BCL-2 Hypermethylation Is a Potential Biomarker of Sensitivity to Antimitotic Chemotherapy in Endocrine-Resistant Breast Cancer

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Abstract

Overexpression of the antiapoptotic factor BCL-2 is a frequent feature of malignant disease and is commonly associated with poor prognosis and resistance to conventional chemotherapy. In breast cancer, however, high BCL-2 expression is associated with favorable prognosis, estrogen receptor (ER) positivity, and low tumor grade, whereas low expression is included in several molecular signatures associated with resistance to endocrine therapy. In the present study, we correlate BCL-2 expression and DNA methylation profiles in human breast cancer and in multiple cell models of acquired endocrine resistance to determine whether BCL-2 hypermethylation could provide a useful biomarker of response to cytotoxic therapy. In human disease, diminished expression of BCL-2 was associated with hypermethylation of the second exon, in a region that overlapped a CpG island and an ER-binding site. Hypermethylation of this region, which occurred in 10% of primary tumors, provided a stronger predictor of patient survival ($P = 0.019$) when compared with gene expression ($n = 522$). In multiple cell models of acquired endocrine resistance, BCL-2 expression was significantly reduced in parallel with increased DNA methylation of the exon 2 region. The reduction of BCL-2 expression in endocrine-resistant cells lowered their apoptotic threshold to antimitotic agents: nocodazole, paclitaxel, and the PLK1 inhibitor BI2536. This phenomenon could be reversed with ectopic expression of BCL-2, and rescued with the BCL-2 inhibitor ABT-737. Collectively, these data imply that BCL-2 hypermethylation provides a robust biomarker of response to current and next-generation cytotoxic agents in endocrine-resistant breast cancer, which may prove beneficial in directing therapeutic strategy for patients with nonresectable, metastatic disease. *Mol Cancer Ther; 12(9); 1874–85. ©2013 AACR.*

Introduction

At least 70% of breast cancers are classified as estrogen receptor (ER)–positive, and consequently, the majority of patients with this disease receive some form of endocrine therapy as part of their adjuvant care (1). Although endocrine therapy reduces the risk of disease recurrence, 28% of patients with luminal A and 43% with luminal B breast cancer will acquire drug resistance and develop distant metastases within 15 years of first receiving treatment (2). Metastases will most commonly form in the bone (19% of luminal A, 30% of luminal B), but also in the brain, lung, and liver, ruling out surgical intervention for most patients (2). Current second-line therapeutic strategies remain limited to the sequential delivery of alternative endocrine therapies and in some instances, targeted therapies to growth factor receptors (3–5). Responses, however, are often short-lived. The median duration of survival from time of relapse is 2.2 and 1.6 years for patients with luminal A and luminal B breast cancer, respectively (2), highlighting the need for more effective therapeutic approaches to metastatic disease, with companion biomarkers of response that can be profiled in the absence of a tumor biopsy.

In an effort to improve traditional chemotherapeutic agents that inhibit mitotic progression, such as taxanes and vinca alkaloids, small-molecule inhibitors that target proteins specific to the mitotic spindle have been developed (6). These include inhibitors of the polo-like kinases (PLK), most commonly Plk1, which block mitotic spindle assembly in dividing cells, thereby causing arrest in the G_2–M phase of the cell cycle. Following mitotic arrest,

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many cancer cells undergo cell death caused by the activation of the intrinsic apoptotic pathway (7). The activation of this pathway can be blocked by BCL-2, an antiapoptotic factor that is overexpressed in many forms of cancer, including 90% of colorectal cancer, 70% of breast cancer, 30% to 60% of prostate cancer, and 80% of B-cell lymphomas (8). In the majority of cancer subtypes, the overexpression of BCL-2 is generally associated with poor survival and reduced sensitivity to cytotoxic chemotherapies (9, 10). In light of this, BCL-2 inhibitors that counteract its prosurvival function and serve to sensitize cancer cells to cytotoxicity have been developed, and are currently undergoing clinical analysis (11).

In breast cancer, however, high BCL2 expression is predictive of favorable prognosis and low tumor grade (12, 13). Although this may seem incongruent, in breast tissue, BCL-2 is regulated by estrogen and is associated with high ER expression, although its expression is not simply a surrogate for ER expression (13–15). Low BCL-2 expression is included in several molecular signatures associated with acquired resistance to endocrine therapy and poor prognosis in primary breast cancer (16–18). Because diminished BCL-2 expression in cancer confers increased sensitivity to cytotoxic chemotherapy, it is possible that breast cancer patients with endocrine-resistant disease could achieve significant therapeutic benefit from cytotoxic agents when used as a second-line treatment.

Although the mechanism for BCL-2 suppression in endocrine-resistant breast cancer has not been established, we and others have shown that following the acquisition of resistance, estrogen-regulated genes suppressed by tamoxifen are subject to permanent epigenetic silencing (19–22), so that well-characterized ER targets such as PgR and pS2 become silenced by promoter hypermethylation in models of acquired tamoxifen resistance (19). Interestingly, a significant proportion of patients who receive adjuvant tamoxifen show reduced BCL-2 expression after 6 months of treatment (23). Because patients typically receive tamoxifen for 5 years, there is considerable potential for further downregulation and possible epigenetic modification in clinical breast cancer; however, there have been no reports linking BCL-2 methylation and expression in endocrine-resistant breast cancer to date.

With the emergence of epigenetic biomarkers for early detection of disease, tumor classification, and response to treatment being explored in the clinic (24), it is possible that methylated BCL-2 could potentially provide a non-invasive biomarker for chemosensitivity in metastatic, nonresectable endocrine-resistant breast cancer. In the present study, we address whether promoter hypermethylation is associated with BCL-2 expression in breast cancer and whether this commonly occurs in antihormone-resistant breast cancer cells. In addition, as low BCL-2 expression is associated with increased apoptotic response in other cancer subtypes, we investigate whether the suppression of BCL-2 could be exploited to confer increased sensitivity to antimitotic agents, including the Plk1 inhibitor, BI2536.

