al., 1992; Garber et al., 1992; Li et al., 1990). Somatic inactivating mutations of the p53 gene are also found in over 50% of all human tumors (Lain and Lane, 2003). Together, these observations and many others support the importance of the p53 pathway in tumor suppression. It is therefore reasonable to assume that naturally occurring polymorphic genetic variants in critical nodes of the p53 pathway might underlie the variation seen between individuals in their susceptibility to cancer and the progression of their disease.

The search for genetic variation in the p53 pathway was begun by looking in the *MDM2* gene, which encodes an important negative regulator of p53. MDM2 directly binds to and inhibits p53 by regulating its location, stability, and activity as a transcriptional activator (Michael and Oren, 2003). *MDM2* is an essential gene in murine development, as a knockout embryo dies before implantation in the uterus. This lethal phenotype is rescued by knocking out the p53 gene, clearly demonstrating an important genetic interaction between these two genes in murine development (Jones et al., 1995; Montes de Oca Luna et al., 1995). Mendrysa et al. (2003) demonstrated the importance of this interaction in the adult mouse by genetically altering mice to express reduced levels of Mdm2. These mice are small, lymphopenic, and radiosensitive, with increased apoptosis in both lymphocytes and epithelial cells. These phenotypes were all shown to be p53 dependent, thereby further demonstrating that Mdm2 is a key negative regulator of p53 in both the developing and mature mouse. In humans, a subset of tumors overexpress MDM2 mRNA and protein; this overexpression is associated with accelerated cancer progression and lack of response to therapy (Freedman and Levine, 1999). In a subset of these tumors, overexpression of MDM2 was mutually exclusive to p53 mutation, which could suggest that overexpression of MDM2 can substitute for inactivating p53 by mutation (Leach et al., 1993; Oliner et al., 1992).

As MDM2 expression levels seem to be vital to a well-regulated p53 response, naturally occurring sequence variations in the *MDM2* promoter may result in altered expression of the MDM2 protein, thereby impacting p53 tumor suppression and potentially cancer in humans. In this report, data are presented which support this hypothesis.

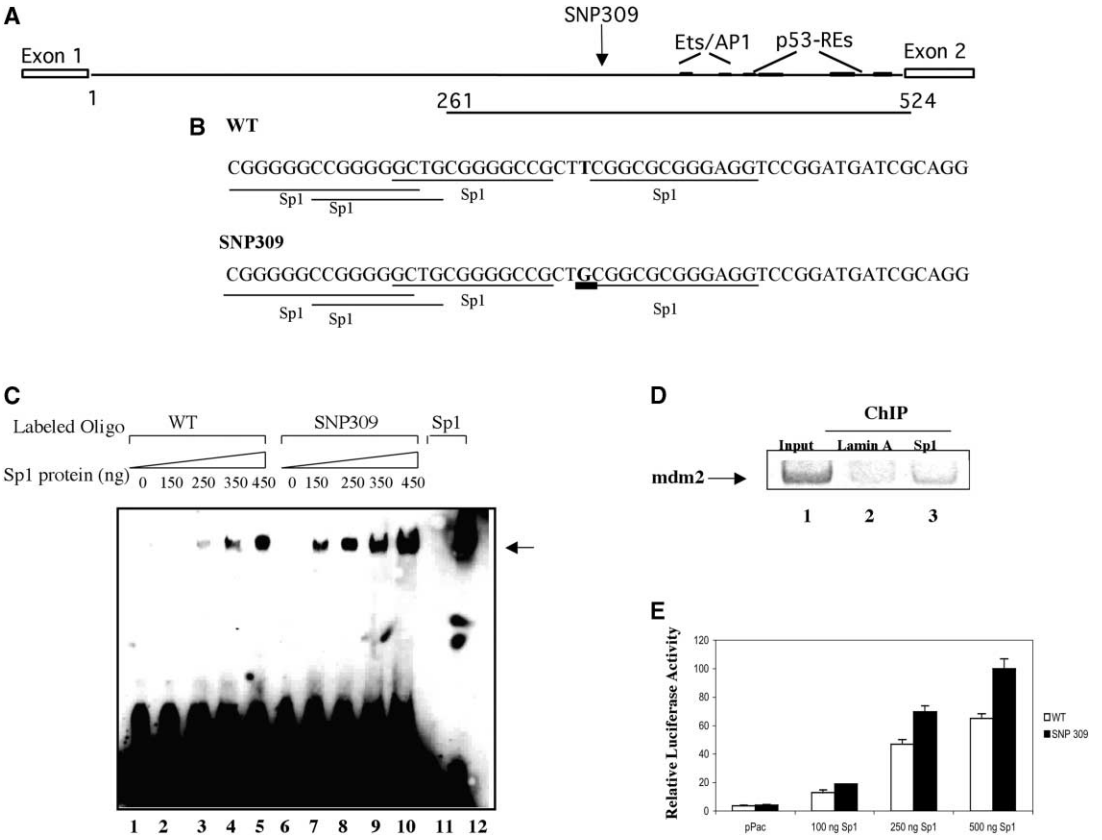

Figure 1. The *MDM2* Promoter Contains a Single Nucleotide Polymorphism Which Alters the Affinity of the Transcriptional Activator Sp1

(A) A schematic diagram depicts the intronic promoter of the *MDM2* gene. The position of SNP309 is indicated in relation to the exon/intron boundaries and the transcription factor binding sites for p53 and Ets/AP-1. The region analyzed for sequence variation is marked by the bar drawn below the diagram.

(B) The analysis of transcription factor binding sites in the region containing SNP309 is depicted. Potential Sp1 sites are underlined.

(C) An autoradiograph of an electrophoretic mobility shift assay is depicted where varying concentrations of purified Sp1 protein were incubated with biotin-labeled oligonucleotides containing either SNP309 (G/G; lanes 6–10), wild-type sequence (T/T; lanes 1–5), or an Sp1 optimal binding site (lane 11, oligo alone, and lane 12, oligo and Sp1). The arrow indicates the specific Sp1-DNA complex.

(D) Chromatin immunoprecipitations (ChIP) were preformed using antibodies against either Sp1 or lamin A. The presence of the *MDM2* promoter was assayed for using PCR. The PCR products are depicted here after electrophoreses on an 8% nondenaturing polyacrylamide gel and subsequent staining with EtBr.

(E) The relative measured luciferase levels are depicted in a bar graph, whereby pGL2 luciferase reporter plasmids containing either SNP309 sequence or wild-type sequence were cotransfected with pPac (control) or pPac-Sp1 expression plasmids. Each value is the average of at least three independent experiments and the error bars represent the standard deviations.

Results

The search for genetic variation in the *MDM2* promoter was focused to a well-characterized region in the first intron of the intronic promoter, which is utilized by both the p53 and ras pathways to activate *MDM2* transcription (Ries et al., 2000; Zauberman et al., 1995). To look for sequence variation in this region, 300 base pairs from genomic DNAs isolated from 50 healthy volunteers were amplified by PCR and sequenced (Figure 1A). Two single nucleotide polymorphisms (SNPs) were found in this region. One of these, SNP309 (a T to G change at the 309th nucleotide in the first intron), was found at relatively high frequency both in the heterozygous state (T/G, 40%) and in the homozygous state (G/G, 12%). The other SNP, SNP344 (a T to A change at the 344th nucleotide in the first intron), is rare and is not further studied (found only in the heterozygous state in 8% of people).

Analysis of this region of the *MDM2* promoter using a computer algorithm (AliBaba) revealed several putative binding sites for the transcription factor Sp1 (Figure 1B). Interestingly, the presence of SNP309, a T to G change, extended the length of one of the putative Sp1 DNA binding sites, suggesting that the presence of SNP309 could increase the affinity of Sp1 to this region of the *MDM2* promoter.

In order to characterize the potential Sp1 binding sites identified by computer analysis and to investigate the functional consequences of SNP309, electro-mobility shift assays (EMSAs) were performed. EMSAs were carried out with purified recombinant human Sp1 protein and labeled double-stranded oligonucleotides containing either the wild-type sequence (T/T) or the SNP309 sequence (G/G). Interestingly, as predicted by the computer analysis, the binding affinity of oligonucleotides containing SNP309 to a range of concentrations (150 ng–450 ng) of purified Sp1 is much higher (2–4-fold) than

that of the wild-type sequence (Figure 1C, lanes 2–5 versus lanes 7–10). The same assays were carried out using HeLa cell nuclear extracts as a source of protein instead of purified Sp1, and similar results were obtained (data not shown). These data suggest that Sp1 can bind to its putative consensus site in the *MDM2* promoter and that SNP309 can greatly enhance its binding affinity of this site.

