Inhibition of the Akt/survivin pathway synergizes the antileukemia effect of nutlin-3 in acute lymphoblastic leukemia cells

Ningxi Zhu,[1] Lubing Gu,[1] Fengzhi Li,[2] and Muxiang Zhou[1]

[1]Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia and [2]Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York

Abstract

The phosphatidylinositol 3-kinase (PI3K)/Akt and p53 pathways play antiapoptotic and proapoptotic roles in cell death, respectively. Cancer cell growth and progression are associated with high levels of PI3K/Akt activation by loss of PTEN expression and the inactivation of p53 by MDM2 overexpression. We report that inhibition of PI3K/Akt, either by the PI3K inhibitor Ly294002 or by expression of PTEN, synergized the ability of the MDM2 antagonist nutlin-3 to induce apoptosis in acute lymphoblastic leukemia (ALL). We used a set of ALL cell lines with wild-type p53 and MDM2 overexpression, but different status of PTEN expression/PI3K/Akt activation, to test the ability of nutlin-3 to induce p53 and apoptosis. Nutlin-3 activated p53 in all the ALL cell lines; however, induction of apoptosis was dependent on PTEN status. Nutlin-3 induced potent apoptosis in cells with PTEN expression but not in those without PTEN, suggesting that PTEN/PI3K/Akt pathway may play a role in this process. Furthermore, nutlin-3 significantly down-regulated survivin expression in PTEN-positive cells but not in PTEN-negative cells. When these nutlin-3–resistant cells were either pretransfected with the PTEN gene or simultaneously treated with the PI3K inhibitor Ly294002, survivin was down-regulated and sensitivity to nutlin-3 was increased. Furthermore, direct silencing of survivin by small interfering RNA also increased the proapoptotic effect of nutlin-3 on the PTEN-negative, nutlin-3–resistant ALL cells. Our results suggest that Akt-mediated survivin up-regulation in PTEN-negative ALL cells may counteract the proapoptotic effect of nutlin-3, and indicate that a combination of MDM2 antagonist and PI3K/Akt inhibitor may be a promising approach for treating refractory ALL. [Mol Cancer Ther 2008;7(5):1101–9]

Introduction

It is well established that the inactivation of p53 plays a critical role in cell transformation and tumor cell growth (1). These p53-inactive cancers progress, developing resistance to apoptosis; therefore, cancer patients with inactivated p53 are usually resistant to the chemoradiation therapy that kills cancer cells through apoptotic pathways (2). Impairment of p53 function may be caused either by p53 gene mutations (3) or by changes in its primary negative regulator MDM2, which is amplified or overexpressed in diverse human malignancies, including leukemia (4, 5). MDM2, a multifunctional oncoprotein, inactivates p53 through different mechanisms. First, MDM2 can bind the transcription domain of p53 and block its ability to activate gene transcription (6). Second, MDM2 functions as an E3 ligase, initiating the ubiquitination and degradation of p53 (7, 8). In addition, MDM2 can promote nuclear export of p53 and inhibit its acetylation (9).

Given the importance of MDM2 in inactivating p53, disruption of the p53-MDM2 interaction seems to be an attractive approach in cancer therapy, at least in cancers having wild-type (wt) p53. Over the years, much effort has been made to disrupt interactions between MDM2 and p53 to facilitate wt p53 function. Strategies included the use of antibodies or inhibitory peptides directed at the MDM2 molecule, and either antisense oligonucleotides or small interfering RNA (siRNA) to inhibit MDM2 expression (10, 11). Recently, a potent and selective MDM2 antagonist called nutlin-3 was discovered (12). This nongenotoxic compound binds to the p53-binding pocket in the MDM2 protein, thus inhibiting the binding of p53 and activating the p53 pathway in cancer cells with wt p53. In fact, treatment with this small-molecule drug selectively induces cell cycle arrest, growth inhibition, and apoptosis in a variety of human cancers with wt p53, including prostate and lung carcinoma (13, 14), neuroblastoma (15), Hodgkin's lymphoma (16), multiple myeloma (17), acute myelogenous leukemia (18), chronic lymphocytic leukemia (19, 20), and acute lymphoblastic leukemia (ALL; ref. 21).

It has been shown that nutlin-3 is cytotoxic to cancer cells due to its ability to increase p53-mediated activity through both induction of proapoptotic factors and repression of antiapoptotic survivin (21). Survivin, a unique member of the inhibitor of apoptosis protein (IAP) family, plays an

Received 2/25/08; accepted 3/26/08.

Grant support: NIH grant R01 CA123490, Leukemia and Lymphoma Society grants 6249-05 and 6033-08, CURE Childhood Cancer, and Hope Street Kids.

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.

Requests for reprints: Muxiang Zhou, Division of Hematology/Oncology, Department of Pediatrics, Emory University School of Medicine, 2015 Uppergate Drive, Atlanta, GA 30322. Phone: 404-727-1426; Fax: 404-727-4455. E-mail: mzhou@emory.edu

doi:10.1158/1535-7163.MCT-08-0179

important role in regulating both apoptosis and cell division (22). The overexpression of survivin has been identified as a negative prognostic factor in various cancer types and was implicated in resistance to apoptosis induction by anticancer agents (23). Overexpressed survivin is associated with activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Evidence for the up-regulation of survivin via the PI3K/Akt pathway was first shown in endothelial cells (24). Later, many other investigational groups further showed that survivin expression is activated by the PI3K/Akt pathway, conferring cell survival and resistance to apoptosis in various malignant cells, including myeloid leukemia (25) and cancers of prostate (26), breast (27), and lung (28–30). These previous study results indicate that activation of the PI3K/Akt pathway is required for the up-regulation of survivin expression. Because nutlin-3 activity is enhanced by the down-regulation of survivin mediated by p53, PI3K/Akt-mediated survivin up-regulation may adversely alter the apoptosis-inducing activity of nutlin-3 in cancer cells.

Because the neoplastic cells from a significant percentage of patients with malignancies have high levels of PI3K/Akt activation due to loss of PTEN expression, we decided to examine the expression of survivin and its association with nutlin-3–induced apoptosis in both PTEN-positive and PTEN-negative leukemia cells of pediatric patients with ALL. By screening a panel of ALL cell lines having wt p53 and MDM2 overexpression, we discovered that the PTEN-expressing ALL cells express relatively low levels of survivin and are sensitive to nutlin-3, whereas, in contrast, the PTEN-negative ALL cells express high levels of survivin and are relatively resistant to nutlin-3. Importantly, we observed that inhibition of the PI3K/Akt/survivin pathway significantly enhances the antileukemic effect of nutlin-3 in PTEN-negative ALL cells.

