

The Receptor Interacting Protein 1 Inhibits p53 Induction through NF- κ B Activation and Confers a Worse Prognosis in Glioblastoma

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Abstract

Nuclear factor- κ B (NF- κ B) activation may play an important role in the pathogenesis of cancer and also in resistance to treatment. Inactivation of the p53 tumor suppressor is a key component of the multistep evolution of most cancers. Links between the NF- κ B and p53 pathways are under intense investigation. In this study, we show that the receptor interacting protein 1 (RIP1), a central component of the NF- κ B signaling network, negatively regulates p53 tumor suppressor signaling. Loss of RIP1 from cells results in augmented induction of p53 in response to DNA damage, whereas increased RIP1 level leads to a complete shutdown of DNA damage-induced p53 induction by enhancing levels of cellular mdm2. The key signal generated by RIP1 to up-regulate mdm2 and inhibit p53 is activation of NF- κ B. The clinical implication of this finding is shown in glioblastoma, the most common primary malignant brain tumor in adults. We show that RIP1 is commonly overexpressed in glioblastoma, but not in grades II and III glioma, and increased expression of RIP1 confers a worse prognosis in glioblastoma. Importantly, RIP1 levels correlate strongly with mdm2 levels in glioblastoma. Our results show a key interaction between the NF- κ B and p53 pathways that may have implications for the targeted treatment of glioblastoma. [Cancer Res 2009;69(7):2809–16]

Introduction

A link between chronic inflammation and cancer has been suspected for over a century (1). A major link between inflammation and cancer is mediated by activation of nuclear factor- κ B (NF- κ B; refs. 2–4). NF- κ B activation is a strong prosurvival signal (5). Constitutive and deregulated activation of NF- κ B is widespread in human cancer (3, 6) and promotes survival of tumor cells and resistance to treatment (7, 8). Furthermore, experimental models support a causal role for NF- κ B activation in inflammation-induced cancer (9, 10). Cross-talk between stress-induced/inflammatory responses and oncogenic signaling pathways is likely to play an important role in cancer. A number of

studies have linked components of the NF- κ B signaling pathway to cell cycle progression and tumorigenesis (11–16).

An intriguing mechanism underlying the pathogenesis of inflammation-induced cancer is the negative regulation of tumor suppressor pathways by inflammatory and stress-induced signals. p53 is a key tumor suppressor altered in a broad range of human cancers, including glioma, and an important outcome of p53 activation is cell cycle arrest or apoptosis after DNA damage (17, 18). Previous studies have documented links between the NF- κ B and p53 networks that have largely been reported to be antagonistic (19) but may also be synergistic (20). There is evidence that components of the NF- κ B signaling network interact with p53 at multiple levels. For example, IKK2 (IKK β) inhibits p53 induction in response to chemotherapeutic drugs via an up-regulation of mdm2 (21), whereas IKK1 (IKK α) interferes with p53-mediated gene transcription by inducing CBP phosphorylation (22). Thus, NF- κ B pathway-mediated inhibition of p53 function may promote the pathogenesis of cancer.

The death domain-containing kinase receptor interacting protein 1 (RIP1, RIPK1) is an essential component of the signaling cascade that activates NF- κ B in response to cellular stress and inflammation (23, 24). Thus, RIP1 is required for tumor necrosis factor α (TNF α)-induced and DNA damage-induced NF- κ B activation (25–28). In addition, RIP1 is also a key component of innate immunity, essential for TLR3-mediated activation of NF- κ B (29). RIP1 is composed of kinase, intermediate, and death domains. RIP1 is involved in the activation of the IKKs via a kinase-independent mechanism (30). Thus, RIP1 seems to function as an adaptor, and it is the intermediate domain of RIP1 that is essential for NF- κ B activation.

Glioblastoma (glioblastoma multiforme) is the most common primary malignant brain tumor in adults and is resistant to treatment (31). The median survival of glioblastoma patients with radiation and chemotherapy was recently noted to be 14.6 months (32). The molecular pathogenesis of glioblastoma includes genetic alterations in pathways mediating proliferation, apoptosis, and cell cycle control (33, 34). Inflammatory responses can be readily detected in glioma in the form of infiltrating macrophages/microglia and lymphocytes, production of inflammatory cytokines, and activation of NF- κ B (35, 36). Importantly, NF- κ B activation may be linked to the resistance of glioblastoma cells to O⁶-alkylating agents (37, 38).

In this study, we show that RIP1 negatively regulates p53 induction in response to DNA damage. RIP1 regulates p53 via the up-regulation of mdm2 levels. We also elucidate a key role for NF- κ B activation in RIP1-mediated regulation of mdm2 and p53

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

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down-regulation. Analysis of glioma patient samples showed that RIP1 is overexpressed in ~30% of glioblastoma (grade IV), but not in grades II and III glioma. Importantly, there was a striking correlation between RIP1 and *mdm2* levels in glioblastoma, consistent with our mechanistic data *in vitro*. Finally, high RIP1 level is an independent negative prognostic indicator in glioblastoma.

Materials and Methods

Plasmids and cell lines. RIP1 plasmid was obtained from Dr. Brian Seed and cloned into pcDNA3.1 with a COOH terminal FLAG tag using standard molecular techniques. *rip1*^{+/+} and *rip1*^{-/-} murine embryo fibroblasts were provided by Dr. Michelle Kelliher (27). In this study, we used primary mouse embryo fibroblasts (MEF) at passages 3 to 4. A p21-LUC promoter was provided by Dr. Bert Vogelstein.

Antibodies, reagents, and Western blotting. p53 (DO1), I κ B α , p65, and extracellular signal-regulated kinase 2 (ERK2) antibodies were obtained from Santa Cruz Biotechnology. The RIP antibody was purchased from BD Biosciences and was used for both immunohistochemistry and Western blotting. *mdm2* (2A10) antibodies were purchased from Calbiochem. p53 (1C12), p21, and FLAG were purchased from Cell Signaling Technology. We used p53 DO1 antibodies for the detection of p53 in (human) glioma cell lines, whereas p53 1C12 antibodies were used for the detection of (mouse) p53 in MEFs. For tumor samples, 30 μ g of protein were loaded on to 7.5% polyacrylamide gels, and Western blotting was performed according to standard protocols.

