

ORIGINAL ARTICLE

The BH3-only protein Bad confers breast cancer taxane sensitivity through a nonapoptotic mechanism

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Antimitotic agents such as taxanes (paclitaxel and docetaxel) have greatly advanced the treatment of breast cancer, although variable patient response and drug toxicity are major limitations. Lack of validated predictive markers for taxane responsiveness precludes *a priori* identification of patients who are most likely to respond to treatment; thus, a subset of patients endure toxic side effects with marginal benefit. Mechanistic insights into taxane therapeutic activity may lead to rational therapeutic improvements. In this paper we report that the proapoptotic BH3-only protein Bad has a major role in taxane-induced cell death *in vitro*, and clinically is a prognostic indicator for overall survival of breast cancer patients after adjuvant taxane chemotherapy. Unexpectedly, Bad did not induce the mitochondrial apoptotic machinery in response to taxane treatment. Instead, Bad indirectly facilitated cell death by stimulating cellular proliferation. As dividing cells are the targets of taxane therapy, Bad-stimulated proliferation may be a marker of taxane sensitivity. Our studies indicate that quantification of Bad protein levels may have value as a diagnostic tool. They also suggest that cells expressing Bad are more sensitive to taxanes because of their altered cell cycle dynamics and reveal a clinically relevant proliferative role of Bad in breast cancer.

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Introduction

Taxane drugs, paclitaxel and docetaxel, are front-line chemotherapeutic agents used in the treatment of breast,

ovarian and lung cancers. Despite their widespread use, substantial shortcomings include myelosuppression, neurotoxicity and the frequent development of resistance (Perez, 1999; McGrogan *et al.*, 2008). Improvements to taxane-based therapies are hampered by a lack of mechanistic knowledge regarding its therapeutic activity: taxanes alter microtubule dynamics and cause arrest at the G2/M phase of the cell cycle (Jordan *et al.*, 1993; Yvon *et al.*, 1999), but the manner in which this mitotic arrest results in cell death is not clear (Pellegrini and Budman, 2005; Weaver and Cleveland, 2005; Gascoigne and Taylor, 2009). Mechanistic insights into taxane-induced cytotoxicity will have two major clinical benefits. First, each effector molecule has the potential to predict taxane responsiveness in breast cancer patients. Identification of predictive markers is of major importance, as currently there is no rational selection of those patients most likely to benefit from taxane therapy (Aapro, 2001; Noguchi, 2006). Second, knowledge of the protein–interaction networks that modulate cellular responses to taxanes may identify targets for future drug development or combination therapy. Therefore, our objective in this study was to elucidate the mechanism of action of the prototypic taxane, paclitaxel, in breast cancer cells and to seek clinical confirmation of these findings.

Extensive literature shows that paclitaxel-induced cell death converges on the mitochondria and is regulated by the Bcl-2 family of proteins. Paclitaxel-induced mitochondrial dysfunction is initiated by the BH3-only Bcl-2 family member Bim, as shown in mouse model systems (Bouillet *et al.*, 1999; Tan *et al.*, 2005) and in certain human cell lines (Sunters *et al.*, 2003; Li *et al.*, 2005), but not in breast cancer cell lines (Czernick *et al.*, 2009). Species- and cell-type specific differences likely dictate those signalling molecules that are activated in response to paclitaxel, and we uncovered evidence to test whether Bad, another BH3-only protein, was involved in paclitaxel-mediated cell death of clinically relevant breast cancer cells.

Bad was originally identified as a Bcl-2-interacting protein (Yang *et al.*, 1995). Bad mediates cell death in response to survival signal downregulation and has a

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key role in growth factor-regulated apoptosis of developing nervous and immune systems (Zha *et al.*, 1996; Datta *et al.*, 2002). Growth factor-stimulated kinases phosphorylate Bad at serine residues 112, 136 and 155 (mouse numbering), resulting in attenuation of Bad prodeath activity through sequestration by 14-3-3 proteins (Zha *et al.*, 1996; Datta *et al.*, 2000; Lizcano *et al.*, 2000; Virdee *et al.*, 2000). Loss of survival signalling results in dephosphorylation of Bad (Chiang *et al.*, 2003; Klumpp *et al.*, 2003; Roy *et al.*, 2009), release from cytosolic 14-3-3 proteins (Zha *et al.*, 1996; Peruzzi *et al.*, 1999; Datta *et al.*, 2000; Shimamura *et al.*, 2000; Tan *et al.*, 2000; Zhou *et al.*, 2000) and subsequent migration to the mitochondria, in which Bad functions to repress prosurvival proteins Bcl-2, Bcl-XL and Bcl-w (Danial *et al.*, 2008; Letai, 2008; Youle and Strasser, 2008). Because Bad induces cell death through inhibition of antiapoptotic proteins, Bad is described as an 'indirect' activator of apoptosis. It is through this mechanism that Bad induces apoptosis of breast cancer cells in response to loss of survival signalling mediated by epidermal growth factor (Gilmore *et al.*, 2002) and oestrogen (Fernando and Wimalasena, 2004). Therefore, we initially hypothesized that Bad is similarly stimulated in response to paclitaxel treatment.

To address this hypothesis, we studied paclitaxel-treated cultured breast cancer cells to explore the mechanism of Bad-dependent cell death. As described below, we found that the BH3-only protein, Bad, contributes to paclitaxel-induced cytotoxicity of breast cancer cell lines. Surprisingly, we found that, unlike growth factor withdrawal, Bad contributed to paclitaxel-induced apoptosis using a mechanism that was independent of interactions with mitochondria or Bcl-XL, Bcl-2 or Bcl-w. Instead, we observed that Bad stimulated G1 exit with subsequent progression into G2/M. This proproliferative signal ensured that cells underwent mitotic arrest, which is a requirement for paclitaxel to trigger a cell death signal.

Clinical findings further confirmed the importance of these results. In immunohistochemical analysis of pretreatment clinical samples from 180 docetaxel-treated women with breast cancer, high levels of Bad protein within carcinoma cells were associated with improved patient survival. We conclude that Bad is an important effector molecule for docetaxel responsiveness in the clinical setting. *In vitro* mechanistic insights suggest that Bad contributes to taxane-induced cell death through a proproliferative activity of Bad, distinct from its well-known proapoptotic role.

Results

To investigate those BH3-only proteins that are involved in paclitaxel-induced apoptosis, we analysed a panel of five validated breast cancer cell lines acquired from Dr Gordon Mills (MD, Anderson Cancer Center, University of Texas); validated cells were used to avoid any controversy with respect to lineage authenticity

(Graham *et al.*, 1986; Osborne *et al.*, 1987). We treated each cell line with 25 nM of paclitaxel, which induces a clinically relevant intracellular accumulation of the drug in tissue culture conditions (Jordan *et al.*, 1996; Derry *et al.*, 1998). After the indicated amount of time, we assessed apoptosis by measuring mitochondrial electrochemical potential loss (TMRE loss) and phosphatidyl serine externalization (annexin V positivity; Figure 1a). We also used colony formation assays to measure long-term clonogenic survival after paclitaxel treatment (Figure 1b). The relative paclitaxel sensitivities of the various breast cancer cell lines in rank order of increasing sensitivity were MDA-MB-231, T47-D, MDA-MB-468, MCF-7 and SKBR-3. It is noteworthy that, whereas the MCF-7 cell line showed a robust loss of mitochondrial potential in response to paclitaxel, phosphatidyl serine externalization was blunted because these cells do not express caspase 3 (Janicke *et al.*, 1998); however, on the basis of mitochondrial dysfunction, clonogenic survival and cellular morphology, it was clear that paclitaxel induced significant death of these cells. We sought correlations between the expression levels of Bcl-2 family proteins in these cell lines with drug sensitivity. As can be seen in Figure 1c, protein expression levels were widely variable. Of the BH3-only proteins, only the expression of Bim and Bad correlated with sensitivity to paclitaxel. As we had previously reported that Bim depletion did not protect these cells from paclitaxel-induced cytotoxicity (Czernick *et al.*, 2009), we pursued loss-of-function studies to assess whether Bad expression contributed to cellular sensitivity to paclitaxel.