Materials and Methods

TCGA breast cancer data analysis

Expression data in the form of RPKM-normalized RNASeq data for BCL-2 were obtained from The Cancer Genome Atlas (TCGA) breast cohort (25) via the cBio data portal ($n = 774$ tumors; http://www.cbioportal.org/public-portal/index.do; ref. 26). Methylation data in the form of probe-level β values from Infinium HumanMethylation450 BeadChips were obtained from the Data-Matrix section of the TCGA data portal ($n = 522$ tumors; $n = 96$ normal tissue; https://tcga-data.nci.nih.gov/tcga/), and the subset of probes with valid data spanning BCL-2 was identified ($n = 42$). Clinical data including follow-up were obtained from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) and analyzed using R (v2.15.1). The association between methylation and RNASeq ($n = 485$) was assessed using the maximal information coefficient (MIC; ref. 27). We chose MIC rather than Spearman or Pearson correlation because MIC is capable of identifying mutual exclusivity relationships (27), which matched our expectation that expression would only be seen when methylation is low, and *vice versa*. Survival analyses were conducted using the survival package in R (28), using Cox models built against penalized-spline predictors of methylation levels ($n = 522$) or RNASeq data ($n = 774$), with disease-specific survival (DSS) as the clinical endpoint. Because of fitting penalized regression splines, we report the likelihood ratio test P value, where $P < 0.05$ defined our level of statistical significance. Correlation between BCL-2, PLK1, and ER expression levels was assessed using the Spearman rank correlation test.

Cell culture and retroviral infection

MCF7 cells were originally obtained from AstraZeneca and their tamoxifen-resistant (TAMR), fulvestrant-resistant (FASR), and estrogen-deprived (MCF7X) derivatives were cultured as previously described (29–31). All cell lines were authenticated by short-tandem repeat (STR) profiling (Cell Bank, Australia) and cultured for less than 6 months after authentication. TAMR stably expressing the EcoR protein were generated by transfection with the retroviral vector pQCXIN (Clontech; ref. 32) and subsequently infected with pQCXIP-BCL-2α retrovirus (33). Expression was confirmed by Western blot analysis.

Proliferation and apoptosis assays

Relative cell number was assessed using a crystal violet–based colorimetric assay. Data are presented as average absorbance per sample, corrected for background. Cell-cycle analysis was achieved by flow-cytometric analysis of propidium iodide–stained, ethanol-fixed cells. Apoptotic cell populations were determined by staining methanol-fixed cells with the M30 CytoDEATH antibody (Enzo Life Sciences). Inhibitors used included BI2536 (Selleck Chemicals), paclitaxel (Taxol; LC Laboratories), nocodazole (Sigma), doxorubicin (Sigma), and ABT-737 (Selleck Chemicals).

Gene expression analysis

Quantitative reverse transcriptase PCR (qRT-PCR) used the High Capacity RT Kit and inventoried TaqMan probes BCL-2 (Hs00608023_m1, which spans exon 2 and 3, that is, specific for BCL-2α), Plk1 (Hs00153444_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs02758991_g1; Applied Biosystems).

Western blot analysis

Briefly, cell lysate samples (40 μg) were denatured, subjected to electrophoretic separation on SDS-PAGE, and trans-blotted onto a polyvinylidene difluoride (PVDF) membrane. Blots were blocked then incubated with the following primary antibodies: BCL-2 (α-isoform specific, i.e., 26 kDa; 124; DakoCytomation), BCL-xL (#2764; Cell Signaling Technology), ER-α (sc-543; Santa Cruz Biotechnology), Plk1 (#4535; Cell Signaling Technology), pPlk1 (Thr210; #5472; Cell Signaling Technology), PARP (#9542; Cell Signaling Technology), and β-actin (AC-15; Sigma). Secondary antibodies were applied [horseradish peroxidase–conjugated sheep α-mouse and donkey α-rabbit (Amersham Biosciences)] and specific proteins were visualized by chemiluminescence.

Clonal bisulfite sequencing

The methylation status of a total of 32 CpG sites spanning the BCL-2 promoter from the 3′ end of the (reverse strand) coding DNA sequence (CDS) of exon 2 and ending just after the upstream ER-binding site (chr18:-60,985,184-60,985,640; GRCh37/hg19) was interrogated. The bisulfite reaction was carried out on up to 1 μg of extracted genomic DNA for 12 hours at 55°C, under conditions previously described (19, 34). PCR amplifications were conducted using the following bisulfite conversion primers: BCL-2 FW: 5′-gagaaatattgaaggggtattatttattattatattttat-3′; BCL-2 RV: 5′-acccrataccacctataatccaccta-3′. After cloning and sequencing, the methylation state of the individual clones was analyzed using BiQ Analyzer software.

Statistical analysis

The statistical significance of pairwise comparisons was derived using paired/unpaired t tests where applicable. Where multiple independent factors were present, data were analyzed using one-way ANOVA with *post hoc* tests ($P < 0.05$ was deemed statistically significant).

Results

BCL-2 methylation in human breast cancer

To determine whether BCL-2 expression in human breast cancer correlated with DNA methylation, we examined Illumina 450k Methylation Array and RNASeq data from TCGA breast cohort (25). Forty-two methylation probes spanned the *BCL-2* gene (Supplementary Fig. S1A), with 27 overlapping the large promoter-associated CpG island (CpG 133; Supplementary Fig. S1B), 7 overlapping the second exon and a smaller CpG island (CpG 27; Supplementary Fig. S1C), 6 spanning the intron, and the remaining 2 overlapping exon 3 (Supplementary Fig.

S1A). The relationship between *BCL-2* gene expression and methylation status is shown for each probe in Supplementary Fig. S2 ($n = 485$), which illustrates the marked variability in both the extent of methylation, and the association with gene expression. This variability occurred in a manner that was consistent with the genomic location of the individual probes. Probes overlapping the small CpG island within exon 2 (CpG_27, probes 11–13) featured a clear inverse relationship between methylation and expression, with a distinct subgroup of patients exhibiting hypermethylation and complete repression of expression (illustrated for probe 11 in Fig. 1A). Interestingly, probes 11 and 12 are in close proximity to an ER-binding site within exon 2 (Supplementary Fig. S1C). The remaining probes spanning exon 2 (i.e., probes 9, 10, 14, and 15) showed a similar relationship between hypermethylation and loss of expression (Supplementary Fig. S2). In marked contrast, the promoter-associated CpG island (spanned by probes 16–42) exhibited low overall methylation and no correlation with expression, whereas the intron and third exon (spanned by probes 1–8) displayed near-maximal hypermethylation but no correlation with expression (Supplementary Fig. S2).

To determine whether this was a cancer-specific event, the methylation level of each probe spanning exon 2 was compared in cancer ($n = 522$) and normal ($n = 96$) samples. Using a conservative cutoff of the 95th percentile of methylation in the normal samples (dashed red line in Fig. 1A and B), the region of DNA interrogated by probe 11 was hypermethylated in 55 of 522 (10.5%) of breast cancers (Fig. 1B), with a similar proportion for probes 12 and 13 (7.5% and 13.4%). As with the relationship between hypermethylation and loss of expression, this pattern was only observed for probes overlapping exon 2, and was strongest for those probes that overlapped the CpG island (i.e., probes 11–13; Supplementary Fig. S3).