Materials and Methods

Cell Lines and Cell Cultures

Six established cell lines originating from children with ALL were studied. Two of these lines (EU-1 and EU-3) were established at Emory University (Atlanta, GA), and three (Sup-B13, Sup-B15, and UOC-B11) were obtained from Dr. Stephen D. Smith (University of Kansas, Kansas City, KS). The Reh ALL line was obtained from Dr. C. Rosenfeld (Institut National de la Sante et de la Recherche Medicale, Villejuif, France). The phenotypes of these cell lines, including p53 status and expression levels of MDM2, PTEN, and PI3K/Akt, were characterized in prior publications (31). These cells were cultured in standard culture medium (RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 50 units penicillin, and 50 μg/mL streptomycin) at 37°C in 5% CO_2.

Reagents and Cell Treatment

The small-molecule MDM2 antagonist nutlin-3, purchased from Cayman Chemical, was dissolved in ethanol to create a 10 mmol/L stock solution, which was stored in small aliquots at −20°C. The ALL cells were exposed to 0 to 10 μmol/L of nutlin-3 for the time period indicated, with the final ethanol concentration kept constant in each experiment. It is of note that the solution is a combination of equimolar amounts of nutlin-3a (active) and nutlin-3b (inactive) isomers. Thus, concentrations of active isomer are equal to 50% of the total concentration. The specific PI3K inhibitor Ly294002, purchased from Cell Signaling, was dissolved in DMSO to create a 10 mmol/L stock solution and also stored in small aliquots at −20°C. Cells were exposed to 0 to 20 μmol/L of Ly294002 for the time period indicated, with the final DMSO concentration kept constant in each experiment.

Gene Transfection

For stable PTEN gene transfection, EU-1 cells in exponential growth were transfected with a wt PTEN expression plasmid (pCMV-PTEN) or a catalytically inactive PTEN mutant (PTENm) plasmid (pCMV-PTEN-C124S) provided by Dr. D.J. Tindall (Mayo Foundation, Rochester, MN). Transfection was done by electroporation at 300 V, 950 μF. The cells were seeded into culture dishes 48 h after transfection for the selection of G418-resistant colonies.

Survivin siRNA and Transfection

The siRNA was designed to target survivin mRNA, with a target sequence of 5′-TGGGCGCCGCGAATCCGGC-3′. A scrambled siRNA was used as a control. EU-1 cells were transfected with 50 to 200 nmol/L of chemically synthesized siRNA (Dharmacon) using Lipofectamine 2000 Reagent (Invitrogen). Briefly, EU-1 cells were seeded into 12-well plates at a density of 1×10^6 per well and cultured overnight. The next day, the siRNA solution was mixed with Lipofectamine 2000 Reagent in Opti-MEM I medium for 20 min following the manufacturer's protocol and then added to EU-1 cells in a total volume of 0.5 mL. After a 4-h incubation, 1 mL of normal medium was added to the cell culture.

Western Blot Analysis

Cells were lysed for 30 min at 4°C in a buffer composed of 150 mmol/L NaCl, 50 mmol/L Tris (pH 8.0), 5 mmol/L EDTA, 1% (v/v) NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 25 μg/mL leupeptin. Equal amounts of protein extracts (10 μg) were resolved by SDS-PAGE. Following transfer to a nitrocellulose filter, it was blocked for 1 h at room temperature with buffer containing 20 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, and 5% nonfat milk; incubated with specific antibodies for 3 h at room temperature; washed; and incubated with a horseradish peroxidase–labeled secondary antibody for 1 h. Finally, the blots were developed using an enhanced chemiluminescence detection system (Amersham Life Science).

Water-Soluble Tetrazolium Salt Assay

The cytotoxic effect of nutlin-3 and doxorubicin on ALL cells was determined with the water-soluble tetrazolium salt (WST) assay. Briefly, cells were cultured in 96-well microtiter plates with different concentrations of nutlin-3 for a 44-h period. Then, WST (25 μg/well) was added and the cells were incubated for an additional 4 h. Following

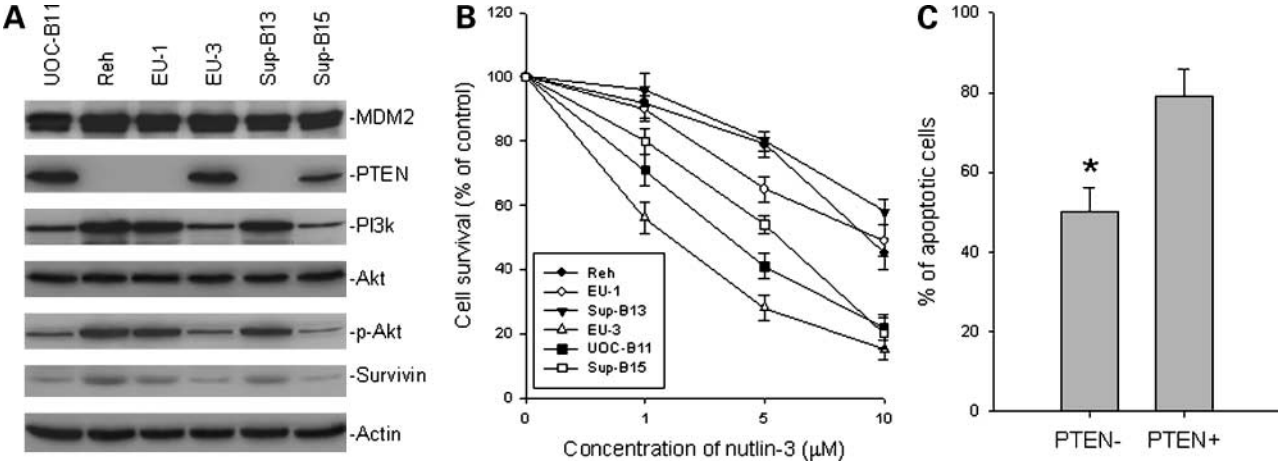

Figure 1. MDM2, PTEN, and survivin expression and Akt activation of ALL cell lines as well as their response to nutlin-3 treatment. **A,** Western blot assay for expression of MDM2, PTEN, and the PTEN-associated gene products PI3K, Akt, and p-Akt, as well as survivin, in six wt p53 ALL cell lines. Protein (20 μg) from cell lysates was electrophoresed in SDS-PAGE gels, transferred to nitrocellulose, and probed with antibodies as indicated. Actin serves as a control for equal protein loading and protein integrity. **B,** cytotoxic effect of nutlin-3 on ALL cell lines. The six ALL lines as studied in **A** were treated with different doses of nutlin-3 for 48 h, and cell viability was determined by WST assays. **C,** *columns,* mean percentage of Annexin V – positive cells, detected by flow cytometry, after treatment with 10 μmol/L nutlin-3 for three PTEN-negative ALL cell lines compared with three PTEN-positive lines; *bars,* SD. *, $P < 0.05$.

incubation, the absorbance of the wells was read with a microplate reader set at a test wavelength of 450 nm and a reference wavelength of 620 nm. Appropriate controls lacking cells were included to determine background absorbance.