To verify the presence of Sp1 on the *MDM2* promoter in vivo, a chromatin immunoprecipitation (ChIP) was performed. Lysates were prepared from growing Manca cells, homozygous for SNP309 (G/G), and immunoprecipitations were carried out using antibodies against either Sp1 or lamin A. After extensive washing and elution, the DNA was purified and the presence of the *MDM2* promoter was assayed for using PCR. As seen in Figure 1D, the *MDM2* promoter was detected in the ChIP using the Sp1 antibody (lane 3) but not using the lamin A antibody (lane 2). These data suggest that Sp1 binds this region of the *MDM2* promoter in vivo.

To investigate a possible role of Sp1 in the transactivation of the *MDM2* promoter and the influence of SNP309, *Drosophila* SL2 cells, which are deficient in Sp-related proteins, were utilized. SL2 cells were transiently transfected with an Sp1 expression vector (pPac-Sp1) and a luciferase reporter plasmid driven by the *MDM2* promoter either wild-type (T/T) or homozygous (G/G) for SNP309. As shown in Figure 1E, cotransfection of Sp1 strongly stimulated luciferase expression of the reporter plasmid driven by the *MDM2* promoter, as measured by luciferase activity, suggesting that Sp1 can bind to this region of the *MDM2* promoter and activate transcription. Interestingly, the presence of SNP309 in the reporter plasmid consistently showed higher Sp1-induced luciferase expression ($\sim$50%) over the presence of wild-type sequence in the reporter plasmid, as the increased binding affinity of Sp1 to SNP309-containing oligonucleotides would have predicted. Similar results were obtained when both reporter plasmids were transfected into the mammalian HeLa cell line which has an abundance of Sp1. The reporter plasmid containing SNP309 (G) yielded significantly higher luciferase levels (60%) than the plasmid containing the wild-type sequence (T) (data not shown).

Together, these data suggest that Sp1 can bind to the *MDM2* promoter and activate transcription and that the presence of SNP309 further stimulates the activity of Sp1 by enhancing its DNA binding affinity to the *MDM2* promoter. If true, individuals homozygous for SNP309 (G/G) should show heightened levels of MDM2 when compared to individuals wild-type for SNP309 (T/T). To address this, cells in culture were employed. Forty-three tumor-derived cell lines were genotyped for SNP309. SNP309 was present at a frequency similar to that found in the normal volunteers. MDM2 RNA and protein levels were compared in four cell lines homozygous for SNP309 (G/G) to the levels found in four cell lines wild-type for SNP309 (T/T). To compare MDM2 RNA levels, total RNA was isolated from growing cells. MDM2 RNA levels were measured by real-time PCR (TaqMan). The presence of SNP309 correlated with high expression of the MDM2 transcript (on average 8-fold) when compared to the levels seen in cells wild-type for SNP309 (T/T) (Figure 2A). MDM2 protein levels were also found to be significantly higher (on average 4-fold) in cell lines homozygous for SNP309 (G/G), as seen in the Western blot analysis of total cell lysates (Figure 2B). Interestingly, three of the four cell lines homozygous for SNP309 (A875, CCF-STTG1, and T47D) have been previously reported to overexpress MDM2 when compared to cell lines derived from similar tumor types (Landers et al., 1997; Lu et al., 2002; Phelps et al., 2003). MDM2 protein levels were found to be intermediate in four heterozygous (T/G) cell lines (MDA-231, MCF-7, WM-9, and CACL-7336): on average 1.9-fold higher than T/T cells (data not shown). Thus, together these data support an association of SNP309 with the increased levels of MDM2.

To test if the Sp1 transcription factor is indeed responsible for the heightened levels of MDM2 in cells homozygous for SNP309, endogenous Sp1 was inhibited, and the resulting effect on endogenous MDM2 levels was analyzed. Sp1 was inhibited using two different approaches. First, Sp1 levels were reduced by transfecting siRNAs specific to Sp1 RNA. Second, Sp1 activity was inhibited by treating cells with mithramycin A, an aureolic antibiotic that has been shown to selectively inhibit Sp transcription factor-mediated transcriptional activation (Blume et al., 1991).

Sp1 siRNA reduced the protein levels of Sp1 over 2-fold in all three cell lines tested, as shown in Figures 3A and 3B, when compared to Sp1 levels in cells transfected with either a nonspecific siRNA (NS, Figure 3A, lanes 3, 6, and 9) or siRNAs targeted against lamin A/B transcripts, although lamin A/B protein levels were dramatically reduced (Figure 3A, lanes 1, 4, and 7). Sp1 siRNA had no effect on another Sp family member, Sp3 (Figure 3A). As expected, reduction of Sp1 levels lead to the reduction of the protein levels of one of its known target genes, cyclin D1, in all three cell lines tested (Figure 3A; Grinstein et al., 2002). As predicted, reduction of Sp1 levels dramatically decreased MDM2 levels, up to 3-fold in cells homozygous for SNP309 (A875 and T47D), while in cells wild-type for SNP309 (HeLa), reduction of Sp1 levels had no significant effect on MDM2 levels (Figures 3A and 3B). Inhibition of Sp1 activity by treating cells with mithramycin A showed similar effects on MDM2 levels. A cell line homozygous for SNP309 (T47D) and a cell line wild-type for SNP309 (HL60) were treated with various concentrations of mithramycin A for 24 hr, and MDM2 protein levels were analyzed as shown in Figure 3C. In cells homozygous for SNP309 (T47D), mithramycin A treatment significantly decreased MDM2 levels. Specifically, 200 nM mithramycin A reduced MDM2 levels more than 5-fold in T47D, while no significant effect was seen in cells wild-type for SNP309 (HL 60). Similar effects were seen in other cell lines as shown in Figure 3D. In two more cell lines homozygous for SNP309 (Manca and A875), mithramycin A treatment significantly decreased MDM2 levels. However, mithramycin A only slightly reduced the MDM2 levels in two more cell lines wild-type for SNP309 (HeLa and ML-1). In summary, both methods of Sp1 inhibition can preferentially reduce the heightened levels of MDM2 in cells homozygous for SNP309, thereby supporting the hypothesis that the Sp1 transcription factor is indeed re-

A

		Mdm2 Levels (comp. ng)		Average
T/T	H460	6.3	(±0.41)	
	Tera 2	11.3	(±0.49)	12.6
	Ml-1	14.4	(±1.51)	
	Saos 2	18.3	(±0.54)	

		Mdm2 Levels (comp. ng)		Average
G/G	A875	8.8	(±0.33)	
	T47d	46.6	(±6.96)	103.4
	Manca	47.0	(±2.26)	
	CCF	311.2	(±36.7)	

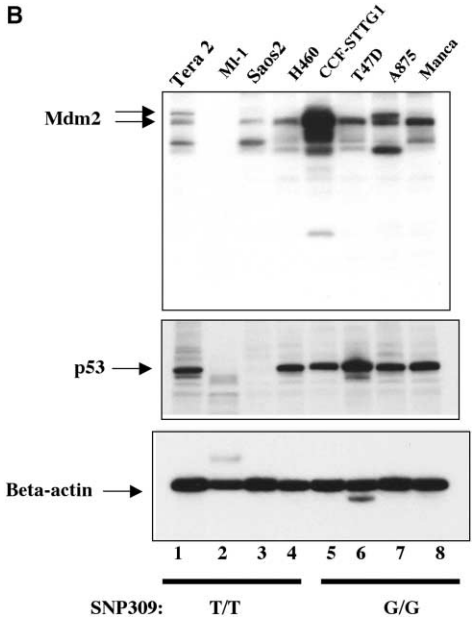

Figure 2. SNP309 Associates with the Overexpression of MDM2

(A) The levels of *MDM2* RNA from tumor-derived cell lines are shown as measured by real-time PCR (TaqMan). All values have been normalized to the level of GAPDH and are the averages of three independent readings. The standard deviation is given in the parenthesis next to each value. (B) A Western blot analysis of MDM2 levels found in total cell lysates is shown. The upper panel is a Western blot using a monoclonal MDM2 antibody (SMP-14). Full-length MDM2 protein runs as a doublet and is indicated by arrows. The middle panel is a Western blot of the same filter using a monoclonal antibody to p53 (Do-1) and the lower panel is a Western blot of the same filter using a monoclonal antibody to actin.

sponsible for the heightened levels of MDM2 in cells homozygous for SNP309.