To establish whether BCL-2 promoter methylation was significantly associated with DSS, Cox regression models were fitted to the tumor-specific methylation level of each individual probe across the cohort ($n = 522$). A p-spline transformation of the methylation data were applied to permit nonlinear relationships between methylation and hazard to be detected, and to smooth out the variation in the methylation data, thus providing a more robust predictor. The region of hypermethylation most significantly associated with DSS was that spanning exon 2, with $P < 0.05$ for 3 of 7 probes (e.g., $P = 0.02$ for probe 11, likelihood ratio test) and $0.05 < P < 0.1$ for a further 2 probes (Fig. 1C, dashed red line; $P = 0.05$). Outside this region, just three of the remaining 35 probes (probes 16, 25, and 41) reached borderline significance ($0.05 < P < 0.1$); however, each of these probes displayed low levels of maximal methylation (Fig. 1C, purple line), and thus these results are unlikely to represent a true association between hypermethylation and DSS at these loci.

To examine the relationship between methylation and DSS more closely, the Cox regression results were
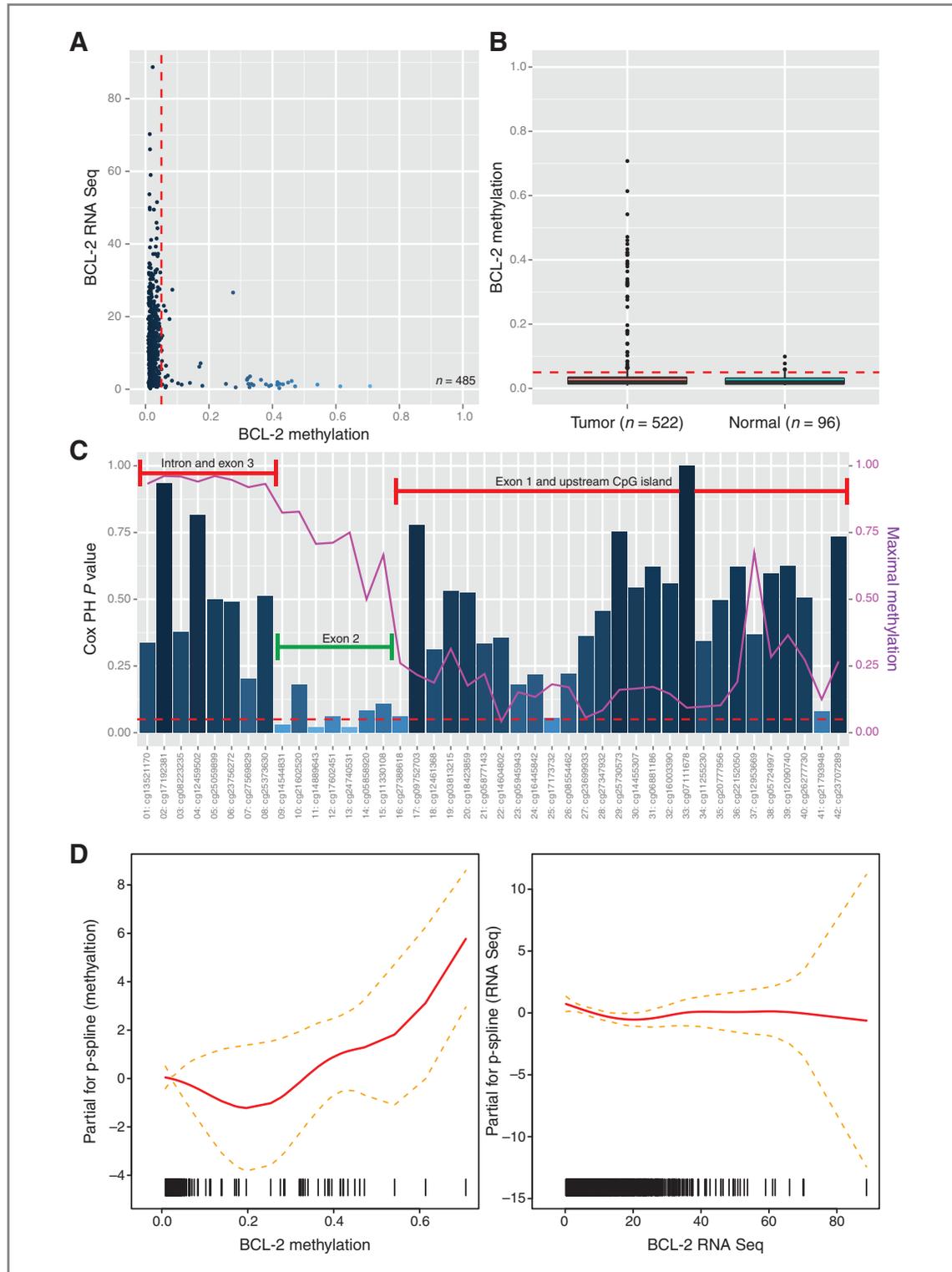
Figure 1. BCL-2 expression is silenced by the DNA methylation of exon 2 in human breast cancer and correlates with poor survival. A, the relationship between BCL-2 methylation and expression is illustrated by data retrieved for probe 11. B, the methylation of BCL-2 in tumor ($n = 522$) versus normal ($n = 96$) samples (data from probe 11). The dashed red line represents the 95th percentile of BCL-2 methylation in the normal samples. C, Cox proportional hazard P values for the 42 methylation probes that span BCL-2. The red dashed line represents $P = 0.05$. The purple line represents the maximal methylation recorded for each probe. D, term-plot for BCL-2 methylation (probe 11) versus risk of hazard (left) and BCL-2 gene expression versus risk of hazard (right). The orange dashed lines represent 95% confidence intervals and the black bars represent individual samples.

visualized in a term plot (Fig. 1D and Supplementary Fig. S4). For probes spanning the CpG island within exon 2, a nonlinear trend of increasing hazard with increasing methylation was identified, that is, the risk of hazard accelerated with increasing methylation. This relationship was statistically significant for probe 11 ($P = 0.02$; Fig. 1D) and 13 ($P = 0.019$), and borderline significant for probe 12 ($P = 0.06$). Term-plots for the majority of probes outside of exon 2 approximated a horizontal line, indicating that there was no strong relationship between increasing methylation and DSS (Supplementary Fig. S4). The relationship between BCL-2 expression by RNASeq and DSS, while approaching significance ($P = 0.078$), was weaker and showed no trend toward increasing hazard with lower expression levels (Fig. 1D), indicating that BCL-2 methylation was a more precise predictor of survival.