Annexin V Assay

Quantification of apoptotic cells was done using Annexin V staining and flow cytometry. Cells with or without treatment were washed once with PBS before staining with FITC-Annexin V and propidium iodide according to the manufacturer's instructions. Samples were analyzed with a FACScan (Becton Dickinson) and WinList software (Verity Software House).

Results

ALL Cells with Differing PTEN Expression Have a Differential Response to Nutlin-3–Induced Cytotoxicity and Apoptosis

The six ALL cell lines used in this study have been previously characterized for their immunophenotype, p53 status, and expression of MDM2 and PTEN (31, 32). All six cell lines have the same immunophenotype of B-cell precursor (BCP-ALL), wt p53, and MDM2 overexpression. Of the six lines, three (Reh, EU-1, and Sup-B13) lack PTEN expression and the other three (EU-3, UOC-B11, and Sup-B15) express PTEN. The expression of PI3K and activation of Akt [phosphorylated form of Akt (p-Akt)] in these lines were correlated with the expression of PTEN in a functionally sequential manner. Cell lines without PTEN expression expressed a higher level of PI3K and a constitutively higher level of p-Akt than the lines with PTEN expression. Interestingly, the PTEN-negative ALL lines expressed relatively higher levels of survivin than did the PTEN-expressing lines (Fig. 1A).

First, we examined the effect of nutlin-3 on the viability and cell survival of these cell lines using the WST cytotoxicity assay. Nutlin-3 exhibited dose-dependent cytotoxic activity in all six cell lines (Fig. 1B). However, the degrees of response to nutlin-3–induced cytotoxicity differ between the PTEN-positive and PTEN-negative lines. The average percent of cell survival at 10 μmol/L nutlin-3 for 48-h treatment for PTEN-positive cell lines was 19 (range, 15–22) compared with 51 (range, 45–58) for PTEN-negative cell lines. To clarify whether the cell death induced by nutlin-3 in these wt p53 ALL cells was indeed due to induction of apoptosis, the Annexin V apoptosis assay was used. Nutlin-3 treatment induced apoptosis in these cell lines. Consistent with the WST results, nutlin-3 induced stronger apoptosis in the PTEN-positive lines than in the PTEN-negative lines. As shown in Fig. 1C, treatment of nutlin-3 at concentration of 10 μmol/L for 48 h induced apoptosis in ~79% of PTEN-positive cells compared with 51% apoptosis in PTEN-negative cells ($P < 0.05$).

Ly294002 Synergizes Nutlin-3 in Inducing Cell Death and Apoptosis in ALL Cells Lacking PTEN Expression

Because the high level of Akt activation due to a deficit in PTEN expression affects the antisurvival and proapoptotic effects of nutlin-3, we investigated whether inhibition of Akt activation by a specific PI3K inhibitor would increase the response of PTEN-negative ALL cells to nutlin-3. We used a well-characterized PI3K inhibitor, Ly294002, to inhibit the phosphorylation of Akt. We evaluated the effect of Ly294002 on nutlin-3–induced cytotoxicity in two PTEN-negative ALL cell lines, Reh and EU-1, using WST analysis. Although Ly294002 alone did not show an obvious cytotoxic effect on both cell lines, cotreatment of Ly294002 with nutlin-3 significantly increased the cytotoxic effect of nutlin-3. As shown in Fig. 2A and B, a significant

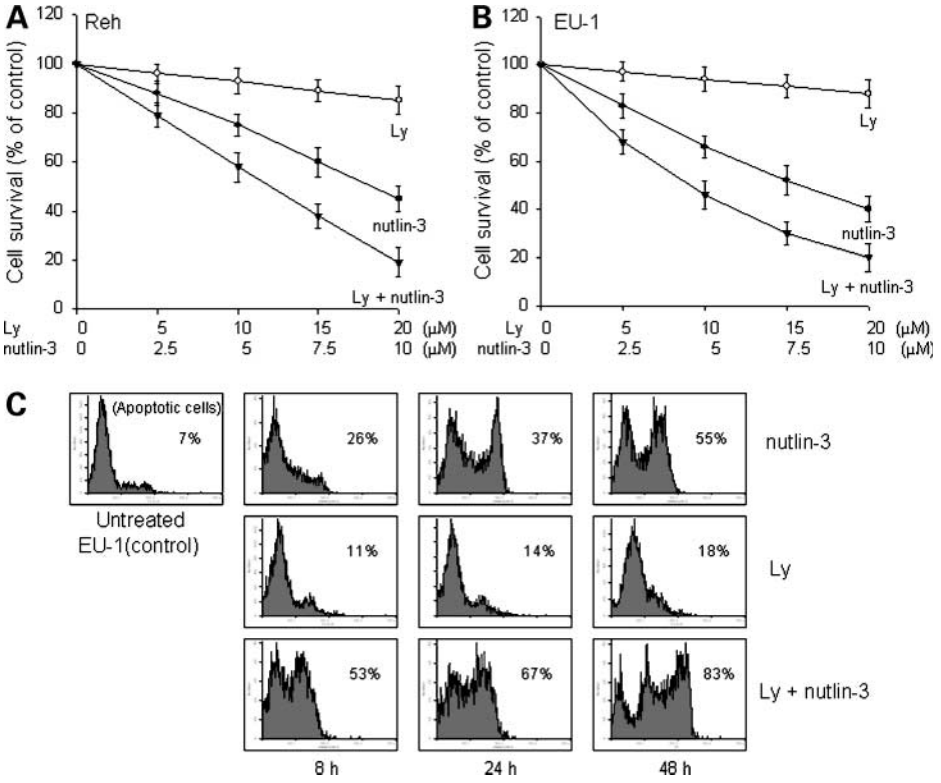

Figure 2. The PI3K inhibitor Ly294002 (*Ly*) synergizes the cytotoxic and apoptotic effect of nutlin-3 on PTEN-negative ALL cells. **A** and **B,** the PTEN-negative ALL lines Reh (**A**) and EU-1 (**B**) were treated with different concentrations of nutlin-3 and Ly294002 alone or with a fixed amount (15 μmol/L) of Ly294002 and different doses of nutlin-3, as indicated. Following 48 h of incubation, cell survival was determined by WST assay. **C,** time course of apoptosis in EU-1 cells induced by nutlin-3 and Ly294002 alone or their combination (nutlin-3, 10 μmol/L; Ly294002, 15 μmol/L; or equal amounts of nutlin-3 plus Ly294002 for the indicated time). Apoptotic cells were detected by Annexin V staining using flow cytometry.

difference in mean cell survival was noted between cells treated for 48 h with nutlin-3 alone and the same cell type treated with a combination of nutlin-3 and Ly294002 ($P < 0.05$). Consistent with these observations, a quantitative confirmatory apoptosis assay using flow cytometry revealed that a significantly increased percentage of EU-1 cells respond to treatment with a combination of nutlin-3 and Ly294002. At 48 h after treatment, the EU-1 cells treated with the drug combination became mostly Annexin V positive (from 7% apoptotic cells to 83%) compared with those treated with nutlin-3 alone (from 7% to 55%; see Fig. 2C).

