

Genistein, a Dietary Isoflavone, Down-Regulates the MDM2 Oncogene at Both Transcriptional and Posttranslational Levels

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Abstract

Although genistein has chemopreventive effects in several human malignancies, including cancers of the breast, colon, and prostate, the mechanisms of action are not fully understood. Herein we report novel mechanisms whereby genistein down-regulates the MDM2 oncogene, perhaps explaining some of its anticancer activities. In a dose- and time-dependent manner, genistein reduced MDM2 protein and mRNA levels in human cell lines of breast, colon, and prostate cancer; primary fibroblasts; and breast epithelial cells. The inhibitory effects were found at both transcriptional and posttranslational levels and were independent of tyrosine kinase pathways. We found that the NFAT transcription site in the region between -132 and +33 in the MDM2 P2 promoter was responsive to genistein. At the posttranslational level, genistein induced ubiquitination of MDM2, which led to its degradation. Additionally, genistein induced apoptosis and G₂ arrest and inhibited proliferation in a variety of human cancer cell lines, regardless of p53 status. We further showed that MDM2 overexpression abrogated genistein-induced apoptosis *in vitro* and that genistein inhibited MDM2 expression and tumor growth in PC3 xenografts. In conclusion, genistein directly down-regulates the MDM2 oncogene, representing a novel mechanism of its action that may have implications for its chemopreventive and chemotherapeutic effects. (Cancer Res 2005; 65(18): 8200-8)

Introduction

Epidemiologic studies have shown that diet, which can vary substantially from one country to another, is one of the major factors in cancer etiology and may account for up to 35% of the differences in cancer rates among different countries (1). Consumption of soybeans reduces the risk for breast, prostate, stomach, colorectal, and lung cancers (2). For example, in societies where the consumption of isoflavone-containing soy foods is substantially higher than in the United States, the risk of breast cancer has been historically low (2). Increasing consumption of soy products is positively correlated to a reduction in breast cancer risk (3, 4). Isoflavones are present in high concentrations (1-5 mg/g) in soy products (5) and the American groundnut (*Apios americana*; ref. 6). In societies that consume soy-based foods as a regular part of their diet, an inverse relationship exists between isoflavone intake and the risk for several types of cancer (7, 8).

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Two isoflavones, genistein (5,7,4'-trihydroxyisoflavone) and daidzein (7,4'-dihydroxyisoflavone), are likely predominant in the cancer preventive activity of soybeans. Genistein is now considered to be the primary anticancer component of soybeans; its *in vitro* and/or *in vivo* activities include the antagonism of estrogen, inhibition of protein tyrosine phosphorylation, suppression of angiogenesis, inhibition of hydrogen peroxide formation induced by tumor promoters, inhibition of topoisomerases, induction of apoptosis and cell differentiation, scavenging of free radicals, and inhibition of carcinogenesis and tumor promotion (2, 9-13).

In animal models of mammary gland, liver, colon, skin, prostate, and stomach carcinogenesis, soy consumption reduces the development of cancers (7). Dietary genistein reduces the multiplicity of mammary and prostate tumors that develop in carcinogen-dosed murine models (14, 15). In mammary tumors, the protective effects are seen for both estrogen-dependent and -independent tumors. Dietary genistein also reduces the incidence of aberrant crypts and colon cancer in carcinogen-dosed rats (16). In addition to its cancer preventive effects, genistein has a role in cancer therapy (17, 18). The mechanisms of action for its chemopreventive and chemotherapeutic effects, however, are not fully understood.

Considering the inhibitory effects of genistein on carcinogenesis and tumor growth, its potential effects on the regulation of oncogenes should be investigated. The mouse double minute 2 (MDM2) oncoprotein is a negative regulator of tumor suppressor p53, blocking p53 transcriptional activity and promoting its proteasome-mediated degradation (19). Nevertheless, the p53-independent effects of MDM2 have attracted increasing attention in recent years. MDM2 interacts with various cellular proteins, including p19/14^{ARF}, E2F1, p300, ribosomal L5/L11/L23 proteins, and p73. The MDM2 oncoprotein is overexpressed in many human malignancies, and high MDM2 levels are associated with poor clinical prognosis (19). Antisense inhibition of MDM2 expression results in substantial *in vitro* and *in vivo* antitumor activities in breast, colon, and prostate cancer models (20-24). The present study was designed to test our hypothesis that genistein may down-regulate the expression of the MDM2 oncogene.

Our results indicate that genistein has a novel mechanism of action, whereby it specifically down-regulates MDM2 at both transcriptional and posttranslational levels. At the transcriptional level, the NFAT site in the MDM2 promoter is important for the effects of genistein. At the posttranslational level, genistein induces MDM2 ubiquitination, which is relevant to down-regulation of the MDM2 protein. We further show the importance of this MDM2 inhibitory effect relative to the antitumor activities of genistein *in vitro* and *in vivo*. We also provide evidence of genistein-mediated, p53-independent up-regulation of the tumor suppressor p21^{Waf1/CIP1} at the posttranslational level, which may be associated with MDM2 down-regulation.

Materials and Methods

Plasmids and reagents. Human full-length, deleted, and ETSa, AP1, and ETSa-AP1 double-mutated P2 MDM2 promoter reporters were kind gifts from Dr. J.P. Blaydes (Southampton General Hospital, United Kingdom). P2 promoter reporters with MEF2, NFAT, and ETSa-AP1-NFAT triple mutations were generated by proofreading PCR and verified by sequencing. The vectors for wild-type MDM2 and mutant MDM2 (C464A) without E3 ligase activity were kindly provided by Dr. J. Chen (Moffitt Cancer Center) and Dr. C.G. Maki (University of Chicago). The human MDM2 cDNA insert was digested with *Bam*HI and *Xho*I and subcloned into the same sites of pcDNA3. To construct short interfering RNA (siRNA) expression plasmids under the control of the U6 promoter, selected oligonucleotides were cloned into pBabe-U6 at *Bam*HI and *Xho*I sites for expression of siRNA *in vivo*. One pair of siRNA oligonucleotides from p53 were synthesized and cloned into pBabe-U6. The target sequence of the oligonucleotides for p53 knockdown (derived from the *p53* gene) was 5'-GACTCCAGTGGTAATCTAC. Genistein was purchased from Sigma (St. Louis, MO).

Cell lines and cell culture. HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells were kindly provided by Dr. B. Vogelstein (Johns Hopkins) and maintained in McCoy's 5A medium. Human primary fibroblasts (IMR90) were gifts from Dr. S. Lee (Harvard; refs. 25, 26). MCF10A, MCF-7, and PC3 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD) and cultured in DMEM and Ham's F-12, respectively. All culture media contained 10% fetal bovine serum and 1% penicillin-streptomycin. To establish an MCF-7 cell line with stable p53 knockdown, MCF-7 cells were transfected with pBabe-U6-p53. Cell clones positive for the vector were selected, maintained with puromycin (0.5 µg/mL), and confirmed by immunoblotting. For the stable MDM2-overexpressing PC3 cell line, PC3 cells were transfected with pcDNA3-MDM2 followed by addition of the selective reagent, neomycin (450 µg/mL). The positive clones were confirmed by immunoblotting and maintained in medium containing neomycin.

Reverse transcription-PCR. Total RNA was extracted using the Trizol reagent from Invitrogen (Carlsbad, CA), quantified by UV spectrophotometry, and used to create cDNA with the SuperScript reverse transcription-PCR (RT-PCR) kit from Invitrogen. The PCR coamplification of MDM2 with β -actin was accomplished using the method described previously (24).

