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Contrasting effects of an *Mdm2* functional polymorphism on tumor phenotypes

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Abstract

MDM2, an E3 ubiquitin ligase, is a potent inhibitor of the p53 tumor suppressor and is elevated in many human cancers that retain wild type p53. *MDM2 SNP309G* is a functional polymorphism that results in elevated levels of *MDM2* (due to enhanced SP1 binding to the *MDM2* promoter) thus decreasing p53 activity. *Mdm2^{SNP309G/G}* mice are more prone to spontaneous tumor formation than *Mdm2^{SNP309T/T}* mice, providing direct evidence for the impact of this SNP in tumor development. We asked whether environmental factors impact *SNP309G* function and show that *SNP309G* cooperates with ionizing radiation to exacerbate tumor development. Surprisingly, ultraviolet B light or Benzo(a)pyrene exposure of skin shows that *SNP309G* allele actually *protects* against squamous cell carcinoma susceptibility. These contrasting differences led us to interrogate the mechanism by which *Mdm2 SNP309* regulates tumor susceptibility in a tissue-specific manner. While basal *Mdm2* levels were significantly higher in most tissues in *Mdm2^{SNP309G/G}* mice compared to *Mdm2^{SNP309T/T}* mice, they were significantly lower in *Mdm2^{SNP309G/G}* keratinocytes, the cell type susceptible to squamous cell carcinoma. The assessment of potential transcriptional regulators in ENCODE ChIP-seq database identified transcriptional repressor E2F6 as a possible negative regulator of *MDM2* expression. Our data

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CONFLICTS OF INTERESTS

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show that E2F6 suppresses *Mdm2* expression in cells harboring the *SNP309G* allele but not the *SNP309T* allele. Thus, *Mdm2 SNP309G* exhibits tissue-specific regulation and differentially impacts cancer risk.

Keywords

IR; B(a)P; UVB; p53; E2F6; SNP

INTRODUCTION

A wealth of evidence exists associating single nucleotide polymorphisms (SNPs) and disease susceptibility (1–3); however, the detailed mechanisms behind this susceptibility are relatively unexplored. Functional SNPs are positioned within regulatory or protein coding sequences and can alter gene expression or protein structure, thus altering function and contributing to disease risk (4–10). Functional SNPs in tumor suppressor pathways exist and mechanistic studies to determine how these genetic variants destabilize cell homeostasis and contribute to cancer risk may provide insight for prevention and treatment of human cancer.

The *TP53* gene is a tumor suppressor and guardian of the genome (11). In response to stress signals, p53 transactivates many genes involved in processes that are critical for maintaining cell homeostasis including cell cycle arrest, DNA repair, and apoptosis (12–15). Thus, *TP53* is the most highly altered gene in human cancers (16). The importance of p53 function is further exemplified by the observation that slight differences in activity can lead to significant effects on cell function, tumor susceptibility, and survival (17, 18); consequently, p53 levels and function are tightly regulated. In addition to *TP53* mutations and deletions, alternate mechanisms also disrupt the p53 pathway (19). MDM2 is an E3 ubiquitin ligase that ubiquitinates p53 targeting it for proteasomal degradation (20). Previous studies have illustrated the interrelationship between these two proteins; p53 transcriptionally activates the *Mdm2* gene, which in turn produces a protein that binds and inhibits p53 activity creating a negative feedback loop (21). In addition, mouse models with *Mdm2* deletion exhibit embryonic lethality, a phenotype that is completely rescued by *p53* deletion (22, 23). Moreover, tumor studies revealed a mutually exclusive relationship between MDM2 and p53; tumors harboring *MDM2* amplification seldom carry *TP53* mutations (19, 24).

MDM2 SNP309G is a functional polymorphism in the p53 pathway that associates with increased cancer risk in many cancer types, including lung, colon, pancreas, endometrium, and head and neck (25–29). Furthermore, genetically engineered mice harboring the homozygous *Mdm2 SNP309G* allele are more susceptible to spontaneous tumor formation and exhibit a significantly lower overall survival compared to homozygous *Mdm2 SNP309T* mice (30). Mechanistically the *SNP309G* sequence creates a stronger binding site for the transcription factor SP1 in the *MDM2* P2 promoter, which results in elevated *MDM2* expression leading to dampened p53 activity (26, 31). However, recent studies have identified a protective role of *SNP309G* in some cancers (28, 32–34). For example, multiple studies associate the *SNP309G* allele with a decreased risk and late onset for prostate cancer compared to patients harboring the *SNP309T* allele (28, 34). Thus, *SNP309* appears to

differentially impact cancer susceptibility in different cancers for reasons that are currently unknown.

