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Therapeutic Discovery

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A 71-Gene Signature of TRAIL Sensitivity in Cancer Cells

Jun-Jie Chen[1], Steen Knudsen[2], Wiktor Mazin[2], Jesper Dahlgaard[2], and Baolin Zhang[1]

Abstract

TNF-related apoptosis inducing ligand (TRAIL) is a promising anticancer agent because of its ability to selectively induce apoptosis in cancer cells but not in most normal cells. However, some cancer cells are resistant to TRAIL cytotoxicity thereby limiting its therapeutic efficacy. Using genome-wide mRNA expression profiles from the NCI60 panel and their differential sensitivities to TRAIL-induced apoptosis, we have identified 71 genes whose expression levels are systemically higher in TRAIL-sensitive cell lines than resistant lines. The elevated expression of the 71 genes was able to accurately predict TRAIL sensitivity in the NCI60 training set and two test sets consisting of a total of 95 human cancer cell lines. Interestingly, the 71-gene signature is dominated by two functionally related gene families—interferon (IFN)-induced genes and the MHC genes. Consistent with this result, treatment with IFN-γ augmented TRAIL-induced apoptosis. The 71-gene signature could be evaluated clinically for predicting tumor response to TRAIL-related therapies. *Mol Cancer Ther; 11(1); 34–44. ©2011 AACR.*

Introduction

Drug resistance is a major cause of cancer treatment failure. Nonresponsive patients do not benefit from the treatment but may still suffer from the side effects. A biomarker predicting tumor response to a drug will enhance the ability to individualize patient treatment, thus making development of cancer therapies more effective and safe.

TRAIL induces apoptosis through death receptors 4 and/or 5 expressed on the surface of target cells (1). Compared with TNFα, which was associated with severe toxicities after systemic administration, TRAIL appears to selectively kill cancer cells while leaving the normal cells largely unaffected (2, 3). Recombinant human TRAIL (rhTRAIL) and its agonistic antibodies are being clinically evaluated as a potential cancer therapy (4). However, some tumor cells, including those from breast cancer, are found to be resistant to TRAIL-induced apoptosis via intrinsic or acquired mechanisms (5–8). There is an unmet need for a biomarker that can predict tumor response to TRAIL-related therapies.

The most common approach in identifying biomarker is a top–down approach where knowledge of the putative target of a treatment is used to look for alterations in the target itself or related signaling components that render the treatment effective or ineffective. By this approach, it has been shown that TRAIL-resistance is associated with genetic defects in TRAIL signaling components, including loss of functional DR4 and DR5 on the cell surface (7, 8), *O*-glycosylation status (9), and elevated expression of antiapoptotic proteins c-FLIP (10), Bcl-2 (1), and IAP family proteins (11). However, these mechanisms are not generally applicable to different cancer types. In this study, we took a universal bottom–up approach that is based on measuring the response in cell lines and correlating differences in their gene expression profiles. This approach does not require knowledge of the putative target(s), and it has shown potential in predicting tumor response to chemotherapy treatments (12–15). Using NCI60 panel as a training set, we have identified a set of 71 genes whose elevated expression predicts the *in vitro* sensitivity of cancer cells to TRAIL-induced apoptosis.

Materials and Methods

Cell lines and reagents

The NCI60 panel of human cancer cell lines used for the testing of TRAIL sensitivity were maintained and tested for identity and Mycoplasma at the U.S. National Cancer Institute (NCI; http://dtp.nci.nih.gov). Human breast cancer cell lines including HCC1428 and HCC1143 were obtained from the American Type Culture Collection (ATCC), where the cell lines were

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tested and authenticated by growth rate, morphology, isoenzymology, short tandem repeat profiling, and Mycoplasma testing (www.ATCC.org). All cell lines were cultured as per vendors' recommendation and tested for sensitivity to TRAIL within 3 months from the date of purchase from ATCC. rhTRAIL was from R & D systems, which contains 168 amino acids corresponding to the extracellular domain of human TRAIL (Val114–Gly281), expressed by *Escherichia coli* and purified as a homotrimeric protein.