Luciferase assay. Cells were cotransfected with full-length, deleted, or mutated human MDM2 P2 promoter vectors with *Renilla* luciferase reporter (as internal control; Promega, Madison, WI) for 12 hours followed by incubation with genistein for 24 hours. The luciferase activity of the MDM2 promoter reporters was determined with the Dual-Luciferase Reporter Assay System (Promega) according to the provided protocol.

The MDM2 reporter activity was normalized to the corresponding *Renilla* luciferase reporter activity.

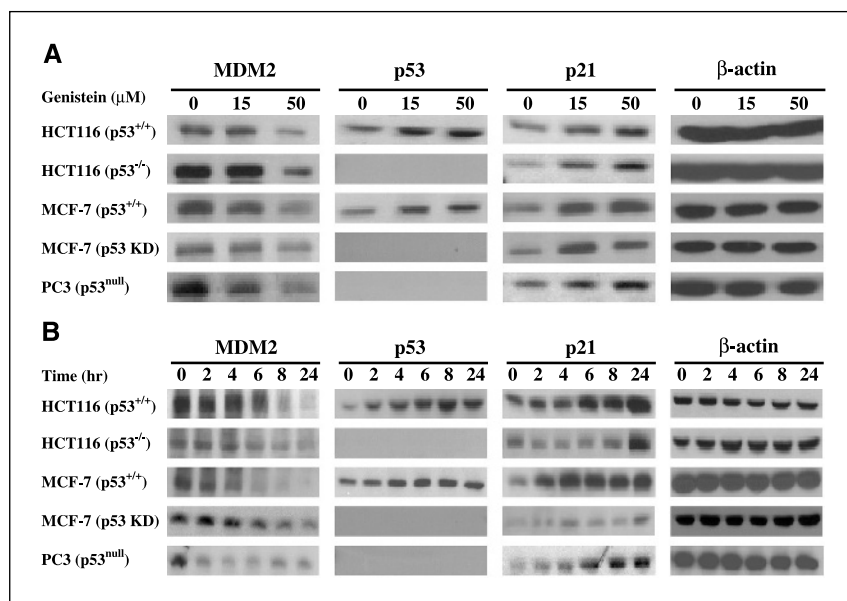
Assays for apoptosis, cell proliferation, cell cycle distribution, and clonogenicity. The methods used to detect apoptotic cells and determine the extent of cell proliferation (bromodeoxyuridine, BrdUrd, incorporation assay) were described previously (24). For cell cycle distribution assay, cells were trypsinized, washed with PBS, and fixed in 1.5 mL 95% ethanol at 4°C overnight followed by incubation with RNase and staining by propidium iodide (Sigma). The DNA content was determined by flow cytometry. To determine the long-term effects, cells were treated with genistein at various concentrations for 24 hours. After being rinsed with fresh medium, cells were allowed to grow for 14 days to form colonies that were stained with crystal violet (0.4 g/L; Sigma).

Xenograft models. The PC3 xenograft model was established using the methods described previously (23, 24). Male, 4- to 6-week-old athymic nude mice (*nu/nu*), were obtained from Frederick Cancer Research and Development Center (Frederick, MD). Cultured cells were washed with and resuspended in serum-free medium. Portions of the suspension (5×10^6 cells in 0.2 mL/mouse) were then injected into the left inguinal area of the mice. The mice were monitored by measuring tumor growth and body weight and by general clinical observation (23, 24). Tumor-bearing mice were randomly divided into multiple treatment and control groups ($n = 5$ mice per group). Genistein, dissolved in cottonseed oil, was given by gavage at doses of 5 mg/kg/d, 5 days/wk for 4 weeks. The control group received cottonseed oil only. Gemcitabine (160 mg/kg) was given by i.p. injection, once per week for 3 weeks. In combination therapy, genistein was given at 5 mg/kg level with gemcitabine as described above.

Results

Genistein inhibits MDM2 expression in human cancer and primary cell lines, regardless of p53 status. The effects of genistein on MDM2 expression were first analyzed in cancer cells containing wild-type p53. HCT116 and MCF-7 cells were treated with various concentrations of genistein for 24 hours or with 50 µmol/L genistein for varying times. MDM2 protein levels were decreased in a time- and concentration-dependent manner (Fig. 1A and B). Most likely as a result of the inhibition of MDM2, the p53 protein level was elevated (Fig. 1A and B). The expression level of *p21*^{Waf1/CIP1}, a p53 target gene, was also elevated (Fig. 1A and B). To test whether the inhibitory effects of genistein on MDM2 require p53, HCT116 (p53^{-/-}), MCF-7 (p53KD; p53 was knocked down by

Figure 1. Effects of genistein on MDM2 expression in human cancer cells. **A**, HCT 116 cells with wild-type (p53^{+/+}) or without p53 (p53KD), MCF-7 cells with wild-type (p53^{+/+}) or without p53 (p53KD), and PC3 cells (p53^{null}) were treated with various concentrations of genistein for 24 hours, and the target proteins (MDM2, p53, p21, and β -actin) were detected by immunoblotting with specific antibodies. **B**, these same cell lines were treated with 50 µmol/L of genistein for various times, and the target proteins were detected by immunoblotting. KD, knockdown.



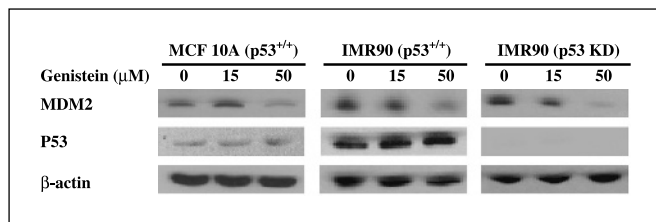


Figure 2. Down-regulation of MDM2 by genistein in human primary cells. IMR90-EEA and MCF10A cells were treated with various concentrations of genistein as above. Target proteins were detected by immunoblotting. To test whether this effect depends on p53 or not, IMR90-EEA cells were transfected with p53 knockdown siRNA plasmids followed by the treatment with various concentrations of genistein for additional 24 hours. Target proteins were examined as above.

siRNA), and PC3 (p53^{null}) cells were treated with genistein as above. In a dose- and time-dependent manner, MDM2 expression was inhibited in all the cell lines (Fig. 1A and B). p21^{Waf1/CIP1} expression levels were elevated (Fig. 1A and B), independent of the expression of p53, indicating that genistein may inhibit the p21 regulatory function of MDM2, as was previously noted when MDM2 antisense oligonucleotides were used (21–24). The modulatory effects of genistein on MDM2 in the presence of p53 were further confirmed

in primary human fibroblasts (IMR90) and human breast epithelial cells (MCF10A), which were treated with various concentrations of genistein as above. MDM2 was down-regulated by genistein dose dependently (Fig. 2). To test whether this effect depends on the presence of p53, a p53 knockdown siRNA plasmid was transfected in IMR90 cells. Later (24 hours), these cells were treated with various concentrations of genistein for 24 hours; MDM2 expression was still shown to be inhibited by genistein (Fig. 2).