Functional SNPs may exacerbate cancer risk by exposure to environmental factors, yet these mechanisms are also poorly defined (35, 36). Our current study tested the hypothesis that *Mdm2* *SNP309G* allele cooperates with environmental factors to exacerbate spontaneous cancer risk. Low dose ionizing radiation (IR), a source of reactive oxygen species (ROS) and DNA damage, exacerbates spontaneous tumor formation in the *Mdm2*^{*SNP309G/G*} mouse compared to *Mdm2*^{*SNP309T/T*} mice. Conversely, exposure to ultraviolet B (UVB) light or Benzo(a)pyrene (B(a)P), a carcinogen found in tobacco products, increased tumor risk in the skin of *Mdm2*^{*SNP309T/T*} mice compared to *Mdm2*^{*SNP309G/G*} mice. Here our investigations show that *SNP309G* directly alters *Mdm2* basal expression in a tissue-dependent manner. In skin keratinocytes, E2F6 represses *Mdm2* expression specifically from the *SNP309G* allele. These findings suggest that the *SNP309G* allele cooperates with environmental and cellular factors to alter cancer risk in a tissue-dependent manner.

RESULTS

SNP309G allele exacerbates tumor risk after low-dose IR

IR generates DNA double strand breaks and reactive oxygen species (ROS), direct mediators of p53 activation (37). Since *SNP309G* dampens p53 activity, we tested whether the *SNP309G* allele cooperates with low dose IR to increase spontaneous tumor risk in mice. First, we treated *Mdm2*^{*SNP309G/G*} and *Mdm2*^{*SNP309T/T*} mice in a C57BL/6 background with a single dose of 1Gy IR 2 days postpartum, a regimen previously shown to activate acute p53 response in C57BL/6 mouse tissues (38), and analyzed p53 activity. As expected, untreated spleen samples from *Mdm2*^{*SNP309G/G*} mice showed significantly higher *Mdm2* basal levels ($p=0.005$) as compared to spleens from *Mdm2*^{*SNP309T/T*} mice. The spleens of *Mdm2*^{*SNP309G/G*} mice showed 7.8-fold increase in *Mdm2* levels ($p<0.001$) 3 hours post IR compared to *Mdm2*^{*SNP309T/T*} spleens [Figure 1A] with concomitant decrease in p53 targets *Cdkn1a* and *Bbc3* consistent with elevated Mdm2 levels and an attenuated p53 response [Figure 1B].

To determine the effects on cell survival, we examined the onset of apoptosis post IR. *Mdm2*^{*SNP309G/G*} mouse lymphatic tissues exhibit a delay in activation of apoptosis compared to *Mdm2*^{*SNP309T/T*} after low-dose IR as measured by the number of cells staining positively for cleaved caspase-3 [Figure 1C, 1D]. At 6 hours post IR, spleen and thymi from *Mdm2*^{*SNP309T/T*} mice showed the presence of apoptotic cells compared to none in the same tissues of *Mdm2*^{*SNP309G/G*} mice; however, by 12 hours post IR, spleens and thymi from *Mdm2*^{*SNP309G/G*} mice showed increased apoptosis compared to *Mdm2*^{*SNP309T/T*} tissues, indicating a delayed response to IR. Next, we utilized the same regimen to examine long-term effects of low-dose IR on tumorigenesis. We generated a cohort of irradiated *Mdm2*^{*SNP309G/G*} and *Mdm2*^{*SNP309T/T*} mice in a C57BL/6 background and monitored for tumor development. Tumors developed significantly more quickly in irradiated *Mdm2*^{*SNP309G/G*} mice than in irradiated *Mdm2*^{*SNP309T/T*} mice ($p<0.001$) [Figure 2A]. *Mdm2*^{*SNP309G/G*} mice exhibited a median survival of 74 weeks; in contrast, the majority of *Mdm2*^{*SNP309T/T*} mice survived without tumor development and thus the median survival

could not be calculated. *Mdm2^{SNP309G/G}* mice had a proclivity to develop lymphomas and sarcomas; however, a number of mice also developed other tumors, including mammary carcinomas, glioblastomas, and histiocytic sarcomas [Figure 2B and Table 1]. In addition, *Mdm2^{SNP309G/G}* mice treated with 1Gy IR had significantly increased tumor multiplicity compared to *Mdm2^{SNP309T/T}* mice ($p<0.001$) [Figure 2C]. Only irradiated *Mdm2^{SNP309G/G}* mice had three or more tumors per mouse. We further interrogated the lymphoma phenotype due to its high penetrance [Figure 2D]. Here, we observed that irradiated *Mdm2^{SNP309G/G}* mice developed lymphomas significantly faster ($p<0.001$) compared to *Mdm2^{SNP309T/T}* mice, of either T cell (CD3 marker) or B-cell (B220 marker) origin [Figure 2E]. These experiments demonstrate that the *SNP309G* allele impacts p53 activity and exacerbates spontaneous tumor development after lowdose IR compared to the *SNP309T* allele.

