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Research Article

Molecular Cancer Therapeutics

Identification of Expression Signatures Predictive of Sensitivity to the Bcl-2 Family Member Inhibitor ABT-263 in Small Cell Lung Carcinoma and Leukemia/Lymphoma Cell Lines

Stephen K. Tahir, John Wass, Mary K. Joseph, Viswanath Devanarayan, Paul Hessler, Haichao Zhang, Steve W. Elmore, Paul E. Kroeger, Christin Tse, Saul H. Rosenberg, and Mark G. Anderson

Abstract

ABT-263 inhibits the antiapoptotic proteins Bcl-2, Bcl-x_L, and Bcl-w and has single-agent efficacy in numerous small cell lung carcinoma (SCLC) and leukemia/lymphoma cell lines *in vitro* and *in vivo*. It is currently in clinical trials for treating patients with SCLC and various leukemia/lymphomas. Identification of predictive markers for response will benefit the clinical development of ABT-263. We identified the expression of Bcl-2 family genes that correlated best with sensitivity to ABT-263 in a panel of 36 SCLC and 31 leukemia/lymphoma cell lines. In cells sensitive to ABT-263, expression of Bcl-2 and Noxa is elevated, whereas expression of Mcl-1 is higher in resistant cells. We also examined global expression differences to identify gene signature sets that correlated with sensitivity to ABT-263 to generate optimal signature sets predictive of sensitivity to ABT-263. Independent cell lines were used to verify the predictive power of the gene sets and to refine the optimal gene signatures. When comparing normal lung tissue and SCLC primary tumors, the expression pattern of these genes in the tumor tissue is most similar to sensitive SCLC lines, whereas normal tissue is most similar to resistant SCLC lines. Most of the genes identified using global expression patterns are related to the apoptotic pathway; however, all but Bcl-rambo are distinct from the Bcl-2 family. This study leverages global expression data to identify key gene expression patterns for sensitivity to ABT-263 in SCLC and leukemia/lymphoma and may provide guidance in the selection of patients in future clinical trials. *Mol Cancer Ther; 9(3); 545–57. ©2010 AACR.*

Introduction

Bcl-2 overexpression has been reported in 55% to 90% of small cell lung carcinoma (SCLC) patients as well as other cancer types (1). Enhanced Bcl-2 expression in these tumors not only permits cell growth and survival in the presence of cellular apoptotic signals associated with the transformed phenotype, but increased Bcl-2 can also lead to failure of chemotherapeutic strategies (2–10). Bcl-2 is the founding member of a family of proteins that contain one or more Bcl-2 homology (BH) domains (11). The antiapoptotic family members (Bcl-x_L, Bcl-2, Bcl-w, A1, Mcl-1) have four BH domains and block the proapoptotic family members (Bax and Bak, which have 3 BH domains) from mediating cytochrome *c* release from the mitochondria and subsequent activation of apoptosis through caspase cleavage via the intrinsic apoptotic pathway (12). Additional proapoptotic members contain only one BH domain (Bad, Bid, Bim, Hrk, Bmf, Noxa, and Puma), and they are referred to collectively as BH3-only proteins. The BH3-only proapoptotic proteins act as molecular sensors of cellular stress or damage. Bcl-2 family members function through interactions with each other, and the balance between the antiapoptotic (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1) and the proapoptotic (BH3-only) members is critical for preventing or initiating apoptosis (13, 14).

Recently, we described the activity of ABT-263, a potent Bcl-2 family member inhibitor that is orally bioavailable (15). ABT-263 binds with high affinity ($K_i \leq 1$ nmol/L) to Bcl-2, Bcl-x_L, and Bcl-w, but not to Mcl-1 and A1 (K_i = 0.35 and 0.55 μmol/L, respectively), which is similar to a related Bcl-2 family member inhibitor ABT-737 ($K_i \leq 1$ nmol/L for Bcl-2, Bcl-x_L and Bcl-w; $K_i > 1$ μmol/L for Mcl-1 and A1; ref. 15). In preclinical models ABT-263 exhibits single-agent activity against a variety of SCLC and leukemia cell lines and primary patient-derived cells *in vitro* and induces complete, maintained tumor regressions in SCLC animal tumor models (15–17). Phase 1/2b clinical trials with

Authors' Affiliation: Global Pharmaceutical Product Research Division, Abbott Laboratories, Abbott Park, Illinois

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Corresponding Author: Mark G. Anderson, Abbott Laboratories, Cancer Research, Global Pharmaceutical Product Research Division, AP9-2, 100 Abbott Park Road, Abbott Park, IL 60064-6099. Phone: 847-937-8926; Fax: 847-937-2625. E-mail: mark.g.anderson@abbott.com

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ABT-263 are ongoing in patients with SCLC and lymphoid malignancies.

The identification of predictive markers of response to chemotherapy can greatly facilitate the development of a new compound by identifying patients that are sensitive to a given therapy. One approach is to look at individual genes, especially those that are targeted by a given chemotherapeutic to better assess the probability a patient will respond to therapy (18–21). Another approach is to use gene expression profiles, such as those derived by microarray analysis, to generate molecular signature sets that can also be used for patient selection during clinical trials and beyond (22–24).

To support the clinical development of ABT-263, we undertook a study to identify expression markers that are predictive of sensitivity to ABT-263 using a panel of SCLC and leukemia and lymphoma cell lines. In our investigation, we determined which Bcl-2 family members correlated best with sensitivity to ABT-263 in each of the cell line panels. Next, we investigated the relationship between the global expression pattern and sensitivity to ABT-263 to identify optimal predictive expression signatures using different statistical approaches. We validated the predictive power of these signatures using independent cell line sets. Finally, we compared the expression pattern of the signature genes in cell lines to the expression pattern in primary SCLC patient tumor samples and normal surrounding tissue.

Our results reveal that high Bcl-2 and low Mcl-1 expression levels correlated with sensitivity to ABT-263 in the SCLC and leukemia/lymphoma cell panels used for this study. Furthermore, we have identified, by different statistical approaches, optimal signature sets that are predictive of sensitivity to ABT-263 which were derived from either the global expression pattern or just using the Bcl-2 family members. Optimal signature sets from the global expression pattern included a number of apoptosis-related genes.

Materials and Methods

Cell Culture and Viability Assays

The SCLC and leukemia/lymphoma cell lines (American Type Culture Collection, Deutsche Sammlung von Mikroorganismen und Zellkulturen, or generous gift from Dr. Louis Staudt, National Cancer Institute) were cultured as previously described (15, 20). Cells were treated at 10,000 or 50,000 per well for adherent or suspension cells, respectively, in 96-well microtiter plates in the presence of 10% human serum for 48 h with or without ABT-263 in a humidified chamber with 5% CO_2. ABT-263 was synthesized as previously described (15, 25), and cell cytotoxicity EC_{50} values were assessed using CellTiter Glo (Promega).

RNA Isolation and Microarrays

RNA from eight SCLC tumor samples (lot numbers A805144, A701047, A701062, A701046, A805145, A701061, A610276, and A609162) and six matched normal adjacent tissue (lot numbers A701047, A701062, A701046, A805145, A701061, and A610276) were purchased from Biochain. Naive cell line samples were lysed, and total RNA was isolated using TRIzol reagent (Invitrogen) and purified on RNeasy columns (Qiagen). To increase statistical power, replicates (between 2 and 4) of each cell line were grown in parallel and analyzed separately. Labeled cRNA was prepared according to the microarray manufacturer's protocol and hybridized to human U133A 2.0 arrays (Affymetrix). The microarray data files were loaded into Rosetta Resolver (Rosetta Biosoftware) software for analysis, and the intensity values for all probe sets were normalized using Resolver's Experimental Definition tool.