We used small interfering RNAs (siRNAs) to test the contribution of Bad expression towards paclitaxel cytotoxicity. siRNA duplexes targeted against Bad, Bim and Bid were transiently transfected into the MCF-7 breast cancer cell line. Knockdown efficiency was monitored by western blotting (Figure 2a, upper). Paclitaxel-mediated cell death as assessed by loss of mitochondrial electrochemical potential was significantly reduced only in cells transfected with Bad-targeted siRNA and not with siRNA targeted against Bim, Bid, a negative control sequence (Figure 2a, see red arrow; and Figure 2b) or with siRNAs to Bim and Bid together (Supplementary Figure S1). Significant reduction of paclitaxel-mediated apoptosis was also induced by an independent siRNA target sequence to Bad (Figure 2c, Bad2). Finally, to determine whether multiple breast carcinoma cell lines were dependent on Bad expression for sensitivity to paclitaxel, we tested SKBR-3 and MDA-MB-468 in addition to MCF-7 cells (Figure 2d). Depletion of Bad protected all these cell lines from paclitaxel-induced cytotoxicity, indicating that Bad has a significant role in paclitaxel-induced cell death in multiple breast cancer cell lines.

Given that Bad levels contributed to paclitaxel responsiveness in cell line model systems, we pursued clinical evaluation of Bad protein levels as a prognostic marker in taxane-treated breast cancer. We queried a data set compiled by Chang *et al.* (2003) that comprised

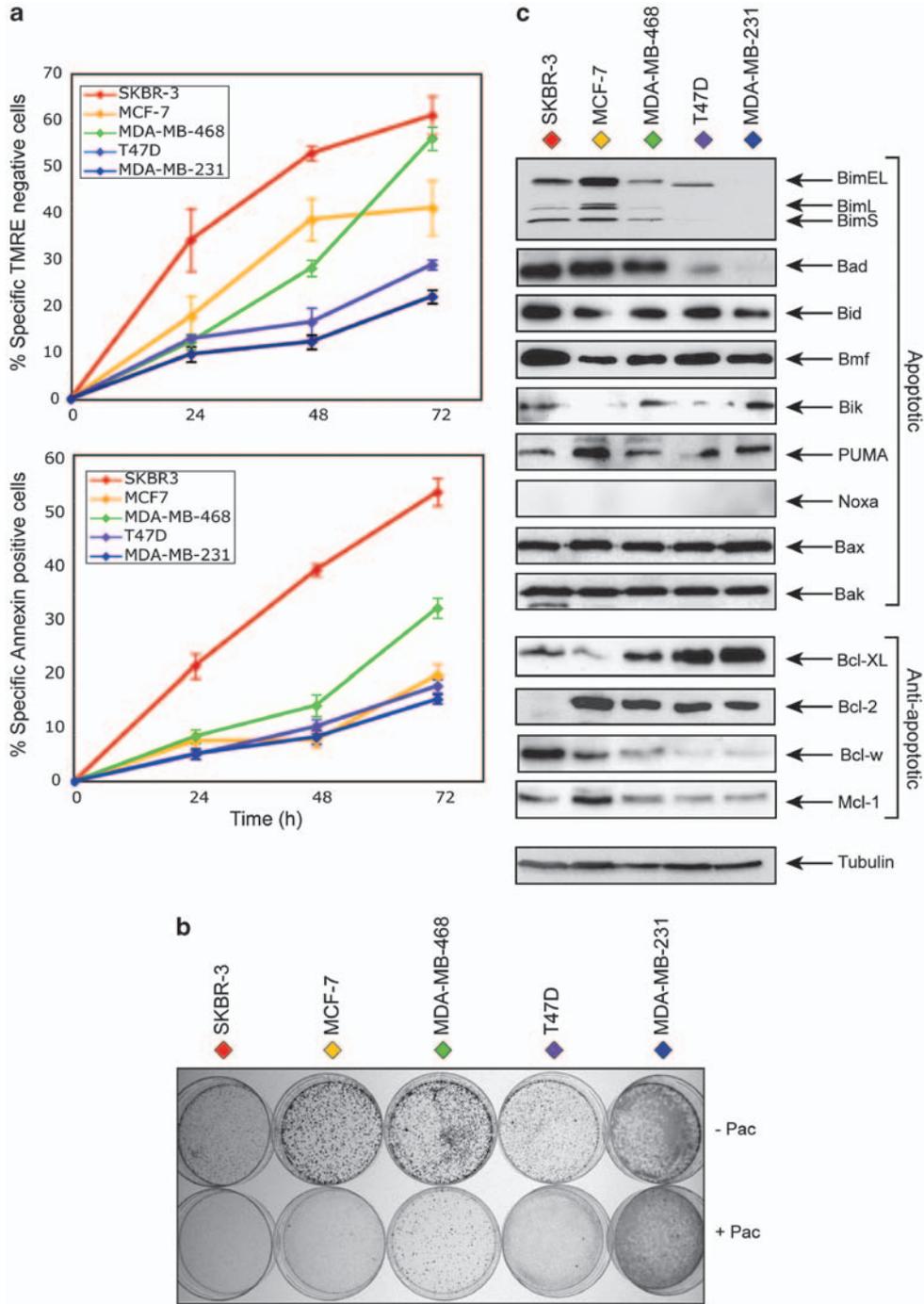


Figure 1 Expression levels of proapoptotic Bim and Bad correlate with paclitaxel sensitivity. **(a)** Indicated breast carcinoma cell lines were treated with 25 nM paclitaxel (Pac) for the indicated amount of time and assessed for mitochondrial depolarization (upper: TMRE negative). Data are graphed relative to control untreated cells (% specific TMRE-negative cells = %TMRE-negative paclitaxel-treated cells minus %TMRE-negative untreated cells). Phosphatidyl serine externalization (lower: annexin V positive) was also determined in a similar manner. Data are represented as mean \pm s.d. Shown is an average of three independent experiments. **(b)** Indicated cell lines were treated with 25 nM paclitaxel for 48 h, after which 5000 untreated and 500 000 paclitaxel-treated cells were plated and allowed to grow for 8 days before staining with crystal violet. **(c)** Untreated whole-cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis western blot and analysed with the indicated antibodies.

gene expression data from 24 tumour samples from breast cancer patients before neoadjuvant docetaxel treatment. After treatment, the samples were stratified as sensitive or resistant based on residual tumour

volume of less than or greater than 25%, respectively. We assessed expression levels of Bcl-2 family members and, similar to Chang *et al.* (2003), observed elevated levels of Bax mRNA in association with tumour