NCI60 anticancer drug screen

The NCI60 panel anticancer drug screen was carried out at the NCI/NIH Developmental Therapeutics Program (see details at http://dtp.nci.nih.gov/branches/btb/ivclsp.html; refs. 16, 17). Briefly, cells were seeded in 96-well plates at plating densities ranging from 5,000 to 40,000 cells per well depending on the doubling time of individual cell lines. After 24-hour incubation, some of the wells were processed to determine a time zero density. To the rest of the plates, rhTRAIL was added at 5 different doses (6, 12, 25, 50, or 100 ng/mL). Plates were incubated for another 24 hours, then fixed with trichloroacetic acid and stained with sulforhodamine B (SRB), and measured for absorbance at 515 nm. SRB binds on protein basic amino acid residues for measuring relative total protein amount and cell viability. Growth inhibition is calculated relative to cells without drug treatment and the time zero control. The use of a time zero control allows the determination of cell killing as well as net growth inhibition. Growth inhibition of 50% (GI_{50}), which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation, is calculated from $[(T_i - T_z)/(C - T_z)] \times 100 = 50$ using absorbance at 515 nm at time zero (T_z), in the absence of TRAIL (C), and in the presence of TRAIL (T_i).

Predictor development based on NCI60 gene expression data

Gene expression profiles of 58 of the 60 cell lines in the NCI60 panel were obtained from Shankavaram and colleagues (18). First, the gene expression measurements were logit normalized, that is, for each array the transformation $\text{logit} = \log[(x - \text{background})/(\text{saturation} - x)]$ was carried out followed by a Z-transformation to mean zero and SD 1 and dChip expression index was calculated by summarizing the 11 independent perfect match probes into one probeset corresponding to one gene. The resulting expression value for each gene in each of the 58 cell lines was then correlated to the measured GI_{50} values in the same cell lines to look for genes that can explain the observed difference in growth inhibition, that is, $\text{correlation} = \text{cor}(\text{expression}, -\log GI_{50})$. Genes with a Pearson correlation above 0.25 were considered potential markers of response and retained as a response profile for TRAIL. To reduce the number of false-positive markers passing the Pearson correlation cutoff, we applied a statistical bootstrapping filter and a biologic relevance filter that mapped sub-networks of markers known to interact.

Finally, we selected the number of genes that carried out best in predicting the growth inhibition/apoptosis in the training set of NCI60. All statistical analysis was carried out in R (www.r-project.org) using the Affy library of Bioconductor (www.bioconductor.org).

Prediction of TRAIL sensitivity in cancer cell lines

The identified gene signature from the NCI60 training set was evaluated in 2 independent test sets consisting of 119 human cancer cell lines from different cancer types (9) and 18 human breast cancer cell lines, respectively, based on gene expression data from Wagner and colleagues (9) and Hoeflich and colleagues (19). In addition, we examined the gene signature for prediction of TRAIL sensitivity in 4 independent cohorts of human breast tissues, including 2 cohorts of normal breast tissues (GEO accession numbers: GSE20437 and GSE9574) and 2 cohorts of breast tumor tissues (GSE20194 and GSE12093; http://wwww.ncbi.nlm.nih.gov/geo). After logit normalization and dChip expression index calculation of array data from independent data sets (cell lines or primary tissues), the expression value of each gene in the response profile for TRAIL were averaged to yield a quantitative score of TRAIL sensitivity on a scale from 0 to 100 on the basis of the lowest average expression (Min) and the highest average expression (Max). Statistical significance of the prediction was calculated as Pearson product moment correlation between the prediction scores of cell lines and their measured TRAIL sensitivity (GI_{50}). When resistant cell lines were compared with sensitive cell lines, statistical significance was calculated using a Wilcoxon test. In some comparisons, the quantitative TRAIL sensitivity score was converted to a categorical prediction (sensitive or resistant) by applying a cutoff to the sensitivity score. Using the above defined 0 to 100 gene expression scale, 50 or (Min + Max)/2 of a specific data set was used as a cutoff between TRAIL-resistant and TRAIL-sensitive cells. In the NCI60 panel, for example, a cell line is predicted to be TRAIL sensitive when the average of mRNA expression of the 71 genes is higher than (Max + Min)/2, that is, half the sum of the maximum (A498) and minimum (SK-OV-3) values of the average expression of the 71 genes in all NCI60 cell lines. Otherwise the cell line is predicted to be TRAIL resistant. Similarly, the (Min + Max)/2 value of individual test set was used a cutoff for prediction TRAIL sensitivity (resistant or sensitive) of samples in the data sets.

