Microenvironment and Immunology

Cancer Research

The Human *TLR* Innate Immune Gene Family Is Differentially Influenced by DNA Stress and *p53* Status in Cancer Cells

Maria Shatz, Daniel Menendez, and Michael A. Resnick

Abstract

The transcription factor p53 regulates genes associated with a wide range of functions, including the Toll-like receptor (*TLR*) set of innate immunity genes, suggesting that p53 also modulates the human immune response. The TLR family comprises membrane glycoproteins that recognize pathogen-associated molecular patterns (PAMP) and mediate innate immune responses, and TLR agonists are being used as adjuvants in cancer treatments. Here, we show that doxorubicin, 5-fluorouracil, and UV and ionizing radiation elicit changes in *TLR* expression that are cell line- and damage-specific. Specifically, treatment-induced expression changes led to increased downstream cytokine expression in response to ligand stimulation. The effect of DNA stressors on TLR expression was mainly mediated by p53, and several p53 cancer-associated mutants dramatically altered the pattern of *TLR* gene expression. In all cell lines tested, *TLR3* induction was p53-dependent, whereas induction of *TLR9*, the most stress-responsive family member, was less dependent on status of p53. In addition, each of the 10 members of the innate immune *TLR* gene family tested was differentially inducible. Our findings therefore show that the matrix of *p53* status, chromosome stress, and responsiveness of individual *TLRs* should be considered in TLR-based cancer therapies. *Cancer Res; 72(16); 3948–57. ©2012 AACR.*

Introduction

The p53 tumor suppressor is a master regulatory transcriptional factor activated in response to several stress signals including DNA damage, resulting in transactivation of target genes. It functions in a variety of biologic processes including cell-cycle arrest, apoptosis, senescence, embryo implantation, and nutritional stress (1). p53 binds *in vitro* as a tetramer to a DNA motif composed of 2 RRRCWWGYYY decamer half-sites (R = A, G; W = A, T; and Y = C, T) separated by a spacer of up to 14 bases (2). Using a combination of yeast and human cell systems (3), we established "rules" whereby p53 was also found to function from noncanonical sequences comprising only a decamer 1/2-site or a 3/4-site (4–6). Using these rules for p53 binding and transactivation, we recently identified p53-binding sites in the promoter region of most human Toll-like receptors (*TLR*) and showed a direct role for p53 in regulation of this entire family of genes in primary human immune cells (7).

TLRs are a group of highly conserved integral membrane glycoproteins that recognize a variety of distinct pathogen-associated molecular patterns (PAMP; ref. 8). The recognition of PAMPs by TLR-expressing cells leads to quick and acute responses required to eliminate pathogens in the host. Upon ligand stimulation, TLRs recruit adaptor molecules MyD88, TIRAP, TRIF, and TRAM leading to activation of NF-κB, interferon responsive factors, and mitogen-activated protein kinases that, in turn, results in ligand-dependent patterns of gene expression including inflammatory cytokines, chemokines, and interferons (reviewed in ref. 8). In addition, endogenously produced ligands, referred to as damage-associated molecular patterns (DAMP) or alarmins that include self-DNA and intracellular proteins, signal tissue injury through TLRs when DAMPs are released from cells (9). In both cases TLR pathway activation mediates immune/inflammatory responses. Ten *TLRs* (*TLR1–10*) have been identified in humans. *TLRs* are expressed in several types of immune cells including spleen, T and B lymphocytes, dendritic cells, and macrophages (10). In addition, TLRs function in nonimmune tissues such as airway and gut epithelial cells which have direct contact with pathogens (11, 12).

Altered *TLR* expression has been associated with autoimmune and chronic inflammatory diseases including atherosclerosis, type I diabetes, inflammatory bowel disease, liver diseases, rheumatoid arthritis, and systemic lupus erythematosus (13, 14). In recent years, cancer-related functions of the human TLR gene family have become apparent. Impaired expression and signaling by *TLR7* and *TLR9* may contribute to reduced innate immune responses during chronic viral infections and oncogenesis (15). In addition, the loss-of-function allele *Asp299Gly* of *TLR4* is associated with breast cancer (16).

Authors' Affiliation: Chromosome Stability Group, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences (NIEHS), NIH, Research Triangle Park, North Carolina

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

M. Shatz and D. Menendez contributed equally to this work.

Corresponding Author: Michael A. Resnick, National Institute of Environmental Health Sciences (NIEHS), NIH, Chromosome Stability Group, Laboratory of Molecular Genetics, Research Triangle Park, NC 27709. Phone: 919-541-4480; Fax: 919-541-7593; E-mail: resnick@niehs.nih.gov

doi: 10.1158/0008-5472.CAN-11-4134

For cancer cells expressing *TLRs*, activation of the pathway can negatively affect growth and viability (17) including direct killing of tumor cells via induction of TNFα (18, 19) and, therefore, can be used in treatment. For instance, TLR7 agonist imiquimod is used in treating basal cell and cutaneous malignancies; also, synthetic oligodeoxynucleotides containing unmethylated CpG motifs that activate TLR9 are in clinical trials (20, 21). Stimulation of the TLR pathway in antigen-presenting cells can induce and potentiate an effective immune response against tumor cells (22–25). In addition, TLR ligands conjugated to tumor-derived proteins or immunogenic peptides are used for therapeutic vaccination to treat cancer (reviewed in ref. 26).

Surprisingly, despite the importance of TLRs in human health, disease, and therapy, relatively little is known about their transcriptional regulation. Only a few studies suggest involvement of transcriptional factors such as PU.1, activator protein, interferon response factors, and Forkhead Box P3 in regulation of specific TLRs in defined cellular systems (27, 28).

Because we have shown in primary cells that activated p53 can regulate expression of the various *TLRs* differentially, depending in part on the agent, the *p53* status could influence expression and function of *TLRs* in cancer cells (7). The *TP53* gene is mutated in over half of human tumors (29, 30). Most mutations are missense and are found within the central core DNA-binding domain of the protein resulting in loss of transcriptional function. However, approximately one-third of cancer-associated p53 mutants retain transactivation capability and exhibit change-in-spectrum of transactivated genes (4, 5). Therefore, differential effects on *TLR* gene expression might be expected from the various *p53* mutations.

So far, only *TLR3* was shown to be a p53 target (31). On the basis of our results with primary human cells where nearly all *TLR* genes are in the p53 network, we have investigated p53-dependent regulation of the entire *TLR* gene family in a panel of cancer cell lines and the consequences of p53 mutations on *TLR* expression. We focused on human cells because there are significant differences in regulation, expression, and specificity of TLRs between humans and rodents (reviewed in ref. 32), especially as relates to p53 responsiveness (7). Here, we establish that most *TLR* genes are responsive to p53 and genotoxic stress in human cancer cells. The considerable variability in responses of specific *TLR* genes that is dependent on cell line, specific p53 mutations, and stressors underscores the importance of considering these factors in TLR-based cancer treatments.

Materials and Methods

Reagents and antibodies

All reagents were from Sigma unless stated otherwise. The primary antibodies used were against p53 (DO-1), TLR5 (H1-27), actin (C-11), β-tubulin (H-300), and lamin B1 (H-90; Santa Cruz Biotechnology), p21 (SXM30, BD Biosciences Pharmigen), TLR2 (#2229, Cell Signaling).