***Mdm2^{SNP309T/T}* mice exhibit increase risk of skin squamous cell carcinoma after exposure to UVB compared to *Mdm2^{SNP309G/G}* mice**

Ultraviolet (UV) radiation is the major environmental cause of skin cancer. Exposure to UVB radiation induces intracellular reactive oxygen species, which elicit a robust p53 response (39, 40) and leads to skin cancer development (41). The FVB mouse strain is susceptible to insult-induced skin tumorigenesis; hence, this model is a powerful tool in studying progressive squamous cell carcinoma (42). To test if the *SNP309G* allele cooperates with UVB exposure to alter p53 activity in skin, we first backcrossed *SNP309* mice to the FVB background for five generations. Isolated primary keratinocytes from *Mdm2^{SNP309G/G}* and *Mdm2^{SNP309T/T}* mouse epidermis were treated them with a single dose of UVB (100mJ/cm²), a dose previously shown to activate acute p53 response in skin tissue (43), and analyzed for p53 activity. In contrast to studies in the spleen [Figure 1A], keratinocytes isolated from *Mdm2^{SNP309T/T}* mice showed greater than two-fold higher *Mdm2* levels ($p=0.001$) 24 hours post UVB treatment compared to *Mdm2^{SNP309G/G}* keratinocytes [Figure 3A]. To further examine p53 activity in skin tissue, we treated 6-week old *Mdm2^{SNP309G/G}* and *Mdm2^{SNP309T/T}* mice with UVB and analyzed p21 expression. Consistent with the elevated *Mdm2* expression levels in keratinocytes from *Mdm2^{SNP309T/T}* compared to *Mdm2^{SNP309G/G}* mice after UVB treatment, *Mdm2^{SNP309G/G}* keratinocytes displayed significantly higher number of cells staining positive for p21 ($p=0.019$) compared to *Mdm2^{SNP309T/T}* keratinocytes [Figure 3B]. To examine skin cancer risk after UVB exposure, we generated a cohort of *Mdm2^{SNP309G/G}* and *Mdm2^{SNP309T/T}* mice in a FVB background and treated with an established UVB regimen (UVB radiation three times per week for 30 weeks) (43) and monitored for spontaneous tumor development for squamous cell carcinoma (SCC). Tumor-free survival analysis of UVB treated mice also showed that *Mdm2^{SNP309T/T}* mice died significantly sooner than *Mdm2^{SNP309G/G}* mice ($p<0.001$) [Figure 3C] with a median survival of 28 weeks compared to with 36 weeks. Thus, UV treatment led to a dampened p53 response and shorter tumor latency in *Mdm2^{SNP309T/T}* mice compared to *Mdm2^{SNP309G/G}* mice, in contrast to IR treatment. These data suggest that either the type of DNA damage or the cell type account for the differences in tumorigenesis. We therefore repeated the experiment using fibroblasts, a cell type that previously displayed higher basal *Mdm2* levels in *SNP309G* than in *SNP309T* genotype (30). Fibroblasts were treated with the same regimen of UVB and the results showed 1.9-fold higher ($p=0.032$) increase in *Mdm2* expression 24 hours post UVB treatment in *SNP309G* compared to

SNP309T fibroblasts [Figure 3D], suggesting that the contrasting differences were cell-type dependent.