MDM2 transcription is repressed by genistein. To address the possible underlying mechanisms of MDM2 inhibition by genistein at the transcriptional level, HCT116 (p53^{-/-}), MCF-7 (p53KD), and PC3 (p53^{null}) cells were treated with various concentrations of genistein for 24 hours, and the MDM2 mRNA expression levels were determined by RT-PCR. As shown in Fig. 3A, MDM2 mRNA levels were decreased by genistein in all three cell lines in a concentration-dependent manner. To confirm these results, a human MDM2-P2 promoter reporter (Luc01) was transfected into PC3 cells that were subsequently treated with genistein (Fig. 3B1). The luciferase activity of the MDM2 reporter was decreased 70% by genistein (50 μmol/L); there were no apparent changes in the cells transfected with the corresponding empty vector reporter (Fig. 3B2). We next identified the genistein-responsive element on the MDM2-P2 promoter. The reporter vectors used in this

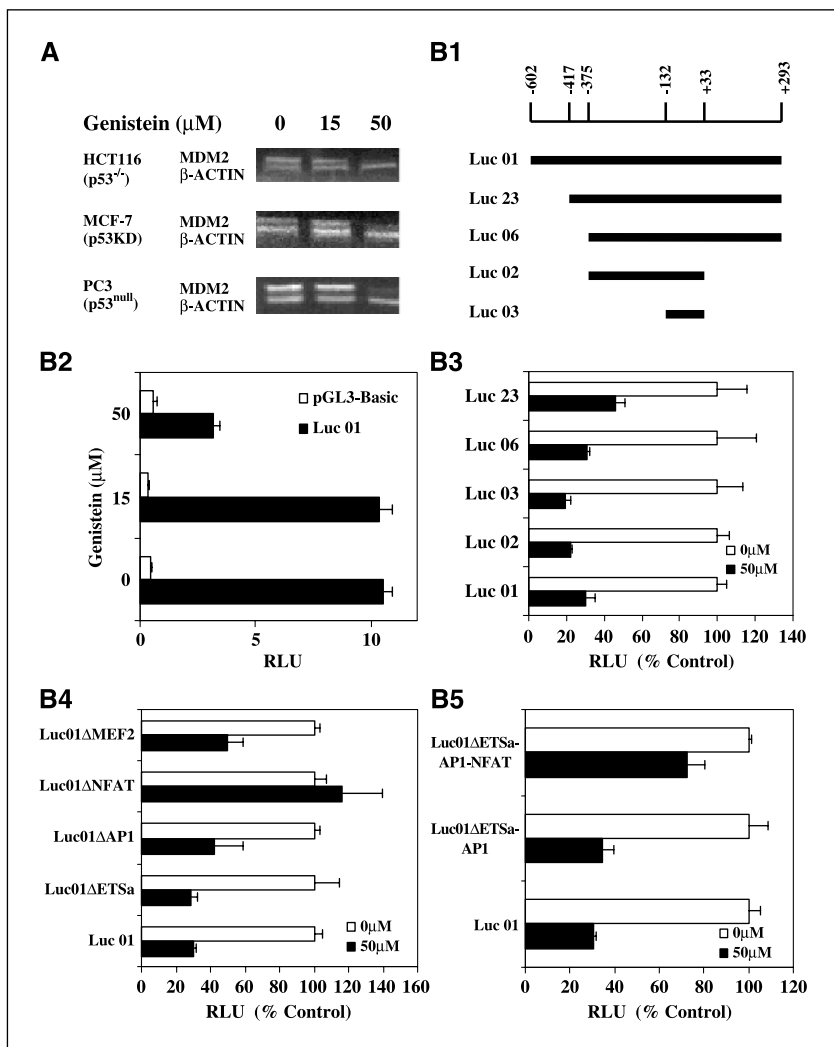
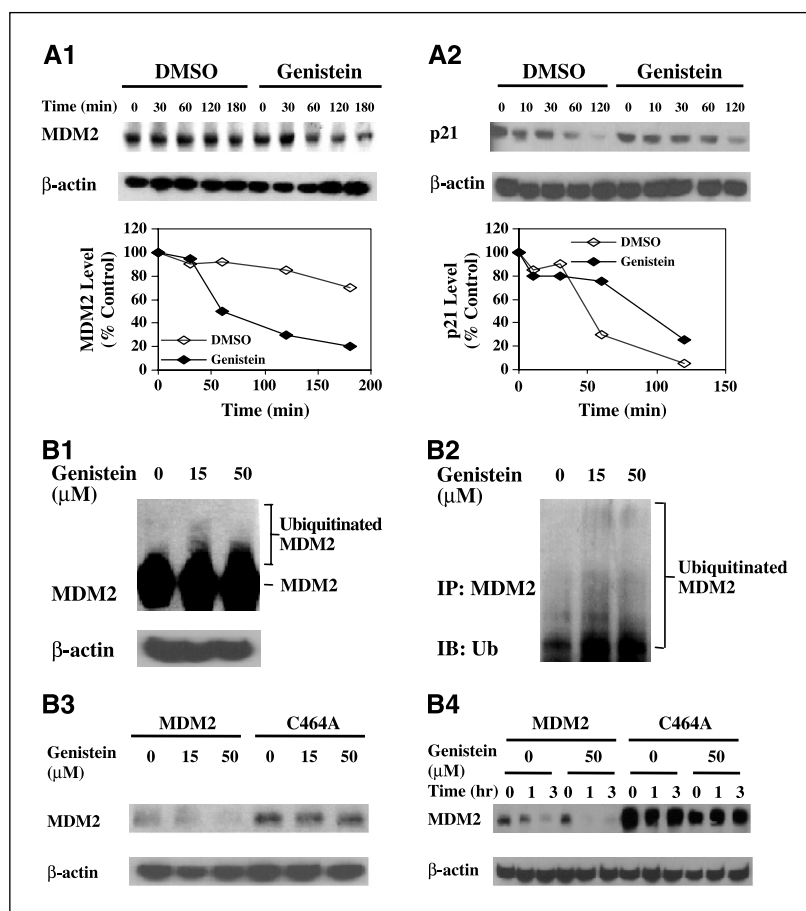


Figure 3. Effects of genistein on MDM2 expression at the transcriptional level, independent of p53. A, HCT 116 cells without p53 (p53^{-/-}), PC3 cells (p53^{null}), and MCF-7 cells without p53 (p53KD) were treated with various concentrations of genistein for 24 hours followed by the extraction of total RNA. MDM2 mRNA was coamplified with β-actin mRNA. B1, comparison of structures of deleted MDM2 promoters with the full-length promoter. B2, PC3 cells were transfected with the MDM2 reporter construct (Luc 01) or corresponding empty vector (pGL3-Basic) for 12 hours followed by incubation with various concentrations of genistein for an additional 24 hours, before luciferase activities were quantified. Columns, means of duplicate assays; bars, ±SD. B3, PC3 cells were transfected with MDM2 deletion reporters (Luc 02, Luc 03, Luc 06, and Luc 23) in parallel with the full-length reporter (Luc 01) for 12 hours followed by incubation with 0 or 50 μmol/L of genistein for an additional 24 hours. The luciferase activity of each reporter treated with 50 μmol/L of genistein is a percentage of that obtained for the control (no genistein). Columns, means of duplicate assays; bars, ±SD. B4, PC3 cells were transfected with MDM2 mutation reporters (Luc 01ΔMEF2, Luc 01ΔETSa, Luc 01ΔAP1, and Luc 01ΔNFAT) in parallel with full-length reporter (Luc 01) for 12 hours followed by the incubation with 0 or 50 μmol/L of genistein for an additional 24 hours. The luciferase activity of each reporter treated with 50 μmol/L of genistein is a percentage of that obtained for the control (no genistein). Columns, means of duplicate assays; bars, ±SD. B5, PC3 cells were transfected with MDM2 mutation reporters (Luc 01ΔETSa-AP1 and Luc 01ΔETSa-AP1-NFAT) in parallel with full-length reporter (Luc 01) for 12 hours followed by incubation with 0 or 50 μmol/L of genistein for additional 24 hours. Luciferase activity of each reporter treated with 50 μmol/L of genistein is a percentage of that for the control (no genistein). Columns, means of duplicate assays; bars, ±SD.