Cell lines and treatments

The source and maintenance conditions of cell lines are described in Supplementary Materials and Methods. Where indicated, cells were treated for 24 to 48 hours with the following: Nutlin-3 (10 μmol/L), doxorubicin (0.3–0.6 μg/mL), 5-fluorouracil (5-FU, 300 μmol/L). For ionizing radiation (IR) treatment and UV, cells were irradiated at 1.56 Gy/min or UV at 1 J/m^2/s, respectively. Pretreatment with the p53 inhibitor pifithrin-α (40 μmol/L) was for 4 hours. Doses of IR, UV, doxorubicin, and Nutlin-3 were chosen based on preliminary optimization as shown in Supplementary Fig. S1. None of the treatments resulted in noticeable cell death during the course of the experiments, based on visual observation.

Plasmid constructs, transfections, luciferase assays and chromatin immunoprecipitation (ChIP) assays, immunoblot, and gene expression analysis by real-time PCR were carried out as previously described (7). For details see Supplementary Materials and Methods.

Results

p53 modulates expression of *TLR* genes in breast adenocarcinoma and osteosarcoma cells

The impact of changes in p53 protein levels on *TLR* gene expression was examined in several cancer cell lines. Increases were accomplished using the p53 inducer Nutlin-3 or p53 overexpression. Nutlin-3 inhibits p53 interaction with its ubiquitin ligase MDM2 leading to p53 stabilization and accumulation (33). The p53 inhibitor pifithrin-α or RNA interference were used to reduce p53-mediated activity. Although pifithrin-α can suppress p53-mediated transactivation, the underlying mechanism of p53 inhibition remains to be established (34).

Shown in Fig. 1A are representative Western blot analysis of p53 and p21 proteins in MCF7 breast adenocarcinoma cells (wild-type p53) and an MCF7 cell line stably expressing shRNAi to *p53* (designated as "vector" and "p53i", respectively) treated with Nutlin-3 or vehicle control dimethyl sulfoxide (DMSO) for 48 hours. As expected, Nutlin-3 greatly increased expression of p53 and its target gene p21 in MCF7-vector cells and only to a small extent in MCF7-p53i cells (less than the basal level of p53 in the MCF7-vector cells). Nutlin-induced changes in expression of *TLR* genes in MCF7-vector and MCF7-p53i cells are presented in Fig. 1B. Expression of only 3 of the 10 *TLR* genes was detected in MCF7 cells under our experimental conditions: *TLR2, 5*, and *6*. The *TLR* genes *TLR2, 5*, and *6* were induced 3.5- to 6-fold by Nutlin-3 compared with DMSO-treated control in a p53-dependent manner in MCF7 cells. There was a corresponding increase in TLR2 and TLR5 proteins in the membrane fraction of the MCF7-vector cell lysate following Nutlin-3 treatment (a reliable human TLR6 antibody was not available) as described in Fig. 1C.

In contrast to MCF7 cells, all *TLRs* except *TLR8* are expressed in the osteosarcoma cell line U2OS (*p53*$^+$) and most *TLRs* were induced by Nutlin increases in p53 (see p53 and p21 proteins in Fig. 1D). There was a 2- to 6.5-fold increase in expression of *TLR2, 3, 5, 6, 9*, and *10* genes by 24 hours Nutlin-3 treatment compared with the DMSO-treated control in U2OS cells (Fig. 1E). This induction was prevented by pretreatment with pifithrin-α, confirming a specific role for p53 in *TLR* gene expression.