Benzo(a)pyrene (B(a)P) is a tobacco-related carcinogen that causes DNA damage (44). To determine if the differences in skin tumor onset are limited to UVB insult, we next investigated the effects of B(a)P on p53 activity in the skin of our mice. The epidermis of *Mdm2^{SNP309G/G}* and *Mdm2^{SNP309T/T}* mice was treated with either acetone (control) or B(a)P and total RNA was isolated 24-hour post treatment. *Mdm2* mRNA levels were higher in *Mdm2^{SNP309T/T}* mice compared to *Mdm2^{SNP309G/G}* mice after B(a)P treatment, however the differences were not statistically significant [S1]. To examine tumor risk following B(a)P exposure, we treated *Mdm2^{SNP309G/G}* and *Mdm2^{SNP309T/T}* mice to an established regimen of 317 nmol of B(a)P once a week for 30 weeks (44) and monitored mice for spontaneous tumor formation. Tumor-free survival showed that B(a)P treated *Mdm2^{SNP309T/T}* mice were more susceptible to squamous cell carcinoma than *Mdm2^{SNP309G/G}* mice ($p<0.001$) [Figure 4A] with a median survival of 22 weeks compared to 25 weeks, respectively. Further assessment of the tumor-free survival data showed that the difference was primarily due to gender [Figure 4B]. Tumor development in male *Mdm2^{SNP309G/G}* mice was significantly delayed ($p<0.001$) compared to the other groups. We therefore revisited the *Mdm2* expression levels in epidermis of *Mdm2^{SNP309G/G}* and *Mdm2^{SNP309T/T}* male mice. Similarly, *Mdm2* mRNA levels were significantly lower ($p=0.030$) in the epidermis of male *Mdm2^{SNP309G/G}* mice compared to male *Mdm2^{SNP309T/T}* mice 24-hour post B(a)P treatment, but there were no significant differences between female mice [Figure 4C, 4D]. Moreover, epidermal keratinocytes in skin samples from male *Mdm2^{SNP309G/G}* mice displayed significantly higher number of cells staining positive for p21 ($p=0.049$) as compared to *Mdm2^{SNP309T/T}* keratinocytes after B(a)P treatment [Figure 4E]. Altogether, the data show that keratinocytes from *Mdm2^{SNP309G/G}* mice exhibit significantly lower *Mdm2* expression and increased p53 activity in *Mdm2^{SNP309G/G}* mice compared to *Mdm2^{SNP309T/T}* mice in response to insult. Thus, *Mdm2^{SNP309G/G}* mice, particularly males, are more resistant to developing skin SCC in response to B(a)P treatment.

The *SNP309G* allele regulates *Mdm2* expression in a tissue-dependent manner

To address the contrasting differences in vulnerability to cancer risk after exposure to environmental factors, we evaluated *Mdm2* basal expression in different tissues harvested from *Mdm2^{SNP309G/G}* and *Mdm2^{SNP309T/T}* mice in a C57BL/6 background. *Mdm2* basal levels vary in different tissues in a SNP-dependent manner [Figure 5A]. *Mdm2* mRNA levels in the thymus (the origin of T-cell lymphomas) of *Mdm2^{SNP309G/G}* mice are statistically higher ($p=0.040$) than in *Mdm2^{SNP309T/T}* mice. Conversely, keratinocytes, the cell of origin for SCC, in *Mdm2^{SNP309T/T}* mice exhibit significantly higher *Mdm2* mRNA levels ($p<0.001$) than keratinocytes in *Mdm2^{SNP309G/G}* mice. One other tissue showed significant differences in *Mdm2* levels; the heart tissue exhibits significantly higher *Mdm2* mRNA levels ($p=0.006$) in *Mdm2^{SNP309G/G}* mice than in *Mdm2^{SNP309T/T}* mice [Figure 5A]. Further investigation showed that other tissues exhibited significant differences in *Mdm2* basal levels when evaluated by gender [S2]. To examine if mouse strain contributed to the contrasting differences, we examined *Mdm2* basal expression in fibroblasts and keratinocytes from C57BL/6 and FVB mice. While basal *Mdm2* levels vary somewhat

between strains, keratinocytes from *Mdm2*^{SNP309T/T} mice have more *Mdm2* than *Mdm2*^{SNP309G/G} keratinocytes and fibroblasts from *Mdm2*^{SNP309G/G} mice have more *Mdm2* than *Mdm2*^{SNP309T/T} fibroblasts in both strains [S3]. Thus, these *Mdm2* basal expression differences are independent of strain.