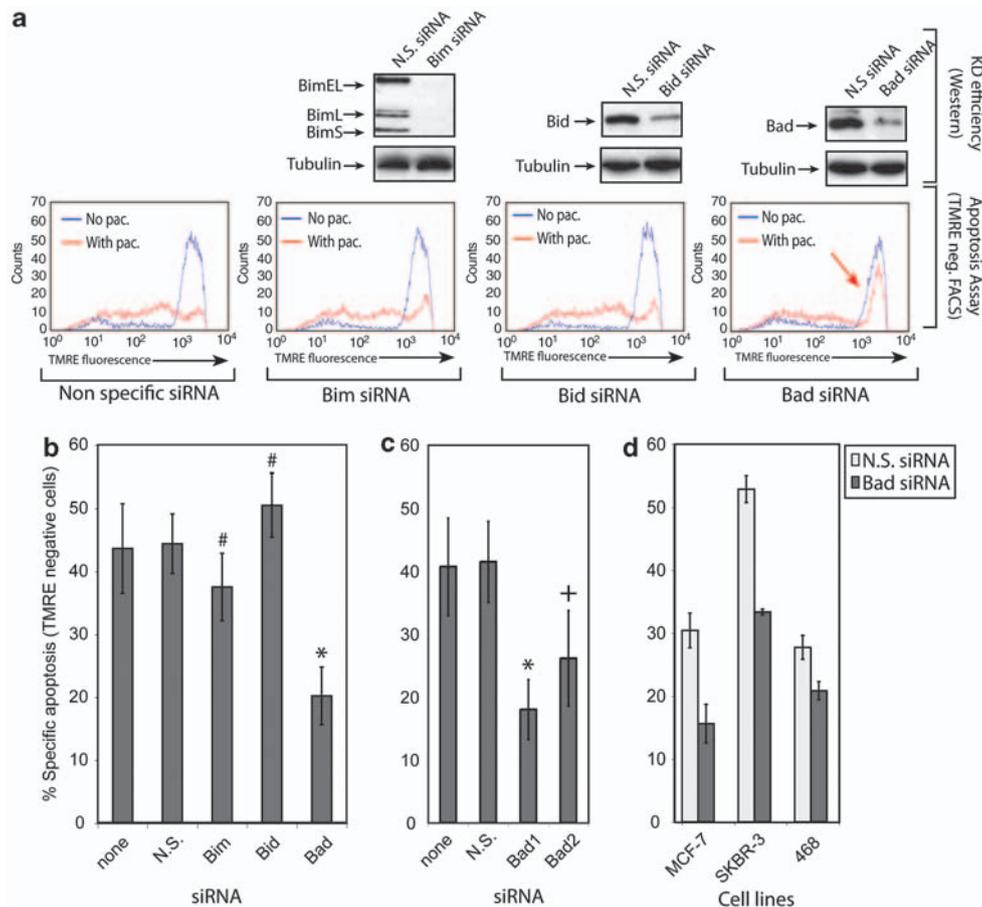


Figure 2 Bad has a significant role in paclitaxel-induced cell death. (a) Knockdown efficiencies in MCF-7 breast carcinoma cell lines transfected with nonspecific (NS) or specific indicated targets were assessed by western blot analyses 72 h after transfection (upper). Apoptotic mitochondrial depolarization in the absence (blue) or presence of 48 h of 25 nM paclitaxel (red) was determined by the measurement of TMRE fluorescent-activated cell sorting analysis (lower). (b) MCF-7 cells were transfected with siRNAs corresponding to NS sequence or the indicated BH3-only target sequences, and apoptosis was assessed after 48 h treatment with paclitaxel. Shown is an average of three independent experiments. # represents $P < 0.1$ compared with nonspecific siRNA control. ** represents $P < 0.0005$ compared with nonspecific siRNA control. (c) similar to (b), using two independent siRNA target sequences to Bad. Shown is an average of three independent experiments. ** represents $P < 0.0005$ compared with non-specific siRNA control. + represents $P < 0.0025$ compared with nonspecific siRNA control (d) similar to (b), assessing the effect of Bad depletion on paclitaxel responsiveness in indicated cell lines. Shown is a representative of two independent experiments carried out in triplicate. All data are represented as mean \pm s.d.

regression. Of the other family members that we tested, only Bad and Bik mRNA levels positively correlated with reduced tumour volume (Figure 3a). Bad mRNA levels on an average were two-fold higher in tumours classified as docetaxel sensitive. To further test the prognostic value of elevated Bad expression, we evaluated Bad protein levels with respect to clinical response to taxane treatment from an independent study. After local research ethics board approval, we retrieved 180 formalin-fixed paraffin-embedded primary tumours from patients who had received adjuvant docetaxel-based chemotherapy at a single institution (Cross Cancer Institute, Edmonton, Alberta, Canada) for whom complete baseline information and long-term outcome data were available. Bad staining was scored semiquantitatively by a breast cancer pathologist blinded to clinical outcomes (Figure 3b). We found significantly increased disease-free survival and overall

survival of individuals with elevated levels of Bad protein ($P = 0.03$ and $P = 0.001$, respectively). In multivariate modelling, only ER and Bad score were independent prognostic factors for disease-free survival and overall survival. Women with low Bad tumour-staining intensity had a higher rate of relapse (hazard ratio 1.96; 95% CI 1.05–3.66) and death hazard ratio (3.65; 95% CI 1.05–3.66). Together, analyses of these two independent data sets showed that elevated Bad levels correlate with patient responsiveness to docetaxel treatment in both the neoadjuvant and adjuvant setting. Further work will involve an independent evaluation of whether Bad levels accurately predict patient response to taxane treatment. Nevertheless, our results, in conjunction with gene expression profiling from other groups (Chang *et al.*, 2008), may lead to the development of validated predictive indicators for taxane responsiveness.

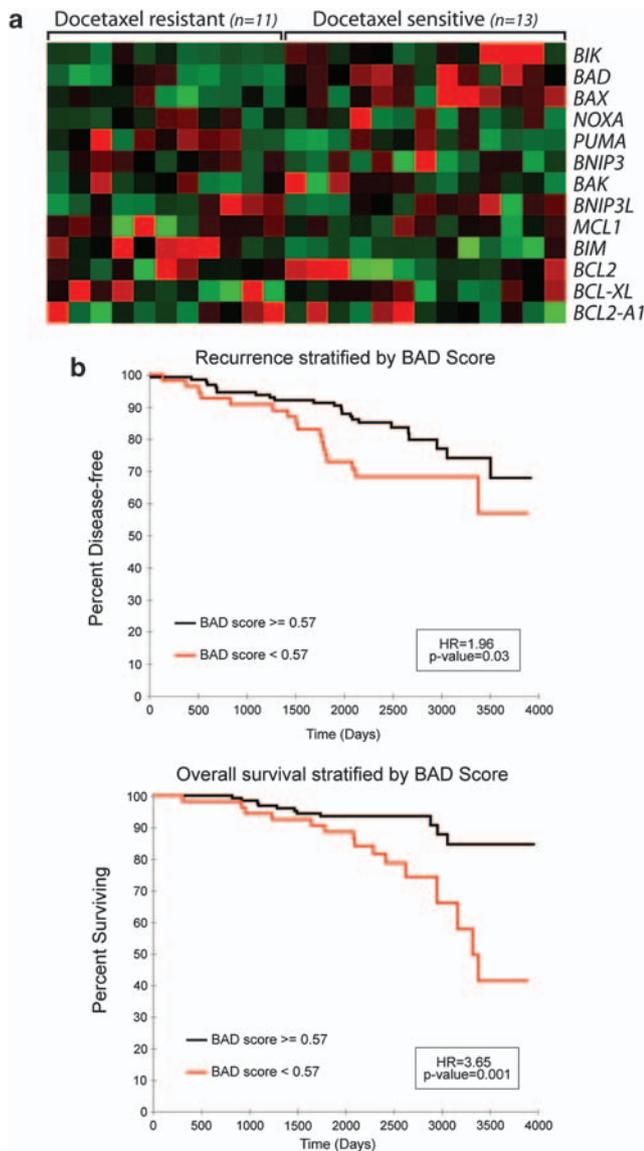


Figure 3 Bad expression correlates with positive clinical outcomes in breast cancer patients. (a) Gene expression analysis for the indicated genes are shown and were obtained from (Chang *et al.*, 2003). Increased or decreased expression is indicated by the intensity of red or green signal, respectively. (b) Kaplan–Meier curves for relapse-free and overall survival for Bad-positive cases compared with those for Bad-negative cases of 180 women with early-stage breast cancer receiving adjuvant docetaxel treatment are shown. Patients were stratified by Bad immunohistochemistry reactivity. Black lines represent 126 patients with Bad-expressing tumours. Red lines represent 54 patients with weak or no Bad expression.