Figure 4. p53-independent effects of genistein on MDM2 expression at the posttranslational level. MDM2 (A1) or p21 (A2) protein levels in 50 $\mu\text{mol/L}$ of genistein- or DMSO-treated PC3 cells were detected by immunoblotting at different time points after the protein synthesis inhibitor cycloheximide (10 $\mu\text{g/mL}$) was added. Graphs (bottom) show quantification of the immunoblotting data. Similar experiments were done at least three times and produced similar results. B1, PC3 cells were transfected with MDM2 followed by treatment with various concentrations of genistein for 24 hours. Before the harvest of protein samples, cells were further exposed to the proteasome inhibitor MG132 (25 $\mu\text{mol/L}$) for 6 hours. Ubiquitinated MDM2 was detected by immunoblotting. B2, PC3 cells, transfected with ubiquitin expressing plasmid, were treated with various concentrations of genistein. After further incubation with MG132 (25 $\mu\text{mol/L}$) for 6 hours, cells were lysed and the cell lysates were subjected to immunoprecipitation with MDM2 antibody. Ubiquitinated MDM2 was detected by ubiquitin antibody. B3 and B4, PC3 cells were transfected with wild-type MDM2 or mutant MDM2 without E3 ligase activity (C464A). After treatment with genistein, cells were either lysed for collection of protein samples (B3) or further exposed to the protein synthesis inhibitor cycloheximide (10 $\mu\text{g/mL}$) for different times (B4). The changes in MDM2 expression were detected by immunoblotting.



approach are described in Fig. 3B1. Deletion mapping of Luc 01 showed that the shortest segment (-132 to $+33$; Luc 03) still retained the response to genistein (Fig. 3B3). There are several transcription factor response sites between -132 and $+33$ in the MDM2 P2 promoter, including sites for ETS, AP1, MEF2, and NFAT. ETS-AP1 is responsible for the activation of the promoter by growth factors dependent on the ras/raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK pathways (27).

To determine which site is involved in the effects of genistein, four P2-luciferase vectors with ETSa, AP1, MEF2, and NFAT mutations, respectively, were transfected into PC3 cells, which were then treated with genistein. The NFAT mutation abrogated the response of the P2 promoter to genistein; the other three mutant vectors retained the capacity to respond (Fig. 3B4). To confirm the results, other P2 promoters with a double mutation ($\Delta\text{AP1-ETSa}$) or triple mutation ($\Delta\text{AP1-ETSa-NFAT}$) were tested following a similar protocol. The ETSa-AP1 mutation had no effect on the response of the P2 promoter to genistein. In contrast, mutation of NFAT in addition to the ETSa-AP1 mutation reduced the response (Fig. 3B5).

Genistein promotes degradation of the MDM2 protein, independent of p53. The effects of genistein on MDM2 regulation were also determined at the posttranscriptional level. In PC3 cells, genistein increased the degradation rate of the MDM2 protein (Fig. 4A1) while prolonging the p21^{Waf1/CIP1} protein half-life (Fig. 4A2). To elucidate the underlying mechanisms, PC3 cells were transfected with MDM2 and ubiquitin, treated with genistein for 24 hours, and then exposed to the proteasome inhibitor MG132 (25 $\mu\text{mol/L}$) for an additional 6 hours. Genistein increased

the ubiquitination of MDM2 (Fig. 4B1). This ubiquitinated MDM2 protein was also detected by immunoprecipitation in a separate study in which PC3 cells were transfected with ubiquitin and treated as above. The ubiquitinated MDM2 in cell lysates was isolated using an MDM2 antibody and detected by a ubiquitin antibody (Fig. 4B2). This increase is due to increased autoubiquitination; an MDM2 mutant (C464A) without ubiquitin E3 ligase activity was resistant to the inhibitory effects of genistein (Fig. 4B3) and was degraded more slowly than the wild-type protein in the presence of genistein (Fig. 4B4).

Inhibitory effects of genistein on tyrosine kinases are not required for the down-regulation of MDM2. Genistein is a potent and pan-tyrosine kinase inhibitor (2), and the MDM2 protein is subject to posttranslational modifications, including phosphorylation, which affects its localization, activities, and stability (28). To determine whether the tyrosine kinase inhibitor activity is involved in the down-regulation of MDM2 by genistein, PC3 cells were pretreated with genistein for 24 hours, then exposed to epidermal growth factor (EGF) for an additional hour. Genistein induced dephosphorylation of extracellular signal-regulated kinase, which was reversed by exposure to EGF (Fig. 5A1). However, down-regulation of MDM2 was not affected by EGF, suggesting that tyrosine kinase inhibition is likely not responsible for the inhibition of MDM2 by genistein (Fig. 5A1). This observation was confirmed using an inhibitor of protein tyrosine phosphatase. The inhibitory effects of genistein were not reversed by pretreatment of PC3 cells with Na_3VO_4 for 3 hours before exposure of the cells to genistein for 24 hours (Fig. 5A2). In a separate experiment, prior inhibition of

tyrosine kinases by lavendustin did not prevent the down-regulation of MDM2 by genistein (Fig. 5B1). HCT116 (p53^{-/-}) cells were pretreated with lavendustin (inhibitor of protein kinase) for 3 hours followed by incubation with genistein for 24 hours (Fig. 5B1). Another protein tyrosine kinase inhibitor, AG18, which was added to PC3 cells 3 hours before genistein treatment, was also unable to block the inhibitory effects of genistein (Fig. 5B2).

Genistein has antitumor effects on apoptosis, cell cycle distribution, and cell proliferation, regardless of cellular p53 status. In a dose-dependent manner, genistein induced apoptosis (Fig. 6A), inhibited cell proliferation (Fig. 6B), and caused G₂-M phase cell cycle arrest (Fig. 6C) in human cancer cells with wild-type p53 (HCT116 and MCF-7) and in cells without p53 expression [HCT116 (p53^{-/-}), MCF-7 (p53KD), and PC3 (p53^{null})].

In vitro antitumor activities of genistein are associated with its capacity to down-regulate MDM2. As illustrated in Fig. 7A, the elevation of p21 protein induced by genistein was inhibited by MDM2 overexpression in PC3 cells; the genistein-induced apoptotic effect was also eliminated by MDM2 overexpression (Fig. 7B). Additionally, MDM2-overexpressing PC3 cells were less responsive to the antiproliferative effects of genistein, as measured by BrdUrd incorporation (Fig. 7C). Moreover, overexpression of MDM2 facilitated the survival of PC3 cells, as shown by clonogenic assays (Fig. 7D1 and D2).

In vivo MDM2 inhibition by genistein shows dose-dependent antitumor activity and chemosensitization, independent of p53. The antitumor activity of genistein was further shown in the PC3 xenograft model, which is p53 null (Fig. 7E1). In this model, genistein also increased the therapeutic effectiveness of gemcitabine (Fig. 7E1). The protein levels of MDM2 were decreased by genistein in a dose-dependent manner, resulting in elevation of p21 (Fig. 7E2).

Discussion

Genistein, a naturally occurring isoflavone, is of interest because of its potent chemopreventive activities. Although there is a large body of evidence supporting the existence of multiple mechanisms for its biological activities (2, 29, 30), details about these

mechanisms and the molecular targets of genistein remain to be clarified. Despite the lack of information about how genistein acts at the molecular level, a phase I clinical study in cancer patients has been initiated (31).