BCL-2 methylation in cell models of endocrine resistance

Collectively, our analyses of TCGA data led to the identification of a specific region of the *BCL-2* gene whose methylation status correlated with reduced gene expression and reduced patient survival in breast cancer. Data permitting the analysis of the relationship between BCL-2 methylation and response to endocrine therapy were, however, not available for this cohort. Thus, to determine whether BCL-2 was silenced by DNA methylation in endocrine resistance, we used well-characterized cell line models of endocrine resistance. These models were generated by long-term culture of MCF7 hormone-responsive breast cancer cells in the presence of the clinically relevant antiestrogens tamoxifen (TAMR; ref. 29) or fulvestrant (FASR; 30), or in the absence of estrogen (MCF7X) to provide a model of acquired resistance to estrogen-deprivation strategies (31). In contrast to the parental MCF7 cells, BCL-2 expression was significantly suppressed in the MCF7X cell-model and barely detectable in TAMR and FASR cell lines (Fig. 2A and B). Critically, BCL-2 suppression was not associated with reduced expression of BCL-xL, a closely related antiapoptotic factor, nor did it correlate with ER status (Fig. 2B).

The methylation status of 32 CpG residues in a 456 bp region of DNA, which spanned from the 3′ end of exon 2 to beyond the ER-binding site (overlapping 450K methylation probes 9 to 11—chr18:-60,985,184-60,985,640, GRCh37/hg19), was interrogated using clonal bisulfite sequencing (Fig. 2C). Relative to the MCF7 cells, there was an increase in DNA methylation detected in the resistant cell lines, particularly at the first four CpG sites (Fig. 2C). Of the first four CpG sites interrogated, only 5 of 72 (i.e., 18 clones per CpG site) were methylated in the MCF7 cells (7%), in contrast with the MCF7X (24 of 72; 33%), TAMR (43 of 72; 60%), and FASR cells [14 of 44 (11 clones per CpG site); 32%]. The greatest increase in methylation was observed in the TAMR cells, particularly at the BCL-2 ER-binding site, which was almost entirely methylated (Fig. 2C).

Effects of antimitotic agents on endocrine-resistant cell lines

Because treatment options are limited in endocrine-resistant breast cancer, we next investigated whether BCL-2 suppression in cell line models of endocrine resistance potentiated the effects of antimitotic agents. We focused on the Plk1 inhibitor, BI2536 (Fig. 3A), one of a new generation of antimitotic therapies specifically targeting mitotic proteins, thus potentially offering therapeutic advantages over currently used antimitotic therapies that target tubulin (6, 7). The therapeutic value of Plk1 inhibition in endocrine-resistant breast cancer is further highlighted by the strong association of PLK1 expression with early relapse in patients with ER-positive breast cancer receiving endocrine therapy (Cox, $P = 1.13 \times 10^{-8}$; see Supplementary Methods and Supplementary Fig. S5). Furthermore, while TCGA data showed a positive correlation between BCL-2 and ER expression ($\rho = 0.61$) and a negative correlation between PLK1 and ER expression ($\rho = -0.35$), BCL-2 and PLK1 expression were also inversely related ($\rho = -0.43$; Spearman rank correlation test). Of the 46 breast cancers in which low BCL-2 expression was associated with DNA methylation (Fig. 1A), 36 (i.e., 78%) had robust PLK1 expression (Supplementary Fig. S5B).

The antiproliferative effect of BI2536 (1–100 nmol/L) was assessed in all cell lines after 48 hours of treatment (Fig. 3B). Maximal inhibition was achieved following treatment with 10 nmol/L Bl2536; however, the magnitude of the maximal response varied (Fig. 3B). The parental, endocrine-sensitive MCF7 cells were the least responsive, displaying a 30% decrease in cell number (compared with nontreated control cells), whereas the cell number decreased by approximately 45%, 55%, and 60% for MCF7X, FASR, and TAMR cells, respectively (Fig. 3B). This difference in sensitivity was not due to the overexpression of Plk1 in the resistant cells, or the failure of the drug to reduce the expression of active, phosphorylated Plk1 (Fig. 3C). All cell lines were arrested in the G_2–M phase of the cell cycle in response to BI2536 (Fig. 3D). Although there was little difference in the cell-cycle phase distribution between the cell lines, there was a greater increase in the sub-G_1 population of endocrine-resistant cell lines compared with the MCF7 cells following treatment (Fig. 3D), suggesting that increased apoptosis might account for the greater decrease in cell number.