PTEN Gene Transfection Increases the Cytotoxic and Apoptotic Effects of Nutlin-3

As described above, the loss of PTEN expression seemed to be associated with activation of Akt and to the resistance of ALL cells to cytotoxicity and apoptosis induced by nutlin-3. To investigate this relationship further, we transfected the *PTEN* gene into an EU-1 cell line that lacks endogenous PTEN expression to confirm the role of PTEN in regulating both Akt activation and the antileukemic activity of nutlin-3. Transfection of a phosphatase-inactive PTENm plasmid into EU-1 was also done as a control. As shown in Fig. 3A, both the transfected PTEN and PTENm proteins were stably expressed. We found that the activation of Akt, as shown by the expression of p-Akt, was reduced in the wt PTEN–transfected cells but not in PTENm-transfected cells. When we compared the EU-1 cells expressing PTEN with EU-1–expressing PTENm and EU-1 transfected with empty neo plasmid (vehicle) with respect to their responses to nutlin-3, we discovered that the EU-1/PTEN cells showed significantly increased cell death compared with EU-1/vehicle and EU-1/PTENm cells. As is shown in Fig. 3A, an obvious difference was noted in mean cell survival after 48 h of nutlin-3 treatment at concentrations of ≥5 mmol/L ($P < 0.05$). Consistent with the WST results, our flow cytometric apoptosis assays showed that an increased percentage of the EU-1/PTEN cells (from 10% to 80%) underwent apoptosis after 48-h nutlin-3 treatment compared with EU-1/vehicle and EU-1/PTENm cells (from 4% to 55% and from 5% to 58%, respectively; Fig. 3B).

Survivin Is Involved in Nutlin-3–Induced Apoptosis, Which Is Regulated by the PTEN/PI3K Pathway

It is well shown that nutlin-3 induces apoptosis through activation of wt p53 and its target genes, including the proapoptotic genes *Bax* and *PUMA*. We investigated the effect of nutlin-3 on the expression of these genes in our ALL cell lines. In particular, we wondered whether nutlin-3 also has a different effect on activation of wt p53 and its target genes in ALL cells with or without PTEN expression and whether this difference is responsible for the previously observed degrees of response to nutlin-3–induced growth inhibition and apoptosis. We compared the induction of p53, p21, Bax, and PUMA by nutlin-3 treatment between two different cell lines, EU-1 (PTEN negative) and EU-3 (PTEN positive). These two cell lines were chosen because they express similar levels of MDM2

to rule out the influence of different amounts of MDM2 on the induction of p53 by nutlin-3 (21). Interestingly, we found that p53, p21, Bax, and PUMA were similarly induced by nutlin-3 treatment in both EU-1 and EU-3 cells (Fig. 4A). Quantitative reverse transcription-PCR further showed that induction of these genes occurs at transcriptional levels, and the levels of their induction are similar in both EU-1 and EU-3 cell lines (data not shown). However, a test comparing their *survivin* gene activities did identify a difference in *survivin* gene down-regulation by nutlin-3 between these PTEN-positive and PTEN-negative cells. As shown in Fig. 4B, treatment with 10 μmol/L nutlin-3 for 12 h significantly reduced survivin expression in all three PTEN-positive cell lines tested but not in the three PTEN-negative lines.

In addition, we compared the kinetic expression of survivin after nutlin-3 treatment of EU-1 cells that were either transfected with the *PTEN* gene or a control. In the EU-1 cells transfected with control vector, no significant change in survivin expression was noted. However, in the PTEN-transfected cells, survivin expression was found to be remarkably down-regulated 4 h after nutlin-3 treatment, and it became barely detectable after 12 h of nutlin-3 treatment (Fig. 4C). We also did a test to assess the specificity of this response. When two other IAP members, cIAP-1 and cIAP-2, were tested for their expression levels following nutlin-3 treatment, they remained unchanged.

As had been shown in previous studies, activation of the PI3K/Akt pathway up-regulates survivin expression, whereas inhibition of PI3K/AKT down-regulates survivin expression. However, in this study, our results showed that the PI3K inhibitor Ly294002 (at a similar concentration) produced no significant down-regulation of survivin expression in EU-1 cells, although it inhibited the activation of Akt (Fig. 5A). In contrast, a combination of Ly294002 and nutlin-3 significantly reduced survivin expression in EU-1 cells, indicating that there is a synergy between these two inhibitors that can regulate survivin gene expression (Fig. 5B). No additional or synergetic effect was found between nutlin-3 and either of the mitogen-activated protein kinase pathway inhibitors SB203580 or PD98059. These findings indicated that, in PTEN-negative EU-1 cells, the lack of survivin down-regulation on treatment with nutlin-3 is at least partially attributed to a high level of Akt activation.

Inhibition of Survivin Expression by siRNA Enhances Nutlin-3–Induced Cytotoxicity and Apoptosis

To further confirm that the down-regulation of survivin plays a critical role in nutlin-3–induced cytotoxicity and apoptosis, we investigated whether direct inhibition of endogenous survivin by siRNA could increase the cytotoxic and apoptotic effect of nutlin-3 in PTEN-negative ALL cells. We used chemically synthesized survivin siRNA. As seen in Fig. 6A, transfection of survivin siRNA into EU-1 cells down-regulated survivin expression in a dose-dependent manner. WST assay showed that the introduction of siRNA mediated the down-regulation of survivin and sensitized EU-1 cells to nutlin-3–induced inhibition of proliferation (Fig. 6B). In fact, a significant difference was noted in mean cell survival after 48 h of nutlin-3 treatment between EU-1 cells treated with control siRNA and the same type of cells treated with survivin siRNA.