To investigate the mechanism by which the *SNP309* alleles regulate *Mdm2* expression in a tissue specific manner, we first interrogated potential transcription factor binding to the *SNP309* allele. Analyses of the ENCODE ChIP-seq database (45) in genome browser (<https://genome.ucsc.edu>) showed that transcriptional repressors E2F4 and E2F6 were candidates because of their binding proximity to *SNP309* [Figure 5B, S4]. Additionally, previous studies associated these repressors as key regulators of keratinocyte proliferation (46). The cell lines utilized in the ENCODE ChIP-seq experiments for E2F4 and E2F6 were MCF10A and K562, respectively. Sequence analysis of these cell lines showed that MCF10A cells harbor a homozygous *SNP309T* genotype and K562 cells harbor a *SNP309T/G* genotype [S5]. To examine if E2F4 and E2F6 regulate *Mdm2* expression in a *SNP309* allele dependent manner, we generated MEFs from *Mdm2*^{SNP309G/G} and *Mdm2*^{SNP309T/T} C57BL/6 mice and transfected them with plasmids expressing GFP and either E2F4, E2F6, or SP1, a transcriptional activator that increases *Mdm2* expression in *SNP309G* cells. As expected, sorted cells expressing GFP and SP1 preferentially increased *Mdm2* expression in *SNP309G* MEFs ($p < 0.001$) [Figure 5C]. E2F4 transfection increased *Mdm2* expression slightly, but there were no significant differences between genotypes [S6]. E2F6 transfection statistically repressed *Mdm2* levels in *SNP309G* MEFs compared to *SNP309T* MEFs ($p < 0.001$) [Figure 5D]. To test the efficiency of transfections, we performed RT-qPCR on *Fgf21* and *Brca1*, known targets of SP1 and E2F6, respectively. No significant differences in expression of *Fgf21* and *Brca1* were observed in *SNP309T* MEFs compared to *SNP309G* MEFs, indicating similar transfection efficiencies. We also examined protein levels of SP1 and E2F6 in thymus and keratinocytes harvested from *Mdm2*^{SNP309G/G} and *Mdm2*^{SNP309T/T} mice and showed that SP1 is expressed in thymus and not detectable in keratinocytes and vice versa E2F6 is clearly expressed in keratinocytes but not in thymus samples [Figure 5E]. These differences were independent of genotype. We next examined the binding specificity of E2F6 to the *MDM2*-P2 promoter in keratinocytes harvested from homozygous *SNP309G* and *SNP309T* newborn pups by performing chromatin immunoprecipitation assays (ChIP). ChIP assays using an E2F6 antibody showed that E2F6 bound more significantly ($p < 0.001$) to the *MDM2*-P2 promoter in keratinocytes of *SNP309G* compared to *SNP309T* [Figure 5F]. We also performed ChIP assays on the *Brca1* promoter, an establish target of E2F6, and saw no difference in E2F6 binding to *SNP309G* or *SNP309T* samples. In addition, a sequence downstream of the *Mdm2* promoter not thought to bind E2F6 showed no difference in E2F6 binding. Collectively, these data show that *SNP309G* regulates *Mdm2* basal expression in a tissue-dependent manner utilizing alternative transcription factors.

DISCUSSION

Functional polymorphisms alter gene function, and disease risk. Environmental stress can have a synergistic effect on some SNPs and exacerbate disease; however cooperative mechanisms between functional polymorphisms and environmental stress have not been well

defined. In this study, we utilize a well-known functional polymorphism involved in the p53 pathway, *SNP309*, to investigate gene-environment interactions in modulating tumor susceptibility. This study used relevant environmental stress models that affect the p53 pathway: humans are consistently exposed to low dose IR via cosmic rays and medical imaging procedures (47), UV from the sun, and the B(a)P carcinogen from smoking (48).

Our study shows that the *SNP309G* allele further increased *Mdm2* expression and dampened p53 activity in spleen and thymus post IR treatment compared to *SNP309T* allele and compared to non-irradiated mice, resulting in exacerbated tumor risk in *Mdm2^{SNP309G/G}* mice compared to *Mdm2^{SNP309T/T}* mice. These results are consistent with previous data, which show that the *Mdm2^{SNP309G/G}* mice have increased susceptibility to tumors compared to *Mdm2^{SNP309T/T}* mice, affirming that *SNP309G* is an 'at risk' allele for spontaneous tumor formation (30).

Conversely, both UVB and B(a)P skin carcinogenesis models in a FVB background demonstrated the opposite effect and highlight increased *Mdm2* expression and attenuated p53 activity is observed in the epidermal skin cells of *Mdm2^{SNP309T/T}* mice compared to *Mdm2^{SNP309G/G}* mice. Consequently, *Mdm2^{SNP309T/T}* mice exhibit increased risk of skin squamous cell carcinoma compared to *Mdm2^{SNP309G/G}* mice after UVB or B(a)P treatment. In this study, one irradiated *Mdm2^{SNP309G/G}* C57BL/6 mouse presented with a squamous cell carcinoma, as well as histiocytic sarcoma and lymphoma; this squamous cell carcinoma represents only ~1% of all tumors present in irradiated *Mdm2^{SNP309G/G}* C57BL/6 mice. In comparison, a larger number of UVB and B(a)P treated mice showed both genotypes develop squamous cell carcinoma but *Mdm2^{SNP309T/T}* mice were more susceptible.