Increased apoptosis in Bl2536-treated endocrine-resistant cells was confirmed by flow-cytometric analysis of cells stained using an antibody specific for caspase-cleaved cytokeratin 18 (M30; Fig. 4A) and by measurement of PARP cleavage (Fig. 4B). Although the percentage of M30-positive MCF7 cells increased 6-fold following 48 hours of incubation with BI2536 (10 nmol/L), the increase in M30 positivity was more marked in the MCF7X (8-fold), TAMR (13-fold), and FASR (11-fold) cells (Fig. 4A). In MCF7 cells, PARP cleavage was not observed following Bl2536 treatment, consistent with the muted cytotoxic response of these cells. However, PARP cleavage was
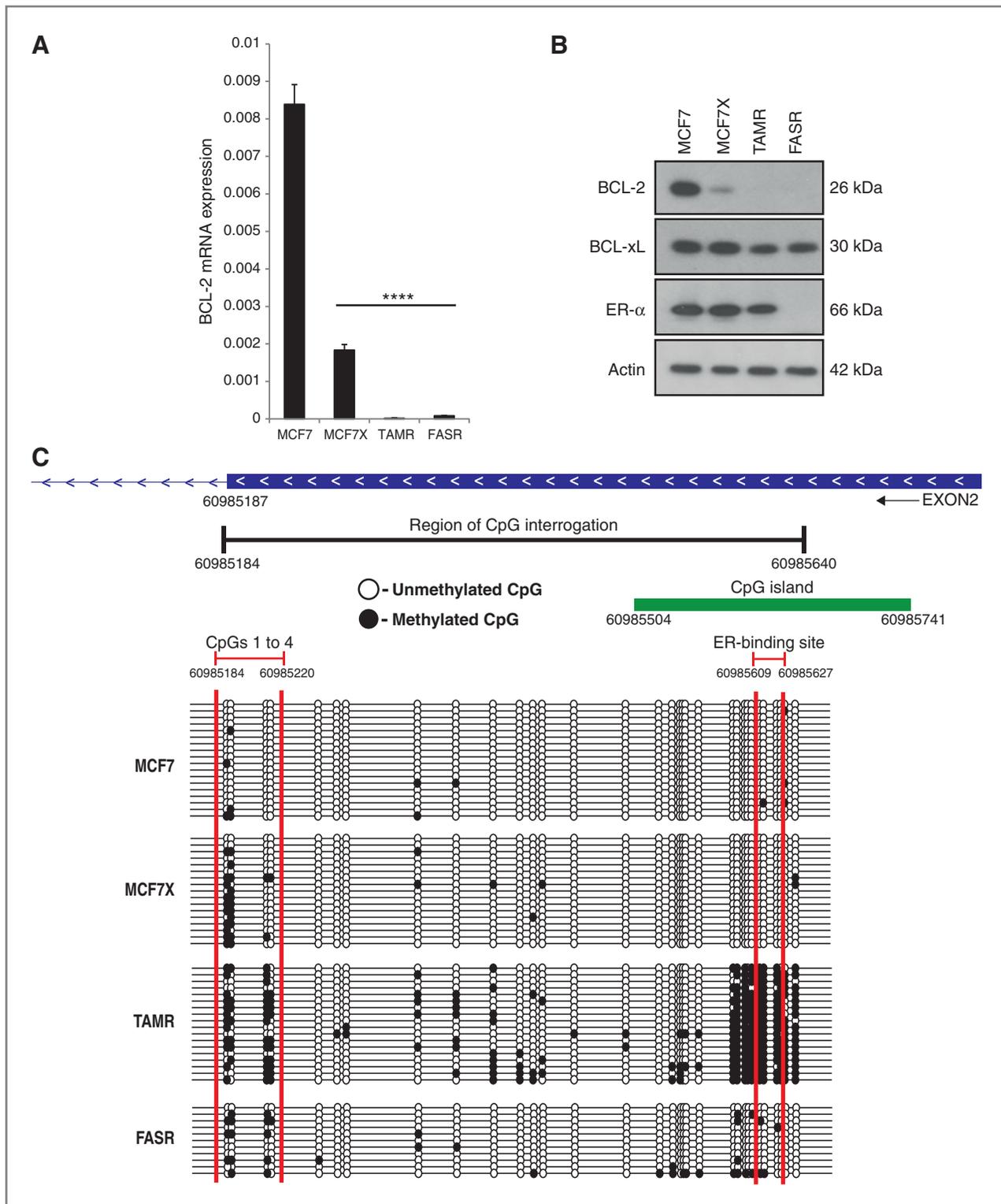
Figure 2. BCL-2 expression is suppressed in cell line models of endocrine resistance and is associated with increased DNA methylation of exon 2 in endocrine-resistant cells. A, BCL-2 mRNA expression in MCF7 cells and the endocrine-resistant sublines MCF7X, TAMR, and FASR cells (****, $P < 0.001$, paired t test). B, BCL-2, BCL-xL, and ER-α protein expression in MCF7 cells and endocrine-resistant sublines. C, clonal bisulfite sequencing data for the interrogation of 32 CpG sites located in exon 2 of the *BCL-2* gene (chr18:-60,985,184-60,985,640). Bisulfite maps determined by direct sequencing of individual clones ($n = 18$ for MCF7, MCF7X, and TAMR; $n = 11$ for FASR) show the density of methylated CpG sites (black circles) and unmethylated CpG sites (white circles) at individual CpG residues. The red lines highlight two regions of differential methylation, that is, the first four CpG residues of exon 2 and those that overlap the ER-α–binding site.
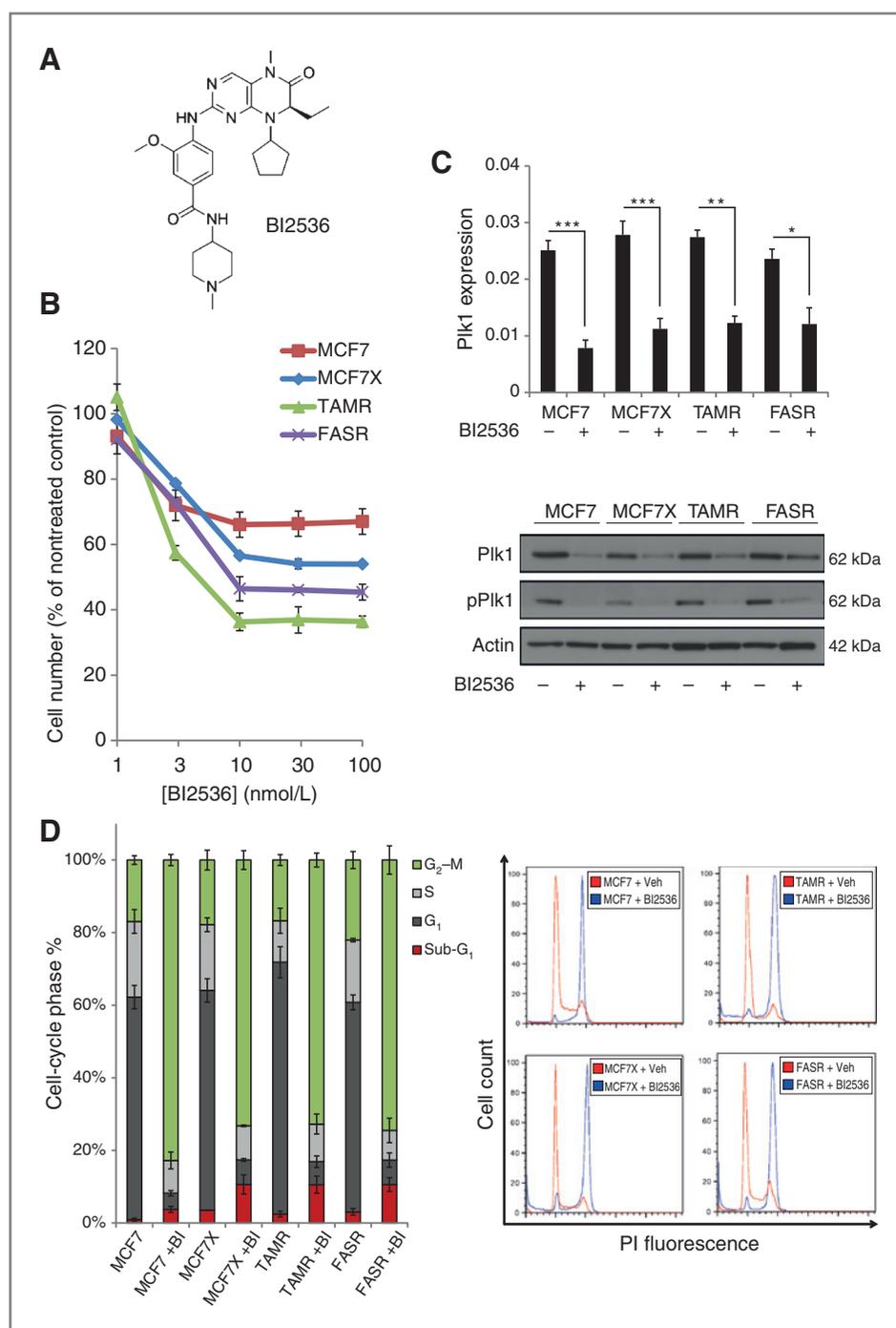
Figure 3. BI2536 (BI) causes G_2–M cell-cycle arrest in endocrine-sensitive and -resistant cell lines. A, the chemical structure of BI2536. B, the antiproliferative effect of BI2536 (1, 3, 10, 30, and 100 nmol/L) was assessed in MCF7, MCF7X, TAMR, and FASR cells after 48 hours using a crystal violet–based assay. Data points represent the mean $\pm$ SD ($n = 3$). C, Plk1 mRNA expression (top) and Plk1 and phosphorylated Plk1 (Thr 210) protein expression (bottom) were measured by qRT-PCR and Western blot analysis, respectively. Gene expression data represent the mean $\pm$ SD ($n = 3$; ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$, paired t test). D, flow cytometry was used to measure cell-cycle phase distribution. Data are presented as percentage of cells in each phase relative to the total population (left, data represent mean $\pm$ SD $n = 3$) and representative DNA histograms (right). Veh, vehicle.