The purpose of this present study was to determine whether genistein might affect the expression of the MDM2 oncoprotein, and, if so, whether this effect has biological consequences on the antitumor activities of genistein. We have generated at least five novel results. First, genistein down-regulates MDM2 expression at both the transcriptional and posttranslational levels, independent of p53, in both human cancer and primary cells. At the transcriptional level, the transcription factor NFAT binding site in the MDM2 promoter is important; at the posttranslational level, the genistein-induced MDM2 ubiquitination plays a role. Second, the down-regulation of MDM2 by genistein apparently does not require tyrosine kinase activity. Third, the inhibition of MDM2 by genistein is essential for its antitumor activities, including apoptosis and cell cycle arrest. Fourth, genistein up-regulates the tumor suppressor p21^{Waf1/CIP1} independent of p53, an effect that may be associated with the inhibition of MDM2. Fifth, genistein has antitumor effects and activities inhibiting MDM2 expression *in vivo*.

Human MDM2 expression is transcriptionally activated by p53, and there are evolutionally conserved p53 response elements in its promoter (32). A number of functional, p53-independent response elements in the MDM2 promoter are present, including a thyroid response element and a combination 5' ETS binding site and composite API-ETS site (33). Our observations that genistein decreases MDM2 mRNA levels in cells without functional p53 expression [i.e., PC3 cells (p53^{null}), HCT116 cells (p53^{-/-}), and MCF-7 cells (p53^{-/-})] supports the p53-independent transcriptional control of MDM2 and indicates that the effects of genistein are p53 independent. By luciferase assays, we identified the region of the MDM2 promoter that was responsive to genistein (Luc 03; -132 to +33), offering clues to the mechanisms of genistein-induced decreases in MDM2. For example, the estrogen receptor is recruited to this element to activate the MDM2 promoter (34), and genistein can function as an estrogen antagonist. However, the inhibition of both MDM2 mRNA and promoter activity in HCT116 and PC3 cells does not support antagonism of the estrogen

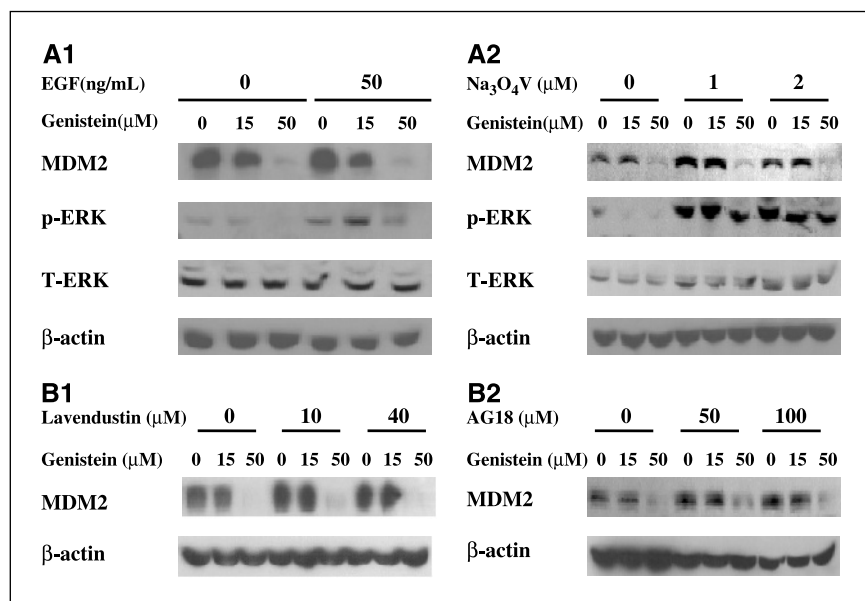


Figure 5. Down-regulation of MDM2 by genistein, independent of tyrosine kinase activity. *A1*, PC3 cells, preincubated with various concentrations of genistein for 24 hours, were further stimulated with EGF (50 ng/mL). MDM2, phosphorylated ERK, total ERK, and β -actin were detected by immunoblotting. *A2*, PC3 cells, treated with genistein for 24 hours, were further incubated with tyrosine phosphatase inhibitor Na₃VO₄ for 3 hours. Target proteins were detected by immunoblotting. *B1*, HCT116 (p53^{-/-}) cells, which were preincubated with various concentrations of lavendustin A for 3 hours, were treated with genistein for additional 24 hours. MDM2 protein was examined by immunoblotting. *B2*, after pretreatment with the tyrosine kinase inhibitor AG18 for 3 hours, PC3 cells were exposed to the various concentrations of genistein for 24 hours. MDM2 and β -actin were examined as above.