Luciferase assays. U87MG cells (70K) were plated in 24-well dishes followed by transfection with either p21-LUC along with RIP plasmid or empty vector, using calcium phosphate. A dual-luciferase reporter assay system was used according to the instructions of the manufacturer (Promega). Firefly luciferase activity was measured in a luminometer and normalized on the basis of *Renilla* luciferase activity. Experiments were done in triplicate and repeated twice.

Production of adenovirus expressing RIP. Preparation of adenovirus expressing RIP1 has been described previously (39). An multiplicity of infection (MOI) of 10 to 100 was used in the experiments. Adenovirus-null or adenovirus I κ B α mutant (I κ B α M) were obtained from Vector Biolabs and used at an MOI of 50.

Primary tumors/immunohistochemistry. All studies were conducted according to Institutional Review Board–approved protocols at University of Texas Southwestern Medical Center. Frozen tissue specimens of human gliomas were stored after resection. Mirror tissue was processed for paraffin sections and examined to ensure that the frozen sample is tumor. Frozen samples available were analyzed by Western blotting. Paraffin-embedded sections were cut at 4- μ m thickness and deparaffinized. The sections were incubated in 3% H₂O₂ for 10 min to block endogenous peroxidase activity. For antigen retrieval, the sections were placed in a Pascal programmable pressure cooker (DakoCytomation) containing EDTA solution (Biocare Medical), with the target temperature and time set at 125°C for 30 s. The sections were exposed to the mouse monoclonal RIP antibody (BD Biosciences) at a dilution of 1:50 for 30 min. The detection system was Envision Dual Link (DakoCytomation). The sections were counterstained lightly with hematoxylin. The immunohistochemical staining intensity for RIP1 was scored semiquantitatively as 0, 1 (+), 2 (++), and 3 (+++). The final immunohistochemistry score was derived from immunohistochemical staining intensity multiplied by the percentage of tumor cells showing positive staining.

RIP small interfering RNA knockdown. RIP1 small interfering RNA (siRNA) were obtained from Dharmacon. Three sequences directed against RIP1 mRNA were used (D-004445-03 RIPK1, D-004445-04 RIPK1, and D-004445-06 RIPK1). A negative control siRNA (nonsequence, Silencer Negative Control 1 siRNA, AM4611) was obtained from Ambion. siRNA was performed according to the manufacturer's protocol using Lipofectamine 2000 reagent (Invitrogen). Lysates were prepared 72 h after siRNA for Western blot.

Statistical analysis. Error bars represent the means \pm SDs of three independent experiments. The RIP1 level was dichotomized into high and

low groups based on a RIP1 value of 1.2, which represents a 50% increase over the levels found in normal brain. To investigate whether RIP1 level (ratio between RIP1 and ERK2) is associated with the clinical outcome, we applied a proportional hazard model using both overall survival time and progression-free survival time as the outcomes (censored by the last known number of follow-up months). The multivariate proportional hazard model was used to analyze the effect of additional prognosis factors, such as age and Karnofsky scores, with the RIP1 level. The survival curves were fitted using the Kaplan-Meier method. The log-rank test was used to compare different survival curves. Fisher's exact test was used to calculate the *P* value for association between RIP1 and *mdm2* in glioblastoma. The survival analysis was performed using the survival package in SAS software.

Results

RIP1 influences p53 induction in response to DNA damage.

Previous studies have documented interactions between components of the NF- κ B signaling network and p53. Thus, we investigated whether the cellular level of RIP1 could influence p53 induction in response to DNA damage. Exposure of RIP1^{-/-} primary MEFs to ionizing radiation (IR) resulted in a significantly greater induction of p53 compared with wild-type cells (Fig. 1A). Similarly, exposure of RIP1^{-/-} MEFs to UV radiation elicited a greater induction of p53 (Supplementary Fig. S1A). Next, we investigated the effect of increased RIP1 expression in the human glioblastoma cell line U87MG, which expresses wild-type p53. To overexpress RIP1, we used a tetracycline-inducible adenoviral expression vector (tet-off). An empty adenoviral vector (Ad-null) was used as an additional control (Supplementary Fig. S1C). Cells were exposed to IR (10 Gy) and then followed by Western blot. Whereas p53 and p21 are induced in response to IR in control cells, overexpression of RIP1 completely blocked IR-mediated induction of p53 and p21 in these cells (Fig. 1B). Indeed, increased expression of RIP1 also down-regulates the steady-state levels of p53 in these cells. Similarly, in A172 cells, a second glioblastoma cell line, with wild-type p53, increased RIP1 expression–inhibited basal and IR-mediated induction of p53 and p21 (Supplementary Fig. S1B). RIP1 also inhibited p53 induction in response to UV radiation (Supplementary Fig. S1D).

siRNA knockdown of RIP1 resulted in increased basal level of p53 and p21 levels (Fig. 1C). Furthermore, RIP1 silencing results in an increase in p53 induction in response to IR (Supplementary Fig. S1E). We used three different siRNA sequences directed against different parts of the RIP1 mRNA with similar results. Similarly, siRNA knockdown of RIP1 in A172 cells increased p53 and p21 levels (Fig. 2D).

Next, we investigated whether the kinase activity of RIP1 was required to regulate p53. We introduced wild-type RIP1 and mutant RIP1 with either the kinase (DKD) or intermediate domain (DID) deleted into U87MG cells. We find that the DKD mutant efficiently down-regulated p53 whereas the DID mutant did not (Fig. 1D). Loss of the intermediate domain increases the levels of the RIP1 protein dramatically and RIP1 DID fails to down-regulate p53, although it is expressed at higher levels. When a lower MOI is used for the DID to equalize expression with DKD, DID fails again to down-regulate p53 (Supplementary Fig. S2A).