apparent in BI2536-treated MCF7X cells, and readily detectable in TAMR and FASR cells (Fig. 4B), paralleling the relative increase in M30 detection (Fig. 4A). To determine whether the enhanced response of the endocrine-resistant cell lines to BI2536 was characteristic of other antimitotic agents, we treated cells with paclitaxel and nocodazole (Fig. 4C). Again, a significant increase in apoptosis was observed in all three endocrine-resistant cell lines compared with the parental MCF7 cells (Fig. 4D). However, there was no significant change in the

apoptotic response to cell cycle nonspecific chemotherapeutic agent, doxorubicin, in the endocrine-resistant cell lines compared with the endocrine-sensitive MCF7 cells (Fig. 4D).

Relationship between BCL-2 expression and sensitivity to antimitotic agents

To determine whether increased apoptosis following treatment with antimitotic agents was dependent on BCL-2 expression, TAMR cells expressing BCL-2

Figure 4. BI2536 and other antimitotic drugs induce apoptosis in endocrine-resistant, but not endocrine-sensitive cell lines. A, flow cytometry was used to measure the percentage of cells undergoing apoptosis in response to BI2536 challenge (10 nmol/L for 48 hours). Data are presented as mean $\pm$ SD (****, $P < 0.001$; ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$, paired t test; $n = 3$; left) and representative DNA histograms (right). B, PARP cleavage