Western Blot Analysis

Total cell lysates (40 μg) were run on 4% to 12% bis-Tris gels and transferred to polyvinylidene difluoride membranes using the iBlot dry blotting system (Invitrogen) according to the manufacturer's instructions. Blots were stained with the following primary antibody: Bcl-2 and Bcl-x_L (R&D Systems); Mcl-1, Bad, and Bax (BD Biosciences); Bak (Epitomics); Noxa (Imgenex); caspase-9 (Cell Signaling Technology); and actin (Abcam), diluted in Odyssey Blocking Buffer (LI-COR Biosciences) with 0.1% Tween 20 overnight at 4°C, except for actin, which was stained for 30 min at room temperature. Blots were stained with the appropriate secondary antibodies (goat anti-mouse or anti-rabbit conjugated to Alexa 680 IgG; Invitrogen) that were diluted 1:500 in Odyssey Blocking Buffer for 30 min at room temperature. Blots were scanned using an Odyssey imager.

Statistical Analysis

To divide the training set cell lines into sensitive and resistant categories, a bivariate fit curve was generated for the ABT-263 EC_{50} versus rank and fitted with a smoothing spline ($\lambda = 1$), and the maximum increase in the slope was at ~5 μmol/L for both sets, which is approximately the same as the maximum concentration *in vivo*. This segregated the SCLC lines into 26 sensitive and 10 resistant lines, and the leukemia/lymphoma cell lines into 25 sensitive and 6 resistant cell lines. To compare the expression of the small subset of genes related to the target (Bcl-2 family members), a *P*-value filter of 0.05 was used, with the expression data from all cell lines. To identify global expression markers, we made expression ratios comparing the sensitive to the resistant lines within Resolver's Experimental Definition tool and then sorted based on *P* value (Method 1). The top 100 probe sets that varied between the sensitive and resistant cell lines by ANOVA were further filtered by discriminant analysis in JMP (version 6.0, SAS) to identify the best group of probe sets for predicting sensitivity and resistance to ABT-263. These groups were further tested by a leave-one-out cross-validation in SAS, and an error

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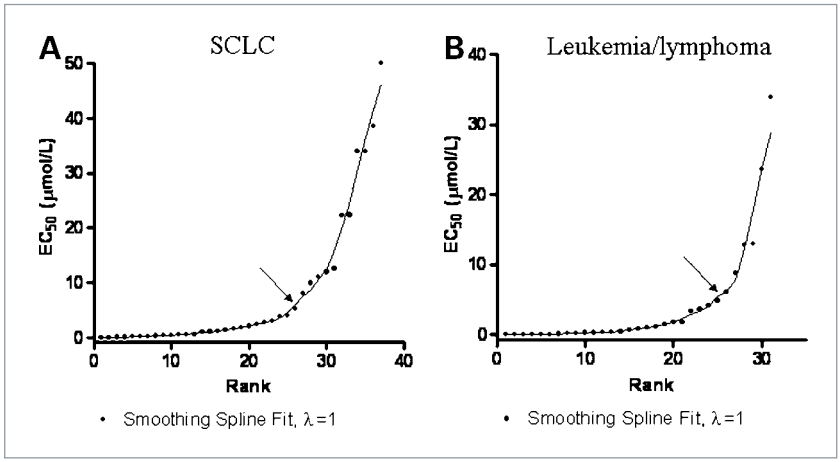

Figure 1. EC_{50} values plotted versus rank. Calculated EC_{50} values (in μmol/L) were plotted based on rank, and a curve was fit using a fitting spline with λ equal to 1 using JMP software for SCLC cell lines (A) and leukemia/lymphoma cell lines (B).

rate was calculated for the cell lines that were left out each time. As a validation step, an additional test set of 14 SCLC cell lines (10 sensitive and 4 resistant) was profiled as the other lines were and tested in discriminant analysis as unknowns. A similar process was done with a panel of nine mantle cell lymphomas (eight sensitive, one resistant).

To use the entire data set to identify additional gene signatures, several derivation procedures and model fitting algorithms (random forests, Bayesian trees, neural nets, and support vector machines) were tested. Overall, based on accuracy of prediction, we found the performance of signatures to be optimal from the diagonal linear discriminant analysis (DLDA) with simulated annealing algorithm. Using this approach (Method 2), genes that were significant on their own based on permutation-based Wilcoxon test in the significance analysis of microarrays method were first identified before deriving the signatures. This entire procedure of filtering out the important genes, deriving the optimal signature, and model fitting was evaluated using 10 replications of 5-fold stratified cross-validation. In the 5-fold stratified cross-validation procedure, the cell lines were randomly divided into five equal parts (folds) and stratified to ensure approximately similar prevalence of resistant and nonresistant cell lines within each fold. Each fold was left out one at a time whereas the gene filtering–signature derivation–model fitting process was carried out in the remaining four parts, and the results were then used to determine whether the cell lines in the left-out fold were predicted to be resistant or nonresistant. The predictions from each of the left-out folds were then aggregated to determine the overall accuracy of this procedure. This entire evaluation was repeated 10 times, and the mean percentage of cell lines correctly identified from these replications was determined with the best performing sets reported. This same analysis was then repeated on the leukemia/lymphoma cell lines. All of these analyses were carried out using programs written in R, version 2.7. Gene network analyses were generated through the use of ingenuity pathways analysis (Ingenuity Systems[1]).

Results

Division of Cell Lines into Sensitive and Resistant Categories

Markers for sensitivity and resistance to ABT-263 will help identify specific tumors and tumor types where the drug can be more effective while also identifying additional targets for therapy. To identify gene expression patterns that can predict sensitivity to ABT-263 as well as genes that might contribute to resistance to treatment, we determined RNA expression profiles for a panel of SCLC cell lines as well as a panel of leukemia and lymphoma cell lines. RNA from untreated cells from these lines was profiled on Affymetrix HG-U133A microarrays that contain over 22,000 probe sets. We next divided these lines into sensitive and resistant sets by plotting their ABT-263 EC_{50} values (15) versus their rank to identify an EC_{50} break point. A bivariate fit curve was generated, and the maximum increase in the slope was between the 4.1 and 8.1 μmol/L EC_{50} values for the SCLC panel and 4.8 and 6.0 μmol/L EC_{50} values for the leukemia/lymphoma panel (Fig. 1), which is in the range of maximal concentration achieved in clinical studies. Therefore, SCLC cell lines with EC_{50} values of <4.1 μmol/L and leukemia/lymphoma cell lines with EC_{50} values of <4.8 μmol/L were categorized as sensitive. Using these criteria, 26 of 36 SCLC cell lines and 25 of 31 leukemia/lymphoma cell lines were categorized as being sensitive to ABT-263 (Supplementary Tables S1 and S2).