Similar results were found by testing with other methods. Transfection of survivin siRNA was able to sensitize EU-1 cells to nutlin-3–induced apoptosis, as measured by Western blot assay for both activation of caspase-3 and cleavage of the death substrate poly(ADP-ribose) polymerase. As is shown in Fig. 6C, significant cleavage of both caspase-3 and poly(ADP-ribose) polymerase was detected in survivin siRNA–transfected cells 8 h after nutlin-3

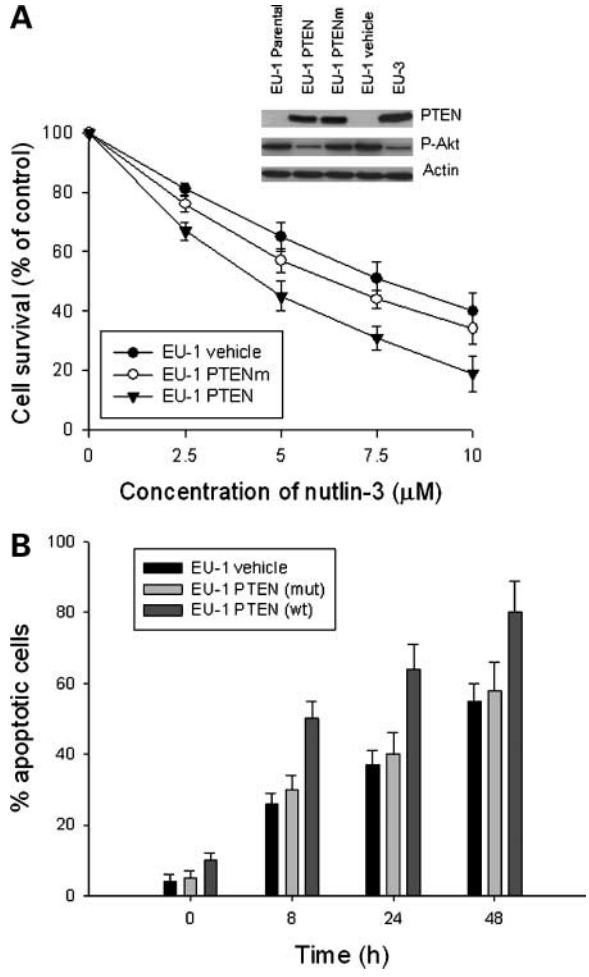

Figure 3. Transfection of PTEN genes and their effects on cytotoxic and apoptotic activity of nutlin-3 in the EU-1 ALL cell line. **A,** comparison of nutlin-3 sensitivity in PTEN-transfected and control-transfected EU-1 cells. EU-1 cells lacking PTEN expression were stably transfected with the PTEN expression plasmid and control plasmids, including a catalytically inactive PTENm and an empty neo plasmid (vehicle). Transfected cells were cultured with different concentrations of nutlin-3 for 48 h, and then cell survival was determined by WST assay. *Inset,* expression of p-Akt and either transfected PTEN in EU-1 or endogenous PTEN in EU-3 (control) was detected by Western blot assay. **B,** time course of nutlin-3–induced apoptosis in different PTEN-transfected EU-1 cells. Cells were treated with nutlin-3 (10 μmol/L) for the indicated time, and apoptotic cells were stained with Annexin V and detected by flow cytometry.

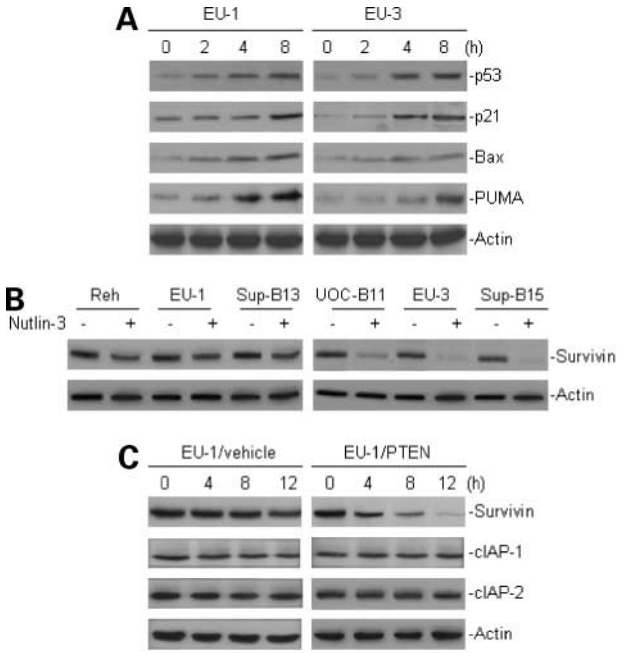

Figure 4. Expression and regulation by nutlin-3 of p53 and its target genes *p21, Bax, PUMA*, and *survivin* in ALL cells. **A,** comparison of p53 induction and activation of its target genes *p21, Bax*, and *PUMA* by nutlin-3 in PTEN-negative EU-1 and PTEN-positive EU-3 cells. Cells treated with 10 μmol/L nutlin-3 for the indicated time had protein expression detected by Western blot assay. **B,** comparison of survivin regulation by nutlin-3 in PTEN-negative (Reh, EU-1, and Sup-B13) and PTEN-positive (UOC-B11, EU-3, and Sup-B15) ALL cells. Cells treated with 10 μmol/L nutlin-3 for 12 h had survivin expression detected by Western blot assay. **C,** kinetic expression of survivin regulated by nutlin-3 treatment in PTEN-transfected EU-1 (EU-1/PTEN) and control-transfected (EU-1/vehicle) cells. Cells were incubated with 10 μmol/L nutlin-3 for the indicated time. Expression of survivin as well as cIAP-1 and cIAP-2 was detected by Western blot assay. Data in each panel are representative of at least three independent experiments.

treatment, whereas cleavage of these two proteins was not observed in the control siRNA–treated cells treated in a similar way with nutlin-3. Consistent with these observations, a quantitative apoptosis assay (Annexin V) proved that, 48 h after nutlin-3 treatment, a much greater number of EU-1 cells that were treated with survivin siRNA were Annexin V positive (83%) than those treated with control siRNA (55%; Fig. 6D).