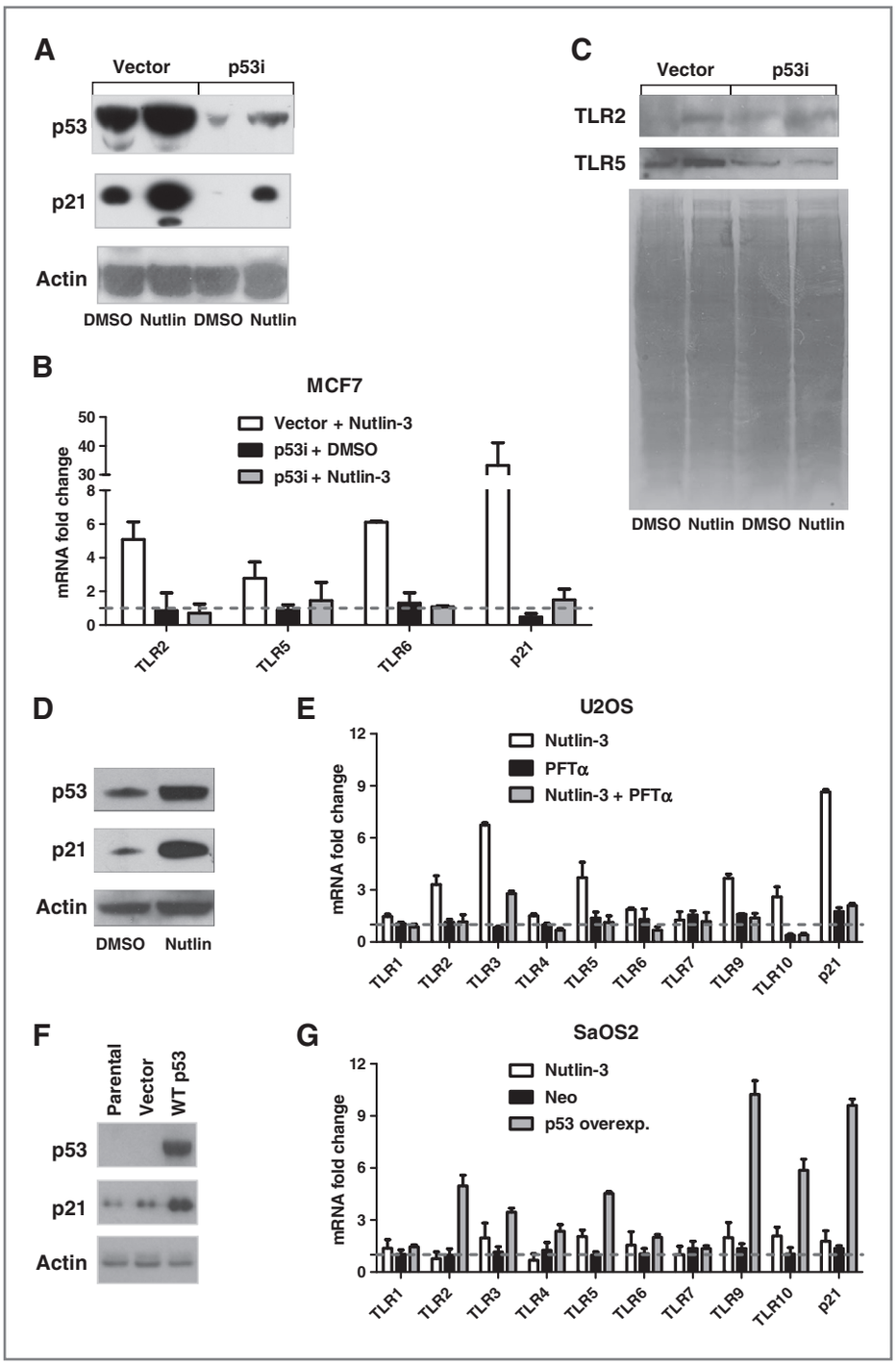


Figure 1. The p53 activator Nutlin-3 induces expression of *TLR* genes. MCF7-vector or MCF7-p53i cells were incubated with 10 μmol/L of Nutlin-3 or 0.1% DMSO for 48 hours. Following treatment, cells were harvested and total RNA and protein were isolated. A, p53 and p21 activation were assessed by Western blot analysis. The exposure was extended long enough to allow visualization of and direct comparison between all samples. Actin was used as a loading control. B, expression of *TLRs 2, 5, 6,* and *p21* following Nutlin-3 treatment was assessed using real-time PCR and normalized to expression of the housekeeping gene *GUSB*. Expression of each gene in DMSO-treated control cells was set as one. C, cell lysates were subjected to subcellular fractionation and 100 μg of protein from each membrane fraction was resolved by SDS-PAGE. Protein levels of TLR2 and TLR5 were detected by Western blotting; gel staining was used for the loading control. D, p53 and p21 induction in U2OS cells following 24 hours of incubation with Nutlin-3 was assessed by Western blot analysis. E, *TLR* gene expression in U2OS cells incubated with either Nutlin-3, pifithrin-α, a combination of both for 24 hours relative to DMSO-treated control was assessed by real-time PCR. F, SaOS2 cells were transfected either with empty vector or with wild p53 or left untreated. Expression levels of p53 and p21 were examined by Western blotting 24 hours posttransfection. G, *TLRs* mRNA expression levels following this transfection or Nutlin-3 (10 μmol/L, 24 hours) treatment were examined by qPCR; p21 was used as a positive control. Gene expression in DMSO-treated sample was set as one. Shown are averages of 3 independent experiments. WT, wild-type.

To further investigate a direct connection between *p53* and *TLR* gene expression, wild-type p53 was overexpressed in *p53*-null osteosarcoma SaOS2 cells (Fig. 1F). The pattern of induction of *TLR* genes differed from both U2OS and MCF7 cells. At 24 hours posttransfection, the levels of *TLR2–6, 9*, and *10* mRNAs were increased 2- to 10-fold (Fig. 1G) relative to untransfected cells. The *TLR1* and *7* gene expression was not changed and *TLR8* mRNA was not detected in SaOS2 cells.

Thus, we establish that many *TLR* genes in cancer cells are responsive to p53. However, there are dramatic differences in the spectra of *TLRs* transactivated suggesting that a profile of individual cancers needs to be considered if TLR-related therapies are to be used.

Functional evaluation of proposed p53 response elements in *TLR* promoters

On the basis of rules for functional p53 binding, we identified several canonical and noncanonical p53-response elements (RE) located in the promoter vicinity of almost all human *TLR* genes (summarized in ref. 7; and Supplementary Table S1). Luciferase reporter constructs containing selected response elements from *TLR* promoter areas were transfected into SaOS2 cells in the presence (solid bars) or absence (open bars) of a vector expressing wild-type p53 and reporter induction was assessed 48 hours posttransfection. Consistent with our previous report, all proposed p53-RE sequences from the *TLRs* could support p53-driven transcription of a luciferase reporter. For most the levels induced were similar to the moderately responsive p53 target response element of p53-regulated apoptosis-inducing protein 1 (*TP53AIP1*), which was used as an internal control (Fig. 2A).

The ability of several of these p53-REs to function as p53 target sequences was investigated further using ChIP assays of MCF7 cells treated for 24 hours with Nutlin-3 or doxorubicin, another well-established activator of p53. The p53 occupancy at the *TLR* promoters was assessed by ChIP analysis and quantified by SYBR Green qPCR, as described in Fig. 2B and Supplementary Fig. S1. The PCR reaction specificity was confirmed using product dissociation curve analysis and by visualization of PCR product on ethidium bromide–stained gels (Supplementary Fig. S2). Binding to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter or a region in the *TLR5* promoter distant from the p53-RE provided a negative control. The binding of p53 to RE sequences in the promoter regions of the *TLR2, TLR5,* and *TLR6* genes was enriched 3- to 6-fold at 24 hours after Nutlin-3 or doxorubicin treatments compared with the control (untreated or DMSO-treated cells). This is similar to the binding enrichment at the well-established p53-REs in *p21* or *PUMA* promoters. Thus, as found in primary cells, *TLRs* can be direct transcriptional targets of p53 in cancer cells.

Role of p53 in doxorubicin and 5-FU–induced upregulation of *TLRs* in MCF7 and U2OS cells

Many clinical anticancer treatments rely on induction of DNA damage as well as p53 activation. Because several TLR ligands have been used as adjuvant treatments in combination with anticancer agents (35), profiling *TLR* gene expression in response to p53 in different cancer cell lines is expected to be relevant to therapeutic treatments. We, therefore, determined whether DNA damage induced by the commonly used anticancer agents doxorubicin, 5-FU, and IR can alter expression of TLR genes in cancer cell lines and what role is played by p53.

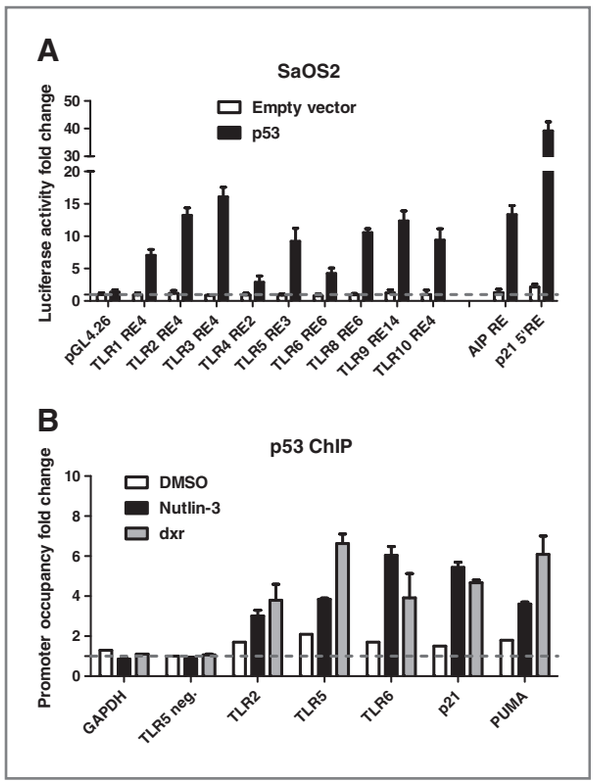


Figure 2. Functional evaluation of proposed p53 response elements (RE) in TLR promoters. A, SaOS2 cells were transfected with luciferase reporter constructs containing p53-RE sequences from different TLRs in the presence (solid bars) or absence (open bars) of the vector expressing wild-type p53. At 48 hours posttransfection, induction of the luciferase reporter was assessed. Relative luciferase activity was compared with the pGL4 plasmid lacking the p53-RE (empty vector) or constructs that contained REs of established p53 targets. Shown is an average of 3 independent experiments each carried out in triplicate. B, MCF7 cells were treated with 0.3 μg/mL of doxorubicin (dxr) or 10 μmol/L of Nutlin-3 for 24 hours. Occupancy of p53 at promoters of *TLRs* 24 hours later was assessed by ChIP assay and quantified by qPCR.