Because RIP1 is commonly overexpressed in human glioblastoma (see below), it is important to note that levels of RIP1 overexpression in these experiments were similar to that detected in human glioblastoma (Fig. 4D).

We also tested the effect of RIP1 on the transcriptional activity of p53 in U87MG cells. In this experiment, we tested the ability of IR

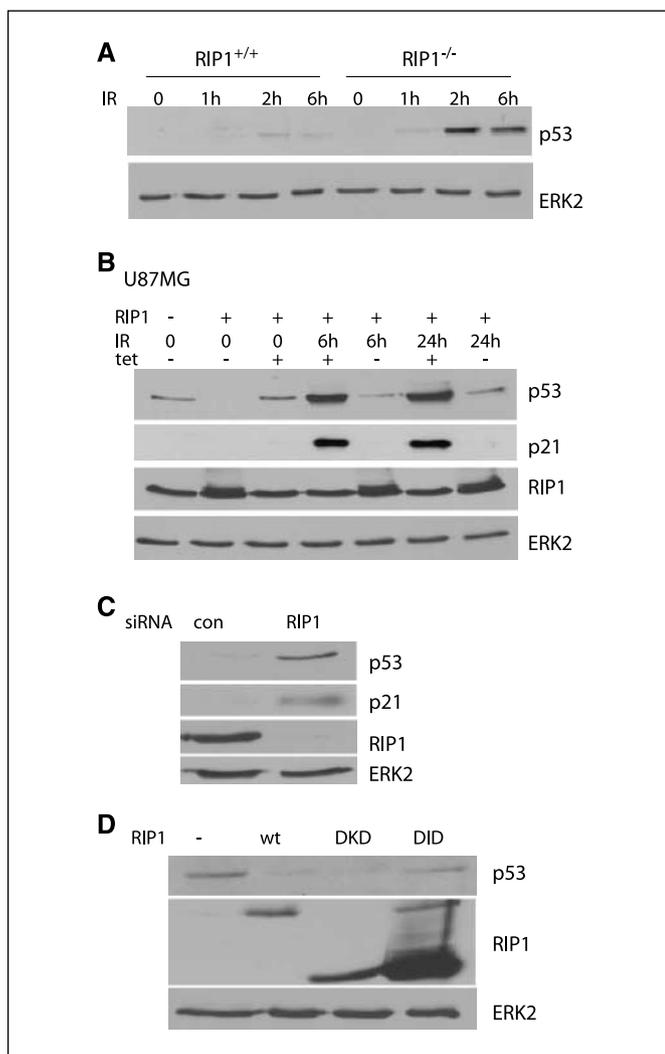


Figure 1. RIP1 regulates p53 levels. *A*, Western blot showing p53 induction at various times in RIP1^{-/-} primary MEFs compared with wild-type MEFs in response to IR (10 Gy). The Western blot was probed with p53 antibodies (1C12). ERK2 was used as a loading control. *B*, U87MG cells were infected with RIP1 adenovirus (MOI, 50) in a tet-off system followed by IR (10 Gy) and preparation of lysates as indicated. As a control, tetracycline (*tet*, 1 μg/mL) was added with RIP1 virus to prevent RIP1 expression. Western blots were probed with a p53 (DO1) and p21 antibodies. Increased RIP1 blocks p53/p21 induction in response to radiation. *C*, U87MG cells were transfected with control or RIP1 siRNA. At 72 h later, lysates were prepared followed by Western blot. Loss of RIP1 augments p53 and p21 levels. Similar results were obtained with two other RIP1 siRNA oligos. *D*, U87MG cells were infected with wild-type or mutant RIP1 for 24 h and then followed by Western blot. *DKD*, deletion of kinase domain; *DID*, deletion of intermediate domain. *Center*, expression of the RIP1 wild-type (*upper band*) and truncated mutants (*lower band*).

to activate a p53-responsive promoter (p21-LUC). We find that coexpression of RIP1 with p21-LUC completely blocked the basal and the IR-induced p53-induced transcriptional activity of the p21 promoter (Supplementary Fig. S1F).

RIP1 regulates the cellular levels of mdm2. mdm2 is a major regulator of p53 and plays a key role in promoting ubiquitination and degradation of p53. We investigated whether RIP1 regulated levels of cellular mdm2. Indeed, increased expression of RIP1 resulted in the up-regulation of mdm2 in U87MG cells (Fig. 2A). Conversely, silencing RIP1 in U87MG cells resulted in a decrease in levels of cellular mdm2 (Fig. 2B). Similarly, increased expression of

RIP1 in A172 cells resulted in increased mdm2 levels (Fig. 2C) while silencing RIP1 down-regulated mdm2 levels (Fig. 2D).

RIP1-mediated activation of NF-κB regulates p53 and mdm2 level. Previous studies have documented that increased expression of RIP1 is sufficient to activate NF-κB in various cell types (40, 41). First, we confirmed that NF-κB was activated by increased RIP1 expression in U87MG cells, as shown in other cells. We tested the