The effect of varying cutoffs on sensitivity and specificity of prediction was determined in a receiver operating characteristic (ROC). The predictive power is measured as

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an area under the curve (AUC) in a ROC plot, where and AUC of 1 means perfect prediction for all cutoffs and AUC of 0.5 is no better than tossing a coin to predict resistance to TRAIL. The ROC library for R at Bioconductor (www.bioconductor.org) was used.

Functional pathway analysis

Ingenuity Pathway Analysis (https://analysis.ingenuity.com/pa/launch.jsp) was used to derive curated molecular interactions, including both physical and functional interactions, and pathway relevance. The databases and software toolsets weigh and integrate information from numerous sources, including experimental repositories and test collections from published literatures.

Results

Identification of a 71-gene expression signature of TRAIL sensitivity

To identify potential biomarkers for predicting TRAIL sensitivity, we examined TRAIL-induced cytotoxicity in the NCI60 panel of human cancer cell lines, including leukemia, melanoma, lung, colon, brain, ovary, breast, prostate, and kidney. By measuring cell viability as a function of rhTRAIL concentrations (see examples in Fig. 1), we obtained GI_{50} values for individual cell lines (Table 1). The results classify 60% (35 of 60) of the cell lines as highly or moderately sensitive (regarded as sensitive) with GI_{50} less than 100 ng/mL. All other cell lines (40%, 25 of 60) exhibited less than 20% growth inhibition in the presence of 100 ng/mL rhTRAIL after 24 hours incubation and are being regarded as resistant ($GI_{50} > 100$ ng/mL) in the following discussion. Next, the measured GI_{50} values were correlated with the genome-wide gene expression data in each of the NCI60 cell lines using Pearson analysis. By applying a 0.25 cutoff value of correlation coefficient (CC) for each gene and a biologic relevance filter, we identified 91 probesets corresponding to 71 genes whose expressions were positively correlated with the measured TRAIL sensitivity, meaning that the 71 genes were systemically expressed at higher levels (~1 to