[1] www.ingenuity.com

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Correlation of Bcl-2 Family Member Expression Levels and Cellular Response

We have shown previously that the expression of certain Bcl-2 family members correlated with the cellular response to ABT-737, a highly related Bcl-2 family member inhibitor (20). To determine which Bcl-2 family members' expression levels best correlate to sensitivity to ABT-263, we focused on the 14 apoptosis-related genes in the Bcl-2 family that are significantly detected on the microarrays. Expression values were compared between the sensitive and resistant cell lines, and the results are shown in Table 1. Here we found that Bcl-2 and PMAIP1 (or Noxa, which can induce Mcl-1 degradation) are expressed at just over 2-fold higher and Mcl-1 expression is slightly lower (30%) in the sensitive SCLC cells compared with the resistant SCLC cells. These results are consistent with previous studies wherein Bcl-2 and Noxa levels were shown to be higher and Mcl-1 levels were lower both at the mRNA and protein levels in SCLC cells sensitive to ABT-737 (20). In addition, expression of Bcl-w is slightly lower (40%) in sensitive SCLC cells.

Similarly, in sensitive leukemia/lymphoma cells, Bcl-2 and Noxa expression is significantly higher and Mcl-1 is lower (Table 1). Expression of Bcl-rambo and the antiapoptotic gene A1 is also lower in sensitive leukemia/lymphoma cell lines, whereas expression of the proapoptotic genes BAD, BAK1, and BAX is higher in sensitive leukemia/lymphoma cell lines (Fig. 2A). By Western blot analysis, Bcl-2 and Noxa protein levels tended to be higher and Mcl-1 levels tended to be lower in sensitive leukemia/lymphoma cells compared with resistant cells (Fig. 2B). We did not observe any clear differences in BAD, BAK, or BAX between sensitive and resistant cells at the protein level. We were unable to discern Bcl-rambo or A1 by Western blot analysis (data not shown).

To compare the expression patterns of the genes between normal and primary tumor tissue, we compared expression of the Bcl-2 family genes in eight SCLC tumor tissue samples to six normal lung tissue samples taken from the same set of patients. Significantly, we found that, overall, the comparison of the Bcl-2 family genes expressed in primary SCLC tumor tissue to the normal lung tissue paralleled the comparison of sensitive to resistant cell lines. That is, Bcl-2 and Noxa expression is higher whereas Mcl-1, Bcl-w, A1, and Bcl-rambo expression levels are lower in tumor/sensitive samples (Table 1).

In addition to examining individual Bcl-2 family members and their correlation to sensitivity and resistance, we sought to identify gene signatures based on the global RNA expression levels from the microarrays that might prove to be better predictors of response. We used two separate and rigorous approaches to identify the best predictive sets for sensitivity/resistance to ABT-263 starting with all of the data from the microarrays, regardless of their relation to the Bcl-2 family of proteins. The first method used discriminant analysis and leave-one-out validation, whereas the second method used a DLDA with simulated annealing algorithm (as described in Materials and Methods).

Identification and Validation of Gene Signatures as Predictors of Response to ABT-263: Method 1

For SCLC cells, a training set of 15 sensitive and 7 resistant cell lines was created to identify the best sets

Table 1. Average differential expression of Bcl-2 family members

Gene	SCLC (sensitive versus resistant)		Leukemia/lymphoma (sensitive versus resistant)		Primary tissue (SCLC versus normal)	
	Fold change*	*P*	Fold change	*P*	Fold change	*P*
BCL2 (Bcl-2)	−2.1	0.021	−2.7	0.026	−1.5	0.02
PMAIP1 (Noxa)	−2.1	5.49E−04	−1.4	0.038	−5.0	4.86E−14
BID						
BCL2L1 (Bcl-x_L)						
BCL2L13 (Bcl-rambo)			1.4	5.04E−03	1.3	0.014
BCL2A1 (A1)			2.9	0.039	6.3	2.53E−06
BCL2L11 (Bim)						
BAD			−1.4	0.029		
BCL2L2 (Bcl-w)	1.4	0.031			1.2	0.013
BBC3 (Puma)						
BIK					−6.2	4.31E−04
BAX			−1.6	0.028		
BAK1			−1.3	0.033		
MCL1 (Mcl-1)	1.3	0.017	1.2	0.042	2.5	6.42E−13

*Differential expression ($P < 0.05$) is shown for all of the resistant cell lines in each panel compared with all of the sensitive cell lines in each panel. A negative number indicates the expression in the sensitive cells is higher (by the indicated fold change) compared with the resistant cells.

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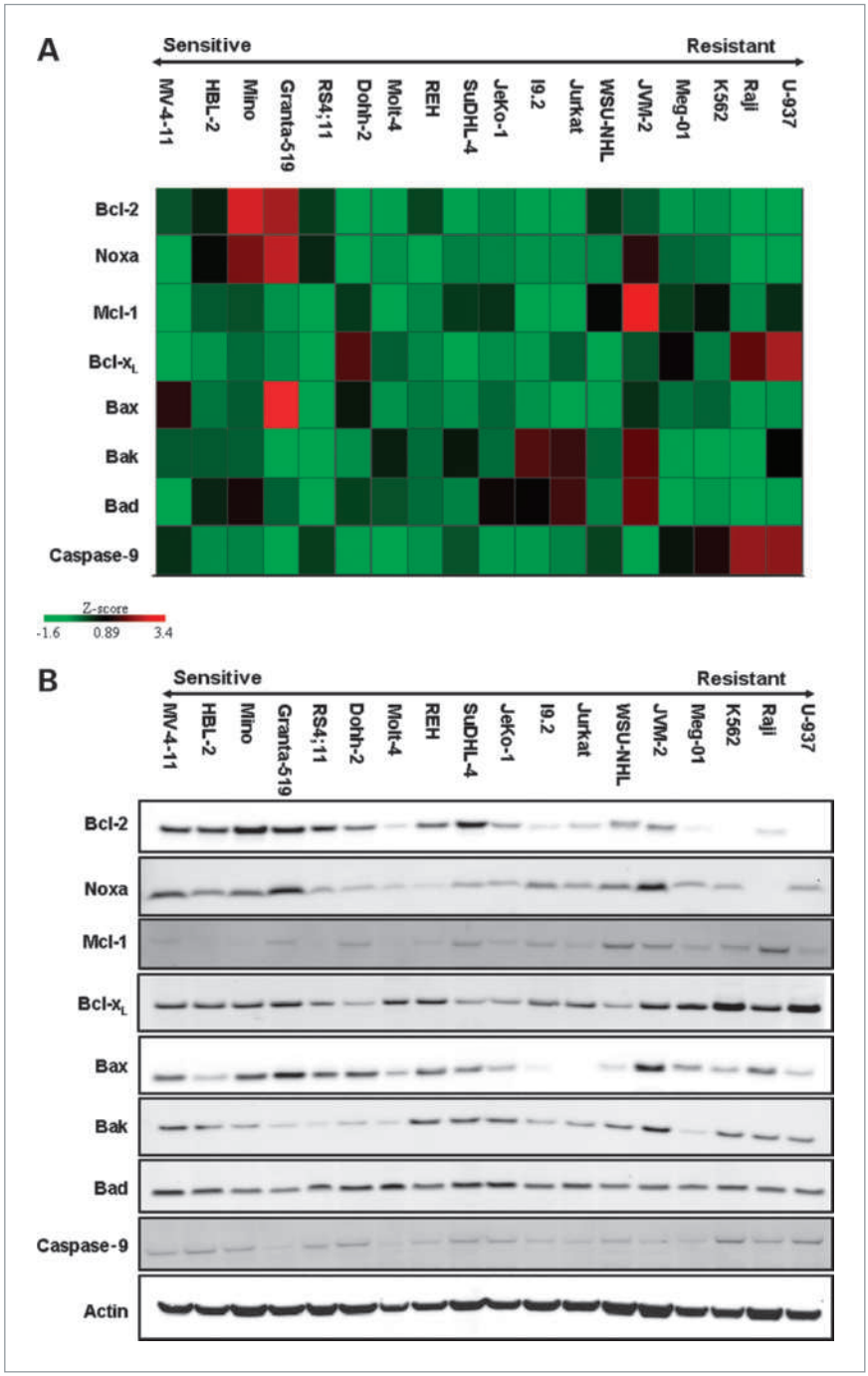