MCF7-vector and MCF7-p53i cells were treated with 0.3 μg/mL doxorubicin for 48 hours and protein (Fig. 3A) or total RNA (Fig. 3B) were isolated. Similar to Nutlin-3, the *TLR2, 5*, and *6* genes were induced by doxorubicin in a p53-dependent fashion. Treatment with 5-FU for 24 hours (Fig. 3C) or IR (Supplementary Fig. S3) also caused p53-dependent induction of *TLR2* and *TLR6*. The lack of *TLR5* induction by 5-FU shows that there are differences between agents in ability to induce *TLRs* in this cancer cell line.

To confirm the role of p53 in regulating of *TLRs* gene expression after DNA damage, U2OS cells were transiently transfected with 25 nmol/L of p53 smart pool (p53i) or scrambled oligos (scr) and subsequently treated for 24 hours with doxorubicin. There was substantial induction of p53 and p21 protein levels

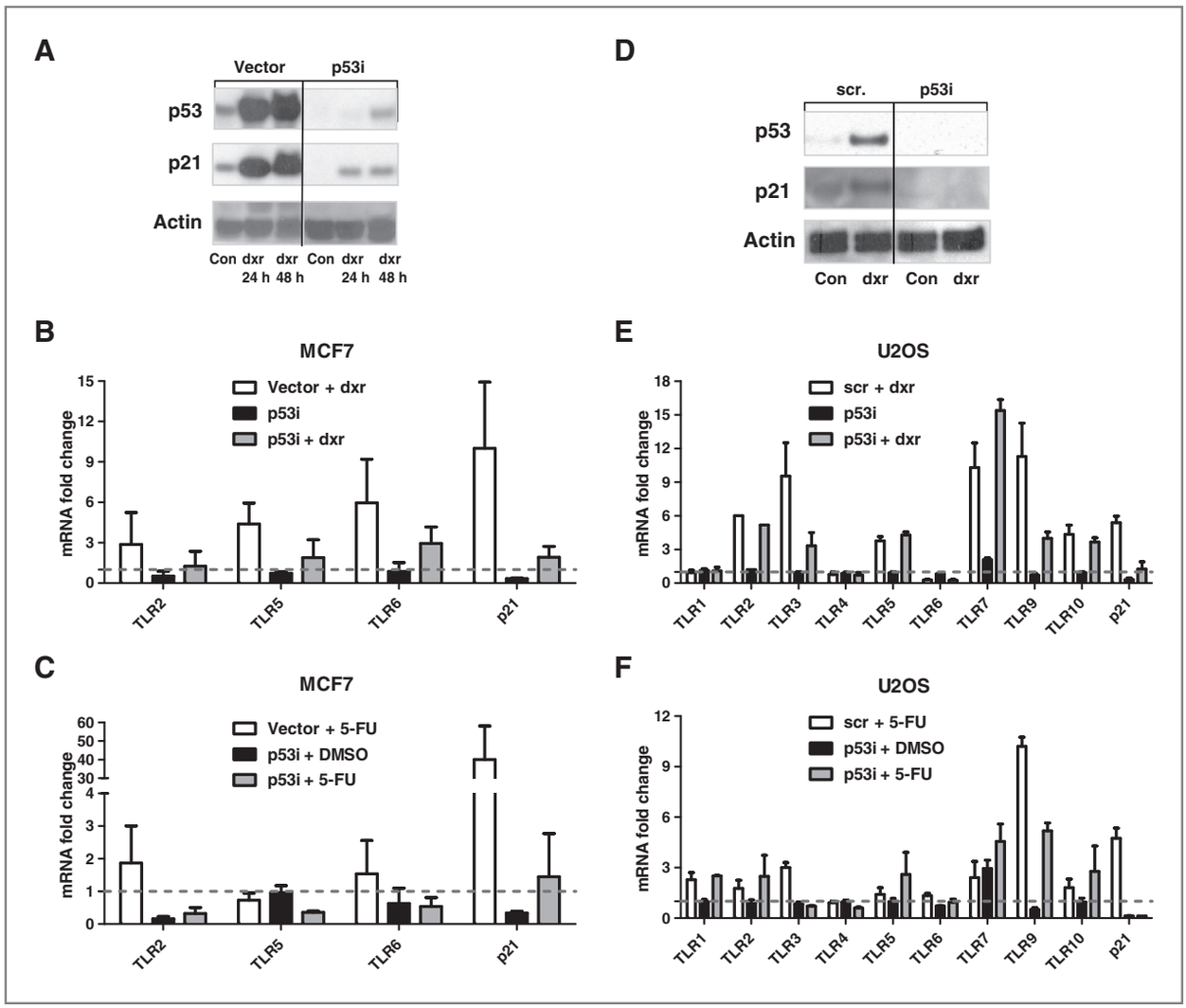


Figure 3. Role of p53 in doxorubicin and 5-FU induced upregulation of TLRs in MCF7 and U2OS cells. A, MCF7-vector and MCF7-p53i cells were treated with 0.3 μg/mL of doxorubicin for 48 hours or left untreated. Following incubation, cells were harvested and subjected to SDS-PAGE analysis to detect p53 and p21 induction. Actin was used as a loading control (Con). B, gene expression of *TLRs 2, 5,* and *6*, and *p21* following treatment with doxorubicin (dxr, 0.3 μg/mL, 48 hours) or with 5-FU (300 μmol/L, 24 hours; C) was assessed using real-time PCR. Shown are averages of 2 to 6 independent experiments. D, U2OS cells transiently transfected with 25 nmol/L of Dharmacon p53 smart pool (p53i) or scrambled oligos (scr) were treated at 24 hours posttransfection with 0.6 μg/mL of doxorubicin for 24 hours. The p53 and p21 protein levels were assessed by Western blotting; actin was used as a loading control. E, presented is the gene expression of TLRs 1 to 10 and p21 following treatment with doxorubicin (0.6 μg/mL, 24 hours) or with 5-FU (300 μmol/L, 24 hours; F). Gene expression was assessed by real-time PCR and normalized to *GUSB* housekeeping gene expression. Expression of each gene in the DMSO-treated control cells was set to one.

upon doxorubicin treatment which was prevented by transfection with p53 siRNA (Fig. 3D). As shown in Fig. 3E, there was a strong (9- to 11-fold) p53-dependent induction of *TLRs 3* and *9*. However, there also may be p53-independent induction of *TLRs 2, 5, 7*, and *10*. The pattern of induction by 5-FU was similar to doxorubicin except that there was p53-independent induction of *TLR1* (Fig. 3F). Altogether, the results show that even though an increase in p53 is sufficient to drive expression of *TLRs* in U2OS cells (as shown for Nutlin-3, Fig. 1), there are cell-specific as well as additional stress-specific factors that can contribute to induced expression of the *TLR* genes.

Doxorubicin-mediated increase in TLR5 enhances flagellin-induced cytokine responses

Next, we determined whether a p53-dependent increase in *TLR* expression affects functional response to TLR ligands downstream of receptor activation. MCF7 cells were treated with doxorubicin to induce TLR5 protein or left untreated for 48 hours (Fig. 4A) and then exposed to the TLR5 ligand flagellin for 3 hours. There was a significant 2- to 3-fold increase in response to flagellin in p53-positive MCF7-vector cells pretreated with doxorubicin as shown by increased production of cytokines interleukin *(IL)-6* and *IL-8* mRNA (Fig. 4B and C).