Discussion

Previously, we studied the activity of the MDM2 antagonist nutlin-3 in the induction of apoptosis in ALL cells. We found that nutlin-3 induces apoptosis in ALL cells with wt p53 and MDM2 overexpression. We also found that nutlin-3–induced apoptosis is involved in the regulation of the antiapoptotic factor survivin, a transcriptional target of p53. The degree of apoptosis induced by nutlin-3 in ALL cells is closely associated with the levels of survivin down-regulation by nutlin-3 (21). Because survivin is tightly regulated by the PI3K/Akt pathway, in the present study, we investigated whether the apoptosis-inducing activity of nutlin-3 differs in ALL cells with and without PI3K/Akt activation. We compared PTEN-positive and PTEN-negative ALL cell lines, assessing the induction of apoptosis by nutlin-3. Our results showed that, despite expression of similar amounts of wt p53 and MDM2, PTEN-expressing ALL cells were sensitive to nutlin-3, whereas PTEN-negative ALL cells were relatively resistant to nutlin-3. Considering that PI3K/Akt is activated in PTEN-negative ALL cells, it could perhaps up-regulate survivin and attenuate nutlin-3 activity, so we treated the PTEN-negative cells with nutlin-3 in combination with the PI3K inhibitor Ly294002, or by prior transfection of PTEN into these PTEN-negative cells, to suppress PI3K/Akt. We discovered that inhibition of Akt significantly synergizes the antileukemic activity of nutlin-3, with concomitant down-regulation of survivin. Furthermore, direct silencing of survivin by siRNA increased the activity of nutlin-3, inducing the apoptosis of PTEN-negative cells. These results indicate that it is the PI3K/Akt/survivin signaling pathway that interferes with apoptosis-inducing activity of nutlin-3 in ALL cells. Knowledge of how these cellular proteins interact may help resolve resistance-related issues for ALL patients.

Survivin is a member of the IAP family. With a single baculovirus IAP repeat domain, survivin has the capability of inhibiting cellular caspase-3, caspase-7, and caspase-9. Overexpression of survivin can lead to a state of resistance to cell death by various types of apoptotic stimuli. In fact, survivin overexpression was found to correlate with poor prognosis in a variety of cancers, including leukemia. A recent study by Troeger et al. (33) showed that survivin overexpression of up to a 10-fold increase above the normal

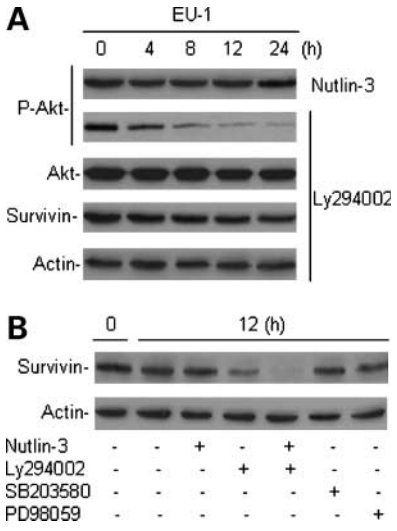

Figure 5. Survivin regulation and state of Akt phosphorylation produced by nutlin-3 and Ly294002 in ALL cells. **A,** EU-1 cells treated with either 10 μmol/L nutlin-3 or 20 μmol/L Ly294002 for the indicated times were processed by Western blot assay to detect expression of Akt, p-Akt, and survivin. **B,** EU-1 cells cultured in the presence of different pharmacologic inhibitors for the indicated times were processed by Western blot assay to detect survivin levels.

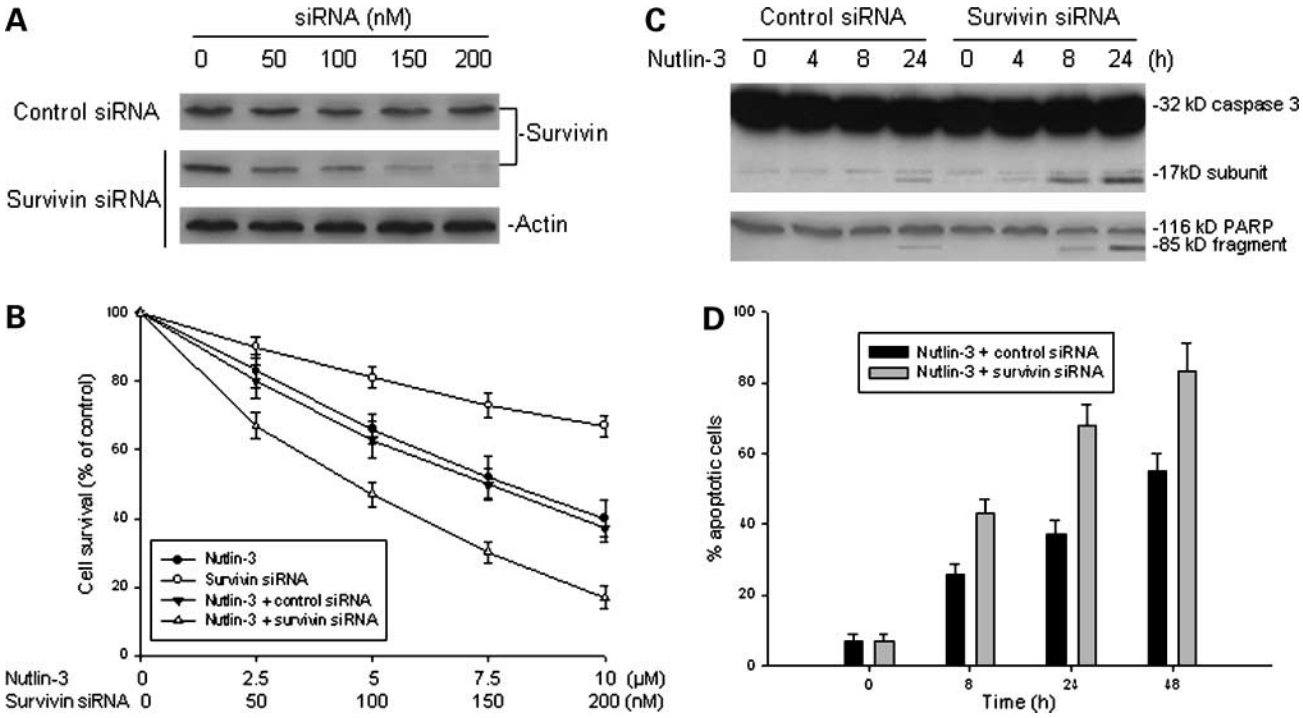

Figure 6. The effects of survivin siRNA on nutlin-3–induced cytotoxicity and apoptosis **A,** EU-1 cells were treated with different concentrations of either survivin siRNA or control siRNA for 24 h. Endogenous survivin expression was detected by Western blot analysis. **B,** EU-1 cells were treated with different concentrations of survivin siRNA and nutlin-3 alone or with a fixed amount (200 nmol/L) of either survivin siRNA or control siRNA and different doses of nutlin-3, as indicated. Following incubation for 48 h, cell viability was determined by WST assay. **C** and **D,** time course of apoptosis induced by nutlin-3 in combination with either survivin siRNA or control siRNA in EU-1 cells. Cells were treated with nutlin-3 (10 μmol/L) plus siRNA (200 nmol/L) for the indicated time. Apoptosis was detected either by examining caspase-3 activation and cleavage of the death substrate poly(ADP-ribose) polymerase (*PARP*; **C**) or by staining with Annexin V and analyzing by flow cytometry (**D**). *Columns,* mean percentage of Annexin V–positive cells from three independent experiments; *bars,* SD.

level was detected in 65% of BCP-ALL samples, so it serves as a significant prognostic marker for this disease.