Figure 2. Expression profile of Bcl-2 family members in leukemia/lymphoma cancer cells. Intensity values (A) from the relevant probe sets for the genes shown in the Western blots (B) were averaged for each cell line, imported into Spotfire, normalized for each gene individually, and displayed using green for low expression and red for high expression, as indicated in the color bar. For Western blots, total cell lysates were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride, and stained as described in Materials and Methods.

of markers to predict sensitivity to ABT-263 using discriminant analysis. The two best predictive sets using this approach are shown (Fig. 3A and B). In a leave-one-out cross-validation test (SAS), we obtained a 2.0% error rate for set 1 and a 7.8% error rate for set 2 for sensitive versus resistant classification of the sample that was left out. Interestingly, none of the Bcl-2 family members were included in these optimized gene signature sets; however, some of these genes are in critical signaling and survival pathways. For example, TCF7L1 and FZD2 had higher expression in resistant cell lines, and these genes are involved in the Wnt/β catenin cell signaling pathway. Also expressed higher in the resistant cells were NOTCH2, which is involved in the Notch signaling pathway, MME and SLC2A3, which are cell surface molecules, and TMBIM1, which contains a Bax inhibitor

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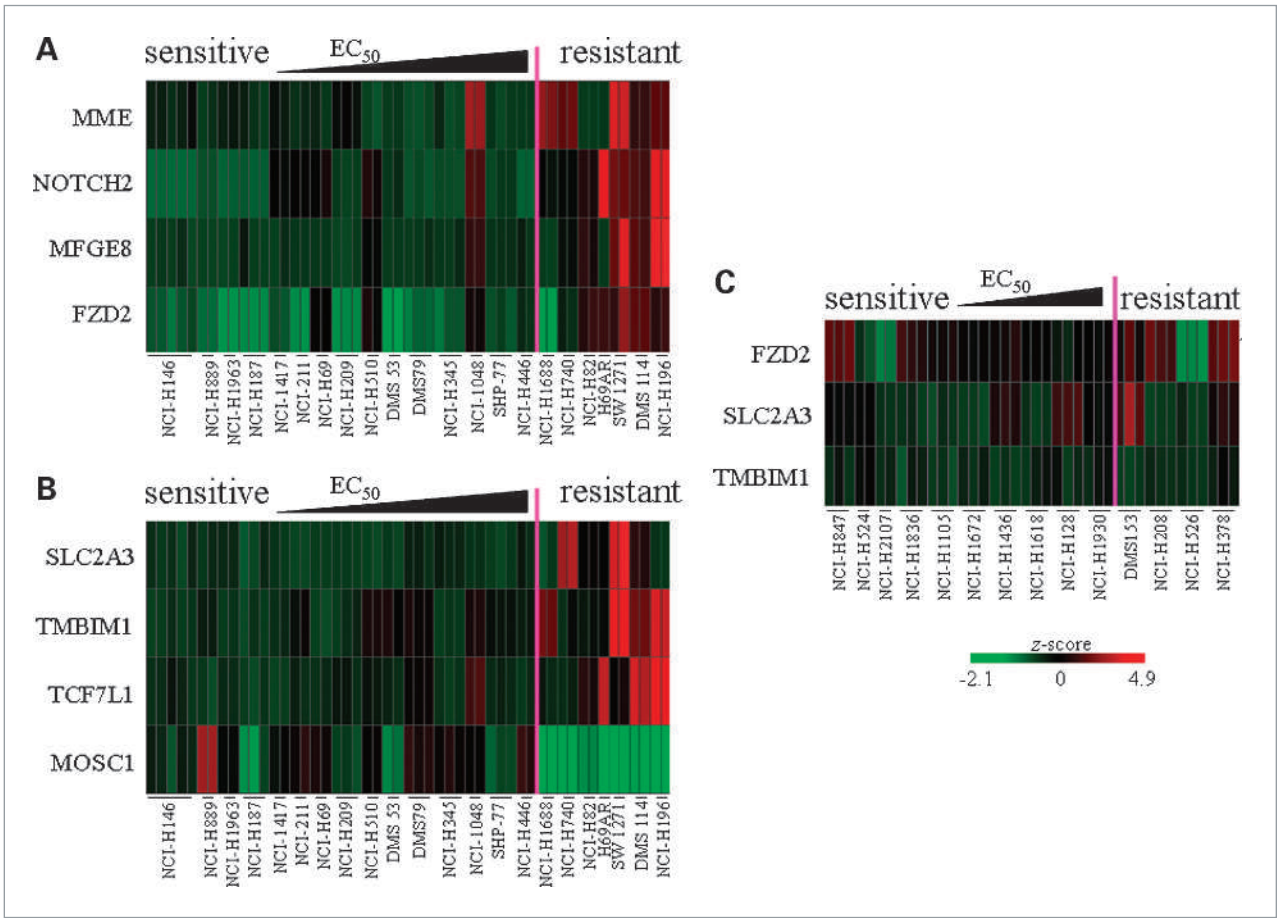

Figure 3. Heat map of genes in SCLC predictor set. Intensity values were calculated and are displayed, as described in Fig. 1, with data from each individual microarray shown in the order of the corresponding EC_{50} value (increasing from left to right). A, expression values for the cell lines in the training set for the genes in predictor set 1. B, expression values for the cell lines in the training set for the genes in predictor set 2. Set 1 and set 2 were combined, and a heat map of the best performing subset (FZD2, SLC2A3, and TMBIM1) is shown for the test cell lines (C).

motif and is part of a family of proteins that may inhibit apoptosis.

We next evaluated the performance of these two predictor sets on a different panel of SCLC cell lines composed of 10 sensitive and 4 resistant lines (Supplementary Table S1). Set 1 identified all lines as sensitive, whereas set 2 classified 62.5% of the arrays for the new lines correctly. Importantly, we were able to obtain much higher accuracy (82.6%) with a three-gene predictor set when we combined TMBIM1 and SLC2A3 from set 1 and FZD2 from set 2 (Fig. 3C). To determine the tissue specificity of these predictor sets, we tested them on the leukemia/lymphoma cell line panel. Set 1 classified 51.3% of the leukemia/lymphoma cell lines correctly, whereas set 2 classified only 40.8% of the leukemia/lymphoma cell lines correctly. Again, the optimized sets of three genes did better (65.8% of the arrays identified correctly).