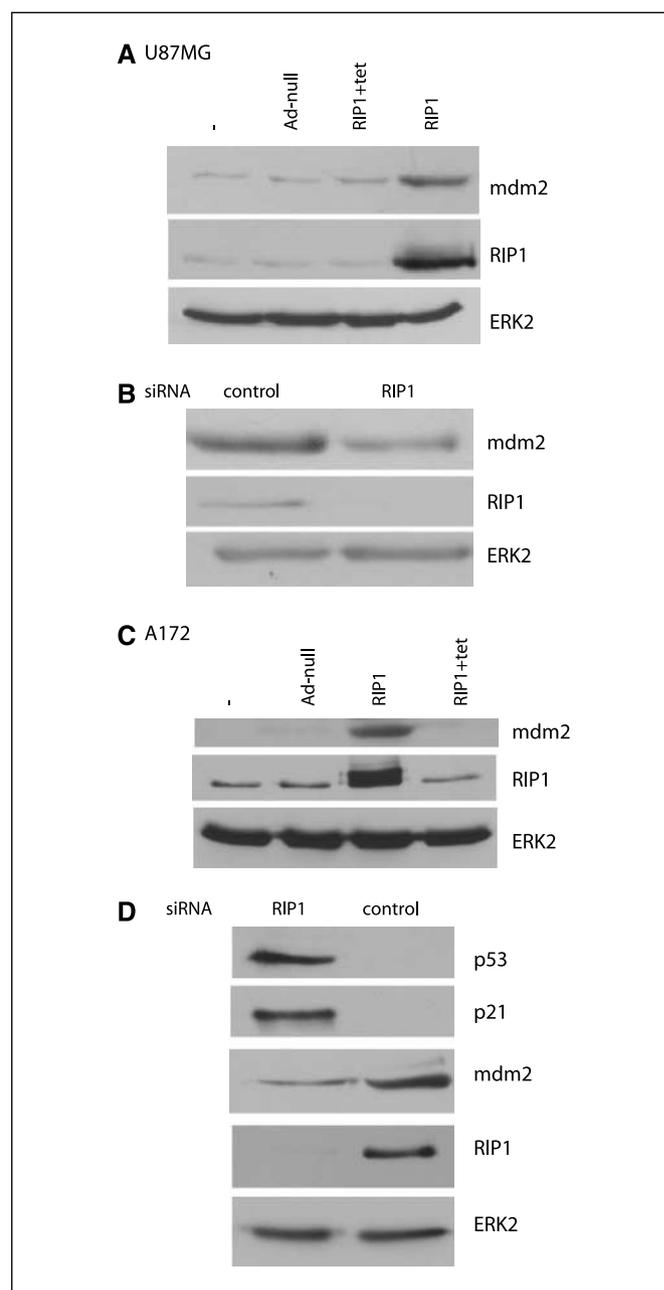
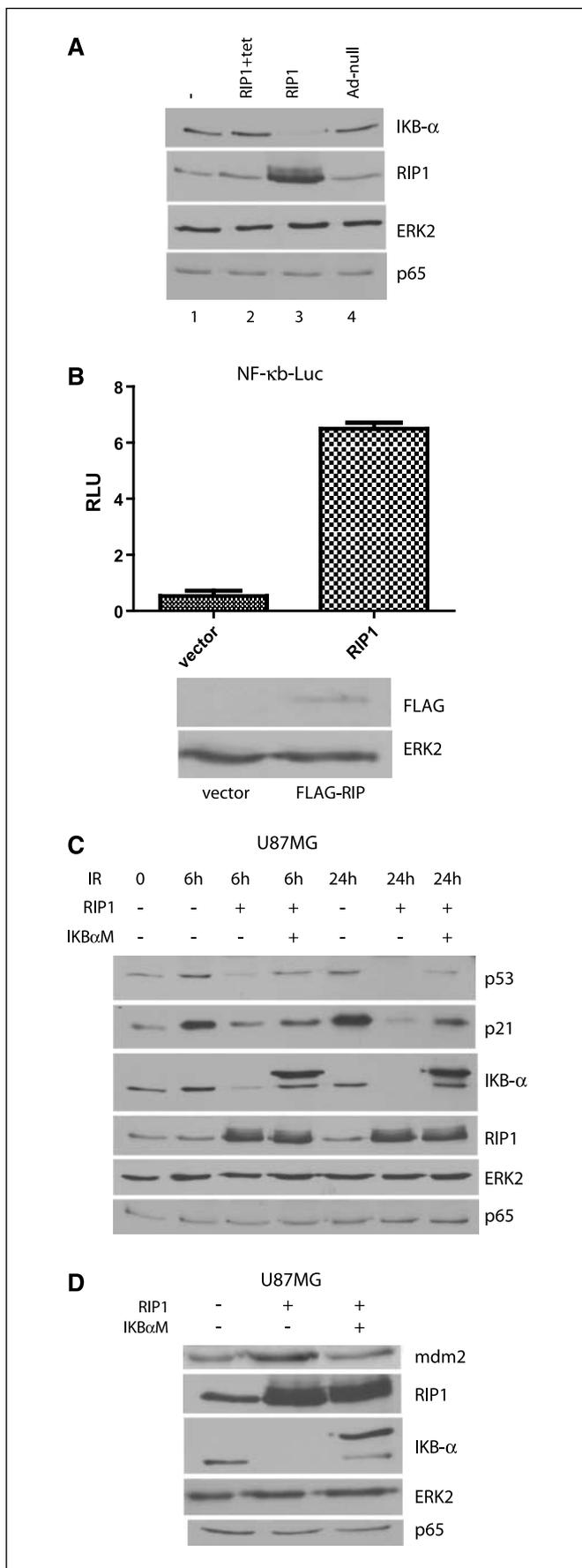


Figure 2. RIP1 regulates mdm2 levels. *A*, increased expression of RIP1 leads to up-regulation of mdm2 levels in U87MG cells. Ad-RIP1 was introduced into cells for 24 h followed by lysate preparation and Western blot. Adenovirus-null and RIP1 + tetracycline were used as negative controls. *B*, siRNA knockdown of RIP1 results in a decrease in mdm2 levels in U87MG cells compared with control (scrambled, nonsequence) siRNA, as shown in the Western blot. *C*, increased expression of RIP1 up-regulates mdm2 in A172 cells. The experiment was performed as described for *A*. *D*, siRNA knockdown of RIP1 results in a decrease in mdm2 levels in A172 cells compared with control (scrambled, nonsequence).



ability of increased RIP1 to down-regulate levels of IκBα. As expected, increased expression of RIP1 in U87MG cells resulted in a complete and persistent down-regulation of IκBα, as shown in Fig. 3A. Furthermore, increased expression of RIP1 was sufficient to increase the transcriptional activity of NF-κB in reporter assays in U87MG cells (Fig. 3B).