A further examination of basal *Mdm2* expression from different tissues harvested from *SNP309* mice in a C57BL/6 background confirms that *SNP309* regulates *Mdm2* basal expression in a tissue-dependent manner. The contrasting difference in basal *Mdm2* expression from skin keratinocytes was observed in C57BL/6 and FVB backgrounds; thus, the skin tissue phenotype is not strain-dependent. Moreover, fibroblasts from FVB mice showed increased *Mdm2* expression in *SNP309G* compared *SNP309T* fibroblast after UVB treatment, indicating that the phenotype is also not insult-dependent. Collectively, these data show that *SNP309* contrastingly regulates basal *Mdm2* expression levels and concomitantly affects p53 activity and cancer risk in a cell-type-specific manner.

To date, epidemiological studies do not associate *MDM2 SNP309* with skin squamous cell carcinoma risk (49, 50). However these study cohorts are modest. Many meta-analysis associating *MDM2 SNP309* to cancer risk are conflicting (25, 51), even in larger studies suggesting that other factors can contribute to tumor risk. We observed differences in expression when we stratified by gender, thus, supporting the hypothesis that other factors cooperate to alter gene expression in a SNP-dependent manner in different tissues. Moreover, a recent study demonstrated a decreased risk of developing oral squamous cell carcinoma (OSCC) for the *MDM2 SNP309G* group (52) with pronounced susceptibility in the *MDM2 SNP309T* group, suggesting *MDM2 SNP309G* may be protective in a cell-dependent manner.

Finally, our data show that *Mdm2* expression is controlled by the *SNP309G* allele in a tissue-dependent manner. The SP1 overexpression experiment supports previous mechanistic studies, both in human and mouse tissues, showing SP1 preferentially binds to *SNP309G* allele to increase *Mdm2* expression (26, 30). In contrast, the E2F6 overexpression experiments show preferential repression of *Mdm2* expression in the *SNP309G* allele, suggesting that this allele will elicit a more potent p53 response and protect mice harboring this SNP from tumor risk. Protein expression of thymi and keratinocytes from *SNP309* mice show a contrasting difference, where E2F6 expression is more pronounced in keratinocyte, thus explaining why E2F6 protein may be more active in this tissue type. The ChIP-seq data from ENCODE confirmed that E2F6 binds to *MDM2*-P2 promoter and our ChIP assay confirmed that E2F6 preferentially binds to the *MDM2*-P2 promoter in keratinocytes of *SNP309G* compared to *SNP309T*. Thus the strength of this study is that we define a specific regulatory mechanism that directly impacts the *Mdm2* *SNP309G* allele resulting in an increased cancer risk in a tissue-dependent manner through discrete mechanisms.

In summary, our data illustrates how *SNP309G* cooperates with environmental stress to exacerbate or protect cancer development in a tissue-dependent manner. Our findings suggest that functional polymorphisms can be multidimensional gene regulators with varying disease risk.

MATERIALS AND METHODS

Mouse Models

Mouse experiments were conducted in compliance with MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC). The *Mdm2*^{*SNP309G/G*} and *Mdm2*^{*SNP309T/T*} mice in C57BL/6 strain have been previously characterized (30). To generate a skin tumor prone FVB strain, the C57BL/6 strains were backcrossed for five generations to FVB.

Environmental stress experiments

Mdm2^{*SNP309G/G*} and *Mdm2*^{*SNP309T/T*} mice in C57BL/6 strain were treated with 1Gy IR 2 days after birth and monitored for tumor incidence and sacrificed upon tumor formation. Six-week old *Mdm2*^{*SNP309G/G*} and *Mdm2*^{*SNP309T/T*} mice in FVB strain were treated with skin carcinogenesis protocols, including B[a]P and UVB, as previously described (43, 53). Non-treated *Mdm2*^{*SNP309G/G*} and *Mdm2*^{*SNP309T/T*} mice were used as controls. The mice were monitored daily for tumor incidence and sacrificed upon tumor formation. Cohorts of C57BL/6 and FVB strain contain male and female mice. Number of animals used for each experiments are present in figures and figure legends.