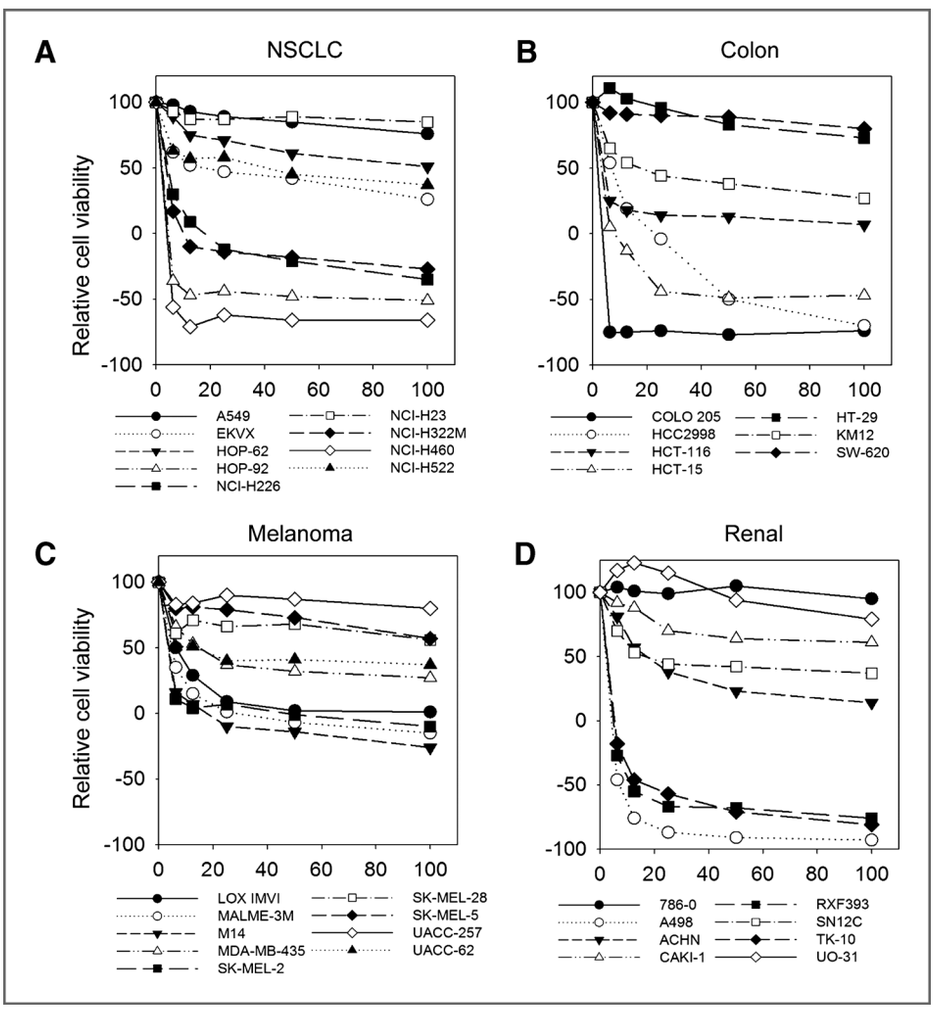

Figure 1. TRAIL cytotoxicity in NCI60 panel. A–D, representatives of cancer types in response to rhTRAIL treatment, including NSCLC (A), colon (B), melanoma (C), and renal carcinoma (D). Cells were seeded in 96-well plates, treated with or without rhTRAIL at the indicated concentrations (6, 12, 25, 50, or 100 ng/mL) for 24 hours, and cell viability was measured by staining with SRB. GI_{50} is calculated from $[(T_i - T_z)/(C - T_z)] \times 100 = 50$, using absorbance at 515 nm measured at time zero (T_z), control (C), and in the presence of TRAIL (T_i). GI_{50} values are listed in Table 1.

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Table 1. TRAIL sensitivity in NCI60 training set

Cancer types	Cell lines	GI_{50}, ng/mL	TRAIL sensitivity	
			Measured[a]	Predicted[b]
Leukemia	CCRF-CEM	69.18	S	R
	HL-60 (TB)	6.31	S	S
	K-562	>100	R	R
	MOLT-4	>100	R	R
	RPMI-8226	6.31	S	S
	SR	6.31	S	S
NSCLC	A549/ATCC	>100	R	R
	EKVX	16.60	S	R
	HOP-62	>100	R	S
	HOP-92	6.31	S	S
	NCI-H226	6.31	S	S
	NCI-H23	>100	R	NA
	NCI-H322M	6.31	S	S
	NCI-H460	6.31	S	S
	NCI-H522	38.02	S	R
Colon cancer	COLO 205	6.31	S	S
	HCC2998	6.76	S	S
	HCT-116	6.31	S	R
	HCT-15	6.31	S	S
	HT-29	>100	R	S
	KM12	16.22	S	R
	SW-620	>100	R	R
CNS cancer	SF-268	>100	R	R
	SF-295	6.31	S	S
	SF-539	6.31	S	S
	SNB-19	>100	R	R
	SNB-75	>100	R	S
	U251	>100	R	R
Melanoma	LOX IMVI	6.31	S	S
	MALME-3M	6.31	S	S
	M14	6.31	S	S
	MDA-MB-435	14.13	S	R
	SK-MEL-2	6.31	S	S
	SK-MEL-28	>100	R	S
	SK-MEL-5	>100	R	R
	UACC-257	>100	R	R
	UACC-62	13.49	S	S
Ovarian cancer	IGROV1	>100	R	R
	OVCAR-3	6.31	S	R
	OVCAR-4	27.54	S	S
	OVCAR-5	21.38	S	S
	OVCAR-8	>100	R	S
	NCI/ADR-RES	>100	R	S
	SK-OV-3	11.48	S	S
Renal cancer	786-O	>100	R	S
	A498	6.31	S	S
	ACHN	16.22	S	S
	CAKI-1	>100	R	S
	RXF393	6.31	S	S
	SN12C	15.49	S	S