To test whether NF-κB activation played a role in RIP1-mediated regulation of p53, we investigated whether introduction of a dominant-negative IκBαM (S³²/S³⁶) that inhibits NF-κB activation would rescue RIP1-mediated suppression of p53 induction. Expression of IκBαM resulted in a substantial reversal of the RIP1-mediated block of p53 induction after IR (Fig. 3C). A similar result was found in A172 cells (Supplementary Fig. S2B). Next, we investigated whether RIP1-mediated up-regulation of mdm2 required NF-κB activation and expressed IκBαM together with RIP1 in U87MG cells. Inhibition of NF-κB activity also blocked the ability of RIP1 to up-regulate mdm2 levels in U87MG and A172 cells (Fig. 3D and Supplementary Fig. 2C). As expected, RIP1 did not influence levels of the p65 subunit of NF-κB.

RIP1 is overexpressed in glioblastoma but not in grades II and III glioma. Previous studies have shown that NF-κB is activated in glioblastoma. Because RIP1 plays a key role in NF-κB activation, we investigated RIP1 levels in glioma. The RIP1 antibody we used has been used in previous studies (30, 42–45). Gliomas are graded I to IV in the order of increasing malignancy, and glioblastoma (grade IV) is the most malignant. We examined 23 gliomas graded II and III, 70 glioblastomas (grade IV), and 1 nontumor brain sample. About 30% of glioblastomas show increased levels of RIP1, whereas RIP1 level among the gliomas graded II and III is low or absent (Fig. 4A and Supplementary Table S1 and S2). Lysates were made directly from resected brain tumors, RIP1 level was detected by Western blots and quantitated by densitometry, and values were normalized for loading using total ERK2 levels. ERK2 is expressed ubiquitously, and we find its level to be stable in glioma. RIP1 levels in glioblastomas were 1.01 ± 0.15 , and levels of RIP1 in nonglioblastomas (grades II–III gliomas) were 0.42 ± 0.07 . The log transformation was applied to the RIP1 level, and the Student's *t* test showed that the RIP1 level in the glioblastoma group was significantly higher than in the nonglioblastoma group ($P = 0.03$; Fig. 4A and C). We also conducted immunohistochemistry in randomly selected tumors with the same RIP1 monoclonal antibody to test the correlation between Western blot and immunohistochemistry. The immunohistochemistry score was significantly associated with the RIP1 expression by Western blotting (Supplementary Table S3). Figure 4B shows levels of RIP1 in normal brain, an

Figure 3. A, introduction of Ad-RIP1 into U87MG cells results in down-regulation of IκBα, which indicates NF-κB activation. Lysates were prepared 24 h after RIP1 infection and adenovirus-null and RIP1 + tet were used as negative controls. B, U87MG cells were transfected with an NF-κB-responsive promoter linked to luciferase (κB-LUC) and cotransfected with FLAG-RIP1 or empty vector. Whereas the activity of the reporter is low in the vector-transfected cells, introduction of RIP1 results in a strong activation of the transcriptional activity of NF-κB. Bottom, expression of FLAG-RIP1 was confirmed by Western blot with FLAG antibodies. C, a dominant-negative IκBα super-repressor mutant blocks the effect of RIP1 on p53 and p21 induction in response to IR. U87MG cells were infected with Ad-RIP1 + Ad-null or Ad-RIP1 + Ad-IκBαM for 24 h followed by IR for 6 or 24 h and Western blot for p53, p21, IκBα, p65, and RIP1 expression. D, a dominant-negative IκBαM blocks the effect of RIP1 on mdm2 up-regulation. U87MG cells were infected with Ad-RIP1 + Ad-null or Ad-RIP1 + Ad-IκBαM for 24 h followed by Western blot for mdm2. Expression of IκBαM blocks the RIP1-mediated increase in mdm2. Western blots were also probed for the p65 subunit of NF-κB.

anaplastic astrocytoma, and intense RIP1 staining in a glioblastoma. Thus, RIP1 levels correlated well with increased malignancy in glioma. RIP1 is expressed mostly in the cytoplasm of tumor cells, but we also detect expression in endothelial cells (Fig. 4B and Supplementary Fig. S3A and B).

In addition, we examined RIP1 levels in four matched pairs of primary low-grade glioma that progressed to secondary glioblastoma. In each case, we found that RIP1 was low in the primary tumor and significantly increased in the secondary glioblastoma (Supplementary Fig. S3C; Supplementary Table S4).

A strong correlation between RIP1 and mdm2 in glioblastoma. Next, we investigated whether there is a correlation between RIP1 and mdm2 levels in glioblastoma. We tested the level of mdm2 by Western blot in 31 glioblastomas, as shown in Fig. 5. mdm2 was detectable by Western blot in 11 of 31 glioblastoma cases. The detection rate of mdm2 was 73% in the high-RIP1 group and 0% in the low-RIP1 group ($P < 0.0001$). Thus, RIP1 levels

correlate strongly with mdm2 in glioblastoma, suggesting that increased RIP1 may up-regulate mdm2 levels in this disease. We further explored the association between p53 and mdm2 levels. The detection rate of mdm2 was 75% in the low-p53 group and 6% in the high-p53 group ($P = 0.0002$). Thus, p53 levels correlate inversely with mdm2 levels in our sample of glioblastomas.

Increased RIP1 expression confers a worse prognosis in glioblastoma. Next, we investigated whether RIP1 expression influenced prognosis in glioblastoma. The clinical outcomes measured were overall survival and progression-free survival ($n = 70$). Survival time was defined as the time from diagnosis to death (or disease progression), which was censored by the last known number of follow-up days. We dichotomized the RIP1 level based on a RIP1/ERK2 level of 1.2, which represents a 50% increase over the levels found in normal brain (Fig. 4B; densitometry data not shown). By this criterion, none of the grades II and III tumors overexpressed RIP1 (Fig. 4A; Supplementary Table S2).