Using the same approach with 17 sensitive and 5 resistant leukemia/lymphoma cell lines, we identified two additional predictor sets (Fig. 4A and B). In a leave-one-out cross-validation test (SAS) of the leukemia/lymphoma cell lines with these gene sets, we obtained a 0% error rate for both sets for sensitive versus resistant classification of the sample that was left out. These sets were distinct from the SCLC predictor sets and included a cell cycle gene (CCNG1/cyclin G1) and two apoptosis genes (BCL2L13/Bcl-rambo and CASP9/caspase-9) as shown in Fig. 4A and B. Similar to the microarray results, the protein levels of caspase-9 were found to be higher in most of the resistant cell lines tested (Fig. 2B). To perform a similar forward validation step for these two predictor sets, we tested a new panel of eight sensitive and one resistant mantle cell lymphoma cell lines (Supplementary Table S2). For each predictor set, six sensitive lines were correctly identified as sensitive; however, one sensitive line and one resistant line were incorrectly identified, for an overall accuracy rate of 78%. We next tested these sets on the SCLC cell line panel. The leukemia/lymphoma predictor set 1 correctly identified 68.1% of the arrays from SCLC cell lines, whereas set 2 did slightly better, correctly identifying 73.6% of the arrays. Thus the predictor sets from the leukemia/lymphoma lines did better on

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the SCLC lines than the predictor sets from the SCLC did on the leukemia/lymphoma cell lines. This may be due to the higher diversity of the cell lines in the leukemia/lymphoma panel.

Identification and Validation of Gene Signatures as Predictors of Response to ABT-263: Method 2

It should be noted that the estimates of sensitivity and specificity from the leave-one-out method can tend to be inflated. Therefore, we did a more rigorous signature selection method wherein we embed the entire signature derivation process within a 5-fold stratified cross-validation, and the resulting estimates of sensitivity and specificity should more closely mimic the performance in a future data set. In this method we used a simulated annealing algorithm within the framework of DLDA and did 10 replications of 5-fold stratified cross-validation on both the SCLC and the leukemia/lymphoma complete data sets, with each analyzed separately, as described in Materials and Methods. This approach identified a set of 10 genes that predicted 66% of the cell line profiles correctly when evaluated using the rigorous 5-fold stratified cross-validation approach on the SCLC cell lines (Table 2). The predictor set derived from the leukemia/lymphoma panel using this approach identified 82% of the leukemia/lymphoma cell line profiles correctly (Table 3). To compare these results to the original predictor sets, we combined the two SCLC Method 1 predictor sets, did a DLDA, and then tested with 10 repetitions of a 5-fold stratified cross-validation. This optimal set of seven genes identified 82% of the cell line profiles correctly (Table 2). An identical analysis for the leukemia/lymphoma predictor set identified eight genes that also did well, with 87% of the cell line profiles identified correctly (Table 3). Lastly we applied DLDA with simulated annealing to identify and test the performance of predictor sets just based on the Bcl-2 family genes. The optimal predictor set, consisting of Bcl-2, Mcl-1, Noxa, and Bid from the SCLC cell panel, identified 70% of the SCLC cell lines correctly. The optimal predictor

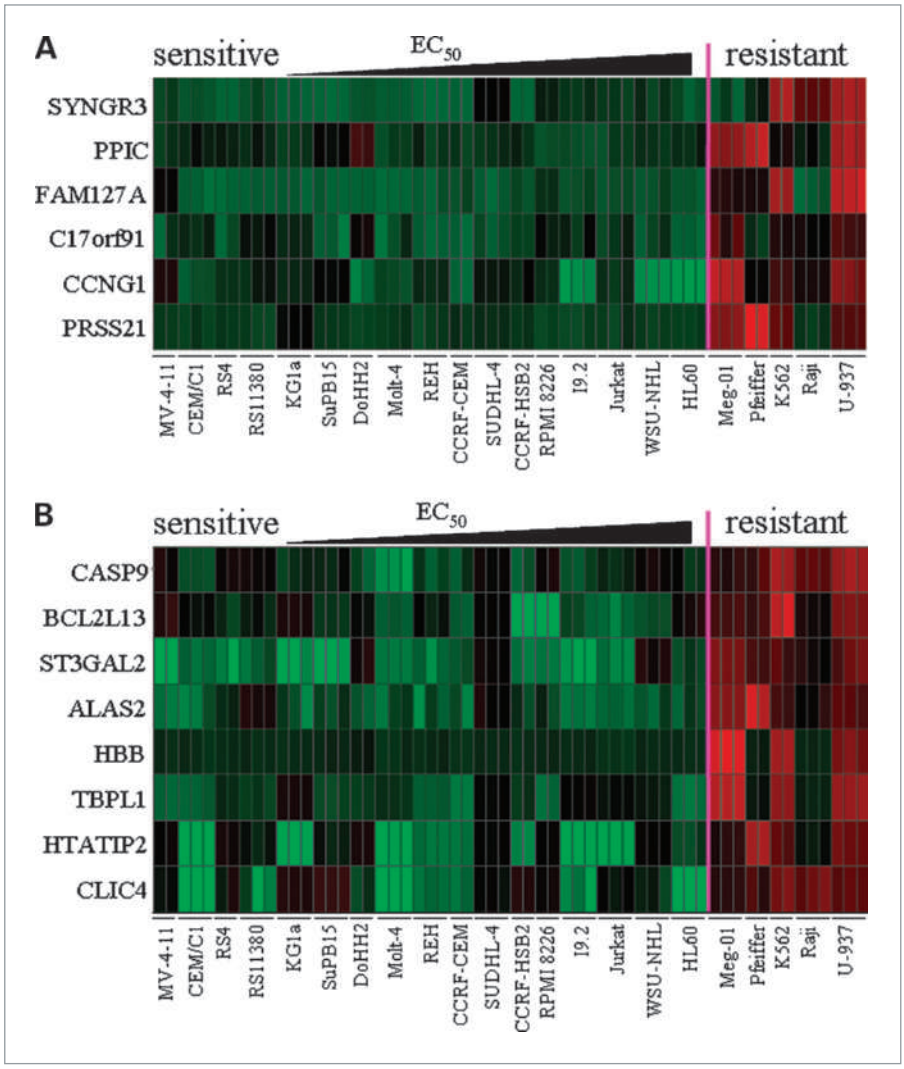

Figure 4. Heat map of genes in leukemia/lymphoma predictor set. Intensity values are displayed, as described in Fig. 3, for the leukemia/lymphoma predictor set 1 (A) and predictor set 2 (B) for the training cell lines.