was assessed in cells treated with BI2536 (10 nmol/L for 48 hours) by Western blot analysis. C, chemical structures of paclitaxel (TAX), nocodazole (NOC), and doxorubicin (DOX). D, flow cytometry was used to measure the percentage of cells undergoing apoptosis in response to BI2536 (10 nmol/L), paclitaxel (10 nmol/L), nocodazole (100 ng/mL), and doxorubicin (1 µmol/L) 48-hour challenge. Data are presented as mean $\pm$ SD ($n = 3$; ****, $P < 0.001$; ***, $P < 0.005$, one-way ANOVA).
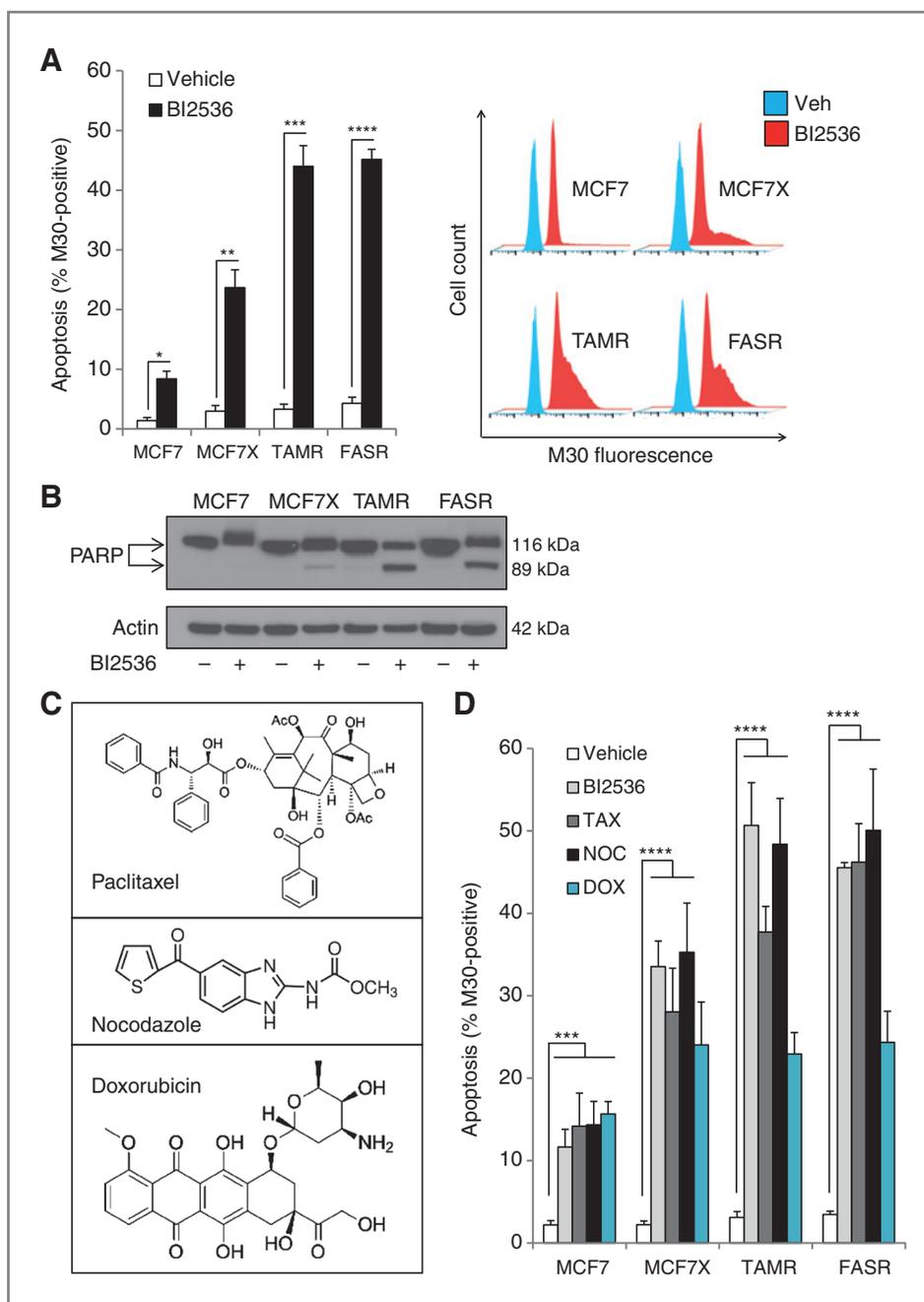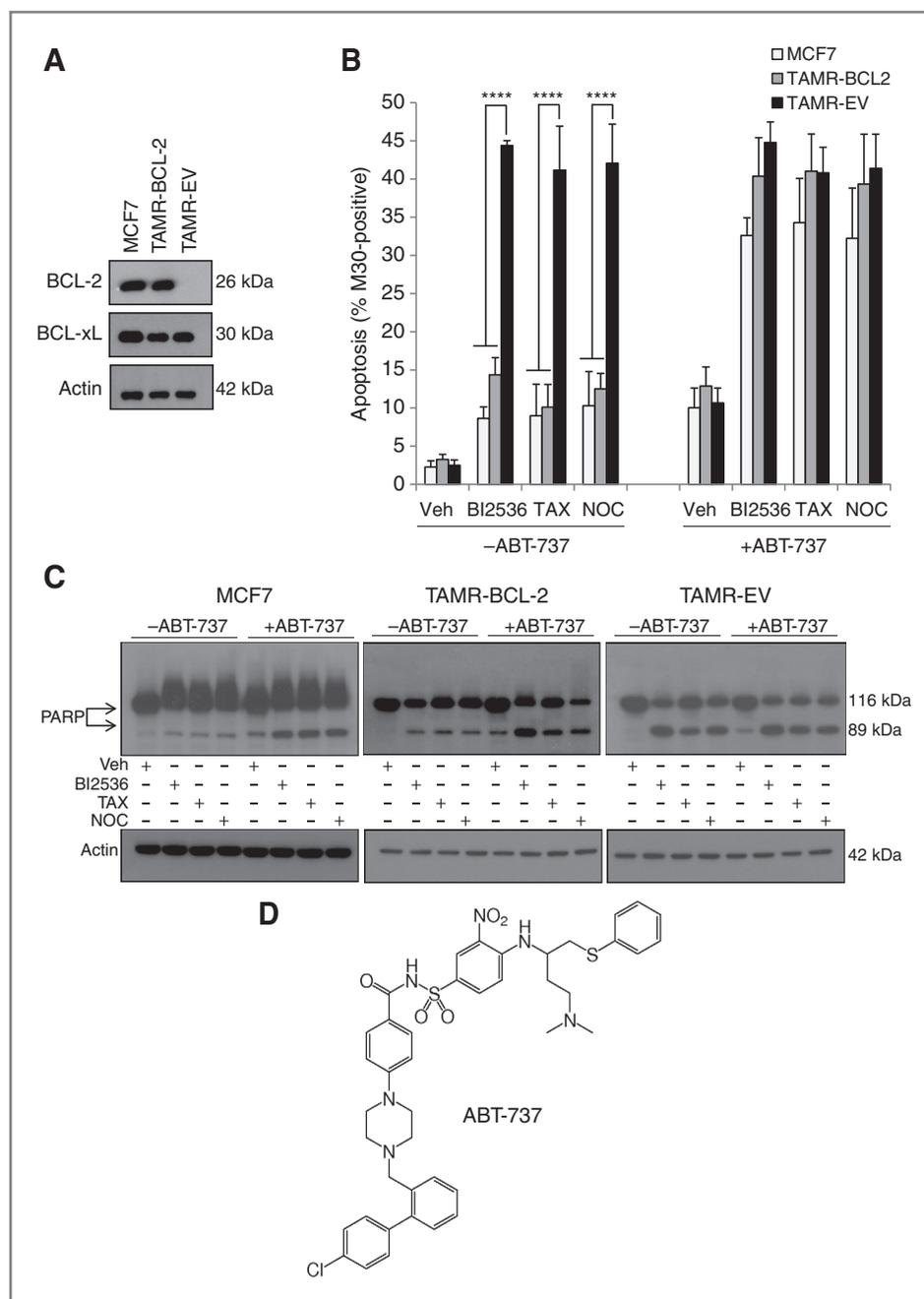
(TAMR-BCL-2) were generated (Fig. 5A). TAMR-BCL-2 cells were significantly less responsive to all antimitotic agents compared with empty vector–transduced TAMR cells (TAMR-EV; Fig. 5B and C) and their apoptotic response comparable with that of the MCF7 cells, as determined by either M30 flow cytometry (Fig. 5B) or PARP expression (Fig. 5C). In contrast, TAMR-BCL-2 and MCF7 cells pretreated with the BCL-2 inhibitor, ABT-737 (Fig. 5D), for 24 hours before exposure to either BI2536, paclitaxel, or nocodazole (for a further 24 hours) exhibited dramatically increased apoptotic responses, comparable with parental TAMR (Fig. 4D)

or TAMR-EV cells (Fig. 5B and C). These cells constitutively express BCL-xL (Figs. 2B and 5A), highlighting BCL-2 expression as a critical determinant of cytotoxic response in these cells (summarized in Supplementary Fig. S6).