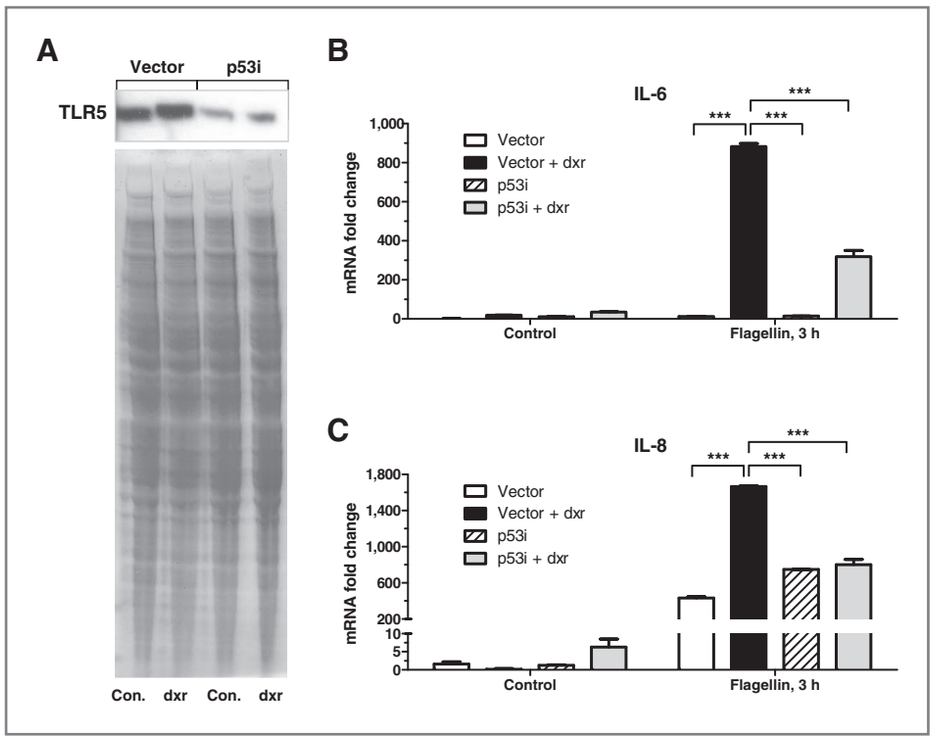


Figure 4. Doxorubicin-mediated increase in TLR5 enhances flagellin-induced cytokine responses. MCF7-vector or p53i cells were incubated with 0.3 μg/mL doxorubicin or left untreated for 48 hours. Cell lysates were subjected to subcellular fractionation and equal amounts of protein from membrane fractions (50 μg per lane) were resolved by SDS-PAGE. TLR5 protein was detected by Western blotting; gel staining was used for the loading control (Con.; A). Flagellin (10 ng/mL) was present in the medium for the last 3 hours. mRNA was purified and *IL-6* (B) and *IL-8* (C) cytokine gene expression was assessed by real-time PCR and normalized to expression of the housekeeping gene *GUSB*. Expression of each gene in control cells was set as one. Statistical analysis was conducted by Graph PadPrizm using 2-way ANOVA and Bonferroni posttests. ***, $P < 0.001$.

Importantly, this effect was p53-dependent based on reduction in the MCF7-p53i cells.

Differential expression profile of *TLR* genes across cancer cell lines

As shown earlier, several *TLR* genes were subject to p53 regulation in the 3 cell lines examined; however, the extent of induction and response to specific agents was variable between cell lines. Because induction of innate immune genes may be important in cancer treatments and differences between cancers could be meaningful, we expanded the panel of cell lines examined for *TLR* responses to DNA-damaging agents and p53 activation. Changes in *TLR* gene expression following 24 hours treatments with Nutlin-3, doxorubicin, 5-FU, IR, and UV (all known to activate p53) are presented in a heatmap format in Fig. 5 and in Supplementary Table S2 (conditions for exposure of MCF7 cells to Nutlin-3 and doxorubicin were modified as described in the table). The red, green, and black squares correspond, respectively, to >2.5-fold induction; less than 0.5 expression (i.e., repression) and modest or no change in expression (0.5- to 2.5-fold).

The *TLR2, 3*, and *9* genes were subject to damage/p53-mediated induction in almost all p53-expressing cell lines. Reintroduction of functional wild-type *p53* in p53-null cancer cells (colon carcinoma HCT116 $p53^{-}$, osteosarcoma SaOS2, and lung cancer H1299) also led to induction of most *TLRs* in at least 2 of the 3 cell lines evaluated. Clearly, there is considerable variation in responsiveness of the *TLR* genes between agents across the many cell lines tested. Importantly, induction is largely dependent on p53.

Effect of p53 mutations on *TLR* expression

Having established that wild-type p53 can regulate expression of *TLR* genes, we assessed the potential for mutant p53 to activate endogenous *TLR* genes as mutants that retain function are frequent in cancers. The osteosarcoma SaOS2 *p53*-null cells were transiently transfected with vectors that express p53 mutant proteins at comparable levels (Fig. 6A).

Seven cancer-associated p53 mutants were analyzed, and the *TLR* expression results are presented as a heatmap in the Fig. 6B. Included is a brief description of the functional transactivation status for each p53 mutant based on yeast reporter assays (36, 37). Also presented is the frequency of these mutations in human somatic and germline tumors. The levels of mRNA from all the *TLR* genes were evaluated 24 hours posttransfection. Consistent with our previous findings, *TLR8* expression was not detected. *B2M* and *p21* gene expression were used as respective negative and positive controls for p53 dependency.

As shown in Fig. 6, 3 patterns of gene expression were observed. Mutants *A138V, P151H*, and *G279R* retain the ability to drive expression of most *TLRs* at levels similar to wild-type p53. However, both *A138V* and *P151H* have decreased ability to drive expression of *TLR3* gene, a strong target of the wild-type protein. The *P151H* was unable to induce *TLR4*. A fourth mutant in this group, *G279R*, had a profile similar to wild-type for *TLRs 1, 2, 3*, and *4* but reduced capacity to transactivate *TLRs 5, 9*, and *10* along with *p21*.

A much greater change-of-spectrum for *TLR* expression was observed for *M133T* and *R337H*. The former exhibited limited transactivation for *TLR4* and *TLR5* only. The *R337H* mutant showed a clear change-in-spectrum of *TLR* expression when compared with wild-type p53. In the third group, the cancer-