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Table 2. Method 2 (DLDA) analysis of SCLC predictor genes

Affymetrix ID	Method 1 genes (82%)*	Symbol	Gene function	Fold change
202443_x_at	notch homologue 2	NOTCH2	cell cycle, transcription, apoptosis	4.3
202499_s_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	carbohydrate transport	5.5
203435_s_at	membrane metalloendopeptidase	MME	proteolysis, cell communication	3.9
210220_at	frizzled homologue 2	FZD2	Wnt receptor signaling pathway	3.4
210605_s_at	milk fat globule-egf factor 8 protein	MFGE8	endocytosis	8.8
217730_at	transmembrane bax inhibitor motif containing 1	TMBIM1	cell, membrane,	3.8
218865_at	moco sulfurase c-terminal domain containing 1	MOSC1	oxidoreductase activity	–3.2
Affymetrix ID	**Method 2 genes (66%)†**	**Symbol**	**Gene function(s)**	**Fold change**
200872_at	s100 calcium binding protein a10	S100A10	cell communication	8.8
201105_at	lectin, galactoside-binding, soluble, 1	LGALS1	apoptosis, cell communication	13.7
201231_s_at	enolase 1	ENO1	glucose metabolic process, transcription	1.7
201477_s_at	ribonucleotide reductase m1 polypeptide	RRM1	DNA metabolic process	–1.4
202088_at	solute carrier family 39 (zinc transporter), member 6	SLC39A6	ion transport	–1.2
209366_x_at	cytochrome b5 type a	CYB5A	electron transport	–1.6
211528_x_at	hla-g histocompatibility antigen, class i, g	HLA-G	immune response	3.7
212063_at	cd44 antigen	CD44	kidney development	5.3
216623_x_at	trinucleotide repeat containing 9	TOX3	transcription	–5.8
217294_s_at	enolase 1	ENO1	glucose metabolic process, transcription	1.8
Affymetrix ID	**Bcl-2 family genes (70%)**	**Symbol**	**Gene function**	**Fold change**
200797_s_at	myeloid cell leukemia sequence 1 (bcl2-related)	MCL1	apoptosis	1.3
203684_s_at	b-cell cll/lymphoma 2	BCL2	apoptosis	–1.8
203685_at	b-cell cll/lymphoma 2	BCL2	apoptosis	–3.3
204285_s_at	phorbol-12-myristate-13-acetate-induced protein 1 (Noxa)	PMAIP1	apoptosis	–2.7
204286_s_at	phorbol-12-myristate-13-acetate-induced protein 1 (Noxa)	PMAIP1	apoptosis	–2.8
211725_s_at	bh3-interacting domain death agonist	BID	apoptosis	–1.5

*Genes from the SCLC discriminant predictor sets (Method 1) were combined and processed by DLDA (Method 2), with the best sets shown. The percentage of arrays correctly identified is shown for the sets listed, with the fold change in resistant compared with sensitive lines.

†The best DLDA predictor gene set is shown for all of the genes, as well as the Bcl-2 family genes.

set for the leukemia and lymphoma cell line panel, consisting of Bcl-2, Mcl-1, Bid, Bcl-x_L, Bcl-w, and Puma, identified 81% of the cell lines correctly (Tables 2 and 3).

Expression Levels for Signature Sets in Primary SCLC Tumors and Normal Lung Tissue

Finally, we compared expression levels of the signature genes in primary patient SCLC tumors to matched normal lung tissue. As can be seen qualitatively in a heat map comparison, the expression pattern in SCLC tumor cells for the SCLC predictor sets 1 and 2 is similar to the sensitive cell lines, whereas the expression pattern in the normal lung tissue is similar to the resistant cell lines (compare Fig. 5A with Fig. 3A and B). In comparison, as expected, the expression pattern for the leukemia/lymphoma predictor sets does not show a consistent

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pattern in either the SCLC tumor or normal lung samples (compare Fig. 5B with Fig. 4A and B). Quantitatively, the SCLC predictor set 1 identified all of the normal tissue as resistant and four of eight SCLC tumors as sensitive, whereas SCLC predictor set 2 also identified all of the normal tissue as resistant and seven of eight of the SCLC tumors as sensitive.

Discussion

Given the complex interaction of the Bcl-2 family members governing apoptosis, it is reasonable to assume that any one or more of the Bcl-2 family members may influence or help predict the cellular response to ABT-263. Indeed, we and others have shown that different family

Table 3. Method 2 (DLDA) analysis of leukemia/lymphoma predictor genes

Affymetrix ID	Method 1 genes (87%)*	Symbol	Gene function	Fold change
201828_x_at	caax box 1	FAM127A	plasma membrane	4.8
205691_at	synaptogyrin 3	SYNGR3	plasma membrane	8.6
207180_s_at	hiv-1 tat interactive protein 2, 30kda	HTATIP2	cell cycle, transcription, apoptosis	3.0
208796_s_at	cyclin g1	CCNG1	regulation of cell cycle,	2.1
209116_x_at	hemoglobin, β	HBB	circulatory system process	18.9
211560_s_at	aminolevulinate, δ-, synthase 2 (sideroblastic/hypochromic anemia)	ALAS2	response to hypoxia, immune response	3.3
214696_at	chromosome 17 open reading frame 91	C17orf91		2.8
217650_x_at	st3 β-galactoside α-2,3-sialyltransferase 2	ST3GAL2	carbohydrate metabolism	2.3
Affymetrix ID	**Method 2 genes (82%)†**	**Symbol**	**Gene function**	**Fold change**
201029_s_at	cd99 antigen	CD99	cell adhesion	–2.2
201288_at	rho gdp dissociation inhibitor β	ARHGDIB	immune response, Rho/Ras signaling	–2.3
201310_s_at	chromosome 5 open reading frame 13	C5orf13	cell communication	–5.9
201347_x_at	glyoxylate reductase/hydroxypyruvate reductase	GRHPR	metabolic process	2.1
206660_at	immunoglobulin λ-like polypeptide 1	IGLL1	immune response	–44.7
208892_s_at	dual specificity phosphatase 6	DUSP6	cell cycle, inactivation of mitogen-activated protein kinase activity	–2.2
209806_at	histone 1, h2bk	HIST1H2BK	nucleosome assembly	5.1
209942_x_at	melanoma antigen family a, 3	MAGEA3	tumor antigen	26.3
211921_x_at	prothymosin, α	PTMA	cell cycle, transcription	–1.6
213515_x_at	hemoglobin, γ a	HBG2	oxygen transport	138.2
Affymetrix ID	**Bcl-2 family genes (81%)**	**Symbol**	**Gene function**	**Fold change**
200796_s_at	myeloid cell leukemia sequence 1 (bcl2-related)	MCL1	apoptosis	1.8
200797_s_at	myeloid cell leukemia sequence 1 (bcl2-related)	MCL1	apoptosis	1.4
200798_x_at	myeloid cell leukemia sequence 1 (bcl2-related)	MCL1	apoptosis	1.7
203684_s_at	b-cell cll/lymphoma 2	BCL2	apoptosis	–1.7
204493_at	bh3 interacting domain death agonist	BID	apoptosis	–1.2
206665_s_at	bcl2-like 1(Bcl-xL)	BCL2L1	apoptosis	2.6
209311_at	bcl2-like 2 (Bcl-w)	BCL2L2	apoptosis	2.6
211692_s_at	bcl2 binding component 3 (Puma)	BBC3	apoptosis	2.2

*Genes from the leukemia/lymphoma discriminant predictor sets (Method 1) were combined and processed by DLDA (Method 2), with the best sets shown. The percentage of arrays correctly identified is shown for the sets listed, with the fold change in resistant compared with sensitive lines.

†The best DLDA predictor gene set is shown for all of the genes, as well as the Bcl-2 family genes.