Given that Bad expression was associated with clinical outcome, we wanted to determine the molecular mechanism of Bad activity. On the basis of our functional *in vitro* assays (Figure 2), it was reasonable to propose that taxanes induced the elimination of breast cancer cells through stimulation of Bad proapoptotic activity. To characterize the mechanism of Bad proapoptotic activity as induced by paclitaxel, we postulated that Bad functioned through its well-established role as an ‘indirect’ activator of the mitochondrial apoptotic

machinery. In this model, an apoptotic signal triggers translocation of Bad from the cytosol to the mitochondria, wherein Bad indirectly stimulates mitochondrial dysfunction by interacting with Bcl-XL, Bcl-2 and Bcl-w. As a first step, we determined the intracellular localization of Bad in response to paclitaxel. Cells were treated with either paclitaxel or the positive control apoptotic inducer staurosporine, both of which induced similar levels of cell death. Cell lysates were fractionated into cytosolic supernatant and mitochondria-containing heavy membrane pellet fractions, and Bad localization was determined by western blotting (Figure 4a). As expected, Bad was cytosolic in untreated cells and migrated strongly to mitochondria in response to staurosporine in MCF-7 cells, with moderate translocation to the membrane fractions in SKBR-3 and MDA-MB-468 cells. On the contrary, paclitaxel treatment did not induce mitochondrial translocation of Bad (Figure 4a, compare green arrows). Furthermore, because nuclear localization of Bad has also been reported (Fernando *et al.*, 2007), we used a more specific subcellular fractionation method to examine whether paclitaxel treatment affected accumulation of Bad to the nucleus. As shown in Figure 4b, again staurosporine, but not paclitaxel treatment, stimulated Bad translocation to mitochondrial-enriched membrane fractions, but there was no detectable nuclear accumulation of Bad under any of the conditions tested. Altogether, these results indicate that paclitaxel did not stimulate translocation of Bad to the mitochondria. As mitochondrial electrochemical potential is dissipated in response to paclitaxel, we propose that a BH3 protein other than Bad triggers mitochondrial dysfunction. Although we cannot rule out a transient interaction of Bad with the mitochondria (‘kiss and run’), our inability to detect these interactions suggests that paclitaxel cytotoxicity is not dependent on Bad interactions with components of the mitochondrial apoptotic machinery.

We next investigated the interaction of Bad with its most relevant downstream target, Bcl-XL (Kelekar *et al.*, 1997; Zha *et al.*, 1997). Coimmunoprecipitation studies showed that Bad interacted with Bcl-XL in untreated cells, and remained as a complex in response to staurosporine treatment. In contrast, paclitaxel treatment resulted in decreased association of Bad and Bcl-XL (Figure 4c compare green arrows), indicating that paclitaxel-induced cell death did not require Bad-dependent inhibition of Bcl-XL. In addition, we determined that paclitaxel also did not enhance the binding of Bad to Bcl-2 or Bcl-w, two other antiapoptotic Bad-binding partners (Figure 4d). Together, these results indicate that paclitaxel induces a Bad-dependent cell death pathway, which is distinct from its well-known role as an inhibitor of Bcl-XL, Bcl-2 or Bcl-w.

Interestingly, we found that paclitaxel-treated cells had reduced levels of Bcl-XL protein; an observation that has been previously reported by others (Liu and Stein, 1997). In fact, mitotic arrest-mediated repression of Bcl-XL contributes to cell death by liberation of Bax (Upreti *et al.*, 2008). However, Bcl-XL levels were similar in Bad-depleted and control paclitaxel-treated