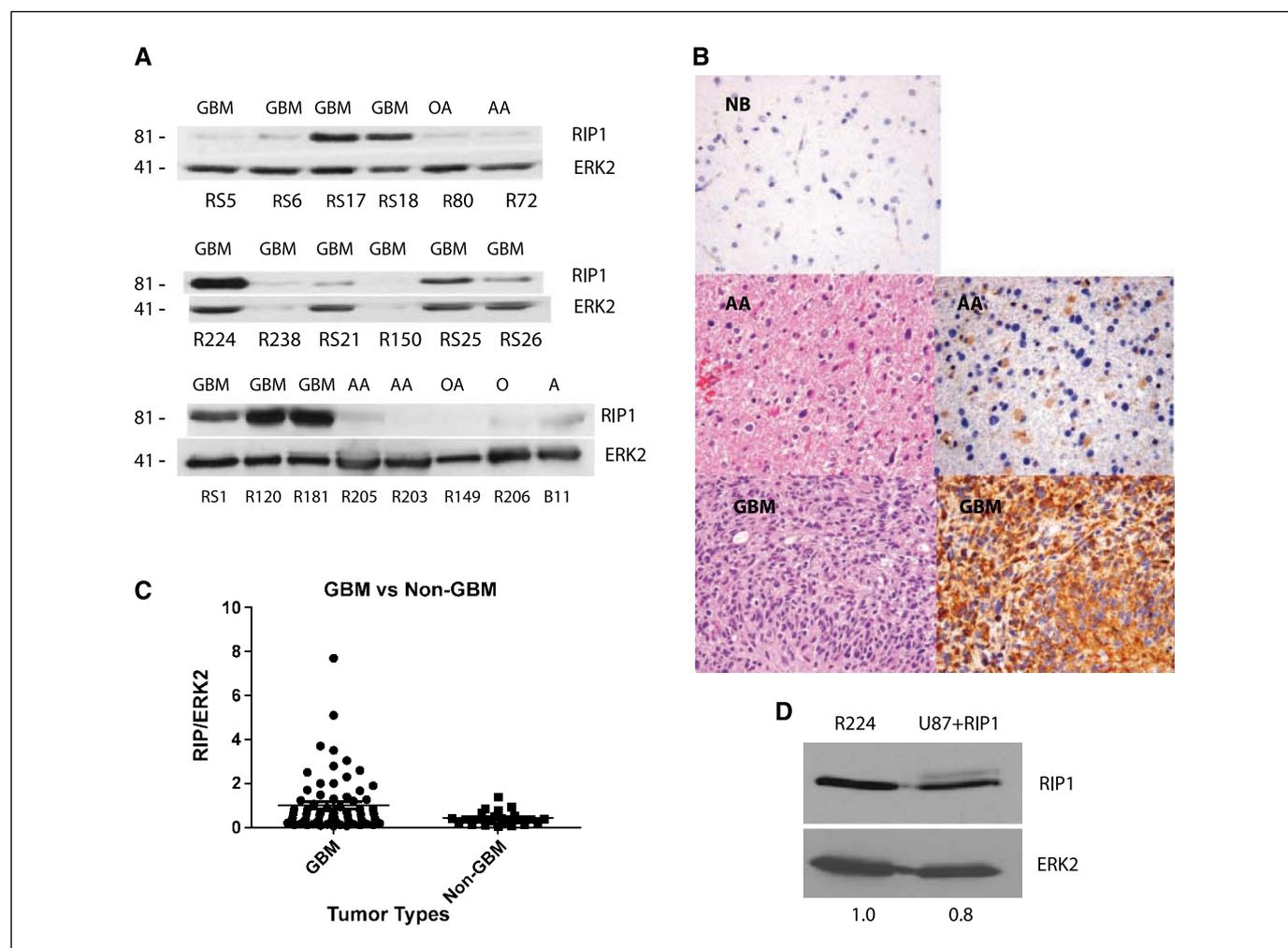


Figure 4. RIP1 expression in glioma. *A*, Western blots using lysates made from resected tumors. Glioblastomas (GBM; grade IV gliomas) include RS5, RS6, RS17, RS18, R224, R238, RS21, R150, RS25, RS26, RS1, R120, and R181. Nonglioblastomas (grades II–III gliomas) include R80 (OA), R72 (AA), R205 (AA), R203 (AA), R149 (OA), R206 (O), and B11 (A). *B*, immunohistochemistry of paraffin-embedded sections. NB, normal brain with absent or low RIP1; AA, anaplastic astrocytoma (R72) with H&E staining (left) and a low level of RIP1 staining (right) in the same section; GBM, glioblastoma (R224) with H&E staining (left) and intense RIP1 staining (right) in this tumor. *C*, levels of RIP1 in glioblastomas versus nonglioblastoma (grades II–III glioma) tumors. The comparison of RIP1 level between 70 glioblastoma samples and 23 nonglioblastoma samples shows that RIP1 level correlates with increased malignancy. *D*, level of RIP1 overexpression achieved by infecting U87MG cells with adenoviral RIP1 (MOI, 50) is similar to expression levels in high RIP1-expressing glioblastomas (R224 is shown here). Protein (10 μ g) was loaded followed by Western blot with RIP1 antibodies. AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; AA, anaplastic astrocytoma; OA, oligoastrocytoma; O, oligodendroglioma; A, astrocytoma.