Overexpression of MDM2 can lead to the inactivation of the p53 pathway (Lain and Lane, 2003). We therefore reasoned that the SNP309-homozygous cell lines, with higher levels of MDM2, should have an attenuated p53 response. To test the p53 response, these cells were treated with the chemotherapeutic drug etoposide (VP16) to induce DNA damage. This DNA damage activates the p53 pathway, leading to DNA repair, cell cycle arrest, and apoptosis. Significant death (20%–35% of the total cell population) was observed in cells that are wild-type at p53 and wild-type for SNP309 (T/T) (H460, ML-1, Tera-2: Figure 4A, lanes 1–3). Interestingly, cells with wild-type p53 that are homozygous for SNP309 (G/G) (Manca, CCF-STTG1, and A875: Figure 4A, lanes 6–8) showed much lower death rates (on average 2%–3% of the total cell population). In fact, the percentage of cells dying after treatment was similar to that seen in cells either mutant (T47D) or null (Saos2) for the p53 gene itself (compare lanes 5 and 4 with 1–3). Four cell lines heterozygous for SNP309 (T/G) and wild-type for p53 were also assayed for their response to etoposide and found to be intermediate in their response: on average 5%–7% of the total cell population (data not shown). Together, these data demonstrate that the DNA damage response pathway is attenuated in the SNP309 homozygous cell lines.

These data support the model that the heightened MDM2 levels in SNP309 cells attenuate the p53 pathway. If true, reducing the MDM2 levels should allow for the p53 pathway to be activated in the SNP309 homozygous cells lines. To test this, MDM2 levels were reduced by blocking Sp1-mediated transcription by mithramycin A treatment, and the activity of the p53 pathway was assessed by measuring the apoptotic response after DNA damage (etoposide treatment). Specifically, Manca (homozygous for SNP309) and ML-1 (wild-type for SNP309) were treated with various concentrations of mithramycin A and etoposide for 48 hr, after which the percentage of cells undergoing apoptosis was measured. Interestingly, as shown in Figure 4B, the percentage of etoposide-induced cell death increased significantly (2–3 fold) in Manca (SNP309 homozygous cell line) with mithramycin A treatment. However, in ML-1 (SNP 309 wild-type cell line), mithramycin A treatment had no obvious effect on etoposide-induced cell death. Similar results were obtained using various incubation times (data not shown). These data demonstrate that reduction of MDM2 levels by inhibiting Sp1 activity can reverse the attenuated p53 response in the SNP309 homozygous cell lines, thereby supporting the model that the heightened MDM2 levels due to increased Sp1 activity on the *MDM2* promoter in SNP309 cells result in the attenuation of the p53 pathway.

p53 responds to DNA damage by activating a transcriptional program (Jin and Levine, 2001). If the low death rates, seen after etoposide treatment in the homozygous SNP309 cell lines, is due to inhibition of p53, the p53 transcriptional program should be weakened in these cells. To test this, cells with wild-type p53 were treated with etoposide, and then the induction of 27 known p53-responsive genes was compared in the cells either homozygous for SNP309 or wild-type for SNP309. RNA was isolated from cells before and after etoposide treatment, and gene expression was analyzed using the

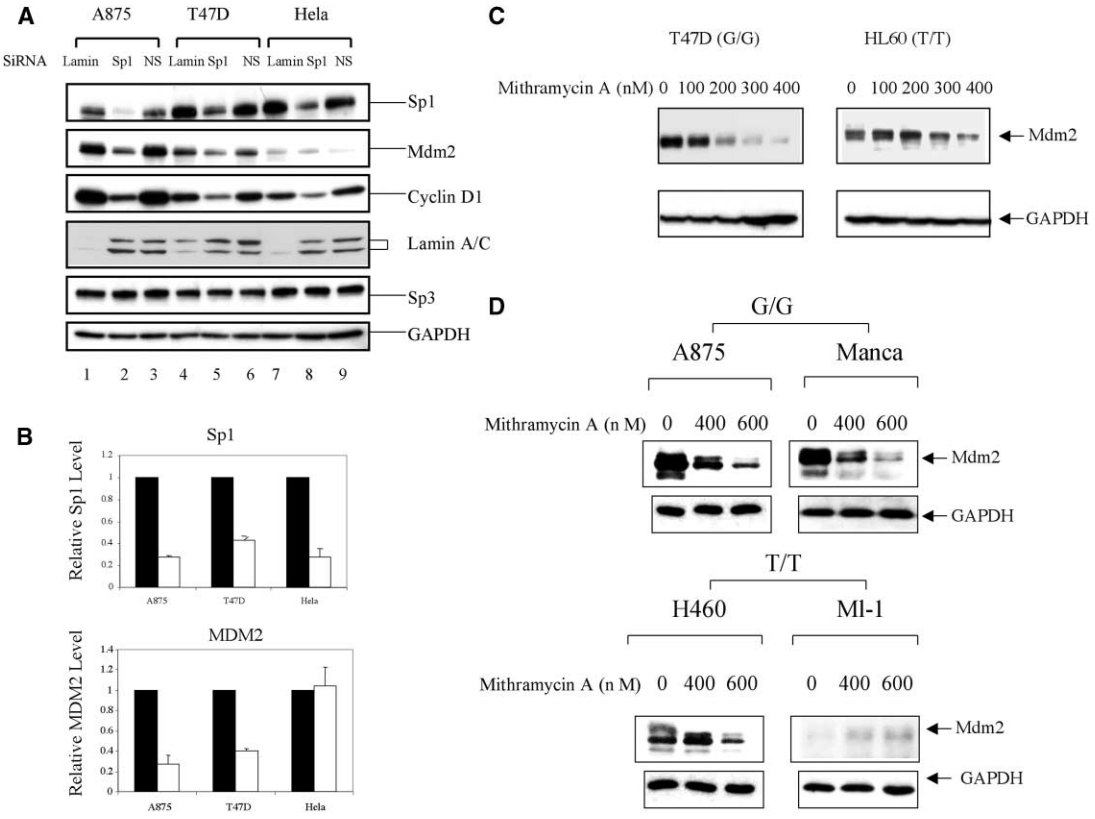

Figure 3. Inhibition of Endogenous Sp1 Can Reduce the Heightened Levels of Endogenous MDM2 in Cells Homozygous for SNP309

(A) Cell lines homozygous for SNP309 (G/G; A875 and T47D) and wild-type for SNP309 (T/T; HeLa) were transfected with siRNA specific for Sp1, for lamin A/B, or a nonspecific siRNA (NS). Depicted here are autoradiographs of Western blots using antibodies specific for Sp1, Sp3, MDM2, cyclin D1, lamin A/B, and Gapdh. Arrows indicate the positions of the proteins.

(B) The bar graphs represent the quantification of Western blot analysis in (A). The relative expression levels in cells transfected with siRNA for Sp1 was determined as the percentage of either Sp1 or MDM2 in the cells transfected with Sp1-siRNA to those transfected with nonspecific siRNA. Each value is the average of at least three independent experiments and the error bars represent the standard deviations.

(C and D) Cell lines either homozygous for SNP309 (G/G; T47D, A875, and Manca) or wild-type for SNP309 (T/T; HL60, H460, and Ml-1) were treated with various concentrations of mithramycin A for 24 hr. Autoradiograms of Western blots using either MDM2 or Gapdh antibodies are shown. Arrows indicate the positions of the proteins.