(Continued on the following page)

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Table 1. TRAIL sensitivity in NCI60 training set (Cont'd)

Cancer types	Cell lines	GI_{50}, ng/mL	TRAIL sensitivity	
			Measured[a]	Predicted[b]
	TK-10	6.31	S	S
	UO-31	>100	R	S
Prostate cancer	PC-3	>100	R	R
	DU-145	>100	R	S
Breast cancer	MCF7	>100	R	R
	MDA-MB-231	7.94	S	S
	HS578T	75.86	S	R
	BT549	6.31	S	S
	T47D	>100	R	R
	MDA-MB-468	>100	R	NA

Abbreviation: NA, gene expression data not available.

[a]Cell lines with a GI_{50} less than 100 ng/mL of rhTRAIL are referred as TRAIL sensitive (S); cell lines with a GI_{50} more than 100 ng/mL of rhTRAIL are referred as TRAIL resistant (R), which exhibited less than 20% growth inhibition in the presence of 100 ng/mL rhTRAIL after 24-hour incubation.

[b]A cell line is predicted to be TRAIL sensitive when the average of mRNA expression of the 71 genes is higher than (Max + Min)/2, that is, half the sum of the maximum (A498) and minimum (SK-OV-3) values of the average expression of the 71 genes in all NCI60 cell lines, otherwise the cell line is predicted to be TRAIL resistant.

2.5 fold) in sensitive cell lines than resistant cell lines. Trying progressively smaller subsets of the 71-gene predictor revealed that the performance drops when less than 71 genes are used (Fig. 2A). We also identified 11 genes whose expression levels were systemically higher in TRAIL-resistant cell lines than in TRAIL-sensitive cell lines, including *EEF1D*, *MAGED4*, *ADARB1*, *CSE1L*, *EVL*, *PAICS*, *RAD51C*, *GATA3*, *SSRP1*, *MARCKSL1*, and *LOC100272216*. However, inclusion of these genes with opposite sign in the prediction of TRAIL sensitivity did not improve the prediction accuracy in our test sets. The identified 71 genes were then used to predict TRAIL sensitivity in 58 of the 60 cell lines on the basis of their available gene expression data, yielding an overall 69% (40 of 58) prediction accuracy, that is, percentage of matches between the predicted and measured TRAIL sensitivity (Table 1). Higher prediction accuracy (>80%) was achieved for cancer cell lines derived from leukemia, central nervous system (CNS), melanoma, and breast carcinoma (Fig. 2B). Figure 2C shows a heatmap of the 71 genes in 58 of the 60 cell lines for which we have gene expression data. Thus, the upregulation of the 71 genes appears to constitute a predictor of TRAIL response in cancer cells (Table 2).

Validation on independent test set of 119 cancer cell lines

To validate the 71-gene predictor, we took advantage of the recently published data on gene expression and TRAIL sensitivity of 119 human cancer cell lines (9). These include 23 pancreatic adenocarcinomas, 42 non–small cell lung carcinomas (NSCLC), 18 malignant melanomas, and 36 colorectal adenocarcinomas. Thirteen of the 119 cell lines are also present in the NCI60 panel used in developing the predictor. The results from Wagner and colleagues (9) showed that 34% (40 of 119) of the cell lines were highly or moderately sensitive to TRAIL-induced killing. We calculated the TRAIL sensitivity on the basis of the expression of 71 genes in these cell lines. The prediction accuracy is poor for NSCLC cell lines, and therefore they were excluded in the following analysis. Figure 3A shows an ROC for the predicted sensitivity of the remaining 77 cell lines (excluding NSCLC cell lines). A Wilcoxon test of difference in predicted TRAIL sensitivity between cell line measured as TRAIL-sensitive and cell lines measured as TRAIL-resistant yielded a P value 0.02. When NSCLC cell lines were omitted, the P value is 0.0006 for the remaining 77 cell lines [see Supplementary Data SI: a comparison between the predicted sensitivity and measured sensitivity for 77 of the 119 cell lines (omitting NSCLC cell lines)]. The prediction accuracy varies between different cancer types, with a better prediction (~70%) in melanoma cell lines (Fig. 3B).