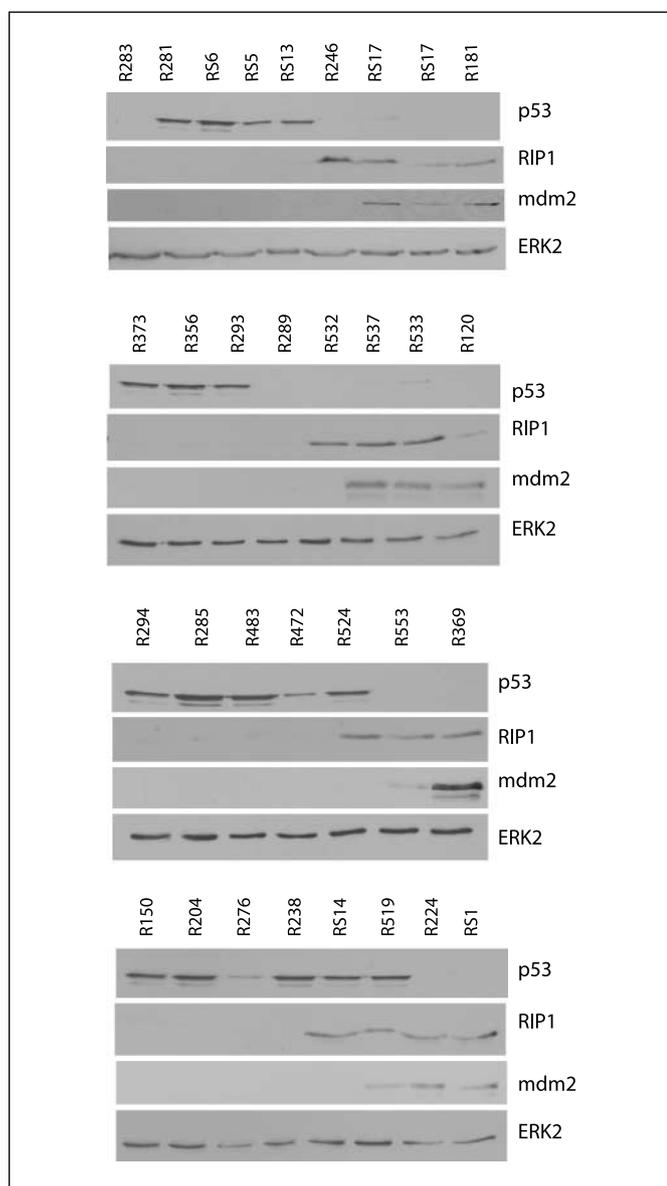


Figure 5. RIP1 levels correlate with mdm2 in glioblastoma. A panel of 31 glioblastomas was tested for mdm2 expression using mdm2 (2A10) antibodies. ERK2 was used to determine loading. The same lysates were also probed for RIP1 and p53. Mdm2 is detectable in ~30% of tumors and strongly correlates with RIP1 levels. An inverse correlation between mdm2 and p53 was also detected.

In the high-RIP1 group, there were 10 of 20 patients dead; in the low-RIP1 group, there were 18 of 50 patients dead within the follow-up months. Figure 6A shows the Kaplan-Meier overall survival curves for the high-RIP1 and low-RIP1 groups, which shows that patients with high-RIP1 levels have significantly shorter survival than patients with low RIP1 levels ($P = 0.014$, log-rank test); the median survival time was 12.4 months for the high-RIP1 group and 26.3 months for the low-RIP1 group. The univariate proportional hazard model showed the dichotomized RIP1 was significantly associated with overall survival time ($P = 0.018$, Cox survival model). The hazard ratio for the dichotomized RIP1 was 2.6, indicating that the risk of death for a patient with a high-RIP1 value would increase ~1.6 fold

compared with a patient with a low-RIP1 value. The dichotomized RIP1 was also significantly associated with progression-free survival time ($P = 0.023$, Cox survival model), the hazard ratio was 2.3, the progression-free median survival time was 7.7 months for the high-RIP1 group and 23.2 months for low-RIP1 group, and the survival curves were shown in Fig. 6B ($P = 0.02$, log-rank test). Progression was defined as the time to detection of recurrent disease on magnetic resonance imaging.

There was no significant association between diagnosis age and RIP1 level ($P = 0.44$, Wilcoxon rank test), and the median ages for high-RIP and low-RIP groups were 48 and 50 years old, respectively.

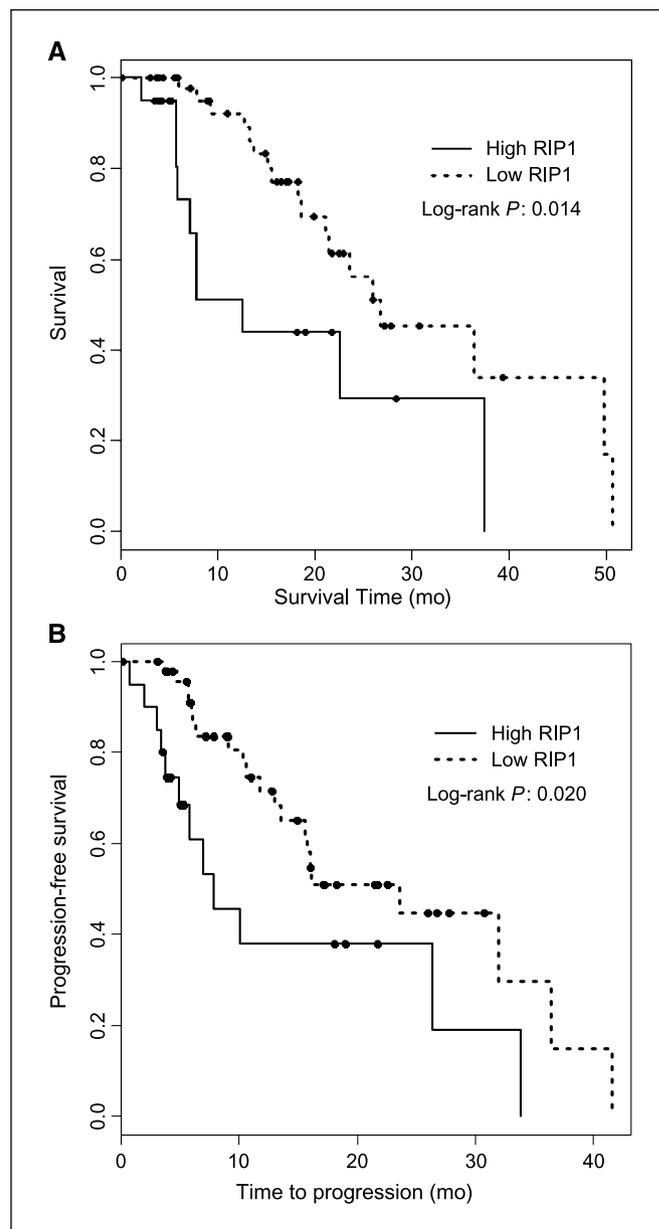


Figure 6. A, Kaplan-Meier estimates of overall survival time in glioblastoma patients, grouped by levels of RIP1. The survival time was significantly longer for patients in low-RIP1 group than those in high-RIP1 group. B, Kaplan-Meier estimates of progression-free survival time in glioblastoma patients grouped by RIP1 levels. The progression-free survival time was significantly shortened for patients who had increased RIP1 levels.