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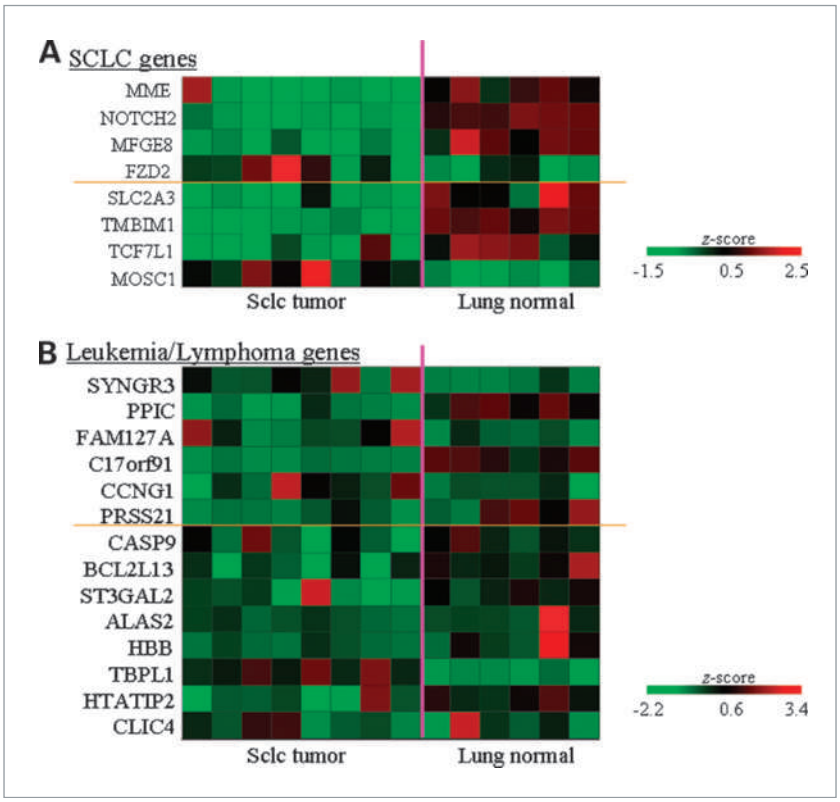

Figure 5. Heat map of expression of predictor set genes in primary SCLC tumors and normal lung tissue. Intensity values are displayed, as described in Fig. 3, for eight SCLC tumor samples and normal adjacent tissue from six of those tumors. The genes are the SCLC predictor sets (A) and the leukemia/lymphoma sets (B).

members can influence or predict the cellular response to another related Bcl-2 family member inhibitor, ABT-737 (20, 26, 27). Elucidating which of the family members are important will help us better identify patients who are more likely to respond to ABT-263 treatment as well as provide guidance for rationally designed combination trials. In both SCLC cell lines and leukemia/lymphoma cell lines, we found that both Bcl-2 and Noxa expression is increased in sensitive cell lines, whereas Mcl-1 expression is lower when compared with resistant cells. This is consistent with the mechanism of ABT-263, as Bcl-2 is one of the targets of ABT-263 whereas Mcl-1, another antiapoptotic family member, is not.

Previous studies have shown that knockdown of Mcl-1 with siRNA, modulating its expression with other chemotherapeutic agents or overexpression of Noxa, sensitizes cells resistant to Bcl-2 family member inhibitors. Therefore, in resistant cells, Mcl-1 not only correlates with cellular response but is a potential resistance factor (15, 20, 28–30). Besides Mcl-1, A1 expression was higher in the resistant leukemia/lymphoma cell lines. A previous study has shown that A1 can confer resistance to ABT-737 in chronic lymphocytic leukemia (24). Thus, this may potentially be another resistance factor with antiapoptotic activity that needs to be considered (31). Interestingly, the expression of the proapoptotic factor Bcl-rambo is higher in the resistant leukemia and lymphoma cell lines. Little has been described about Bcl-rambo; however, enhanced Bcl-rambo expression was identified as being associated with resistance to L-asparaginase (32). Whereas Bcl-rambo was identified as a proapoptotic factor through its unique COOH terminal extension (33), its association with drug resistance and unfavorable long-term clinical outcome in children was postulated to occur through an antiapoptotic activity, possibly mediated by a previously unidentified splice variant (32).

In addition to looking at individual Bcl-2 family members that correlated with cellular response to ABT-263, we also derived optimal predictive gene signatures just from the Bcl-2 family members. These gene signature sets correctly predicted the response in 70% and 81% of the SCLC and leukemia/lymphoma cell lines, respectively. Overall, individually or collectively, Bcl-2 family members may be useful as predictors of response to ABT-263. We are currently evaluating their expression levels in on-going clinical trials to assess their potential as patient stratification biomarkers.

Ideal chemotherapeutics will have a maximal effect on tumor cells while having a minimal effect on the normal tissue. Our comparison of primary SCLC tumors to matching normal adjacent tissue showed a differential expression similar to that seen with the sensitive and resistant cell lines. Specifically, Bcl-2 and Noxa expression was significantly lower in normal tissue compared with tumor tissue, whereas expression of A1 and Mcl-1 is significantly higher in normal tissue, as was seen in the resistant cell lines compared with the sensitive cell lines. Our analysis with the SCLC predictor sets indicated that

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the expression pattern of these genes in normal tissue is also similar to resistant lines, whereas the tumor expression pattern of these genes is similar to the sensitive cell lines. Indeed, when we analyzed the tumor and normal tissues by discriminant analysis with the SCLC predictor sets, the normal tissues were all identified as resistant and the tumors were primarily identified as sensitive (four of eight with set 1 and seven of eight with set 2).

The predictor genes that correlate with resistant cell lines could be directly involved in resistance as has been shown previously with Mcl-1. However, they could be genes that are only indirectly involved yet still be good markers collectively for predicting response to ABT-263. Using ingenuity pathway analysis, we identified several of the signature genes directly related to the Bcl-2 family of proteins (Fig. 6). For example, Bcl-rambo may play a direct role through an antiapoptotic pathway not yet fully delineated or through a more general mechanism of drug resistance (32), as mentioned above. Conversely, the higher CASP9 expression in resistant cells may be indirect, as CASP9 is responsible for proteolytic cleavage during the apoptotic process, and higher expression would be expected to facilitate apoptosis. The higher expression of CASP9 in the resistant cell lines may therefore be possible due to apoptotic pathways being blocked upstream of CASP9.

For some of the predictor genes highly expressed in resistant SCLC cell lines, there is evidence that they play a role in cell growth regulation or in the prevention of apoptosis. For example, MFGE8 promotes tumor progression and resistance to apoptosis through integrin and AKT signaling (34). FZD2 (frizzled homologue 2) is part of the Wnt pathway that includes the TCF7L1 gene that encodes the TCF-3 transcription factor, indicating that Wnt signaling may play a role in resistance. NOTCH2 is overexpressed in B-cell chronic lymphocytic leukemia and might also contribute to the inhibition of apoptosis in B-cell chronic lymphocytic leukemia (35), and constitutively active NOTCH2 decreases protease inhibitor–induced apoptosis in a human B-cell line (36).