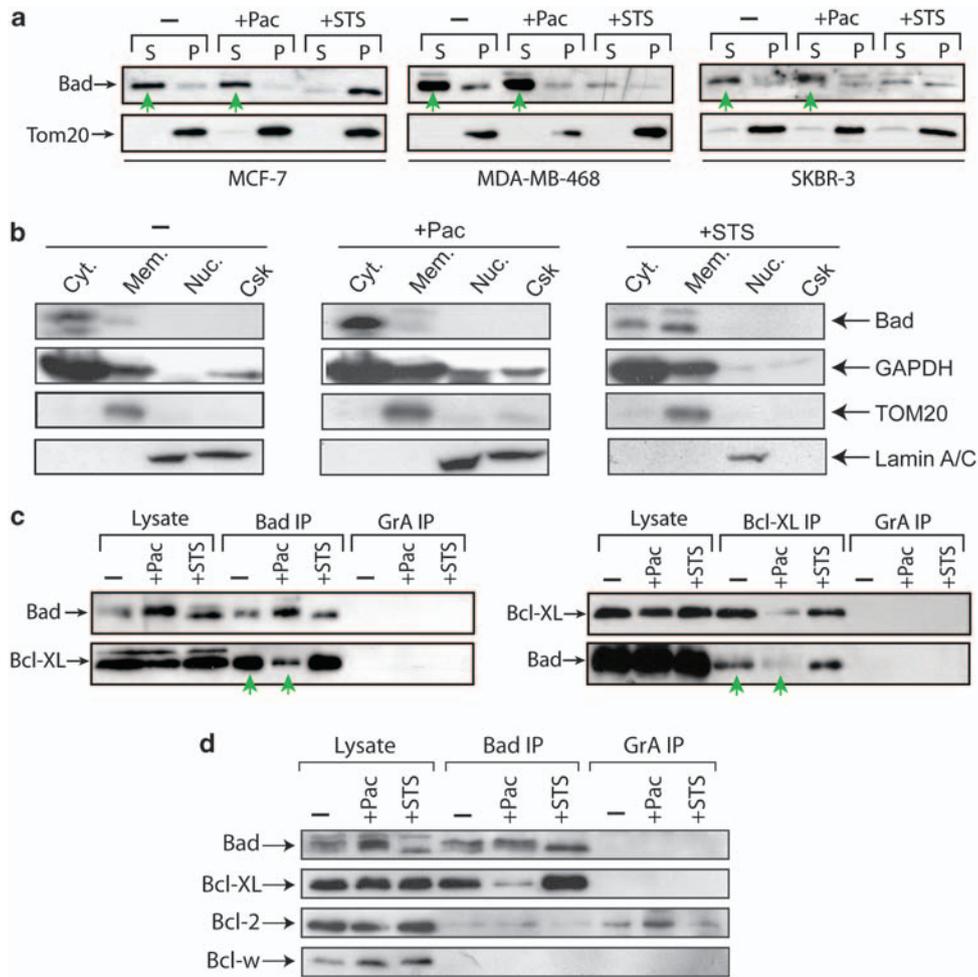


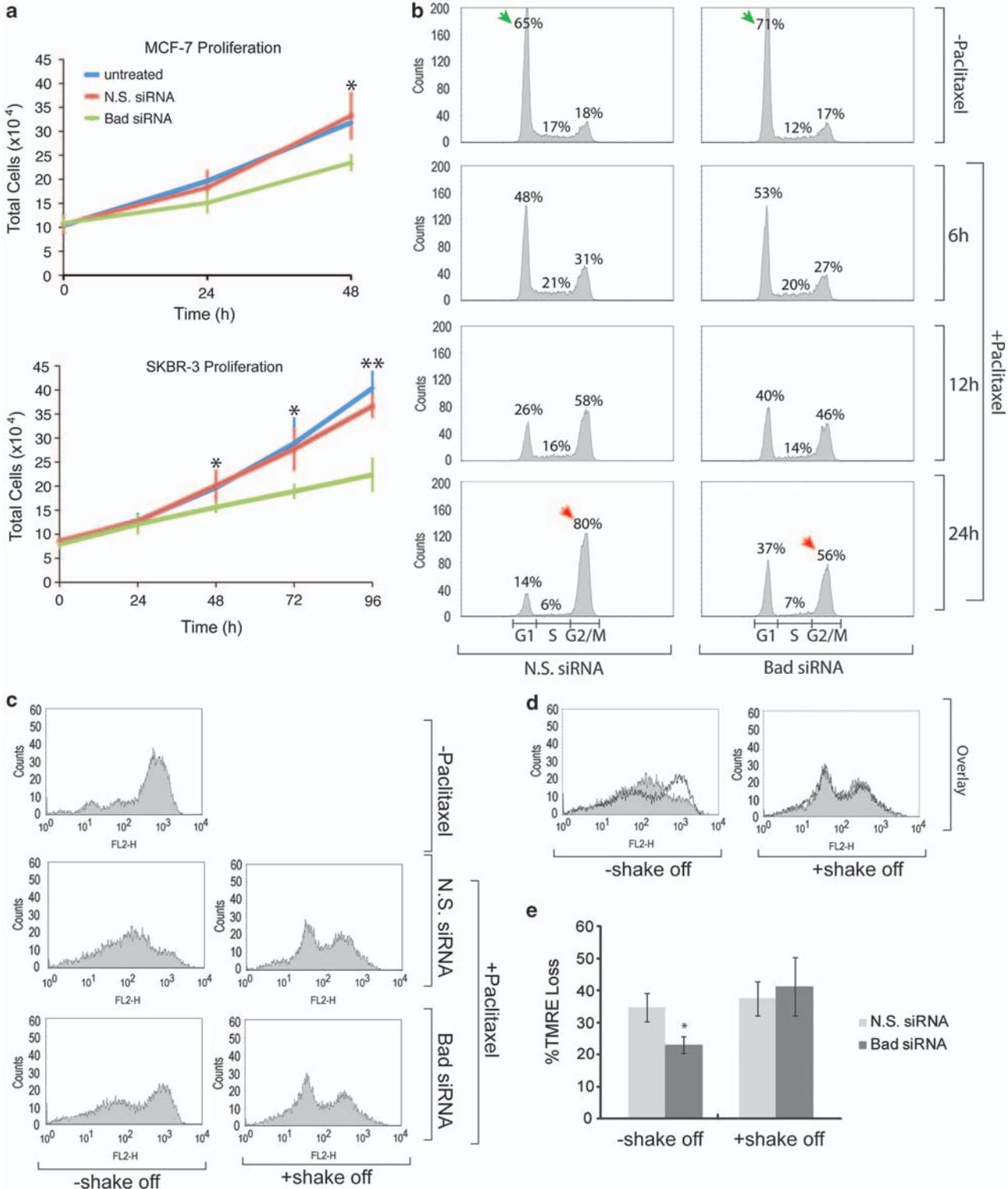
Figure 4 Bad mediates paclitaxel cytotoxicity through a nonapoptotic pathway. **(a)** Paclitaxel does not induce Bad translocation to the mitochondria. MCF-7, MDA-MB-468 and SKBR-3 cells were treated with 25 nM paclitaxel (Pac) for 48 h or with 2.5 μ M staurosporine (STS) for 4 h, and then lysed and fractionated into cytosolic supernatant (S) or heavy membrane pellet (P) fractions. Intracellular localizations of Bad or of mitochondrial membrane control Tom20 were determined by western blot analyses. Green arrows highlight Bad cytosolic localization in response to paclitaxel treatment. **(b)** MCF-7 cells were treated as above and subcellular fractions were obtained with the Q-proteome kit (Qiagen). Intracellular localizations of Bad, mitochondrial membrane marker Tom20, cytosolic marker glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and nuclear marker Lamin A/C were determined by western blot analysis. **(c, d)** Paclitaxel does not induce Bad binding to Bcl-XL or Bcl-2 or Bcl-w. MCF-7 cells that were untreated or treated as above were lysed and subject to immunoprecipitation with antibodies against Bad, Bcl-XL, Bcl-2, Bcl-w or a negative control nonexpressing protein (negative granzyme A), before performing the indicated western blots. Green arrows highlight diminished Bad:Bcl-XL interactions in paclitaxel-treated versus STS-treated cells.

Figure 5 Bad stimulates cell cycle progression that contributes to paclitaxel cytotoxicity. **(a)** MCF-7 and SKBR-3 cells were transfected with no siRNA (untreated), negative control siRNA (NS) or Bad-specific siRNA (Bad). After allowing 24 h for knockdown (labelled 0 h on the graph), the total number of cells was determined over a time course of 48 h (MCF-7) and 96 h (SKBR-3). Data are represented as mean \pm s.d. Shown is an average of at least three independent experiments. * represents $P < 0.05$, whereas *** represents $P < 0.005$ when comparing both the CA and NS treatments to the Bad treatment. **(b)** Bad stimulates G1 exit. MCF-7 cells were transfected with nonspecific (NS) or Bad-specific siRNA, treated with or without paclitaxel for the time points indicated, then fixed, permeabilized and stained with propidium iodide. DNA content was determined by flow cytometry. Green arrows highlight increased proportion of cells in G1 phase of the cell cycle in Bad-depleted cells versus control cells, and red arrows highlight decreased proportion of cells in G2/M phase of the cell cycle in Bad-depleted cells versus control cells, after G1 entry is blocked by paclitaxel treatment. Shown are representative images of three independent experiments. **(c)** Bad sensitizes cells to paclitaxel-induced cell death through its stimulation of cell cycle progression and is not essential for cell death of M-phase-arrested cells. MCF-7 cells were transfected with negative control siRNA (NS) or Bad-specific siRNA. Cells were treated with 25 nM paclitaxel for 39 h, then subjected to a mitotic shakeoff. Cells lifted during the shakeoff (+ shakeoff) were collected and replated. At 48 h of paclitaxel treatment, both + and - shakeoff cells were stained with TMRE and analysed by flow cytometry. **(d)** Overlay of Bad-specific siRNA-treated cells (black line) with NS siRNA cell histogram for results shown in **(c)**. **(e)** Bar graph for results shown in **(c)** and **(d)**. Error bars represent mean \pm s.d. ($n = 3$). * represents $P < 0.05$ compared with NS siRNA -shakeoff cells.

cells (Supplementary Figure S2), suggesting that Bad does not contribute to paclitaxel-mediated apoptosis through Bcl-XL downregulation.