Validation on independent test of breast cancer cell lines

We (7, 8, 20) and others (5, 6, 21) have shown that TRAIL is potentially useful in treating breast cancers. However, a significant number of breast cancer cell lines were found to be resistant to TRAIL killing. We asked whether the 71-gene predictor could also be used to predict TRAIL sensitivity in breast cancer cells. Therefore, we investigated TRAIL sensitivity in a panel of

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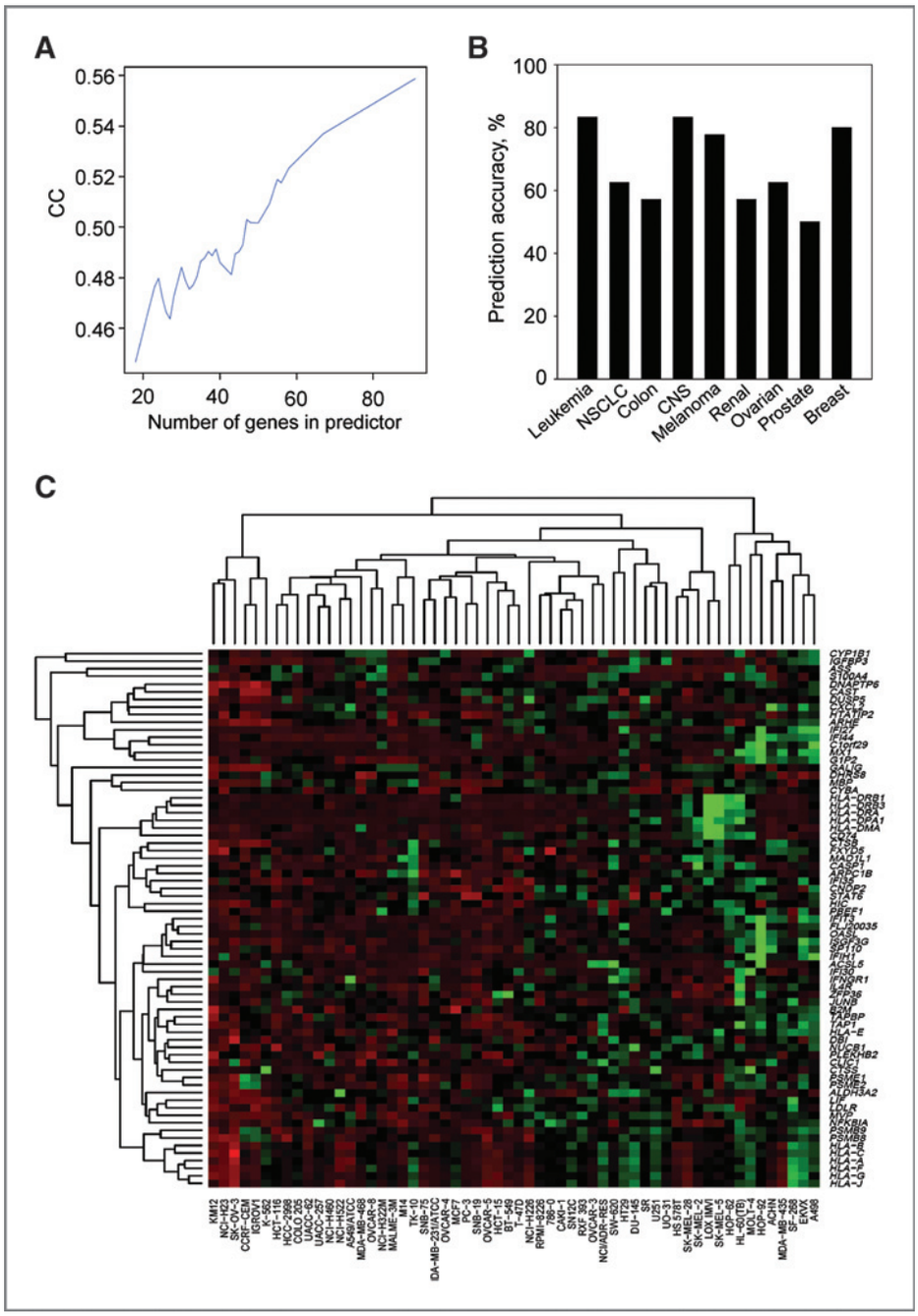