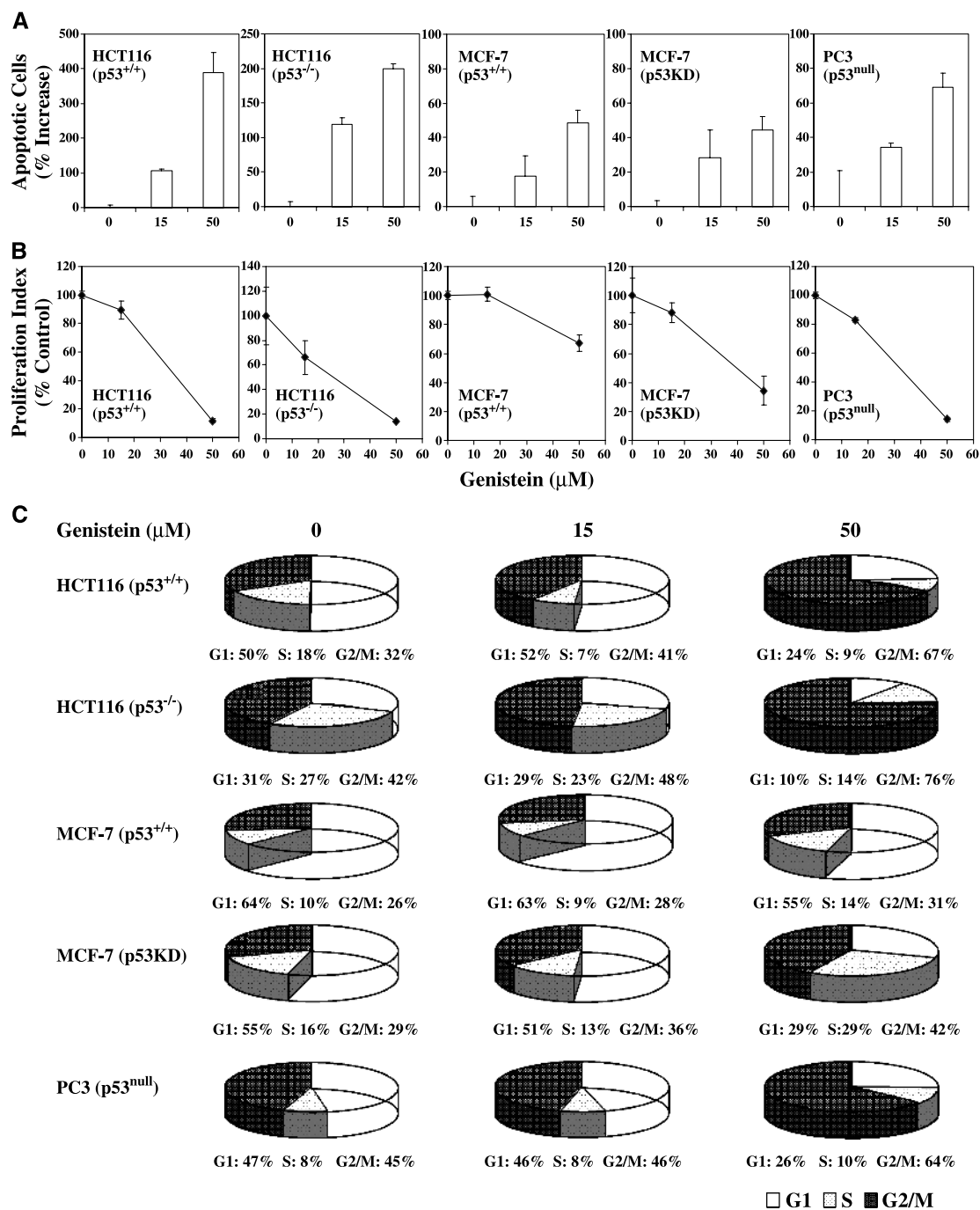


Figure 6. Activities of genistein on apoptosis, cell proliferation, and cell cycle distribution in tumor cell lines. HCT 116 cells with wild-type p53 (p53^{+/+}) or without p53 (p53^{-/-}), MCF-7 cells with wild-type p53 (p53^{+/+}) or without p53 (p53KD), and PC3 cells (p53^{null}) were treated with various concentrations of genistein for 24 hours followed by apoptosis assay (A), cell proliferation assay (B), or cell cycle distribution assay (C). Points, percentages of controls (increases in the number of apoptotic cells and proliferation indices); bars, \pm SD. The cell population in each cell cycle phase is the mean percentage of the total events counted. All assays were done in triplicate.

receptor as the mediator of this effect. In contrast, mutation of the NFAT binding site in the MDM2 promoter eliminates the inhibitory effects of genistein.

NFAT proteins are a family of transcription factors originally identified in T cells as the activators of cytokine gene expression (35). Recently, it has become apparent that NFAT is involved in the

regulation of growth and development of cells in a variety of tissues, not only of the immune system (35). The involvement of NFAT proteins in carcinogenesis is now recognized. As shown *in vitro*, the constitutive mutant NFATc1 transforms 3T3-L1 fibroblasts (36), and NFAT1 and NFAT5 promote tumor invasion (37). NFAT is also involved in the carcinogenic effects of nickel

compounds (38). The *NFATc2* gene is amplified in human pancreatic cancer cell lines and tissue specimens (39). Our data provide evidence that NFAT is involved in the p53-independent up-regulation of MDM2 transcription, which might be one of the mechanisms whereby NFAT proteins promote carcinogenesis. Considering the facts that MDM2 is still expressed or even overexpressed in human cancers lacking functional p53 and the prognosis for patients with cancers having both loss of p53 and MDM2 overexpression is worse than for patients whose cancers have either abnormality alone (40), knowledge about the regulation of MDM2 through other transcription factors, including NFATs, is important for elucidation of the carcinogenesis process.

The posttranslational regulation of the MDM2 protein is complicated. A RING finger domain located in the COOH terminus has ubiquitin E3 ligase function and facilitates the ubiquitination of p53 and MDM2 itself, which consequently leads to proteasome-mediated degradation (41). Because MDM2 facilitates its own ubiquitination, there must be mechanisms decreasing this activity to ensure that MDM2 has sufficient stability to access its targets. Several cellular proteins such as p14^{Arf}, p300, and MDMX interact with the MDM2 protein and affect its E3 ligase function and thus

protein stability (42). Posttranslational modifications, including phosphorylation by protein kinases and sumoylation, also control the balance between the autoubiquitination and substrate ubiquitination of MDM2 (28, 43). Based on our observations, genistein shifts the MDM2 E3 ligase activity to autoubiquitination. The RING finger domain of MDM2 may be involved in the switch from substrate to autoubiquitination, as mutation of this domain at least partially reverses the effects of genistein. In accordance with our data, an MDM2 construct with a heterologous RING finger can catalyze its own ubiquitination but not that of p53 (41). This observation has important clinical relevance because MDM2 is up-regulated after DNA damage to limit the extent of p53 activation and thus the effects of cytotoxic therapies. Moreover, the up-regulation of MDM2 results in a variety of other effects due to its p53-independent activities (24). Genistein promotes the self-degradation of MDM2, providing a rationale for the combination of genistein with DNA-damaging agents to treat human cancers.

Based on the capacity of genistein to inhibit tyrosine kinase activity and the fact that MDM2 is up-regulated by the ras/MEK/MAPK pathway, we hypothesized that the genistein-induced down-regulation of MDM2 was through a tyrosine kinase pathway.