We obtained an understanding of the manner by which Bad contributes to paclitaxel-induced cell death when we observed that Bad stimulated cellular

proliferation. Depletion of Bad decreased the doubling times of MCF-7 and SKBR-3 cells in culture (Figure 5a). Cell-cycle DNA content analysis showed that a consistently higher proportion of Bad-depleted cells was in the G1 stage of the cell cycle (Figure 5b, compare green arrows in the top two plots). These



results suggested that Bad may be required for G1 exit. To verify that the increased proportion of cells in G1 was due to delayed G1 exit and not due to increased G1 entry, we inhibited G1 entry by arresting cells at G2/M with paclitaxel. Cells were incubated for up to 24 h in paclitaxel, which induced mitotic arrest, but was not enough time to induce apoptosis (Czernick *et al.*, 2009). In time course analyses, the progression of cells from G1 through S to G2/M was delayed in Bad-depleted cells relative to control-transfected cells. This delayed cell cycle progression resulted in a lower proportion of Bad-depleted cells in G2/M, relative to control cells (Figure 5b, compare red arrows). These results suggest that Bad contributes to cell cycle progression by facilitating exit from the G1 phase of the cell cycle, enabling cells to enter paclitaxel-induced mitotic arrest and then respond to downstream apoptotic signals.

If it is the proliferative and not apoptotic activity of Bad that contributes to cytotoxicity, we predicted that bypassing the proliferative activity of Bad would mitigate Bad dependence. Thus, the apoptotic rate of cells that had reached M-phase would be similar between control and Bad knockdown cells. Using a mitotic shakeoff strategy, we collected cells that had already entered paclitaxel-induced mitotic arrest and then assessed apoptosis in control versus Bad-depleted cells. As already shown, Bad-depleted cells that had not been enriched for M-phase were protected from paclitaxel-induced apoptosis (Figures 5c–e, –shake off); however, in contrast, once cells had entered mitotic arrest, Bad depletion did not protect cells from paclitaxel cytotoxicity (Figures 5c–e, +shake off). These results support the hypothesis that Bad proliferative activity facilitates paclitaxel-induced cell death.

Discussion

Using two independent data sets, we identified that an elevated level of the Bcl-2 family member Bad is associated with a positive clinical outcome for taxane-treated breast cancer patients. Thus, we have discovered that Bad is a prognostic indicator for breast cancer patient responsiveness to taxane chemotherapy. Furthermore, despite the fact that Bad proapoptotic activity in response to growth factor withdrawal is well documented, our studies showed that, in response to paclitaxel-induced mitotic arrest, Bad did not directly stimulate apoptosis. Instead, the ability of Bad to stimulate proliferation was critical for subsequent paclitaxel-induced cell death. This is because taxanes must induce mitotic arrest to trigger apoptosis (Swanton *et al.*, 2007; Gascoigne and Taylor, 2008). Thus, we have identified a physiologically relevant proliferative role for Bad in breast cancer.

In particular, Bad may be a specific regulator of breast cell growth, is associated with a favourable outcome in breast cancer patients (Al-Bazz *et al.*, 2009) and is therefore a potential drug target for clinical

manipulation. Although we show here that Bad can stimulate proliferation, substantial evidence exists that Bad also mediates apoptosis of breast cells. For example, loss of epidermal growth factor receptor signalling triggers a Bad-stimulated cell death pathway in a mouse model system (Ranger *et al.*, 2003), and in human mammary epithelial cells (Gilmore *et al.*, 2002). In addition, blockade of oestradiol signalling induced Bad-dependent death of MCF-7 cells (Fernando and Wimalasena, 2004), with elevated levels of Bad protein correlating with increased survival of tamoxifen-treated breast cancer patients (Cannings *et al.*, 2007). Bad therefore stimulates an apoptotic pathway in response to epidermal growth factor receptor and ER inhibition, two widely used breast cancer therapies. In normal breast tissue, Bad may also contribute to the development of the mammary gland. Bad is expressed at high levels in breast cells (Kitada *et al.*, 1998), and Bad expression is elevated in apoptotic cells of the mammary gland during involution after pregnancy and weaning (Metcalf *et al.*, 1999; Schorr *et al.*, 1999). Therefore, in terms of clinical applications, stimulation of Bad may confer some selectivity in the quest for targeted elimination of breast cancer cells. Our studies indicate, however, that the mechanics of switching between a progrowth and prodeath role of Bad must first be defined.

Before we consider the clinical manipulation of Bad, we must first fully understand the cellular consequences of stimulating Bad activity. Significantly, Bad is not only a proapoptotic protein but also exhibits progrowth/survival roles in metabolism, autophagy and the cell cycle (Zinkel *et al.*, 2006; Danial, 2008; Danial *et al.*, 2008). Bad overexpression stimulates proliferation, anchorage-independent growth and tumour growth in rodent fibroblasts and prostate cancer cell lines (Chattopadhyay *et al.*, 2001; Smith *et al.*, 2009). In rodent fibroblasts, Bcl-XL mediates this proliferative activity of Bad (Chattopadhyay *et al.*, 2001; Janumyan *et al.*, 2003). Bcl-XL inhibits cell cycle progression in response to growth arrest signals (cell confluency and serum deprivation) by stabilizing the cyclin-dependent kinase inhibitor p27, and by triggering cell cycle exit and arrest in G0 (Janumyan *et al.*, 2008). Although not directly tested, this suggests that Bad indirectly stimulates proliferation by inhibiting Bcl-XL. Upregulation of Bad in some cancer cells (Kitada *et al.*, 1998; Andreeff *et al.*, 1999) could also be an indication of Bad proliferative activity, although it may also be a consequence of oncogene-activated death signals that are stimulated in cancer cells (Letai, 2008). In addition, in liver and pancreatic cells, Bad can also directly increase metabolism by stimulating the glycolytic enzyme glucokinase (GK-Hexokinase IV; Danial *et al.*, 2003, 2008), although glucokinase expression is cell restricted (Matschinsky and Ellerman, 1968) and is not expressed in breast cancer cells.

In summary, our studies into the molecular mechanisms of taxane cytotoxicity may open up new areas for clinical investigation. In particular, identification of Bad as a major factor in paclitaxel-induced cell death *in vitro*,

and correlation with survival of docetaxel-treated patients in the clinic, may lead to the development of Bad as a diagnostic tool. In terms of newer treatment options, because of the requirement for the proliferative activity of Bad in response to paclitaxel treatment, simple stimulation of Bad proapoptotic activity will probably be ineffective in the clinical setting, and at worse, may even promote cancer cell proliferation. Therefore, defining the nonapoptotic Bad-directed signalling pathway is a priority. Detailed understanding may lead to novel drug development and combination therapies.

Materials and methods

Cell culture

Human breast cancer cell lines, MCF-7, SKBR-3, T47-D, MDA-MB-468 and MDA-MB-231, were obtained from Dr Gordon Mills (MD Anderson Cancer Center, University of Texas). Cells were cultured in RPMI 1640 media supplemented with 10% foetal bovine serum.

Reagents

Antibodies against Bim, Bid, Bmf, Bik, PUMA, Noxa, Bcl-XL, Bcl-2 and Bcl-w were from Cell Signaling Technologies (Boston, MA, USA), Bad and Bcl-XL were from Sigma (St Louis, MO, USA) and Cell Signaling Technologies, Bax and Bak were from Santa Cruz (Santa Cruz, CA, USA) and Mcl-1 and tubulin were from Sigma. siRNA duplexes were purchased from Qiagen (Valencia, CA, USA). TMRE, annexin V, DAPI and propidium iodide were purchased from Invitrogen (Carlsbad, CA, USA).