AffyMetrix GeneChip array (Human Genome U95A Array). Cells wild-type for SNP309 induced multiple known p53-responsive genes above 5-fold (Figure 4C). Specifically, nine genes in the lung carcinoma cell line, H460 (Figure 4C, lane 1), six genes in the myeloid leukemic cell line, ML-1 (Figure 4C, lane 2), and four genes in the testicular teratoma cell line, Tera-2 (Figure 4C, lane 3), were induced. In contrast, cells homozygous for SNP309 showed a much-weakened p53-dependent transcriptional response. The melanoma cell line, A875 (Figure 4C, lane 4), did not induce any known p53-responsive gene above 5-fold and both the astrocytoma cell line, CCF-STTG1 (Figure 4C, lane 6), and the Burkitt's lymphoma cell line, Manca (Figure 4C, lane 5), only induced one gene above 5-fold. These data demonstrate that the ability of p53 to act as a transcriptional activator for known target genes is attenuated in cells homozygous for SNP309.

Taken together, these data support a model that cells homozygous for SNP309 (G/G) express higher levels of MDM2, thereby attenuating the p53 pathway. MDM2 has been shown to inhibit p53 minimally by three different mechanisms. One well-studied mechanism is its ability to serve as an E3 ubiquitin ligase, targeting p53 for proteosamal degredation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Upon certain cellular stresses, such as DNA damage, p53 levels rise, as the half-life of p53 dramatically increases. The increase of the half-life of p53 has been attributed to the inability of MDM2 to target p53 for degradation after DNA damage. Specifically, immediately after DNA damage, MDM2 levels decrease dramatically as does its affinity for p53 binding (Michael and Oren, 2003).

To address what effect the elevated MDM2 levels in G/G cells have on p53 levels, the levels of wt p53 in cells before and after stress (DNA damage) were monitored. As seen in Figure 2B there are no significant differences in basal wild-type p53 levels between cells wild-type or homozygous for SNP309 (compare p53 levels in lanes 1, 2, and 3 to those in lanes 5, 7, and 8). To rule out the possibility that MDM2 cannot target p53 for degradation in G/G cells, MDM2 levels were artificially reduced in nonstressed cells, and p53 levels were monitored. As seen in Figure 4D, reduction of MDM2 levels, using siRNAs, in the two G/G cell lines tested led to the significant stabilization of p53. The stabilized p53 is active, as p21 levels also rise, and p21 is a well-known

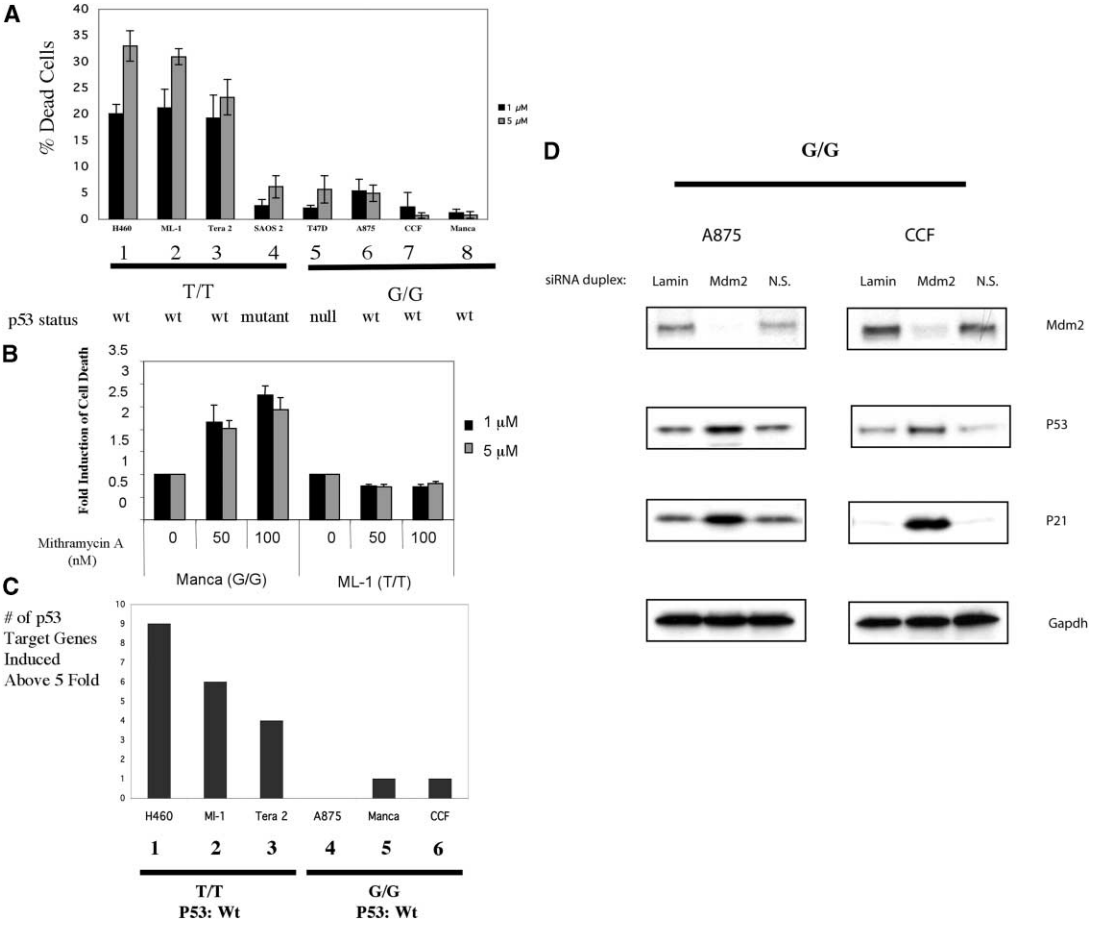

Figure 4. p53 Activity Is Attenuated in Cells Homozygous for SNP309

(A) In the bar graph, the percent of the population of cells which are dead after 24 hr of either 1 μM (black) or 5 μM (gray) etoposide treatment is plotted for each cell line. Each value is the average of three independent experiments and the error bars represent the standard deviations.

(B) Inhibition of Sp1 activity by mithramycin A treatment increases DNA damage-induced cell death in cells homozygous for SNP309. Manca (homozygous for SNP309) and ML-1 (wild-type for SNP309) were treated with either 1 μM (black) or 5 μM (gray) etoposide and various concentrations of mithramycin A for 48 hr. The percentage of the dead cell induced by etoposide and mithramycin A treatment was measured and subtracted with any death induced by mithramycin A treatment alone. Fold induction of cell death is depicted in the bar graph. Each value is the average of at least three independent experiments and the error bars represent the standard deviation.

(C) In the bar graph, the number of known p53 target genes induced after DNA damage (etoposide treatment) above 5-fold from levels found in untreated cells is plotted for each cell line.

(D) MDM2 can inhibit p53 in cell lines homozygous for SNP309 (G/G; A875 and CCF). Cells were transfected with siRNA specific for *MDM2*, for lamin A/B or a nonspecific siRNA (NS). Depicted here are autoradiographs of Western blots using antibodies specific for MDM2, p53, p21, and Gapdh.

p53 transcriptional target. These data suggest that MDM2 can target p53 for degradation in G/G cells, but that, in nonstressed cells, heightened levels of MDM2 in G/G cells do not further reduce the levels of wt p53.

As mentioned above, the heightened MDM2 levels could also inhibit the proper stabilization of p53 in response to stress, thereby attenuating the p53 pathway. To address this possibility, the stabilization of p53 after etoposide treatment was monitored (Figures 5A and 5B). Cells were treated with etoposide and harvested after 1, 2, and 3 hr of treatment. The p53 levels were measured by Western blotting and the fold induction of p53 levels after etoposide treatment was calculated for each cell line and each time point. The values are depicted in the bar graph in Figure 5B. As expected, the p53 levels in cells wild-type for SNP309 (T/T) increased 4-fold or above after only 1 hr of etoposide treatment. Interestingly, there was no significant stabilization of p53 in cells homozygous for SNP309 (G/G) in the times tested. These data support the hypothesis that the heightened levels of MDM2 in SNP309 cells result in the inability to properly stabilize p53 in response to cellular stresses like DNA damage but that the heightened levels of MDM2 do not further reduce the levels of wt p53 in nonstressed cells. The inability to properly stabilize p53 in response to DNA damage provides one possible mechanism for the observed attenuation of p53 pathway in cells homozygous for SNP309 (G/G).