Discussion

Despite decades of research dedicated to deciphering the mechanisms by which endocrine-resistant cells evade the inhibitory effects of antihormone drugs, effective therapeutic strategies for the management of endocrine-resistant breast cancer have yet to be identified. In the

Figure 5. Antimitotic drug-induced apoptosis is dependent on BCL-2 expression. A, TAMR cells were generated to ectopically express BCL-2 (TAMR-BCL-2) or empty vector (TAMR-EV). The overexpression of BCL-2 had no impact on BCL-xL expression, as evidenced by Western blot analysis. B, flow cytometry was used to measure the percentage of cells undergoing apoptosis in response to BI2536, paclitaxel (TAX), or nocodazole (NOC; 24 hours) following 24-hour prior culture $\pm$ ABT-737 (1 μmol/L). Data are presented as mean $\pm$ SD ($n = 3$; ****, $P < 0.001$, one-way ANOVA). C, PARP cleavage was assessed in parallel by Western blot analysis. D, the chemical structure of ABT-737. Veh, vehicle.

present study, we provide evidence that the expression of BCL-2 is diminished in cell models of acquired endocrine-resistant breast cancer in association with increased DNA methylation of the second exon; a region that contains a CpG island and an ER-binding site. The reduced expression of BCL-2 conferred increased sensitivity to cytotoxic agents including the Plk1 inhibitor, BI2536 and paclitaxel; highlighting BCL-2 methylation as a potential biomarker of response to current and next-generation cytotoxic chemotherapy in nonresectable metastatic endocrine-resistant breast cancer.

Proof-of-concept analysis has shown that circulating tumor DNA is an informative, inherently specific, and highly sensitive biomarker of metastatic breast cancer (35). Furthermore, recent publications have shown the prognostic potential of methylated DNA, readily detected in serum samples from patients with breast cancer (36, 37). The detection of methylated DNA as a biomarker of disease has several advantages over protein-based assays as defined regions of cancer-specific hypermethylated-DNA can be readily amplified from samples collected noninvasively using PCR-based technology (38). In

addition, panels of DNA methylation probes specific to multiple genes can be readily assembled to further inform diagnosis, as shown for lung cancer (39), ovarian cancer (40), and prostate cancer (41). Although the detection of methylated BCL-2 has not been assessed in breast cancer patient serum, recent publications describe the detection of BCL-2 methylation in blood samples derived from patients with pancreatic cancer (42), and in urinary samples used to detect and monitor bladder cancer (43).

To our knowledge, the methylation of the DNA sequence encoding the second exon of BCL-2 has not been previously linked to gene expression in breast cancer. Interestingly, methylation of this region was observed in the development of colorectal carcinoma, however, it did not correlate with expression (44). This discrepancy between the relationship of BCL-2 methylation to gene expression in breast and colorectal cancer may be explained by the presence of an ER-binding site within the small CpG island in exon 2. BCL-2 expression is largely dependent on estrogen regulation in breast cancer cells (15), and thus hypermethylation of the ER-binding site is more likely to diminish BCL-2 expression in estrogen-responsive tissues.

BCL-2 expression was silenced in association with increased DNA methylation in approximately 10% of TCGA breast tumor samples. Although this equates to a substantial number of patients due to the high prevalence of the disease, this statistic is reflective of BCL-2 methylation incidence in nonstratified, untreated breast cancer patient samples. We hypothesize that the percentage of patients with silenced BCL-2 would be significantly greater in a cohort of patients with acquired tamoxifen resistance, as reflected in our *in vitro* models of endocrine resistance. Interestingly, BCL-2 methylation was a stronger molecular marker of disease-specific death compared with gene expression. One possible explanation for this observation is that DNA hypermethylation is indicative of permanent epigenetic gene suppression, whereas in patients with no detectable BCL-2 methylation, but low BCL-2 expression, gene suppression may instead be a more transient result of altered regulation. Thus reduced BCL-2 expression likely combines two distinct phenotypes, one of which (BCL-2 promoter methylation) has a greater impact on prognosis, perhaps because it is a marker of more widespread epigenetic gene suppression.

All endocrine-resistant cell lines exhibited increased methylation in the region of BCL-2 DNA interrogated by clonal bisulfite sequencing, and as even the smallest of changes in DNA methylation can have a significant impact on gene transcription, this likely contributes to diminished BCL-2 expression in these cell lines. However, the pattern of methylation varied. In contrast to the TAMR cells, the FASR and estrogen-deprived cells did not exhibit DNA hypermethylation of the BCL-2 ER-binding site. This inconsistency may be attributed to the distinct mechanisms by which different endocrine therapies exert their antiestrogen action. The presence of tamoxifen-bound ER at the gene promoter serves to accelerate the

process of epigenetic silencing through the direct recruitment of corepressors of transcription capable of recruiting DNA-methyl transferases (DNMT; ref. 45). In FASR or estrogen-deprived cells, ER-binding sites remain unoccupied because of diminished levels of receptor or ligand, respectively, and thus epigenetic modifiers are not directly recruited to the site of transcription. It is possible, however, that the BCL-2 ER-binding site is suppressed by early epigenetic changes in the FASR and MCF7X cells, as repressive chromatin modifications are known to accumulate at ER-binding sites in the absence of ER-signaling, as shown in proof-of-principle studies using ER siRNA (46).

Significantly, our data indicate that Plk1 inhibition may provide a novel therapeutic strategy in endocrine-resistant breast cancer, where low expression of BCL-2 serves to lower the apoptotic threshold of these cells (summarized in Supplementary Fig. S6). The potential applicability of this therapeutic strategy to endocrine-resistant breast cancer is further highlighted by the strong association between Plk1 expression and tamoxifen failure in patients with ER-positive breast cancer. However, despite promising preclinical studies, Plk1 inhibitors have had limited success when used as single agents in patients. The most recent clinical trials in non-Hodgkin's lymphoma and pancreatic cancer have proven disappointing, with overall response rates of 9.8% and 2.3%, respectively (47, 48). The failure of Plk1 inhibitors in the clinic is reflective of the lack of proven predictive biomarkers of response. Most recently p53 status was assessed as a predictor of response to Plk1 inhibition. No correlation between p53 expression/mutation and cytotoxic effect was observed, despite reports that the cytotoxicity induced by Plk1 inhibition is elevated in cancer cells with defective p53 (49). Our data suggest that BCL-2 status could potentially provide a robust predictive biomarker of response to Plk1 inhibition. Furthermore, Plk1 inhibitors could be used in combination with BCL-2 inhibitors, such as ABT-737 or the BCL-2–specific inhibitor, ABT-199, to maximize the cytotoxic effect on cancer cells that express BCL-2. Indeed, the antitumor effect of synergistic inhibition of BCL-2 and Plk1 expression has been previously shown in xenograft models using antisense oligodeoxynucleotides (50).

Taken together, these data show that BCL-2 expression is associated with DNA methylation in human breast cancer and this represents a likely mechanism for diminished BCL-2 expression in a proportion of patients with breast cancer with acquired endocrine resistance. As such, BCL-2 hypermethylation may provide a robust biomarker of response to current and next-generation cytotoxic agents, such as BI2536, which has significant therapeutic value in endocrine-resistant disease, given the strong association between Plk1 expression and early relapse in patients receiving endocrine therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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