Apoptosis assays and siRNA-mediated knockdown assays

Briefly, breast cancer cell lines were treated with dimethyl sulfoxide vehicle control or 25 nM paclitaxel, which corresponds to a clinically relevant dose (Jordan *et al.*, 1996; Blagosklonny and Fojo, 1999). After treatment, samples were divided and analysed for apoptosis as previously described (Czernick *et al.*, 2009). siRNA-mediated knockdown was performed as previously described (Czernick *et al.*, 2009).

Coimmunoprecipitation

Cells were treated as indicated and lysed in either 2% CHAPS lysis buffer (10% glycerol, 20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA, 2% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulphonate); Figure 4c) or 1% CHAPS lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% CHAPS; Figure 4d), and protease inhibitor (Roche, Mississauga, ON, Canada). Soluble cell lysate was incubated with indicated antibodies. Immune complexes were recovered by incubation to protein A-sepharose (GE Healthcare, Mississauga, ON, Canada).

Subcellular fractionation

Cells were treated as indicated and then subcellular fractionation was performed as previously described (Goping *et al.*, 1998). Briefly, cells were homogenized in HIM buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes pH 7.4). Nuclei and unbroken cells were removed by centrifugation at $700 \times g$ for 10 min. The cleared lysate was then fractionated into supernatant (cytosol/light membranes) and pellet (heavy membranes).

Microarray analysis

The DataSet SOFT file corresponding to docetaxel-sensitive and -resistant breast cancers (Chang *et al.*, 2003) was downloaded from Gene Expression Omnibus at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS360). Microarray expression data were imported into Excel (Microsoft) and individual expression values were recovered for BCL-2 family members. Cluster analysis was performed using CLUSTER 3.0 (open source software was downloaded from <http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm#ctv>; Eisen *et al.*, 1998; de Hoon *et al.*, 2004) and results were presented using Java Tree View (<http://jtreeview.sourceforge.net/>; Saldanha, 2004).

Immunohistochemistry and survival analysis

Tissue microarray samples were obtained from formalin-fixed breast tissues in triplicate 0.6 mm cores using the TMArrayer (Pathology Devices, Westminster, MD, USA). Tissues on slides were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol to water. Endogenous peroxidase was quenched in 0.3% H₂O₂ for 10 min. For antigen retrieval, slides were placed in boiling citrate buffer pH 6.0 for 10 min, followed by rinsing in water for 10 min. Tissues were incubated with the Bad antibody (Cell Signaling Technology) at a dilution of 1:25 at 4 °C overnight in a humidified container. Slides were washed two times in phosphate-buffered saline for 5 min. For the secondary antibody, tissues were incubated with Anti-Rabbit EnVision + System-HRP (Dako, Glostrup, Denmark) at room temperature for 30 min. Slides were washed two times in phosphate-buffered saline and then tissues were incubated with 3,3'-diaminobenzidine (Dako) for 10 min. Slides were rinsed in water for 10 min, followed by a soak in 1% copper sulphate for 5 min. Haematoxylin was used to counterstain the tissues. Slides were dipped three times in saturated lithium carbonate, rinsed in water, dehydrated in increasing concentrations of ethanol and xylene and coverslipped. Staining intensity was scored as 0 (absent), 1 (weak) or 2 (strong). The average of the three samples was used to define the staining for each patient. Receiver operator curve analysis was used to select the optimal cutoff point to dichotomize a continuous variable. Using ROC, an optimized cutoff point of 0.57 was chosen, and patients were analysed on the basis of this cutoff point. Multivariate analysis including age, stage, grade and ER status was conducted using SAS v 9.1.3 (SAS Institute Inc., Cary, NC, USA).

Conflict of interest

The authors declare no conflict of interest.

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References

- Aapro MS. (2001). Adjuvant therapy of primary breast cancer: a review of key findings from the 7th international conference, St Gallen, February 2001. *Oncologist* **6**: 376–385.
- Al-Bazz YO, Underwood JC, Brown BL, Dobson PR. (2009). Prognostic significance of Akt, phospho-Akt and BAD expression in primary breast cancer. *Eur J Cancer* **45**: 694–704.
- Andreeff M, Jiang S, Zhang X, Konopleva M, Estrov Z, Snell VE *et al*. (1999). Expression of Bcl-2-related genes in normal and AML progenitors: changes induced by chemotherapy and retinoic acid. *Leukemia* **13**: 1881–1892.
- Blagosklonny MV, Fojo T. (1999). Molecular effects of paclitaxel: myths and reality (a critical review). *Int J Cancer* **83**: 151–156.
- Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F *et al*. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* **286**: 1735–1738.
- Cannings E, Kirkegaard T, Tovey SM, Dunne B, Cooke TG, Bartlett JM. (2007). Bad expression predicts outcome in patients treated with tamoxifen. *Breast Cancer Res Treat* **102**: 173–179.
- Chang JC, Makris A, Gutierrez MC, Hilsenbeck SG, Hackett JR, Jeong J *et al*. (2008). Gene expression patterns in formalin-fixed, paraffin-embedded core biopsies predict docetaxel chemosensitivity in breast cancer patients. *Breast Cancer Res Treat* **108**: 233–240.
- Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Elledge R *et al*. (2003). Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet* **362**: 362–369.
- Chattopadhyay A, Chiang CW, Yang E. (2001). BAD/BCL-[X(L)] heterodimerization leads to bypass of G0/G1 arrest. *Oncogene* **20**: 4507–4518.
- Chiang CW, Kanies C, Kim KW, Fang WB, Parkhurst C, Xie M *et al*. (2003). Protein phosphatase 2A dephosphorylation of phosphoserine 112 plays the gatekeeper role for BAD-mediated apoptosis. *Mol Cell Biol* **23**: 6350–6362.
- Czernick M, Rieger A, Goping IS. (2009). Bim is reversibly phosphorylated but plays a limited role in paclitaxel cytotoxicity of breast cancer cell lines. *Biochem Biophys Res Commun* **379**: 145–150.
- Danial NN. (2008). BAD: undertaker by night, candyman by day. *Oncogene* **27**(Suppl 1): S53–S70.
- Danial NN, Gramm CF, Scorrano L, Zhang CY, Krauss S, Ranger AM *et al*. (2003). BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. *Nature* **424**: 952–956.
- Danial NN, Walensky LD, Zhang CY, Choi CS, Fisher JK, Molina AJ *et al*. (2008). Dual role of proapoptotic BAD in insulin secretion and beta cell survival. *Nat Med* **14**: 144–153.
- Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB *et al*. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol Cell* **6**: 41–51.
- Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW *et al*. (2002). Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. *Dev Cell* **3**: 631–643.
- de Hoon MJ, Imoto S, Nolan J, Miyano S. (2004). Open source clustering software. *Bioinformatics* **20**: 1453–1454.
- Derry WB, Wilson L, Jordan MA. (1998). Low potency of taxol at microtubule minus ends: implications for its antimetabolic and therapeutic mechanism. *Cancer Res* **58**: 1177–1184.
- Eisen MB, Spellman PT, Brown PO, Botstein D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* **95**: 14863–14868.
- Fernando R, Foster JS, Bible A, Strom A, Pestell RG, Rao M *et al*. (2007). Breast cancer cell proliferation is inhibited by BAD: regulation of cyclin D1. *J Biol Chem* **282**: 28864–28873.
- Fernando RI, Wimalasena J. (2004). Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt. *Mol Biol Cell* **15**: 3266–3284.
- Gascoigne KE, Taylor SS. (2008). Cancer cells display profound intra- and interline variation following prolonged exposure to antimetabolic drugs. *Cancer Cell* **14**: 111–122.
- Gascoigne KE, Taylor SS. (2009). How do anti-mitotic drugs kill cancer cells? *J Cell Sci* **122**: 2579–2585.
- Gilmore AP, Valentijn AJ, Wang P, Ranger AM, Bundred N, O'Hare MJ *et al*. (2002). Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. *J Biol Chem* **277**: 27643–27650.
- Goping IS, Gross A, Lavoie JN, Nguyen M, Jemmerson R, Roth K *et al*. (1998). Regulated targeting of BAX to mitochondria. *J Cell Biol* **143**: 207–215.
- Graham KA, Trent JM, Osborne CK, McGrath CM, Minden MD, Buick RN. (1986). The use of restriction fragment polymorphisms to identify the cell line MCF-7. *Breast Cancer Res Treat* **8**: 29–34.
- Janicke RU, Sprengart ML, Wati MR, Porter AG. (1998). Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* **273**: 9357–9360.
- Janumyan Y, Cui Q, Yan L, Sansam CG, Valentin M, Yang E. (2008). G0 function of BCL2 and BCL-xL requires BAX, BAK, and p27 phosphorylation by Mirk, revealing a novel role of BAX and BAK in quiescence regulation. *J Biol Chem* **283**: 34108–34120.
- Janumyan YM, Sansam CG, Chattopadhyay A, Cheng N, Soucie EL, Penn LZ *et al*. (2003). Bcl-xL/Bcl-2 coordinately regulates apoptosis, cell cycle arrest and cell cycle entry. *EMBO J* **22**: 5459–5470.
- Jordan MA, Toso RJ, Thrower D, Wilson L. (1993). Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc Natl Acad Sci USA* **90**: 9552–9556.
- Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L. (1996). Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res* **56**: 816–825.
- Kelekar A, Chang BS, Harlan JE, Fesik SW, Thompson CB. (1997). Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. *Mol Cell Biol* **17**: 7040–7046.
- Kitada S, Krajewska M, Zhang X, Scudiero D, Zapata JM, Wang HG *et al*. (1998). Expression and location of pro-apoptotic Bcl-2 family protein BAD in normal human tissues and tumor cell lines. *Am J Pathol* **152**: 51–61.
- Klumpp S, Selke D, Kriegelstein J. (2003). Protein phosphatase type 2C dephosphorylates BAD. *Neurochem Int* **42**: 555–560.
- Letai AG. (2008). Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer* **8**: 121–132.
- Li R, Moudgil T, Ross HJ, Hu HM. (2005). Apoptosis of non-small-cell lung cancer cell lines after paclitaxel treatment involves the BH3-only proapoptotic protein Bim. *Cell Death Differ* **12**: 292–303.
- Liu QY, Stein CA. (1997). Taxol and estramustine-induced modulation of human prostate cancer cell apoptosis via alteration in bcl-xL and bak expression. *Clin Cancer Res* **3**: 2039–2046.
- Lizcano JM, Morrice N, Cohen P. (2000). Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. *Biochem J* **349**: 547–557.
- Matschinsky FM, Ellerman JE. (1968). Metabolism of glucose in the islets of Langerhans. *J Biol Chem* **243**: 2730–2736.
- McGrogan BT, Gilmartin B, Carney DN, McCann A. (2008). Taxanes, microtubules and chemoresistant breast cancer. *Biochim Biophys Acta* **1785**: 96–132.
- Metcalf AD, Gilmore A, Klinowska T, Oliver J, Valentijn AJ, Brown R *et al*. (1999). Developmental regulation of Bcl-2 family protein expression in the involuting mammary gland. *J Cell Sci* **112**(Part 11): 1771–1783.
- Noguchi S. (2006). Predictive factors for response to docetaxel in human breast cancers. *Cancer Sci* **97**: 813–820.
- Osborne CK, Hobbs K, Trent JM. (1987). Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast Cancer Res Treat* **9**: 111–121.