The multivariate proportional hazard survival model was fitted with RIP1, age, and Karnofsky performance score as covariates. After adjusting for the effects of age and Karnofsky scores, high RIP1 level was still significantly associated with short survival time (hazard ratio, 2.3; $P = 0.045$) and progression-free survival time (hazard ratio, 2.2; $P = 0.039$). Almost all patients underwent surgery, radiation, and chemotherapy with temozolomide (Supplementary Table S1).

Discussion

The major finding of this study is that RIP1, an essential component of inflammation and NF- κ B signaling, plays an important role in regulating p53. Our findings indicate that RIP1 activates NF- κ B, resulting in up-regulation of mdm2 and a complete shutdown of the p53 tumor suppressor signaling network. We show that RIP1 is overexpressed in human glioblastoma, the most common adult malignant brain tumor, but not in lower grade glioma, and confers a worse prognosis in this disease. Furthermore, our data suggest that RIP1 may regulate mdm2, lowering p53 function in glioblastoma.

Stressful stimuli, such as DNA damage, results in activation of both p53 and NF- κ B pathways. Cross-talk between the NF- κ B and p53 signaling pathways is well documented and may play important roles in the pathogenesis of stress/inflammation-induced cancer and resistance to treatment. However, specific mechanisms by which NF- κ B and p53 cross-talk are still under intense study. Altered regulation of both the NF- κ B and p53 pathways is established in glioblastoma. p53 function is frequently altered in glioma either by direct mutation or changes in regulatory signals due to mdm2 gene amplification or loss of p14^{ARF}. Our data suggest that increased expression of RIP1 may be an important additional mechanism of regulating p53 in glioblastoma. It is important to note that mdm2 is known to down-regulate both wild-type and mutant p53, which may result in complex biological outcomes (46).

Previous studies have shown that IKK2 or Bcl-3, a protein related to the I κ B family of NF- κ B inhibitors, regulate p53 via augmentation of mdm2 levels (21, 47). In the case of IKK2, up-regulation of mdm2 is mediated by activation of NF- κ B. Our data show that inhibition of NF- κ B activation using a dominant-negative I κ B α M results in a block of RIP1-mediated mdm2 up-regulation and rescues RIP1-mediated p53 inhibition. Also, a RIP1 mutant lacking the intermediate domain known to be deficient in NF- κ B activation failed to inhibit p53 induction. Thus, the negative regulation of p53 by NF- κ B occurs at multiple nodes and may be important in the pathogenesis of cancer. An increase in IKK2 levels has not been reported in glioblastoma, and our data suggest that RIP1 may be the key player conducting the NF- κ B–p53 cross-talk in glioblastoma.

A major finding of this study is that RIP1 is overexpressed in glioblastoma but not in lower grade glioma and confers a worse prognosis in glioblastoma. Increased expression of RIP1 is common in glioblastoma, with ~30% of tumors showing increases in RIP1, and frequently, the increase is substantial. Furthermore, increased expression of RIP1 is uncommon in grades II and III gliomas, showing a correlation of RIP1 with increased malignancy. Importantly, in matched pairs of primary low-grade glioma that progressed to secondary glioblastoma, RIP1 is usually low in the primary low-grade glioma and increased in the secondary glioblastoma. In a study of 70 glioblastomas, we find that increased RIP1 is an independent negative prognostic indicator in glioblastoma. There were no differences in the age, Karnofsky performance status, or treatment

in the low-RIP1 versus high-RIP1 groups. These findings imply that increased expression of RIP1 promotes a more malignant clinical phenotype in glioblastoma. It should be noted that because most patients in our study had a complete resection, our study does not address whether low RIP1 level would confer a survival advantage in those patients with partial resection or biopsy alone.

RIP1 may contribute to the pathogenesis of glioblastoma by multiple mechanisms. Firstly, increased expression of RIP1 is sufficient to activate NF- κ B, as shown in Fig. 3 and reported previously (40, 41). Thus, an increased RIP1 level in cancer is likely to lead to constitutive and deregulated NF- κ B activation. In addition, RIP1 has also been reported to have a role in phosphatidylinositol 3-kinase–Akt activation (39, 48). Thus, an augmented cellular RIP1 level in glioma cells seems sufficient to induce sustained activation of at least two prosurvival signaling pathways of central importance in cancer. In this study, we show that RIP1-mediated NF- κ B activation leads to up-regulation of mdm2 and inhibition of p53 pathways. Thus, cells with increased RIP with activated NF- κ B and Akt and down-regulated p53 would favor oncogenic signaling, resistance to DNA damage, and chemotherapy-induced apoptosis, all favoring a more malignant phenotype.

Paradoxically, RIP1 is also involved in cell death when ectopically expressed in response to inflammatory cytokines or other forms of cellular stress (24). However, apoptosis and proliferation are closely linked, and a number of key oncogenic proteins, such as Ras, c-Myc, and E2F1 can also induce apoptosis or growth arrest (49). The RIP1 knockout phenotype includes failure to thrive and an early death with substantial apoptosis in lymphoid and adipose tissues (27), suggesting an important role for RIP1 in survival signaling. RIP1 knockout MEFs are more sensitive to TNF-induced cell death, and RIP1 protects thymocytes from TNFR-2–induced cell death (50). The data in this study also support an oncogenic and prosurvival role for RIP1 in glioblastoma, but the effect of RIP1 signaling could be quite complex in the heterogenous tumor populations.

Our data suggest that increased expression of RIP1 identifies a group of glioblastoma patients who have a significantly worse prognosis. The clinical utility of this study may lie in the identification of a subgroup of patients with glioblastoma that have high RIP1 levels and worse prognosis and are resistant to standard chemotherapy. We propose that these patients may respond better to drugs targeting the NF- κ B signaling network.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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