It has been well defined that the expression of survivin is transcriptionally regulated by wt p53. The *survivin* gene promoter contains a p53 response element. Increased expression of p53 represses survivin promoter activity, resulting in decreased survivin protein expression (34–36). Mutation of the *p53* gene in BCP-ALL is rare (37), so in most cases of the disease p53 is wt. Differential expression of survivin in BCP-ALL suggests that additional mechanisms are involved in the regulation of survivin expression in these kinds of neoplastic cells. Our results from the present study showed that expression of survivin in BCP-ALL cell lines is associated with expression of the *PTEN* tumor suppressor gene. Despite having similar immunophenotypes, wt p53, and MDM2 expression levels, all the PTEN-negative ALL cell lines studied expressed high levels of survivin, whereas all PTEN-positive cell lines expressed low levels of survivin. Our observations are consistent with the results of a study using tissue microarray in endometrial carcinoma, in which survivin expression had a statistically significant correlation with decreased PTEN expression (38). Because PTEN is a potent inhibitor of the PI3K/Akt pathway, the loss of PTEN expression resulting in elevated survivin expression suggested that survivin expression is regulated by PI3K/Akt signaling.

In fact, it has been noted that activation of the PI3K/Akt pathway is involved in the regulation of survivin, although the precise mechanisms by which PI3K/Akt regulates survivin expression have not been elucidated. Many studies report that inhibition of PI3K/Akt signaling down-regulates survivin expression, enhancing apoptosis induced by tumor necrosis factor-α (39), geranylgeranyltransferase I inhibitor (40), lovastatin (41), and anticancer drugs (42, 43). Because we hypothesized that, in PTEN-negative cells, nutlin-3–induced apoptosis is attenuated by survivin that was up-regulated by PI3K/Akt, we evaluated whether inhibition of PI3K/Akt in these cells would down-regulate survivin and thus increase the apoptotic activity of nutlin-3. Although inhibition of PI3K using its specific inhibitor Ly294002 significantly decreased Akt activity in our ALL cell model, the expression of survivin was only slightly down-regulated. However, when used in combination with nutlin-3, the expression of survivin was remarkably down-regulated compared with the use of either nutlin-3 or Ly294002 alone. Tests on the mitogen-activated protein kinase inhibitors SB203580 and PD98059 did not show any synergistic effect with nutlin-3 with respect to decreasing survivin expression in PTEN-negative

ALL cells. It is of note that all PTEN-negative ALL cell lines in this study express high levels of PI3K and Akt, yet transfection of PTEN into these cells resulted in significant down-regulation of survivin but not other IAP members, such as cIAP-1 and cIAP-2, in response to nutlin-3 treatment. These results indeed show that survivin expression is specifically regulated by the PI3K/Akt pathway and that inhibition of PI3K/Akt signaling synergizes with nutlin-3 treatment to significantly down-regulate survivin.

The p53-mediated nutlin-3 apoptotic activity takes place by two major pathways: p53-induced activation of proapoptotic proteins, including Bax and PUMA, and p53-induced repression of the antiapoptotic protein survivin (21). In PTEN-negative/Akt-activated ALL cells, the survivin pathway that is affected by nutlin-3 activity seems to be predominant. When treated with nutlin-3, we detected similar inductions of Bax, PUMA, and p53 in both the PTEN-positive and PTEN-negative ALL cells. However, expression of survivin was significantly repressed in the PTEN-positive cells, but not in PTEN-negative cells, after treatment with nutlin-3. Cell death as measured by cytotoxic and apoptotic assay showed that the activity of nutlin-3 in PTEN-negative cells could be significantly enhanced by a down-regulation of survivin that was achieved by inhibition of PI3K/Akt. Furthermore, direct silencing of survivin expression by siRNA increased the apoptotic activity of nutlin-3, showing the critical role that survivin down-regulation/silencing plays in achieving success with nutlin-3–induced apoptosis.

ALL is the most common malignant disease in children. Despite the ability of the current treatment regimens to reach relatively high rates of remission induction and survival, approximately 20% to 25% of children with ALL do not respond well to therapy. These ALL patients either fail initial induction therapy or relapse after a short remission (44). Both overexpression of the p53-inhibitory factor MDM2 and loss of PTEN expression that causes high levels of PI3K/Akt activation in ALL cells have been reported as factors that contribute to leukemia cell survival and resistance to chemotherapy, which may be implicated in the incidence of refractory disease (31, 45, 46). Our study has clarified the molecular requirements for nutlin-3 to function well in controlling most types of ALL cells, including those that have previously proved fatal to pediatric patients. As a consequence, we believe that simultaneous inhibition of both MDM2 and PI3K/Akt pathways would be a novel and highly promising approach to improving therapeutic efficacy in refractory ALL cases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307 – 10.

2. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594 – 604.

3. Hollstein M, Sidransky D, Vogelstein B, et al. p53 mutations in human cancers. Science 1991;253:49 – 53.

4. Bond GL, Hu W, Levine AJ. MDM2 is a central node in the p53 pathway: 12 years and counting. Curr Cancer Drug Targets 2005;5:3 – 8.

5. Bueso-Ramos CE, Yang Y, deLeon E, et al. The human MDM-2 oncogene is overexpressed in leukemias. Blood 1993;82:2617 – 23.

6. Momand J, Zambetti GP, Olson D, et al. The MDM-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992;69:1237 – 45.

7. Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 1997;420:25 – 7.

8. Tao W, Levine AJ. Nucleocytoplasmic shuttling of oncoprotein Hdm2 is required for Hdm2-mediated degradation of p53. Proc Natl Acad Sci U S A 1999;96:3077 – 80.

9. Kobet E, Zeng X, Zhu Y, et al. MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins. Proc Natl Acad Sci U S A 2000;97:12547 – 52.

10. Chène P. Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. Nat Rev Cancer 2003;3:102 – 9.

11. Rayburn E, Zhang R, He J, et al. MDM2 and human malignancies: expression, clinical pathology, prognostic markers, and implications for chemotherapy. Curr Cancer Drug Targets 2005;5:27 – 41.