Figure 2. Development of gene expression predictor for TRAIL response. A, performance of the TRAIL response predictor on the training set of 58 cell lines as a function of the number of gene prosets in the predictor. Performance is given as correlation between prediction and measurement in training set. Before removing genes, they were ranked according to the number of times they were selected in a leave-one-out cross-validation. B, prediction accuracy (%) of TRAIL response (sensitive or resistant) in the NCI60 panel determined by the expression of the selected 71 genes and the number of matches with the measured GI_{50} values in each cancer type. C, the resulting 71-gene profile was visualized with a heatmap using the Heatplus library of Bioconductor. The 71 genes (right Y-labeled) and the NCI60 panel cell lines (lower X-labeled) were hierarchically clustered using Euclidean distance on the basis of the expression levels of each gene in specific cell lines relative to its average value in the NCI60 panel. Green color indicates upregulated genes and red color indicates downregulated genes. The color codes represent fold change from average for each gene.

18 human breast cancer cell lines, including 9 cell lines tested for TRAIL sensitivity in our laboratory (MDA-MB-231, SKBR3, BT474, BT549, T47D, MDA-MB-468, MCF7, HCC1143, and HCC1428; ref. 8), of which 7 cell lines were also tested by Rahman and colleagues (22), and 9 additional cell lines tested by Rahman (AU-565, HCC1954, HCC1937, HS578T, MDA-MB-436, HCCC38, HCC1500, MDA-MB-453, and BT20). Using the expression levels of the 71 genes, our calculation predicted that 44% (8 of 18) of the cell lines to be TRAIL sensitive and 56% (10 of 18) of the cell lines to be resistant (Fig. 3C and Supplementary Data SII). When compared with measured TRAIL sensitivity, the predictions matched the actual *in vitro* sensitivity results for 72% (13 of 18) of the cell lines, with a negative predictive value of 80% (8 of 10) and a positive predictive value of 63% (5 of 8). Of note, there was a discrepancy in the measured TRAIL sensitivity for MDA-MB-468 cell line. Consistent with

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Table 2. Functional grouping of the 71-gene predictor of TRAIL sensitivity

Function (no. of genes)	Genes
IFN or viral induced ($n = 13$)	*IFI41*, *IFI44L*, *DDX60*, *MX1*, *G1P2*, *IFIH1*, *IRF9*, *IFI35*, *IFI27*, *IFI44*, *IFI30*, *IFIT3*, and *IFNGR1*
MHC genes ($n = 13$)	*HLA-A*, *HLA-B*, *HLA-C*, *HLA-E*, *HLA-F*, *HLA-G*, *HLA-J*, *HLA-DRB1*, *HLA-DRB3*, *HLA-DMA*, *HLA-DPA1*, *HLA-DRA*, and *CD74*
MHC processing ($n = 9$)	*B2M*, *TAPBP*, *TAP1*, *PSMB8*, *PSMB9*, *PSME1*, *PSME2*, *MBP*, and *CTSS*
Apoptosis ($n = 13$)	*IGFBP3*, *PBEF1*, *ACSL5*, *HTATIP2*, *MVP*, *CTSB*, *CAST*, *CASP1*, *NUCB1*, *STAT6*, *IL4R*, *RhoE*, and *GALIG*
Other ($n = 23$)	*ASS*, *ALDH3A2*, *MAD1L1*, *OASL*, *CXCL2*, *DBI*, *CNDP2*, *DHRS8*, *FXYD5*, *ZFP36*, *JUNB*, *DUSP5*, *LIF*, *PLEKHB2*, *LDLR*, *CLIC1*, *CYBA*, *DNAPTP6*, *S100A4*, *NFKBIA*, *ARPC1B*, *CYP1B1*, and *HIC*

our previous result (8), the 71-gene profile predicted this cell line to be sensitive to TRAIL-induced apoptosis.