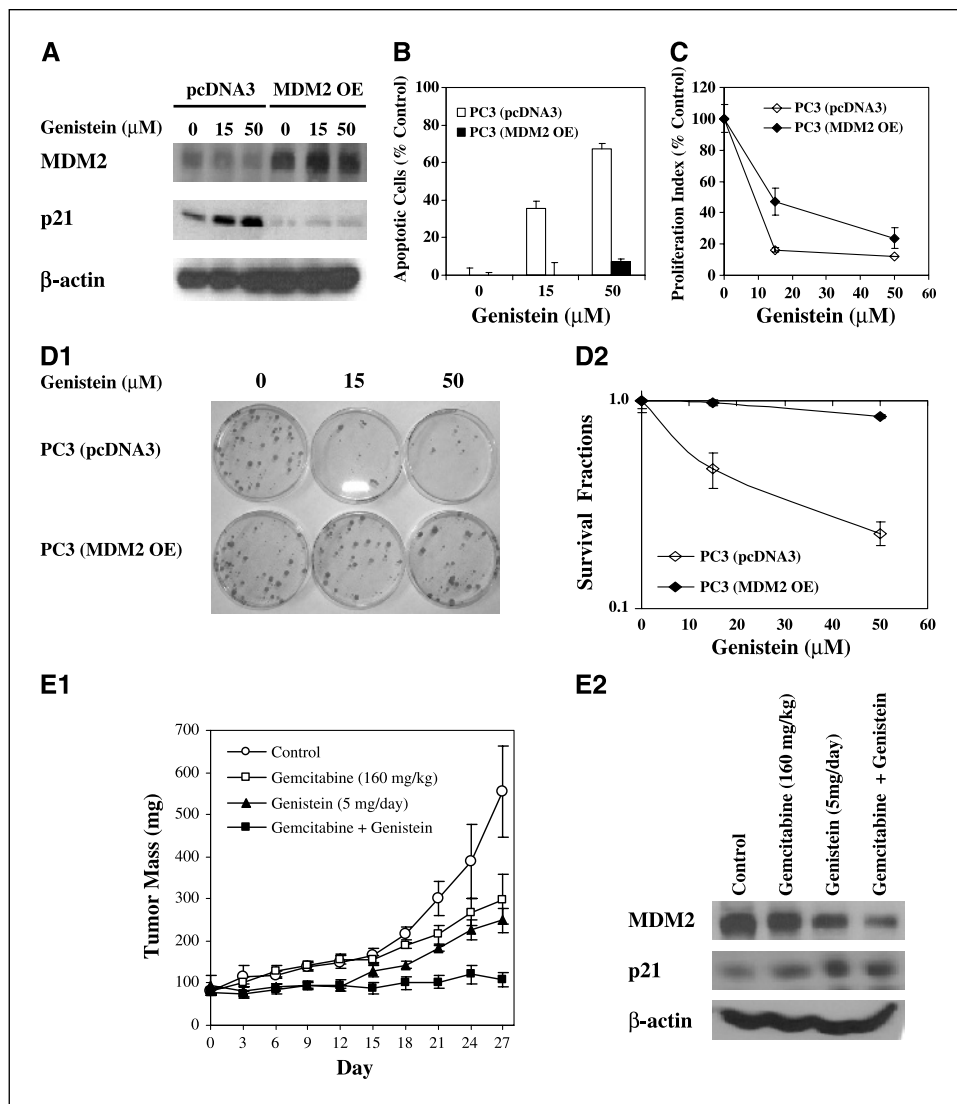


Figure 7. MDM2 expression level relative to the response of PC3 cells to genistein. PC3 cells with stable overexpression (OE) of MDM2 or corresponding empty vector were treated with various concentrations of genistein for 24 hours followed by Western blot analysis (A), apoptosis assay (B), cell proliferation (BrdUrd incorporation) assay (C), or clonogenic assay (D1 and D2). A, genistein-induced p21 elevation was reversed by MDM2 overexpression. B, genistein-induced apoptosis in PC3 cells was inhibited by MDM2 overexpression. C, in the BrdUrd assay, MDM2-overexpressing cells were less sensitive to the antiproliferative effects of genistein. D1 and D2, clonogenic assay was used to elucidate the possible differences in long-term effects of genistein between parental and MDM2-overexpressing cell lines. After exposure to genistein at various concentrations for 24 hours, cells were cultured for an additional 14 days. At the end of the experiments, cell colonies were fixed, stained, and counted. Representative colonies (D1). Quantitative data are means of duplicate assays; bars, \pm SD. *In vivo* antitumor activity of genistein administered alone or in combination with gemcitabine (E1) in nude mice bearing PC3 xenografts and the protein expression profile of xenografts (E2). Genistein (5 mg/kg/d) treatments were initiated on day 0, 5 days/wk for 4 weeks. Gemcitabine (160 mg/kg) was given at days 11, 18, and 25 by i.p. At the end of the treatment, tumor xenografts were removed and proteins of tumor homogenates were analyzed by Western blotting.

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Nevertheless, we observed that EGF, an activator of tyrosine kinases, does not reverse the inhibition of MDM2 by genistein. Still, the possibility that genistein may down-regulate MDM2 through other tyrosine kinase pathways not associated with EGF was not ruled out. Pretreatment with another tyrosine kinase inhibitor, lavendustin, did not affect the down-regulation of MDM2, showing that the down-regulation is not likely to be due to tyrosine kinase inhibition. Further studies are needed, including examination of other isoflavones with structures similar to genistein, to determine if they may also have an effect on MDM2 expression.

The MDM2 oncogene has the capacity to transform cells in culture and is overexpressed in many human malignancies; indeed, high MDM2 levels are associated with poor prognosis and resistance to DNA-damaging agents (19, 44, 45). MDM2 has been suggested as a drug target for human cancer therapy, regardless of the p53 status of the cancer (19–24). In this study, we found that MDM2 is down-regulated by genistein *in vitro* and *in vivo*, which may have an important role in its chemopreventive and chemotherapeutic activities. We found that the concentration- and time-dependent down-regulation of MDM2 by genistein was accompanied by induction of apoptosis, inhibition of cell proliferation, and cell cycle arrest at G₂-M in human breast, prostate, and colon cancer cell lines, regardless of their p53 status. Overexpression of MDM2 in PC3 cells at least partially impaired the response of tumor cells to the genistein treatment. Although the capacity of genistein to induce apoptosis and G₂ arrest has been well documented, the possible mediators of these effects remain unclear (46, 47). More importantly, the *in vitro* genistein activities were recapitulated in a PC3 xenograft model, and genistein also has *in vivo* chemosensitization effects on gemcitabine treatment. These observations provide strong support for the use of genistein as an antitumor agent and chemosensitizer for human cancer treatment. Our *in vivo* data also show that MDM2 is a novel and effective target for cancer therapy, as observed in our other studies using MDM2 antisense inhibitors (20–24).

Our observations suggest that the antitumor activity of genistein is, at least in part, associated with its MDM2 inhibitory effects. Moreover, we also observed that genistein prolonged the half-life of the p21^{Waf1/CIP1} protein, independent of p53. This was accompanied by MDM2 inhibition, suggesting that the increase

in the p21 half-life may be a downstream effect of the p53-independent decrease in MDM2 induced by genistein. To our knowledge, this is the first report showing that p21^{Waf1/CIP1} protein stability is affected by genistein, although it has been previously reported that genistein increases p21^{Waf1/CIP1} mRNA (47). In our previous studies (21–24), we found that the p21^{Waf1/CIP1} protein was a direct target of MDM2 for proteasomal degradation, independent of p53. We report in the current study that overexpression of MDM2 overcomes the effects of genistein on p21^{Waf1/CIP1} activation. Because p21^{Waf1/CIP1} is involved in the genistein-induced apoptosis and G₂ arrest in tumor cells (48), and p21^{Waf1/CIP1} protein is stabilized by genistein, it might be a mediator of the antitumor effects of genistein. In addition, the p53 independence of these effects is clinically relevant because >50% of human cancers, especially advanced stage cancers, have p53 dysfunction (19).

In conclusion, our observations have significance for cancer biology, prevention, and treatment. The present study provides insight into MDM2 regulation in the absence of p53 and provides evidence supporting a previously unrecognized mechanism of action for genistein: direct, p53-independent, down-regulation of the MDM2 oncogene. Considering the numerous activities of MDM2 in cancer growth and progression, as well as in cancer therapy, we speculate that genistein can be used as a chemopreventive and therapeutic agent, alone or in combination with other conventional agents. Continuing research on the p53-independent regulation of MDM2 may also be instrumental in elucidating of the process of cancer initiation and progression.