12. Vassilev LT, Vu BT, Graves B, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004;303: 844 – 8.

13. Logan IR, McNeill HV, Cook S, et al. Analysis of the MDM2 antagonist nutlin-3 in human prostate cancer cells. Prostate 2007;67:900 – 6.

14. VanderBorght A, Valckx A, Van Dun J, et al. Effect of an hdm-2 antagonist peptide inhibitor on cell cycle progression in p53-deficient H1299 human lung carcinoma cells. Oncogene 2006;25:6672 – 7.

15. Van Maerken T, Speleman F, Vermeulen J, et al. Small-molecule MDM2 antagonists as a new therapy concept for neuroblastoma. Cancer Res 2006;66:9646 – 55.

16. Drakos E, Thomaides A, Medeiros LJ, et al. Inhibition of p53-murine double minute 2 interaction by Nutlin-3A stabilizes p53 and induces cell cycle arrest and apoptosis in Hodgkin lymphoma. Clin Cancer Res 2007; 13:3380 – 7.

17. Stuhmer T, Chatterjee M, Hildebrandt M, et al. Nongenotoxic activation of the p53 pathway as a therapeutic strategy for multiple myeloma. Blood 2005;106:3609 – 17.

18. Kojima K, Konopleva M, Samudio IJ, et al. MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy. Blood 2005;106:3150 – 9.

19. Secchiero P, Barbarotto E, Tiribelli M, et al. Functional integrity of the p53-mediated apoptotic pathway induced by the nongenotoxic agent nutlin-3 in B-cell chronic lymphocytic leukemia (B-CLL). Blood 2006;107: 4122 – 9.

20. Coll-Mulet L, Iglesias-Serret D, Santidrian AF, et al. MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. Blood 2006;107:4109 – 14.

21. Gu L, Zhu N, Findley HW, et al. The MDM2 antagonist nutlin-3 is a potent inducer of apoptosis in pediatric acute lymphoblastic leukemia cells with wild-type p53 and overexpression of MDM2. Leukemia. In press 2008.

22. Li F, Ambrosini G, Chu EY, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998;396:580 – 4.

23. Altieri DC. Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer 2008;8:61 – 70.

24. Papapetropoulos A, Fulton D, Mahboubi K, et al. Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. J Biol Chem 2000;275:9102 – 5.

25. Carter BZ, Milella M, Altieri DC, et al. Cytokine-regulated expression of survivin in myeloid leukemia. Blood 2001;97:2784 – 90.

26. Gong J, Lee J, Akio H, et al. Attenuation of apoptosis by chromogranin A-induced Akt and survivin pathways in prostate cancer cells. Endocrinology 2007;148:4489 – 99.

27. Asanuma H, Torigoe T, Kamiguchi K, et al. Survivin expression is regulated by coexpression of human epidermal growth factor receptor 2 and epidermal growth factor receptor via phosphatidylinositol 3-kinase/AKT signaling pathway in breast cancer cells. Cancer Res 2005;65: 11018 – 25.

28. Belyanskaya LL, Hopkins-Donaldson S, Kurtz S, et al. Cisplatin

activates Akt in small cell lung cancer cells and attenuates apoptosis by survivin upregulation. Int J Cancer 2005;117:755 – 63.

29. Semba S, Trapasso F, Fabbri M, et al. Fhit modulation of the Akt-survivin pathway in lung cancer cells: Fhit-tyrosine 114 (Y114) is essential. Oncogene 2006;25:2860 – 72.

30. Dasgupta P, Kinkade R, Joshi B, et al. Nicotine inhibits apoptosis induced by chemotherapeutic drugs by up-regulating XIAP and survivin. Proc Natl Acad Sci U S A 2006;103:6332 – 7.

31. Zhou M, Gu L, Findley HW, et al. PTEN reverses MDM2-mediated chemotherapy resistance by interacting with p53 in acute lymphoblastic leukemia cells. Cancer Res 2003;63:6357 – 62.

32. Zhou M, Yeager AM, Smith SD, et al. Overexpression of the MDM2 gene by childhood acute lymphoblastic leukemia cells expressing the wild-type p53 gene. Blood 1995;85:1608 – 14.

33. Troeger A, Siepermann M, Escherich G, et al. Survivin and its prognostic significance in pediatric acute B-cell precursor lymphoblastic leukemia. Haematologica 2007;92:1043 – 50.

34. Hoffman WH, Biade S, Zilfou JT, et al. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem 2002;277: 3247 – 57.

35. Zhou M, Gu L, Li F, et al. DNA damage induces a novel p53-survivin signaling pathway regulating cell cycle and apoptosis in acute lymphoblastic leukemia cells. J Pharmacol Exp Ther 2002;303:124 – 31.

36. Mirza A, McGuirk M, Hockenberry TN, et al. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 2002;21:2613 – 22.

37. Fenaux P, Jonveaux P, Quiquandon I, et al. Mutations of the p53 gene in B-cell lymphoblastic acute leukemia: a report on 60 cases. Leukemia 1992;6:42 – 6.

38. Pallares J, Martínez-Guitarte JL, Dolcet X, et al. Survivin expression in endometrial carcinoma: a tissue microarray study with correlation with PTEN and STAT-3. Int J Gynecol Pathol 2005;24:247 – 53.

39. Fornaro M, Plescia J, Chheang S, et al. Fibronectin protects prostate cancer cells from tumor necrosis factor-α-induced apoptosis via the AKT/survivin pathway. J Biol Chem 2003;278:50402 – 11.

40. Dan HC, Jiang K, Coppola D, et al. Phosphatidylinositol-3-OH kinase/AKT and survivin pathways as critical targets for geranylgeranyltransferase I inhibitor-induced apoptosis. Oncogene 2004;23:706 – 15.

41. Kaneko R, Tsuji N, Asanuma K, et al. Survivin down-regulation plays a crucial role in 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor-induced apoptosis in cancer. J Biol Chem 2007;282:19273 – 81.

42. Martinelli S, Kostylina G, Niggli V, et al. Targeting survivin via PI3K but not c-akt/PKB by anticancer drugs in immature neutrophils. Oncogene 2006;25:6915 – 23.

43. Hideshima T, Catley L, Raje N, et al. Inhibition of Akt induces significant downregulation of survivin and cytotoxicity in human multiple myeloma cells. Br J Haematol 2007;138:783 – 91.

44. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. N Engl J Med 2006;354:166 – 78.

45. Marks DI, Kurz BW, Link MP, et al. High incidence of potential p53 inactivation in poor outcome childhood acute lymphoblastic leukemia at diagnosis. Blood 1996;87:1155 – 61.

46. Sakai A, Thieblemont C, Wellmann A, et al. PTEN gene alterations in lymphoid neoplasms. Blood 1998;92:3410 – 5.