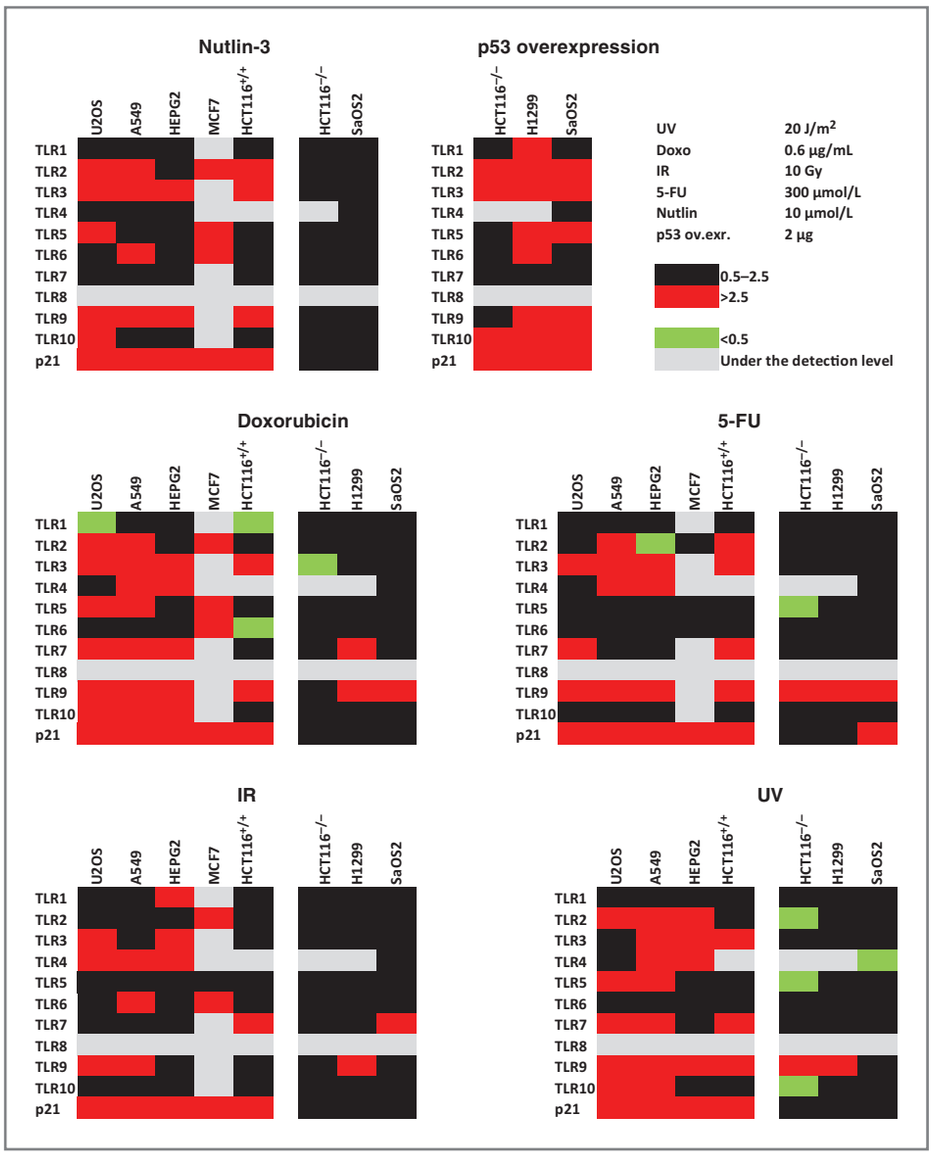


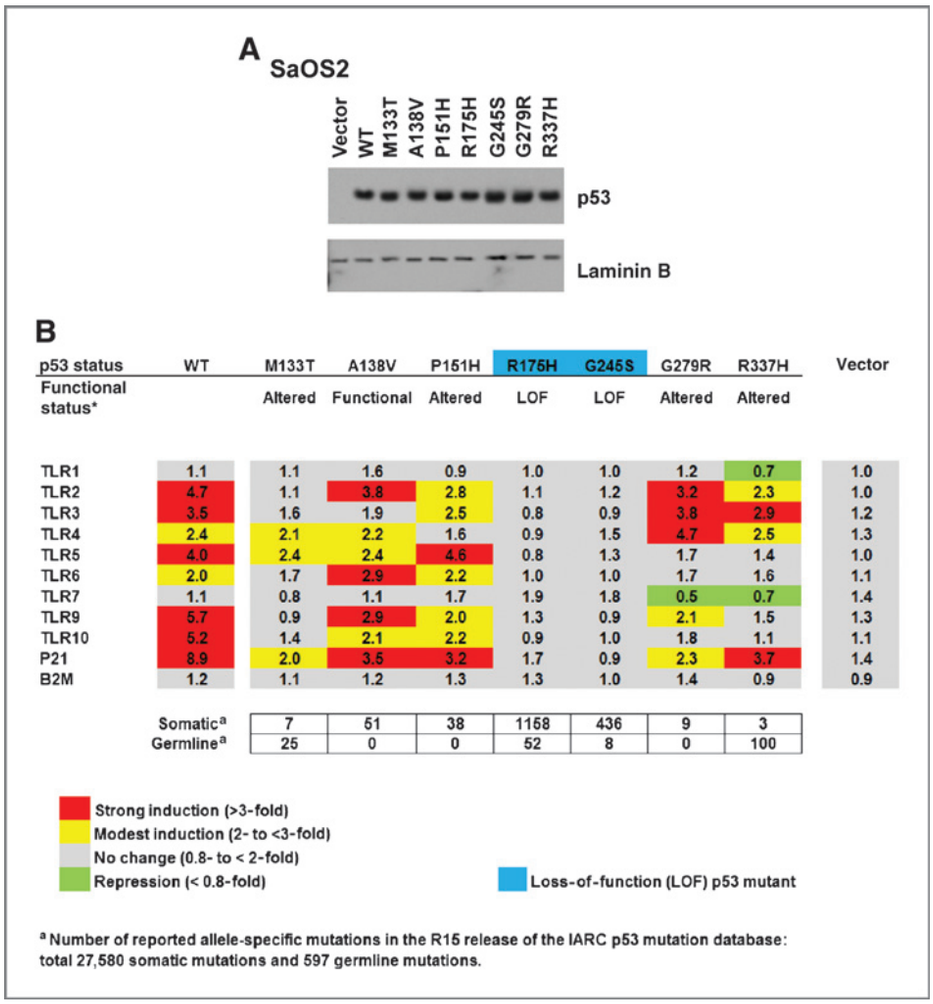
Figure 5. Genotoxic stress and p53 activation differentially affect expression of *TLR1* to *10* genes across a variety of cancer cell lines. Presented is a heatmap of changes in *TLR* gene expression after the following treatments: 24 hours incubation with Nutlin-3, 10 μmol/L; doxorubicin, 0.6 μg/mL; and 5-FU, 300 μmol/L. Cells were also exposed to IR, 10 Gy; UV, 20 J/m^2 and harvested 24 hours after the treatment. MCF7 cells were exposed to Nutlin-3, 10 μmol/L, and to 0.3 μg/mL doxorubicin in MCF7 cells for 48 hours. Gene expression is relative to the untreated control cells, set as one. The red, green, and black squares correspond, respectively, to >2.5-fold induction; less than 0.5 expression (i.e., repression) and modest or no change in expression (0.5- to 2.5-fold). The values are averages of 3 to 6 independent experiments.

associated loss-of-function hotspot p53 mutants *R175H* and *G245S* were transcriptionally inactive toward all *TLR* genes as well as *p21*. Differences in transactivation profiles for the p53 mutants were also evident for the p53 target gene *p21*. Thus, cancer-associated p53 mutants can have dramatically different effects on endogenous expression of the *TLR* family of genes and potentially affect their transcriptional response to stress and downstream signaling.