The data thus far support a model that cells homozygous for SNP309 express higher levels of MDM2, thereby weakening the p53 pathway. Humans who carry a germline p53 mutation in one allele (Li-Fraumeni Syn-

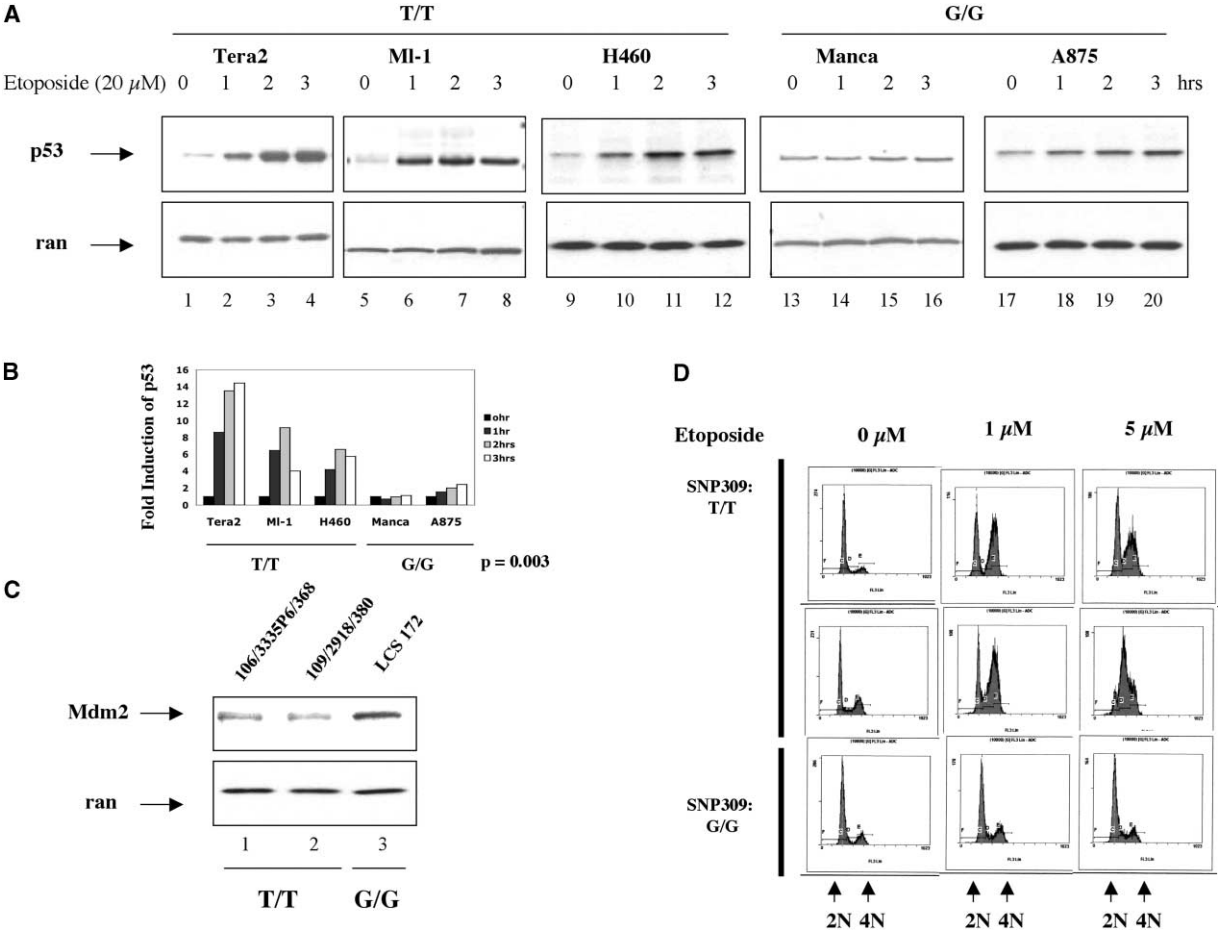

Figure 5. The Stabilization of p53 after DNA Damage Is Impaired in Cells Homozygous for SNP309, and the p53 DNA Damage Response Is Attenuated in Li-Fraumeni Fibroblasts Homozygous for SNP309 with High MDM2 Levels

(A) Cell lines either wild-type (T/T) or homozygous (G/G) for SNP309 were treated with etoposide and harvested after 1, 2, and 3 hr of treatment. Depicted here are autoradiographs of Western blots using antibodies specific for p53 and ran. The autoradiograms are depicted, and exposures were chosen to highlight the induction of p53 after etoposide treatment for each cell line. The p53 levels were measured and normalized to ran levels from the autoradiograms in (A), and the fold induction of p53 after etoposide treatment from basal levels was calculated for each cell line and each time point. The values are depicted in the bar graph in (B).

(C) A Western blot analysis of MDM2 levels found in total cell lysates is shown. The upper panel is a Western blot using a monoclonal MDM2 antibody. Full-length MDM2 protein is indicated an arrow. The lower panel is a Western blot of the same filter using a monoclonal antibody to ran.

(D) The fibroblastoid cell line derived from a germline p53 mutation carrier (Li-Fraumeni) and homozygous for SNP309 has an altered DNA damage response when compared to Li-Fraumeni fibroblastoid cell lines wild-type for SNP309. Histograms are depicted of the DNA content in cells after 24 hr of varying concentrations of etoposide treatment (0, 1, and 5 μM, indicated above the panels).

drome) develop tumors at very high frequencies (Garber et al., 1992; Li et al., 1990). These individuals develop tumors on average at very young ages and can develop multiple primary tumors throughout a lifetime. We hypothesized that increased levels of MDM2 by SNP309 could further weaken the p53 pathway in Li-Fraumeni individuals and further impact tumorigenesis. To test this, the first approach was to analyze MDM2 levels and the DNA damage response (etoposide treatment) in fibroblasts derived from Li-Fraumeni individuals who were either wild-type for SNP309 (T/T) or homozygous for SNP309 (G/G). MDM2 levels were analyzed by Western blotting for two fibroblast cell lines wild-type for SNP309 (T/T) and one fibroblast cell line derived from the Li-Fraumeni individual homozygous for SNP309 (G/G). As predicted by the analysis of tumor-derived cell lines, the G/G fibroblasts showed significantly higher MDM2 levels (above 3-fold) when compared to those found in both T/T fibroblast cell lines (Figure 5C). Upon etoposide treatment, the two fibroblast cell lines wild-type for SNP309 (T/T) showed a characteristic p53-mediated G2 arrest as noted by the accumulation of cells with tetraploid DNA content (Figure 5D). In contrast, the fibroblast cell line homozygous for SNP309 (G/G) did not show such a response. In fact, no significant difference in the DNA content of the SNP309 homozygous (G/G) cells was seen. These data suggested that both MDM2 levels and the p53 pathway in Li-Fraumeni individuals, as measured by the DNA damage response in fibroblasts, could be greatly affected by the presence of SNP309.