- Pellegrini F, Budman DR. (2005). Review: tubulin function, action of antitubulin drugs, and new drug development. *Cancer Invest* **23**: 264–273.
- Perez EA. (1999). Current management of metastatic breast cancer. *Semin Oncol* **26**: 1–10.
- Peruzzi F, Prisco M, Dews M, Salomoni P, Grassilli E, Romano G *et al*. (1999). Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. *Mol Cell Biol* **19**: 7203–7215.
- Ranger AM, Zha J, Harada H, Datta SR, Danial NN, Gilmore AP *et al*. (2003). Bad-deficient mice develop diffuse large B cell lymphoma. *Proc Natl Acad Sci USA* **100**: 9324–9329.
- Roy SS, Madesh M, Davies E, Antonsson B, Danial N, Hajnoczky G. (2009). Bad targets the permeability transition pore independent of Bax or Bak to switch between Ca(2+)-dependent cell survival and death. *Mol Cell* **33**: 377–388.
- Saldanha AJ. (2004). Java Treeview—extensible visualization of microarray data. *Bioinformatics* **20**: 3246–3248.
- Schorr K, Li M, Krajewski S, Reed JC, Furth PA. (1999). Bcl-2 gene family and related proteins in mammary gland involution and breast cancer. *J Mammary Gland Biol Neoplasia* **4**: 153–164.
- Shimamura A, Ballif BA, Richards SA, Blenis J. (2000). Rsk1 mediates a MEK-MAP kinase cell survival signal. *Curr Biol* **10**: 127–135.
- Smith AJ, Karpova Y, D'Agostino Jr R, Willingham M, Kulik G. (2009). Expression of the Bcl-2 protein BAD promotes prostate cancer growth. *PLoS One* **4**: e6224.
- Sunters A, Fernandez de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA *et al*. (2003). FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* **278**: 49795–49805.
- Swanton C, Marani M, Pardo O, Warne PH, Kelly G, Sahai E *et al*. (2007). Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. *Cancer Cell* **11**: 498–512.
- Tan TT, Degenhardt K, Nelson DA, Beaudoin B, Nieves-Neira W, Bouillet P *et al*. (2005). Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell* **7**: 227–238.
- Tan Y, Demeter MR, Ruan H, Comb MJ. (2000). BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. *J Biol Chem* **275**: 25865–25869.
- Upreti M, Galitovskaya EN, Chu R, Tackett AJ, Terrano DT, Granell S *et al*. (2008). Identification of the major phosphorylation site in Bcl-xL induced by microtubule inhibitors and analysis of its functional significance. *J Biol Chem* **283**: 35517–35525.
- Virdee K, Parone PA, Tolkovsky AM. (2000). Phosphorylation of the pro-apoptotic protein BAD on serine 155, a novel site, contributes to cell survival. *Curr Biol* **10**: 1151–1154.
- Weaver BA, Cleveland DW. (2005). Decoding the links between mitosis, cancer, and chemotherapy: the mitotic checkpoint, adaptation, and cell death. *Cancer Cell* **8**: 7–12.
- Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* **80**: 285–291.
- Youle RJ, Strasser A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* **9**: 47–59.
- Yvon AM, Wadsworth P, Jordan MA. (1999). Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol Biol Cell* **10**: 947–959.
- Zha J, Harada H, Osipov K, Jockel J, Waksman G, Korsmeyer SJ. (1997). BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. *J Biol Chem* **272**: 24101–24104.
- Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**: 619–628.
- Zhou XM, Liu Y, Payne G, Lutz RJ, Chittenden T. (2000). Growth factors inactivate the cell death promoter BAD by phosphorylation of its BH3 domain on Ser155. *J Biol Chem* **275**: 25046–25051.
- Zinkel S, Gross A, Yang E. (2006). BCL2 family in DNA damage and cell cycle control. *Cell Death Differ* **13**: 1351–1359.

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