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References

- Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 1981;66:1191–308.
- Fournier DB, Erdman JW Jr, Gordon GB. Soy, its components, and cancer prevention: a review of the *in vitro*, animal, and human data. *Cancer Epidemiol Biomarkers Prev* 1998;7:1055–65.
- Wu AH, Ziegler RG, Horn-Ross PL, et al. Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiol Biomarkers Prev* 1996;5:901–6.
- Shu XO, Jin F, Dai Q, et al. Soy food intake during adolescence and subsequent risk of breast cancer among Chinese women. *Cancer Epidemiol Biomarkers Prev* 2001;10:483–8.
- Coward L, Barnes NC, Setchell KDR, et al. The antitumor isoflavones, genistein and daidzein, in soybean foods of American and Asian diets. *J Agric Food Chem* 1993;41:1961–7.
- Barnes S, Wang CC, Kirk M, et al. HPLC-mass spectrometry of isoflavonoids in soy and the American groundnut, *Apis americana*. In: Buslig BS, Manthey JA, editors. *In Flavonoids in cell function*. New York (NY): Kluwer Academic/Plenum Publishers; 2002. p. 77–88.
- Messina M, Persky V, Setchell KD, Barnes S. Soy intake and cancer risk: a review of *in vitro* and *in vivo* data. *Nutr Cancer* 1994;21:113–31.
- Zhang M, Xie X, Lee AH, Binns CW. Soy and isoflavone intake are associated with reduced risk of ovarian cancer in southeast China. *Nutr Cancer* 2004;49:125–30.
- Krishnan HB. Identification of genistein, an anticarcinogenic compound, in the edible tubers of the American groundnut (*Apis americana medikus*). *Crop Sci* 1998;38:1052–6.
- Barnes S. Effect of genistein on *in vitro* and *in vivo* models of cancer. *J Nutr* 1995;125:777–83S.
- Cai QY, Rahn RO, Zhang RW. Dietary flavonoids, quercetin, luteolin and genistein, reduce oxidative DNA damage and lipid peroxidation and quench free radicals. *Cancer Lett* 1997;119:99–107.
- Barnes S, Grubbs C, Setchell KD, Carlson J. Soybeans inhibit mammary tumors in models of breast cancer. *Prog Clin Biol Res* 1990;347:239–53.
- Hawrylewicz EJ, Huang HH, Blair WH. Dietary soybean isolate and methionine supplementation affect mammary tumor progression in rats. *J Nutr* 1991; 121:1693–8.
- Wang J, Eltoum IE, Lamartiniere CA. Dietary genistein suppresses chemically induced prostate cancer in Lobund-Wistar rats. *Cancer Lett* 2002;186:11–8.
- Lamartiniere CA, Cotroneo MS, Fritz WA, Wang J, Mentor-Marcel R, Elgavish A. Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate. *J Nutr* 2002;132:552–8S.
- Steele VE, Pereira MA, Sigman CC, Kelloff GJ. Cancer chemoprevention agent development strategies for genistein. *J Nutr* 1995;125:713–6S.
- Kallay E, Adlercreutz H, Farhan H, et al. Phytoestrogens regulate vitamin D metabolism in the mouse colon: relevance for colon tumor prevention and therapy. *J Nutr* 2002;132:3490–3S.
- Tamura S, Bito T, Ichihashi M, Ueda M. Genistein enhances the cisplatin-induced inhibition of cell growth and apoptosis in human malignant melanoma cells. *Pigment Cell Res* 2003;16:470–6.
- Zhang R, Wang H. MDM2 oncogene as a novel target for human cancer therapy. *Curr Pharm Des* 2000;6:393–416.

20. Chen L, Agrawal S, Zhou W, Zhang R, Chen J. Synergistic activation of p53 by inhibition of MDM2 expression and DNA damage. *Proc Natl Acad Sci U S A* 1998;95:195-200.
21. Wang H, Nan L, Yu D, Agrawal S, Zhang R. Antisense anti-MDM2 oligonucleotides as a novel therapeutic approach to human breast cancer: *in vitro* and *in vivo* activities and mechanisms. *Clin Cancer Res* 2001;7:3613-24.
22. Wang H, Nan L, Yu D, Lindsey JR, Agrawal S, Zhang R. Anti-tumor efficacy of a novel antisense anti-MDM2 mixed-backbone oligonucleotide in human colon cancer models: p53 dependent and p53-independent mechanisms. *Mol Med* 2002;8:185-99.
23. Wang H, Yu D, Agrawal S, Zhang R. Experimental therapy of human prostate cancer by inhibiting MDM2 expression with novel mixed-backbone antisense oligonucleotides: *in vitro* and *in vivo* activities and mechanisms. *Prostate* 2003;54:194-205.
24. Zhang Z, Li M, Wang H, Agrawal S, Zhang R. Antisense therapy targeting MDM2 oncogene in prostate cancer: effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proc Natl Acad Sci U S A* 2003;100:11636-41.
25. Ohtsuka T, Ryu H, Minamishima YA, et al. ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. *Nat Cell Biol* 2004;6:121-8.
26. Fearhead HO, Rodriguez J, Govek EE, et al. Oncogene-dependent apoptosis is mediated by caspase-9. *Proc Natl Acad Sci U S A* 1998;95:13664-9.
27. Ries S, Biederer C, Woods D, et al. Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. *Cell* 2000;103:321-30.
28. Meek DW, Knippschild U. Posttranslational modification of MDM2. *Mol Cancer Res* 2003;1:1017-26.
29. Li Y, Che M, Bhagat S, et al. Regulation of gene expression and inhibition of experimental prostate cancer bone metastasis by dietary genistein. *Neoplasia* 2004;6:354-63.
30. Wang J, Eltoum IE, Lamartiniere CA. Genistein alters growth factor signaling in transgenic prostate model (TRAMP). *Mol Cell Endocrinol* 2004;219:171-80.
31. Takimoto CH, Glover K, Huang X, et al. Phase I pharmacokinetic and pharmacodynamic analysis of unconjugated soy isoflavones administered to individuals with cancer. *Cancer Epidemiol Biomarkers Prev* 2003;12:1213-21.
32. Wu X, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* 1993;7:1126-32.
33. Qi JS, Yuan Y, Desai-Yajnik V, Samuels HH. Regulation of the mdm2 oncogene by thyroid hormone receptor. *Mol Cell Biol* 1999;19:864-72.
34. Kinyamu HK, Archer TK. Estrogen receptor-dependent proteasomal degradation of the glucocorticoid receptor is coupled to an increase in mdm2 protein expression. *Mol Cell Biol* 2003;23:5867-81.
35. Horsley V, Pavlath GK. NFAT: ubiquitous regulator of cell differentiation and adaptation. *J Cell Biol* 2002;156:771-4.
36. Neal JW, Clipstone NA. A constitutively active NFATc1 mutant induces a transformed phenotype in 3T3-L1 fibroblasts. *J Biol Chem* 2003;278:17246-54.
37. Jauliac S, Lopez-Rodriguez C, Shaw LM, Brown LF, Rao A, Toker A. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. *Nat Cell Biol* 2002;4:540-4.
38. Huang C, Li J, Costa M, et al. Hydrogen peroxide mediates activation of nuclear factor of activated T cells (NFAT) by nickel subsulfide. *Cancer Res* 2001;15:8051-7.
39. Holzmann K, Kohlhammer H, Schwaenen C, et al. Genomic DNA-chip hybridization reveals a higher incidence of genomic amplifications in pancreatic cancer than conventional comparative genomic hybridization and leads to the identification of novel candidate genes. *Cancer Res* 2004;64:4428-33.
40. Ganguli G, Wasylyk B. p53-independent functions of MDM2. *Mol Cancer Res* 2003;1:1027-35.
41. Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* 2000;275:8945-51.
42. Zeng SX, Jin Y, Kuninger DT, Rotwein P, Lu H. The acetylase activity of p300 is dispensable for MDM2 stabilization. *J Biol Chem* 2003;278:7453-8.
43. Hui L, Abbas T, Pielak RM, Joseph T, Bargonetti J, Foster DA. Phospholipase D elevates the level of MDM2 and suppresses DNA damage-induced increases in p53. *Mol Cell Biol* 2004;24:5677-86.
44. Kang CM, Cho HN, Ahn JM, et al. Alteration of gene expression during radiation-induced resistance and tumorigenesis in NIH3T3 cells revealed by cDNA microarrays: involvement of MDM2 and CDC25B. *Carcinogenesis* 2004;25:123-32.
45. Perry ME. Mdm2 in the response to radiation. *Mol Cancer Res* 2004;2:9-19.
46. Xu J, Loo G. Different effects of genistein on molecular markers related to apoptosis in two phenotypically dissimilar breast cancer cell lines. *J Cell Biochem* 2001;82:78-88.
47. Choi YH, Lee WH, Park KY, Zhang L. p53-independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G₂/M arrest by the isoflavone genistein in human prostate carcinoma cells. *Jpn J Cancer Res* 2000;91:164-73.
48. Li Y, Upadhyay S, Bhuiyan M, Sarkar FH. Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. *Oncogene* 1999;18:3166-72.