Discussion

Here, we show that expression of most members of the *TLR* gene family in human cancer cells can be responsive to p53 activation and genotoxic stress. This is expected to increase opportunities in TLR-based cancer treatments, especially as therapies may include both DNA-damaging agents and increased levels of p53. In support of this, we showed that treatment of p53-positive MCF7 cells with doxorubicin before activation of TLR5 by its ligand flagellin leads to increased downstream expression of cytokines *IL-6* and *IL-8*. Interestingly, treatment of breast cancer cells with flagellin was recently shown to reduce cell proliferation in culture and xenograft growth in mice. This effect was in part mediated by flagellin-induced soluble factors (38). Similarly, a combined treatment with flagellin along with a class I phosphoinositide 3-kinase inhibitor resulted in delayed tumor growth and increased animal survival in several mouse models (39). Therefore, an increased expression of cytokines in response to Nutlin-3/flagellin combination might predict better treatment outcome.

We have shown that there is considerable variation in transcriptional responses of specific *TLR* genes between cell lines and stressors. For example, *TLR3* expression was induced by all genotoxic agents tested in almost all the p53-positive cell



p53 status	WT	M133T	A138V	P151H	R175H	G245S	G279R	R337H	Vector
Functional status*		Altered	Functional	Altered	LOF	LOF	Altered	Altered	
TLR1	1.1	1.1	1.6	0.9	1.0	1.0	1.2	0.7	1.0
TLR2	4.7	1.1	3.8	2.8	1.1	1.2	3.2	2.3	1.0
TLR3	3.5	1.6	1.9	2.5	0.8	0.9	3.8	2.9	1.2
TLR4	2.4	2.1	2.2	1.6	0.9	1.5	4.7	2.5	1.3
TLR5	4.0	2.4	2.4	4.6	0.8	1.3	1.7	1.4	1.0
TLR6	2.0	1.7	2.9	2.2	1.0	1.0	1.7	1.6	1.1
TLR7	1.1	0.8	1.1	1.7	1.9	1.8	0.5	0.7	1.4
TLR9	5.7	0.9	2.9	2.0	1.3	0.9	2.1	1.5	1.3
TLR10	5.2	1.4	2.1	2.2	0.9	1.0	1.8	1.1	1.1
P21	8.9	2.0	3.5	3.2	1.7	0.9	2.3	3.7	1.4
B2M	1.2	1.1	1.2	1.3	1.3	1.0	1.4	0.9	0.9
Somatic[a]		7	51	38	1158	436	9	3	
Germline[a]		25	0	0	52	8	0	100	

[a] Number of reported allele-specific mutations in the R15 release of the IARC p53 mutation database: total 27,580 somatic mutations and 597 germline mutations.

Figure 6. Mutations in p53 differentially modify p53 effects on expression of the *TLR* gene family. A, p53-null SaOS2 cells were transfected with a panel of cancer-related p53 mutants. At 24 hours posttransfection, cells were harvested and p53 nuclear levels were evaluated by Western blotting. B, a heatmap of fold change in mRNA expression for *TLRs, p21*, and *B2M* genes following transfection with wild-type (WT) or mutant p53 as compared with the mock-transfected control (set as one). Gene expression was assessed by real-time PCR and normalized to *GAPDH* expression. *, denotes a description of the functional transactivation status for each p53 mutant based on yeast reporter assays (36, 37).

lines examined as well as by overexpression of p53 protein in p53-null cells. *TLR3* was previously shown to induce apoptosis in cancer cells (18, 40), suggesting that a combination of TLR3 ligands with genotoxic drugs could be beneficial for treatment of tumors containing wild-type p53. Alternatively, because DNA damage can induce *TLR9* expression in p53-dependent and -independent manners, it may be possible to amplify *TLR9*-based treatment by genotoxic agents regardless of tumor p53 status. Similarly, enhanced expression of *TLR1* and *TLR7* may be associated with cell type as well as with DNA damage response mechanism. We did not see a dependence on p53 status for treatments leading to enhanced *TLR7*. The *TLRs 2, 4, 5*, and *6* appeared to be less subject to induction across the cell lines examined; however, the induction generally required the presence of functional p53. Previously, we reported that a wide diversity in responses was also seen for p53-induced expression of *TLR* genes in stimulated lymphocytes from healthy individuals (7). Similar differences between individuals in terms of transcriptional response to IR have been observed in derived lymphoblastoid cell lines (41). Therefore, variability between cell lines may originate from either interindividual or tissue type-dependent difference.

The altered function p53 mutations can result in cellular responses that impact genome stability, repair, replication, and programmed cell death (36, 42). Single amino acid changes in p53 that differentially impact transactivation may alter the efficacy of chemotherapeutic agents and diversify cell responses to stress (43). Here, we evaluated the effect of several p53 mutants associated with both somatic and germline tumors, particularly breast and colon cancers [Fig. 5 and the IARC database R15 (ref. 30, http://www-p53.iarc.fr/)], on expression of the *TLR* genes. The changes in TLR transactivation patterns, including change-of-spectrum, are consistent with our previous report in which each of mutant was tested for transactivation at 11 validated human p53-REs using a yeast-based system (4).

While the classic loss-of-function mutants *R175H* and *G245S* were unable to induce any *TLR* gene, the profile of *TLR*-expressed genes for the remaining 5 p53 mutants was altered. The *R337H* mutation resulted in the most dramatic changes including loss or gain of transactivation for different *TLRs* (Fig. 5). Located on the surface of the tetramerization domain of the p53 protein, the *R337H* mutation is considered to have a subtle functional transactivation change causing a pH-dependent effect on folding (44). The differential response of *TLR*

genes to this p53 mutant, which is associated with pediatric adrenal cortical carcinoma, could inform treatments that are TLR ligand–based. In our study, we observed that the *R337H* p53 mutant might have the ability to induce expression of *TLR2*. Other factors also may play a role in the regulation of the adrenal response as shown for *TLR9* and its relation with sepsis response in the presence of bacterial DNA (45). These observations support the view that the specific status of *p53* should be considered in designing adjuvant cancer therapies that use TLR pathways.

Overall, we suggest that a profile of *TLR* gene expression patterns in specific tumors in response to p53 and DNA-damaging agents combined with knowledge of p53 expression and mutation status in these tumors can be an important tool in cancer diagnosis and in strategies that target TLR pathway for cancer therapy. For example, the presence of a p53 mutation that can change a damage-induced *TLR* expression pattern could determine which TLR agonists or antagonists to use for specific tumors as well as the choices of p53 adjuvant inducers of TLRs. Also, we suggest that there are unique opportunities to capitalize on chromosome stress responses in human cancer therapies that might not be apparent from studies in other organisms as the responsiveness of the human set of *TLR* genes to p53 is unique to primates (7).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Shatz, D. Menendez, M.A. Resnick
Development of methodology: M. Shatz, D. Menendez, M.A. Resnick
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Shatz, D. Menendez
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Shatz, D. Menendez, M.A. Resnick
Writing, review, and/or revision of the manuscript: M. Shatz, D. Menendez, M.A. Resnick
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Menendez, M.A. Resnick
Study supervision: D. Menendez, M.A. Resnick

Acknowledgments

The authors appreciate discussions with David Schwartz at early stages of work and critical comments of Carl Anderson, Michael Fessler, and Stavros Garantziotis during manuscript development.

Grant Support

The study was supported by NIEHS intramural research funds, project Z01-ES065079.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 28, 2011; revised May 17, 2012; accepted May 19, 2012; published OnlineFirst June 6, 2012.