Some of the predictor genes highly expressed in resistant leukemia/lymphoma cell lines may also play a direct role in preventing apoptosis. TMBIM1 is a member of a family of proteins with Bax inhibitor motifs, and one of those family members, TMBIM6, has been shown to protect cells from endoplasmic reticulum stress-induced apoptosis by inhibiting endoplasmic reticulum stress proteins as well as the accumulation of reactive oxygen species through heme oxygenase I (37). Furthermore, in the mitochondria, the rate-limiting step of heme biosynthesis (which occurs in all cells) is regulated by the ALAS gene, whereas ALAS2 (the gene from the leukemia

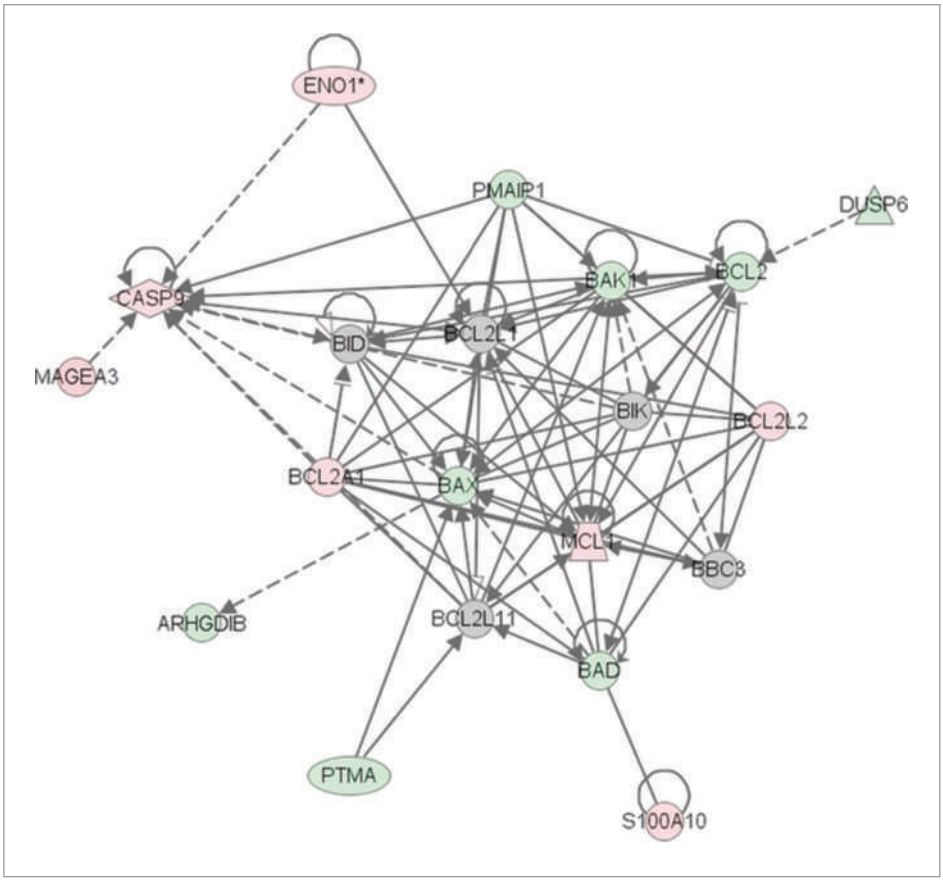

Figure 6. Predictor gene networks with Bcl-2 family members. A network of genes that interact with Bcl-2, Bcl-x_L, and Mcl-1 is shown. Genes that are colored in pink have higher expression in resistant cell lines, whereas genes colored in green have higher expression in sensitive cell lines (genes in gray were not differentially expressed). The genes in the center are Bcl-2 family members and are colored based on the highest expression change indicated in Table 1. The predictor genes on the outside of the figure are similarly colored based on the expression differences in the training set where they were identified.

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lymphoma predictor set) is normally specifically expressed in erythroid cells. Because defective ALAS2 has been linked to sideroblastic anemia and diminished heme production, ALAS2 function can enhance cellular survival, as excess iron promotes reactive oxygen species (38). Therefore, increased ALAS2 may function to decrease reactive oxygen species and promote mitochondrial stability. PPIC, or CypC, is a member of the cyclophilin family of proteins normally located in the endoplasmic reticulum and may have an antiapoptotic function through a chaperone activity that responds to cellular stress (39). Expression of PPRSS21/Testisin, a serine protease, has been shown to increase transformation, whereas siRNA that decreases PPRSS21/Testisin expression increases apoptosis, possibly through its cleavage activity (40).

Some of the genes identified by the DLDA method play a direct role in the regulation of apoptosis, tumor progression, and chemoresistance. For example, knockdown of CD44 (expressed higher in resistant SCLC lines) increased apoptosis and chemosensitivity accompanied by multidrug resistance-1 and Bcl-2 expression decreases in hepatocellular carcinoma cells (41). In addition, CD44 interaction with hyaluronan led to activation of β-catenin–mediated TCF/LEF activation, causing multidrug resistance-1 and Bcl-x_L upregulation in breast tumor cells (42). RRMI (expressed lower in resistant SCLC lines) expression leads to suppression of proliferation, cell cycle arrest, and apoptosis, possibly through DNA damage repair pathways (43).

Similarly, genes from the leukemia/lymphoma set are known to play a role in drug resistance or apoptosis. For example, loss of DUSP6/MKP3, a phosphatase that dephosphorylates extracellular signal-regulated kinase 1/2 (and has tumor suppressive effects), has been shown to increase tumorgenicity and chemoresistance (44). DUSP6 expression is high in 10 of 19 sensitive lines, whereas its expression is at or below background levels in five of six resistant leukemia/lymphoma cell lines, as would be predicted if loss of expression leads to chemoresistance. ARHGDIB/RhoGDI2 is a metastasis suppressor gene that has been shown to contribute to apoptosis in HL60 and K562 (45, 46). Significantly, HL60 cells are more sensitive than K562 to ABT-263, and expression of ARHGDIB/RhoGDI2 is almost 9-fold higher in HL60 cells. CD99, a gene whose expression is lower in resistant leukemia/lymphoma cell lines, contributes to Fas-mediated apoptosis in Jurkat cells (47), whereas ligation of CD99 in Ewing sarcoma cells induces caspase-independent apoptosis (48). Significantly, Jurkat cells, which are sensitive to ABT-263, had the highest expression of CD99 in our panel. MAGEA3 (increased in resistant leukemia/lymphoma lines) has been shown to contribute to fibronectin-mediated cancer progression through enhanced tumor growth (49).

In summary, we have found that high levels of Bcl-2 and Noxa and low levels of Mcl-1 correlated with the cellular response of SCLC and leukemic cell lines to ABT-263. Through expression profiling of untreated cells and comparison of sensitive and resistant SCLC and leukemia/lymphoma cell lines, we have identified expression patterns that are predictive of sensitivity to ABT-263 with high accuracy. This forms a testable hypothesis that can be applied in future clinical trials to determine how well these predictive markers identify patients that are most likely to benefit from ABT-263. The parallels in expression patterns between sensitive models and primary SCLC tumors further substantiate SCLC as an excellent target for ABT-263, and interrogation of these signatures and their predictive potential in ongoing clinical trials could help expedite the delivery of optimal treatment to future patients.

Disclosure of Potential Conflicts of Interest

The authors are employed by Abbott Laboratories and patents are filed on expression signatures.

Acknowledgments

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Identification of Expression Signatures Predictive of Sensitivity to the Bcl-2 Family Member Inhibitor ABT-263 in Small Cell Lung Carcinoma and Leukemia/Lymphoma Cell Lines

Stephen K. Tahir, John Wass, Mary K. Joseph, et al.

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