If SNP309 further debilitates the p53 pathway in Li-

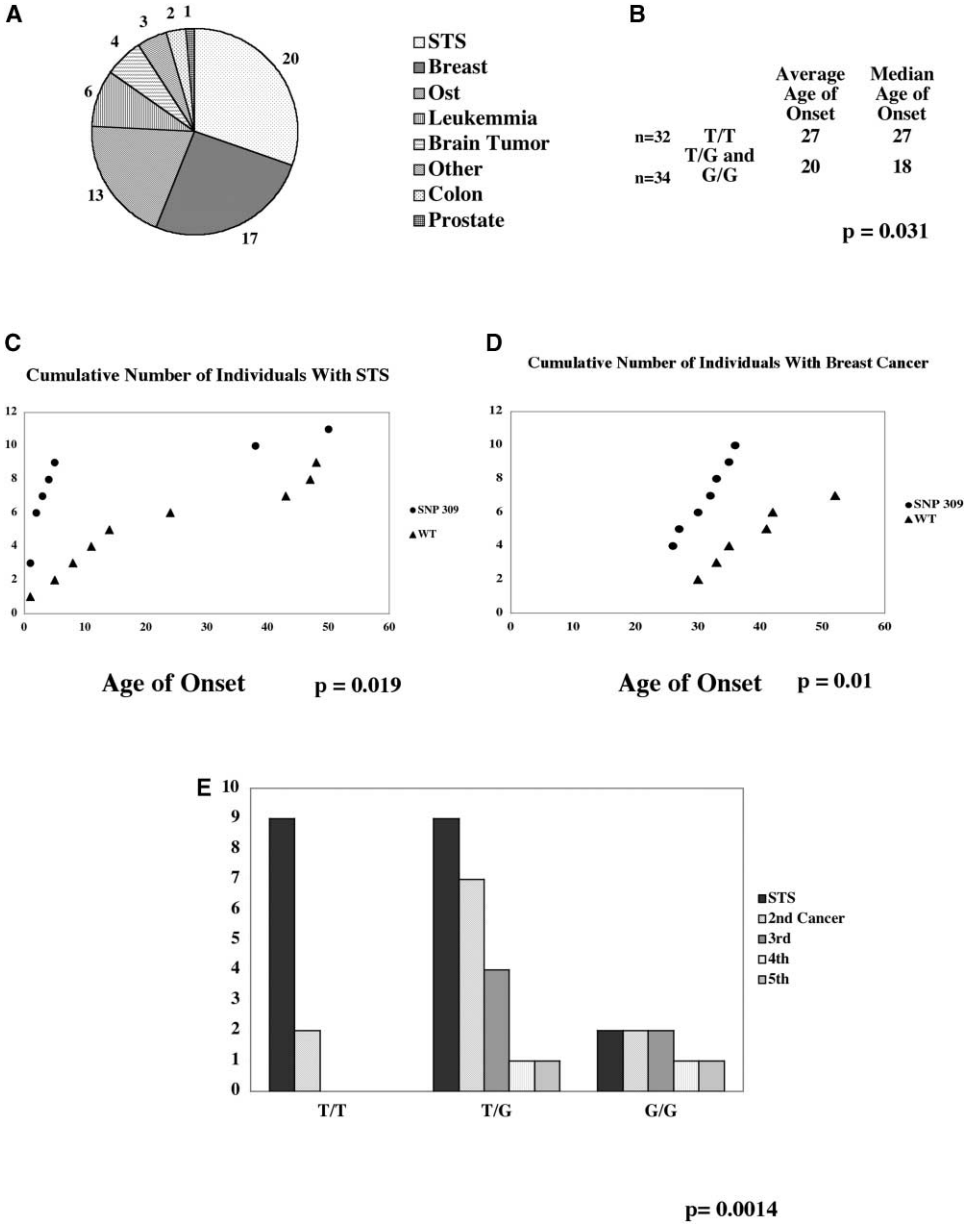

Figure 6. SNP309 Associates with an Accelerated Age of Onset of Tumors and the Occurrence of Multiple Subsequent Tumors in Li-Fraumeni Individuals

(A) Of the 88 individuals in the Li-Fraumeni cohort, 66 were diagnosed with at least one cancer at a median age of 22 years old. The first cancers the 66 individuals were diagnosed with are shown in the pie chart. Individuals either T/G or G/G are diagnosed on average 7 years earlier than wild-type (T/T) individuals as depicted in (B). The cumulative number of individuals either with SNP309 (heterozygous or homozygous) (circles) or wild-type for SNP309 (triangles) is plotted against the age of onset of STS (C) and breast cancer (D).

(E) The bar graph depicts the number of individuals who were first diagnosed with STS (black) and then with subsequent cancers (second, third, fourth, and fifth) for individuals either wild-type (T/T), heterozygous (T/G), or homozygous (G/G) for SNP309.

Fraumeni individuals, tumor development might also be affected. To address this possibility, 88 individuals who are members of Li-Fraumeni families and have germline mutations in one allele of p53 were studied, and the frequency of SNP309 was found to be similar to the frequencies found in the 50 normal volunteers (Hwang et al., 2003). Sixty-six have been diagnosed with at least one cancer so far. Soft tissue sarcomas (STS) (20 individuals), breast cancer (17), and osteosarcomas (13) were the most prevalent first cancers (Figure 6A). Interestingly, individuals who carried SNP309, in either the heterozygous or homozygous state, showed a significantly earlier age of onset for all tumor types (Figure 6B). For individuals with SNP309, the median age of tumor onset was 18 years old, while in wild-type individuals the median age of tumor onset was 27 years old ($p = 0.031$). Specifically, the median age of onset of STS in wild-type individuals was fourteen years old, while in SNP309 individuals the median age of onset was two years old ($p = 0.019$, Figure 6C). In those who developed breast

cancer, the median age of onset in wild-type individuals was 39 years old, and in SNP309 individuals the median age of onset was 29 years old ($p = 0.01$, Figure 6D). Taken together, those carrying SNP309 developed all tumor types 9 years earlier, STS on average 12 years earlier and breast cancer on average 10 years earlier than those who do not carry SNP309. These data support the hypothesis that the presence of SNP309 further debilitates the p53 pathway in Li-Fraumeni individuals, resulting in an even earlier tumor onset.

As mentioned above, another hallmark of the Li-Fraumeni syndrome is the occurrence of multiple primary tumors in a lifetime. Interestingly, the presence of SNP309 also correlated with the occurrence of multiple tumors in those individuals first diagnosed with STS ($p = 0.0014$, Fisher's exact test, Figure 6E). Specifically, both individuals homozygous for SNP309 (G/G) developed a second and a third cancer, and one developed a fourth and a fifth cancer. Of the nine people heterozygous for SNP309 (T/G), seven developed a second cancer, four a third, and one a fourth and a fifth cancer. In contrast, of the nine people wild-type for SNP309 (T/T), only two developed a second cancer and none a third, a fourth, or a fifth cancer. These data indicate an association with the presence of SNP309 and the occurrence of independent subsequent cancers. Together, these data support a model whereby the presence of SNP309 in the promoter of the *MDM2* gene leads to overexpression of the MDM2 protein, which then inhibits p53 and impacts two major hallmarks of the Li-Fraumeni Syndrome, namely the early age of onset of tumors and the occurrence of multiple primary tumors in a lifetime.

The next question raised was if SNP309 caused a weakened p53 pathway, did this SNP act upon sporadic cancers as well as genetically altered individuals with a p53 defect. To address this question, a group of patients who develop sporadic adult STS and had no known hereditary cancer predisposition and no known germline p53 mutation was studied. As seen in the pie chart in Figure 7A, the sarcomas come from a variety of tissues. Interestingly, as in the Li-Fraumeni individuals, SNP309 associates with an earlier age of onset of STS (Figure 7B). Specifically, those individuals homozygous for SNP309 were diagnosed on average 12 years earlier than those individuals without SNP309 ($p = 0.01$, Figure 7C). As seen in Figure 7D, the frequency of the SNP309 G allele was greatly increased in those individuals who developed STS at a young age. Those who developed STS below the age of 41 years old had an allele frequency for SNP309 of 50%, while the allele frequency is only 33% for the whole group ($p = 0.0262$, Figure 7D). These data demonstrate that SNP309 does not require the presence of an inactivating germline p53 mutation to associate with earlier STS tumor formation.

Discussion

Experimental data have been presented in this report which support the hypothesis that single nucleotide polymorphisms in the tumor suppressor p53 pathway can be a part of the genetic variation which underlies the phenotypic variation seen in individuals' susceptibility to cancer. To summarize, the results in biochemical assays in cell culture systems demonstrate that SNP309 in the promoter of the *MDM2* gene increases the binding affinity of the transcriptional activator Sp1, which results in high levels of MDM2 RNA and protein. The heightened MDM2 levels were shown to lead to the attenuation of the p53 DNA damage response, in concordance with the observations that MDM2 is as a key negative regulator of p53. The results of clinical epidemiological studies demonstrated that SNP309 associates with minimally a 9-year earlier onset of tumors in both hereditary and sporadic cancers. Together, these data support a model whereby SNP309 enhances the affinity of the transcriptional activator Sp1 to the promoter of the *MDM2* gene, resulting in heightened transcription. Heightened levels of MDM2 lead to the direct inhibition of p53, which releases the cell from p53 tumor suppression. Inhibition of endogenous Sp1 using either siRNA or mithramycin A can significantly reduce SNP309-related overexpression of MDM2. The experiments presented here demonstrate that reduction of SNP309-related overexpression of MDM2 by mithramycin A treatment can reverse the attenuated p53 response. Mithramycin A is an aureolic antibiotic that has been used in humans to treat several types of cancer and hypercalcemia associated with cancers (Hurtado and Esbrit, 2002). The data presented here suggest that treating cancer patients homozygous for SNP309 with mithramycin A may enhance their response to chemotherapeutic drugs by reducing MDM2 levels to allow for a p53-dependent apoptotic response induced by most chemotherapeutic agents.