References

1. Vousden KH, Ryan KM. p53 and metabolism. Nat Rev Cancer 2009;9:691–700.
2. el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. Nat Genet 1992;1:45–9.
3. Jegga AG, Inga A, Menendez D, Aronow BJ, Resnick MA. Functional evolution of the p53 regulatory network through its target response elements. Proc Natl Acad Sci U S A 2008;105:944–9.
4. Jordan JJ, Menendez D, Inga A, Nourredine M, Bell D, Resnick MA. Noncanonical DNA motifs as transactivation targets by wild type and mutant p53. PLoS Genet 2008;4:e1000104.
5. Menendez D, Inga A, Resnick MA. Estrogen receptor acting in cis enhances WT and mutant p53 transactivation at canonical and non-canonical p53 target sequences. P Natl Acad Sci U S A 2010;107:1500–5.
6. Kato S, Han SY, Liu W, Otsuka K, Shibata H, Kanamaru R, et al. Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. Proc Natl Acad Sci U S A 2003;100:8424–9.
7. Menendez D, Shatz M, Azzam K, Garantziotis S, Fessler MB, Resnick MA. The Toll-like receptor gene family is integrated into human DNA damage and p53 networks. PLoS Genet 2011;7:e1001360.
8. Kawai T, Akira S. TLR signaling. Semin Immunol 2007;19:24–32.
9. Bauer S, Muller T, Hamm S. Pattern recognition by Toll-like receptors. Adv Exp Med Biol 2009;653:15–34.
10. Kabelitz D. Expression and function of Toll-like receptors in T lymphocytes. Curr Opin Immunol 2007;19:39–45.
11. Uematsu S, Jang MH, Chevrier N, Guo Z, Kumagai Y, Yamamoto M, et al. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c +lamina propria cells. Nat Immunol 2006;7:868–74.
12. Muir A, Soong G, Sokol S, Reddy B, Gomez MI, Van Heeckeren A, et al. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. Am J Respir Cell Mol Biol 2004;30:777–83.
13. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 2005;5:446–58.
14. Cook DN, Pisetsky DS, Schwartz DA. Toll-like receptors in the pathogenesis of human disease. Nature Immunol 2004;5:975–9.
15. Hirsch I, Caux C, Hasan U, Bendriss-Vermare N, Olive D. Impaired Toll-like receptor 7 and 9 signaling: from chronic viral infections to cancer. Trends Immunol 2010;31:391–7.
16. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 2007;13:1050–9.
17. Taura M, Fukuda R, Suico MA, Eguma A, Koga T, Shuto T, et al. TLR3 induction by anticancer drugs potentiates poly I:C-induced tumor cell apoptosis. Cancer Sci 2010;101:1610–7.
18. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 2006;176:4894–901.
19. Garay RP, Viens P, Bauer J, Normier G, Bardou M, Jeannin JF, et al. Cancer relapse under chemotherapy: why TLR2/4 receptor agonists can help. Eur J Pharmacol 2007;563:1–17.
20. Bong AB, Bonnekoh B, Franke I, Schon MP, Ulrich J, Gollnick H. Imiquimod, a topical immune response modifier, in the treatment of cutaneous metastases of malignant melanoma. Dermatology 2002;205:135–8.
21. Krieg AM. Development of TLR9 agonists for cancer therapy. J Clin Invest 2007;117:1184–94.
22. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. Nat Rev Cancer 2009;9:57–63.
23. Akazawa T, Ebihara T, Okuno M, Okuda Y, Shingai M, Tsujimura K, et al. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci U S A 2007;104:252–7.
24. He H, Genovese KJ, Nisbet DJ, Kogut MH. Synergy of CpG oligodeoxynucleotide and double-stranded RNA (poly I:C) on nitric oxide induction in chicken peripheral blood monocytes. Mol Immunol 2007;44:3234–42.
25. Kanzler H, Barrat FJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med 2007;13:552–9.

26. Zom GG, Khan S, Filippov DV, Ossendorp F. TLR ligand-peptide conjugate vaccines: toward clinical application. Adv Immunol 2012;114:177–201.
27. Bell MP, Svingen PA, Rahman MK, Xiong Y, Faubion WA Jr. FOXP3 regulates TLR10 expression in human T regulatory cells. J Immunol 2007;179:1893–900.
28. Tsatsanis C, Androulidaki A, Alissafi T, Charalampopoulos I, Dermitzaki E, Roger T, et al. Corticotropin-releasing factor and the urocortins induce the expression of TLR4 in macrophages via activation of the transcription factors PU.1 and AP-1. J Immunol 2006;176:1869–77.
29. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science 1991;253:49–53.
30. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat 2007;28:622–9.
31. Taura M, Eguma A, Suico MA, Shuto T, Koga T, Komatsu K, et al. p53 regulates Toll-like receptor 3 expression and function in human epithelial cell lines. Mol Cell Biol 2008;28:6557–67.
32. Schwartz DA, Cook DN. Polymorphisms of the Toll-like receptors and human disease. Clin Infect Dis 2005;41 Suppl 7:S403–7.
33. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004;303:844–8.
34. Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999;285:1733–7.
35. Adams S. Toll-like receptor agonists in cancer therapy. Immunotherapy 2009;1:949–64.
36. Resnick MA, Inga A. Functional mutants of the sequence-specific transcription factor p53 and implications for master genes of diversity. Proc Natl Acad Sci U S A 2003;100:9934–9.
37. Jordan JJ, Inga A, Conway K, Edmiston S, Carey LA, Wu L, et al. Altered-function p53 missense mutations identified in breast cancers can have subtle effects on transactivation. Mol Cancer Res 2010;8:701–16.
38. Cai Z, Sanchez A, Shi Z, Zhang T, Liu M, Zhang D. Activation of Toll-like receptor 5 on breast cancer cells by flagellin suppresses cell proliferation and tumor growth. Cancer Res 2011;71:2466–75.
39. Marshall NA, Galvin KC, Corcoran AM, Boon L, Higgs R, Mills KH. Immunotherapy with PI3K inhibitor and Toll-like receptor agonist induces IFN-gamma+IL-17+ polyfunctional T cells that mediate rejection of murine tumors. Cancer Res 2012;72:581–91.
40. Paone A, Starace D, Galli R, Padula F, De Cesaris P, Filippini A, et al. Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-alpha-dependent mechanism. Carcinogenesis 2008;29:1334–42.
41. Correa CR, Cheung VG. Genetic variation in radiation-induced expression phenotypes. Am J Hum Genet 2004;75:885–90.
42. Menendez D, Inga A, Resnick MA. The biological impact of the human master regulator p53 can be altered by mutations that change the spectrum and expression of its target genes. Mol Cell Biol 2006;26:2297–308.
43. Temam S, Flahault A, Perie S, Monceaux G, Coulet F, Callard P, et al. p53 gene status as a predictor of tumor response to induction chemotherapy of patients with locoregionally advanced squamous cell carcinomas of the head and neck. J Clin Oncol 2000;18:385–94.
44. Ribeiro RC, Sandrini F, Figueiredo B, Zambetti GP, Michalkiewicz E, Lafferty AR, et al. An inherited p53 mutation that contributes in a tissue-specific manner to pediatric adrenal cortical carcinoma. Proc Natl Acad Sci U S A 2001;98:9330–5.
45. Tran N, Koch A, Berkels R, Boehm O, Zacharowski PA, Baumgarten G, et al. Toll-like receptor 9 expression in murine and human adrenal glands and possible implications during inflammation. J Clin Endocrinol Metab 2007;92:2773–83.