Real-Time RT-PCR

Fresh tissues were necropsied from mice and flash frozen. RNA isolation and Real-Time RT-PCR was performed as previously described (54). RT-PCR primers for *E2f4* (forward 5'-GATCGCTGACAAGCTGATTG -3' and reverse 5'-CTGTTCTGGACGTCCTCAGTG -3'), *E2f6* (forward 5'-CGGAAGAGGCGAGTGTATG -3' and reverse 5'-CAGTTCGATGCCATCCAAG -3'), *Fgf21* (forward 5'-GGGTCTACCAAGCATACCCC

-3' and reverse 5'-GTACCTCTGCCGGACTTGAC-3'), *Bra1* (forward 5'-GCTGCAGCCACGCTTTTC and reverse 5'-GGCGAAGAACGAGAGAATGAA-3'), *Sp1* (forward 5'-GCGAAGCATCTTGGGTGTGT-3' and reverse 5'-TCCTTTCCTCTTCAGTCGCTTT-3). The RT-PCR primers for *Mdm2*, *Bbc3*, *Cdkn1a* (*p21*), and *Rplp0* have been previously described (30). Expression was normalized to *Rplp0*. Experiments were repeated three times for each sample.

Cell culture and transfections

Primary keratinocytes and early passage MEFs from *Mdm2*^{SNP309G/G} and *Mdm2*^{SNP309T/T} mice were generated as previously described (21, 55). Early passage MEFs were transfected with 7 µg of pCMV-GFP vector containing E2F4 cDNA, E2F6 cDNA, SP1 cDNA, or empty vector using Lipofectamine 3000 reagent (Sigma, L3000015). Forty-eight hours later, GFP positive cells were sorted by FACS and RNA was extracted for RT-PCR analysis.

Chromatin immunoprecipitation assay (ChIP)

The ChIP and Real-time PCR assays were executed as previously described (54). The E2F6 antibody (Santa Cruz, Dallas, TX, USA, E20) (6µg) was added overnight at 4°C. RT-PCR primers for *MDM2-P2 promoter* (forward 5'-GATCGCTGACAAGCTGATTG-3' and reverse 5'-CTGTTCTGGACGTCCTCAGTG-3'), *Bra1* (forward 5'-GCAGCCGCAATTACAATCTATC-3' and reverse 5'-ACGTGTCTGGATCTTGTGTTC-3'), *Negative* (forward 5'-ACCTGAGGTCAGGAGTACAA-3' and reverse 5'-AGCTGGGATTATAGGCATGTG-3'). Data was presented and calculated as percentages of the total.

Protein analysis

Protein lysates were prepared by lysing thymi or keratinocytes from *SNP309* mice in NP-40 buffer. Protein estimation was carried out with BCA (Pierce, Waltham, MA, USA #23225). One hundred micrograms of lysate was resolved on 10% SDS-PAGE and immunoblotted with antibodies against SP1 (1:200; Santa Cruz, sc-59), E2F6 (1:200; Santa Cruz #E-20), and GAPDH (1:1000; Abcam, Cambridge, UK #ab9485). Western blots were repeated at least three times with biological replicates.

Immunohistochemistry

Samples were stained for p21 (Dako, Santa Clara, CA, USA #SX118), caspase 3 (R&D Systems, Minneapolis, MN, USA #AF835) CD3 (Abcam #Ab5690), or B220 (BD Pharmingen, San Jose, CA, USA #530286). Digital images of stained sections were captured using the Aperio ScanScope (Aperio Technologies, Vista, CA, USA). Experiments were repeated at least three times with biological replicates.

Statistical analysis

All comparisons were analyzed using GraphPad Prism version 6.00 (GraphPad Software, LaJolla CA, USA). For statistical analysis, experiments were repeated with at least three biological replicates. Differences between two or more groups were analyzed by the Student's *t*-test or analysis of variance followed by the Dunnett's multiple comparison *post-*

hoc test, respectively. A statistical analysis for tumor incidence and tumor multiplicity was performed by Fisher's exact test. A statistical analysis for Kaplan-Meier survival curves was performed by Log-rank test. All statements of significant differences showed a 5% level of probability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Attenuation of the p53 pathway in an *Mdm2*^{SNP309} allele specific manner after IR. A, *Mdm2* expression in spleens of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice 3hrs post 1Gy IR (n = 5, mean ±SD). B, Expression of *Cdkn1a* and *Bbc3* (p53 targets) in spleens of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice 3hrs post 1Gy IR. Expression levels are normalized to that of untreated T/T samples. C, D, Immunohistochemistry of spleens and thymi of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice with caspase-3 antibody. Graphs represent mean value, n = 3, mean ±SD. Statistical significance was determined by two-way analysis of variance (ANOVA). T/T, *SNP309T*, *Mdm2*^{SNP309T/T} mice; G/G, *SNP309G*, *Mdm2*^{SNP309G/G} mice. n= number of biological replicates.