The model proposed here states that the heightened levels of MDM2, due to SNP309, result in accelerated tumor formation in humans. There is much evidence in the literature to support the claim that high MDM2 levels can positively impact tumorigenesis. For example, Jones et al. (1998) created mice which overexpress Mdm2 by using the entire *MDM2* gene as a *trans*-gene. These mice expressed an average of 4-fold more Mdm2 in various tissues relative to nontransgenic mice. Interestingly, 100% of the Mdm2-overexpressing mice developed spontaneous tumors in a lifetime. Lundgren et al. (1997) also showed that targeted overexpression of Mdm2 in the murine mammary epithelium results in tumors, albeit with a lower penitence (16%). These two studies, together with numerous accounts of MDM2 overexpression or amplification in a variety of human cancers, support the idea that heightened levels of MDM2, by SNP309, could positively impact tumor formation (Cordon-Cardo et al., 1994; Momand et al., 1998; Oliner et al., 1992; Taubert et al., 2000).

MDM2 is a key negative regulator of p53. MDM2 can regulate the degradation of p53 as an E3 ubiquitin ligase, targeting p53 for proteosamal degredation (Michael and Oren, 2003). Observations in this report support the hypothesis that the heightened levels of MDM2 in SNP309 cells (G/G) result in the inability to properly stabilize p53 in response to cellular stresses like DNA damage; this hypothesis offers one possible mechanism of p53 attenuation by MDM2 in SNP309 (G/G) cells. Interestingly, the heightened levels of MDM2 do not further reduce the levels of wild-type p53 in nonstressed cells. One explanation for these observations is that the ability of MDM2 to regulate p53 levels may not be limiting in nonstressed cells, so heightened levels of MDM2 would

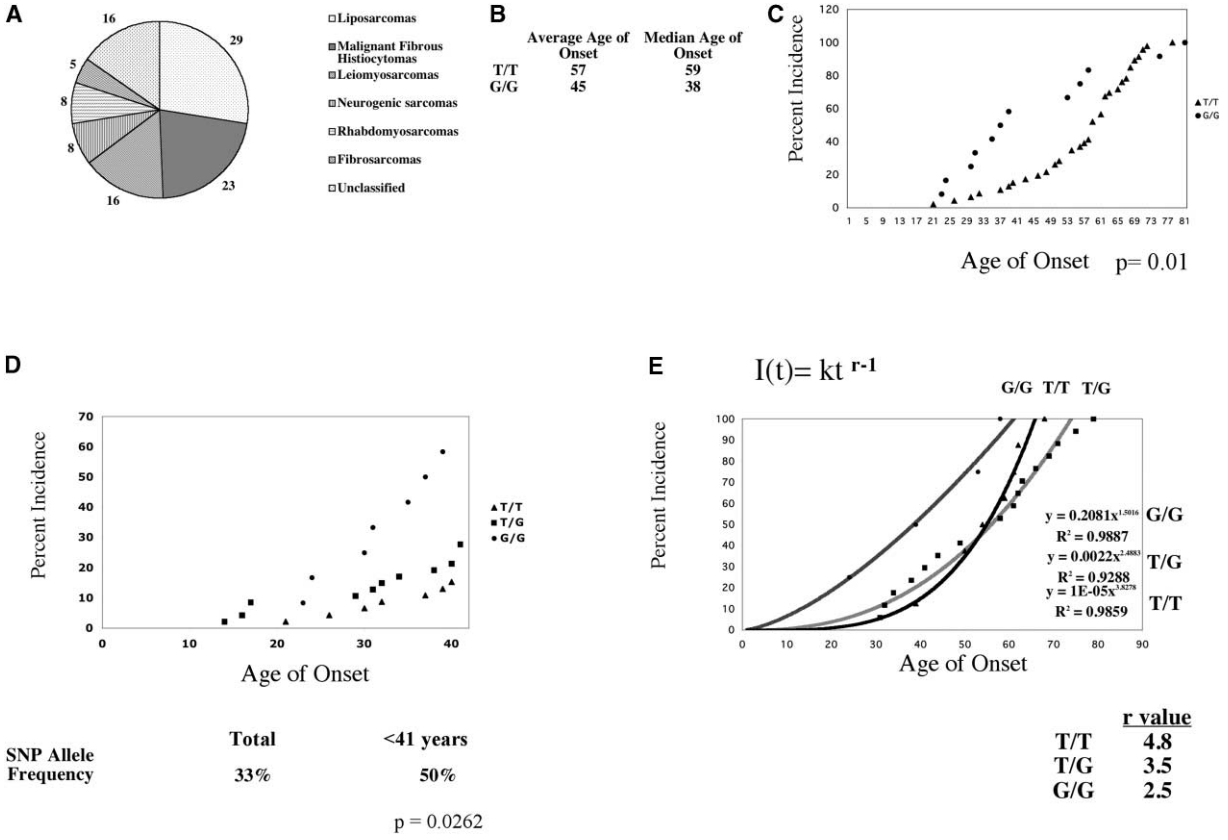

Figure 7. SNP309 Associates with an Accelerated Age of Onset of Tumors in Sporadic Soft Tissue Sarcoma

(A) The subtypes of STS are depicted in the pie chart. Individuals homozygous for SNP309 showed a significantly earlier age of onset of STS, on average 12 years earlier (B). The cumulative number of individuals either homozygous for SNP309 (circles) or wild-type for SNP309 (triangles) is plotted against the age of onset of STS (C).

(D) SNP309 is at a higher frequency in the population with early onset STS. The cumulative number of individuals either homozygous for SNP309 (circles), heterozygous for SNP309 (squares), or wild-type for SNP309 (triangles) is plotted against the age of onset of STS in those who were diagnosed below the age of 41 years old (one standard deviation from the median age of onset).

(E) SNP309 could serve as a rate-limiting event in liposarcoma. The number of rate-limiting events required to create a cancer cell and subsequent tumor was calculated using $I(t) = kt^{r-1}$, whereby r is the number of rate-limiting events. The R value was calculated for each genotype by plotting the cumulative number of individuals either homozygous for SNP309 (circles), heterozygous for SNP309 (squares), or wild-type for SNP309 (triangles) against the age of onset of liposarcoma and performing a nonlinear regression as a power function to obtain the exponent for each curve. The equations for the trend-line, R^2 value, and R value are displayed for each genotype.

have no further impact on p53 levels. Only when MDM2 activity is reduced after cellular stress (or artificially, e.g., siRNA in nonstressed cells, Figure 4D) does MDM2 become limiting, where heightened MDM2 levels would result in less induced p53 (Figures 5A and 5B).

The results presented in this report suggest that SNP309 can impact tumorigenesis in humans who carry a germline inactivating mutation in one p53 allele (Li-Fraumeni Syndrome). The data suggest that SNP309 can further lower the age of onset of tumors in these individuals on average 9 years and increase the occurrence of multiple primary tumors in a lifetime. One possible scenario to explain these observations is that high levels of MDM2 and just one wild-type p53 allele in those Li-Fraumeni individuals with SNP309 produce a severely weakened p53 tumor suppressor pathway resulting in a higher mutation rate, poorer DNA repair processes, and reduced apoptosis leading to faster and more frequent tumor formation.

Fifty years ago it was noted that age-specific incidence of many tumors increases with the power of age, and it was proposed that the age-specific increase could be correlated to the number of rate-limiting steps involved in the formation of a cancer cell and subsequent tumor. Specifically, the function $I(t) = kt^{r-1}$ was used to describe the incidence of cancer (I) observed at a given age (t), whereby r is the number of rate-limiting events which have to occur in cells at the constant rate (k). This equation or derivatives thereof have been successfully used to describe many cancers (Knudson, 2001), most notably in Alfred G. Knudson's description of hereditary and nonhereditary forms of retinoblastoma, which later became known as the two hit theory. As shown in Figures 6 and 7, the age-incidence curves of those patients with SNP309 (G/G) vary greatly from those individuals with a genotype of T/T or even T/G. Interestingly, when the number of rate-limiting events (R value) is calculated for each genotype at the SNP309 locus in sporadic liposarcomas, T/T gives a value of 4.8, T/G 3.5, and G/G 2.5 (Figure 7E). The fact that the heterozygote reduces

the R value by one and the homozygote by two suggests that the G allele can serve as a rate-limiting event in the formation of a sarcoma.

It is not difficult to propose a model to explain why SNP309 could be a rate-limiting event in carcinogenesis. Over the past 50 years, it has become clear that age-specific incidence of cancer is dependent minimally on three factors: the number of rate-limiting mutations required for a given cancer, the mutation rate per mitosis, and the net proliferation rate of the effected cells (cell division rate minus cell death rate; Knudson, 2001). Inhibition of the p53 pathway by SNP309 could potentially affect all three of these factors (Jin and Levine, 2001; Lain and Lane, 2003). The p53 gene itself is thought of as a rate-limiting mutation in many cancer types, as it is found mutated in over 50% of all human tumors, and humans who carry a germline p53 mutation develop cancer with increased incidence and on average early in life. The wild-type p53 pathway is also thought to reduce mutation rates per mitosis, as loss of p53 leads to defective centrosome replication and numerous chromosomal abnormalities. Finally, the p53 pathway also impacts the net proliferation rate of cells, as it functions to arrest the cell cycle and induce apoptosis upon stress signals like DNA damage and oncogene activation. Therefore, inhibition of the p53 pathway by SNP309 could affect all three factors, which have been shown to influence the age-specific incidence of cancer to accelerate carcinogenesis in an individual.

Experimental Procedures

Sequence Analysis

The *MDM2* promoter was analyzed for sequence variation by PCR amplification and subsequent sequencing, primer 1: CGGGAGTT CAGGGTAAAGGT and primer 2: AGCAAGTCGGTGCTTACCTG.

Electrophoretic Mobility Shift Assays (EMSA)

EMSAs were performed with a LightShiftTM Chemiluminescent EMSA Kit (PIERCE, Rockford, Illinois). The binding reactions were performed for 20 min at room temperature in 10 mM Tris-HCl ([pH 7.5] at 25°C), 1 mM $MgCl_2$, 50 mM NaCl, and 0.5 mM DTT, 4% glycerol, 50 μg/ml poly (dI-dC) (dI-dC), 10 fmol biotin 3′-end-labeled double-stranded oligonucleotides, and purified recombinant Sp1 protein. After incubation, samples were separated on a native 4% polyacrylamide gel and then transferred to a nylon membrane. The positions of the biotin end-labeled oligonucleotides were detected by a chemiluminescent reaction with streptavidin-horseradish peroxidase according to the manufacturer's instruction and visualized by autoradiography. The nucleotide sequence of the double-stranded oligonucleotides with either wild-type sequence or the SNP is as follows: 5′-CCGGGGGCTGCGGGGCCGCTT/GCGGCGC GGGAGGTCCGGATG-3′.

Chromatin Immunoprecipitation

Proteins were crosslinked to DNA in 1% formaldehyde. After washing, cells were lysed in detergent lysis buffer. Lysates were washed and sonicated. Two micrograms of antibodies were added and incubated overnight. Protein A/G Plus beads (Santa Cruz) were used, and after extensive washing, crosslinks were removed at 65°C overnight in an elution buffer (1% SDS, 0.1 M $NaHCO_3$). DNA was isolated using the QIAquick PCR purification kit (Qiagen). Ten percent of purified DNA was analyzed by PCR. The entire PCR reaction was analyzed by gel electrophoreses on an 8% nondenaturing polyacrylimide gel and subsequent ethidium bromide staining.

Luciferase Reporter Assays

The *MDM2* promoter-luciferase reporter plasmids containing either the wild-type sequence or the SNP309 sequence were constructed by tandemly inserting two copies of double-stranded oligonucleotides containing the same sequence as used for the EMSAs into pGL2 luciferase reporter plasmid (Promega). The clones were confirmed by DNA sequencing.

Drosophila Schneider's SL2 cells were seeded at 1.5×10^6 cells per well in a six-well plate 24 hr before transfection. Transient transfections were preformed using CellfectAMINE (Invitrogen) according to the manufacturer's instructions. The DNA transfection mixture contained 250 ng reporter plasmid, varying amounts of pPac-Sp1 plasmid, and empty pPac vector to normalize DNA concentrations. The expression plasmids pPac and pPac-Sp1 were kindly provided by Dr. Richard D. Kolodoner (La Jolla, California). Cells were harvested 48 hr after transfection in reporter lysis buffer (Promega) and assayed for luciferase activity. Each extract was analyzed in duplicate, and at least three independent experiments were performed. Luciferase activities were normalized to cellular protein, measured by the Bio-Rad protein assay system.

RNA Analysis

To analyze *MDM2* RNA levels, total RNA was isolated from cell pellets using RNeasy (Qiagen). cDNAs were made using TaqMan reverse transcription reagents from Applied Biosystems. Real-time PCR was carried out using the ABI Prism 7000 sequence detection system. Probe and primer sets for *MDM2* and Gapdh were purchased as predeveloped assays from Applied Biosystems.

Protein Analysis

To analyze protein levels, total cell extracts were made by using a detergent lysis buffer (50 mM Tris [pH 7.5] 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% deoxycholic acid, 5mM EDTA, and a protease inhibitor cocktail [Complete Mini, Roche]). Thirty micrograms of total protein was run on a 4%–20% tris-glycine gel (Invitrogen) and transferred to a PVDF membrane. MDM2 was detected using the mouse monoclonal antibody SMP-14; Sp1 and Sp3 were detected using the rabbit polyclonal antibody PEP2 and D-20, respectively. Lamin A/C, cyclin D1, p53, and actin were detected using the mouse monoclonal antibody 346, DCS-6, Do-1, and C-2, respectively. Gapdh was detected using the goat polyclonal antibody V-18. All antibodies were purchased from Santa Cruz.

Gene Silencing with siRNA

Sp1 siRNA targeted to AATGAGAACAGCAACAACTCC was used to lower Sp1 expression. Two hundred picomoles siRNA duplex was transfected into cells at 30%–50% confluencey using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Control siRNA duplex has no known target in mammalian genomes and was used as follows: sense UUCUCCGAACGUGUCACGUdTdT, antisense ACGUGACACGUUCGGAGAAdTdT. Lamin A/C siRNAs were purchased from Qiagen and *MDM2* siRNAs from Dharmacom. Cells were lysed 48 hr after transfection, and protein levels of Sp1, Sp3, and MDM2 were analyzed as described above.

Mithramycin A Treatment

Logarithmically growing cells were treated with various concentrations of mithramycin A (Sigma) for 24 hr. After treatment, cells were lysed and protein levels of MDM2 were analyzed.

Cell Viability Analysis

To analyze induction of cell death after etoposide treatment, growing cells were treated with either 1 μM or 5 μM etoposide for 24 hr. To analyze etoposide-induced cell death after inhibition of Sp1, growing cells were treated with various concentrations of mithramycin A and etoposide for 48 hr. After treatment, cells were harvested and viability was measured using the Guava ViaCount assay (Guava).

Cell Cycle Analysis

To analyze the DNA damage response in fibroblasts, all cells were treated with two concentrations of etoposide (1 μM and 5 μM) for 24 hr. Cells were harvested and fixed with methanol and kept at −20°C for 40 min or overnight. After washing and equlibration in PBS, the cell pellet was resuspended in 1 ml of staining solution containing 50 μg of RNase A and 0.5 μg of propidium iodide per ml in PBS and kept at room temperature for over 30 min. The cells

were then applied to the fluorescence-activated cell sorter (FACSCalibur; Becton Dickson). The FACSCalibur program was used to sort and count the cells.

Statistical Analysis

A randomization test is employed to determine the statistical significance of the age of onset of cancer between the groups with and without SNP309. The two groups are compared pair-wise, and each instance of an element from the second group greater than an element of the first group adds one to the distance. This total distance is the cutoff. The lists are then randomly permuted, holding fixed the number of elements of each list. The calculated p value is the percent of randomized groups that have a distance less than the cutoff as determined by a large Monte Carlo simulation.

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