Figure 2.

Exacerbation of tumor phenotypes after 1Gy IR. A, Kaplan-Meier survival curve of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice after 1Gy IR. B, Tumor incidence of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice. C, Tumor multiplicity. Statistical analyses for tumor incidence and tumor multiplicity was performed by Fisher's exact test. D, Lymphoma-free survival curve of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice. Statistical analyses for Kaplan-Meier survival curves was performed by Log-rank test. E, Immunohistochemistry of *Mdm2*^{SNP309G/G} lymphoma tissues with CD3 & B220. n= number of biological replicates.

Figure 3.

Tumor development after UVB treatment. A, *Mdm2* expression in primary keratinocytes of *Mdm2*^{SNP309T/T} (T/T) and *Mdm2*^{SNP309G/G} (G/G) mice 24hrs post UVB treatment (100 mJ/cm²) (mean $\pm$ SD; n= 3). B, Immunohistochemistry analysis of p21 in skin tissue samples from 6-week old *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice (n=5) 24hrs post UVB treatment. Statistical analysis was performed using Student's t-test. C, Kaplan-Meier survival curve of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice after UVB treatment (Log-rank test). D, *Mdm2* mRNA expression in primary adult fibroblasts of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice 24hrs post UVB treatment (mean $\pm$ SD; n= 4). Expression levels are normalized to that of untreated samples. Statistical analyses were performed using ANOVA test. n= number of biological replicates.

Figure 4.

Tumor phenotype after B(a)P treatment. A, Kaplan-Meier survival curves of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice after B(a)P treatment. B, Kaplan-Meier survival curves of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice separated by gender. Statistical analyses for Kaplan-Meier survival curves were performed by Log-rank test. C, D, *Mdm2* expression in epidermis of male and female *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice 24hrs post acetone or B(a)P treatment. Data were normalized to expression levels of *Mdm2* in the epidermis tissue samples of acetone treated controls, (n = 3, mean ±SD). Statistical analysis was performed using Two-way ANOVA. E, Immunohistochemistry of skin tissues of 6-week old male *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice with p21 antibody, 24hrs post B(a)P treatment (Student's t-test, n=3). n= number of biological replicates.

Figure 5.

SNP309 modulates *Mdm2* expression in a tissue-dependent manner. A, *Mdm2* expression in different tissues of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice. Statistical analyses were performed using Student's t-test (mean $\pm$ SD, n=8; 4 σ , 4 ϕ). In this experiment, *RplpO* expression was used as a reference gene. B, Predicted SP1 & E2F binding sites in the *MDM2* P2 promoter proximal to *SNP309* site. C, Expression of *Mdm2* and *Fgf21* (a known SP1 target) in MEFs transfected with SP1. D, *Mdm2* and *Brca1* (a known E2F6 target) expression in MEFs transfected with E2F6. Statistical analysis was performed using Two-way ANOVA. E, Western blot analysis of SP1 and E2F6 in thymus and keratinocytes of

SNP309 mice. GAPDH was used as loading control. F, E2F6 ChIP analysis of the *MDM2* P2 promoter in keratinocytes of *Mdm2^{SNP309T/T}* and *Mdm2^{SNP309G/G}* mice. E2F6 ChIP assays for the *Brca1* promoter in keratinocytes was used as positive control. A sequence downstream of the *MDM2* promoter not thought to bind E2F6 was used as a negative control. Statistical analysis was performed using Two-way ANOVA. n= number of biological replicates.

TABLE 1

Tumor spectrum of *Mdm2*^{SNP309T/T} and *Mdm2*^{SNP309G/G} mice after 1Gy IR.

Tumor Type	<i>Mdm2</i> ^{SNP309T/T} (IR) n=11	<i>Mdm2</i> ^{SNP309G/G} (IR) n=46
Lymphoma	4	17
Sarcoma NOS		2
Rhabdomyosarcoma		1
Hemangiosarcoma		3
Osteosarcoma	2	25
Medulloblastoma		1
Glioblastoma		1
Neuroblastoma		1
Adenoma	2	3
Alveolar carcinoma		2
Hepatocellular carcinoma	3	4
Mammary carcinoma		2
Renal tubule		1
Squamous cell carcinoma		1
Histiocytic sarcoma	1	4
Germ cell tumor	1	2
Total tumors	13	70

Abbreviations: Gy, Gray; IR, ionizing radiation; NOS, not otherwise specified