A unique property of TRAIL is that it selectively induces apoptosis in cancer cells without harming most normal cells, albeit the undefined mechanisms. An interesting question was asked whether the 71-gene signature could predict TRAIL sensitivity patterns in normal tissues and tumors. We tested this possibility in breast tissue samples from 4 independent clinical trials with a total of 485 patients, based on their gene expression profiles (see Materials and Methods). The predicted TRAIL sensitivity is shown in Figure 3D. All the normal breast tissues were found to express lower levels of the 71 genes, with average expression values below the cutoff 50 in each cohort, and then were predicted to be resistant to TRAIL. Of the breast tumors, 64% are predicted to be sensitive to TRAIL. Overall, normal breast tissues were predicted to be significantly less sensitive to TRAIL cytotoxicity than breast tumors ($P < 2.2e\text{-}16$). These results support not only the selectivity of TRAIL toward cancer cells but also the use of the 71-gene signature in prediction of TRAIL sensitivity.

Involvement of interferon-induced genes in TRAIL sensitivity

The identified 71 genes fall into at least 5 distinct functional groups, including interferon (IFN) pathways, major histocompatibility complex (MHC), apoptosis, and those of less defined functions (Table 2). Thirteen genes were found to be IFN-related, including *IFI41*, *IFI41*, *IFI44L*, *DDX60*, *MX1*, *G1P2*, *IFIH1*, *IRF9*, *IFI35*, *IFI27*, *IFI44*, *IFI30*, *IFIT3*, and *IFNGR1*. Some of these genes have been shown to be directly upregulated in response to type I (e.g., IFN-α, IFN-β) and/or type II IFN (IFN-γ) in human cancer cells (23). We sought to examine whether treatment with IFN could alter cellular response to TRAIL-induced apoptosis. To this end, we randomly selected 5 cell lines from the NCI60 panel that were predicted to be TRAIL-resistant, including A549, SK-MEL-5, HT-29, K562, and 768-0. All 5 cell lines were confirmed to be resistant to TRAIL-induced killing (Fig. 4A and data not shown). Cells were then pretreated with IFN-γ or IFN-α for 48 hours, followed by incubation with TRAIL, and analyzed for cytotoxicity. Combination with IFN-γ and rhTRAIL significantly reduced cell viability in A549, SK-MEL-5, and HT-29 cell lines (not shown), but not in K562 and 768-0 cells (not shown). The failure of IFN-γ in overcoming TRAIL resistance in some cell lines may reflect the difference in their genetic background and also suggest the involvement of other genes in regulation of TRAIL apoptosis pathways. In contrast, IFN-α had no synergy with TRAIL in the cell lines tested. The mechanism underlying the differential effects between IFN-γ and IFN-α is not clear, but it may be related to their difference in inducing caspase genes (24). Indeed, the IFN-γ enhanced cytotoxicity was correlated with an increase in apoptosis (Fig. 4B) and caspase cleavage (Fig. 4C), which was completely blocked by treatment with a general caspase inhibitor Z-VAD (Fig. 4D). Consistent with our data, there are also reports that IFN-γ augmented TRAIL killing in cell lines derived from colon (25), bladder (26), renal (27), and gastric carcinomas (28). These data show that IFN-γ pathways play a critical role in modulating cellular sensitivity to TRAIL-induced apoptosis.

Functional pathway analysis revealed various types of interactions between MHC genes, IFN pathways, and TRAIL pathways (Supplementary Data SIII). While the molecular mechanisms remain to be elucidated, the upregulation of IFN-γ–regulated genes appears to constitute a critical determinant of TRAIL sensitivity.

Discussion

As with other chemotherapies, TRAIL death receptor targeted agents also encountered resistance in tumor cells (5–8). The ability to identify specific tumors that are potentially responsive to TRAIL or its agonistic antibodies would be very beneficial to the clinical development of these agents. We carried out a comprehensive analysis of the gene expression profiles of NCI60 panel cell lines and their sensitivities to TRAIL-induced cytotoxicity. We have identified 71 genes whose expression levels were systemically higher in sensitive cell lines than in TRAIL-resistant cell lines. Furthermore, we have evaluated the 71-gene

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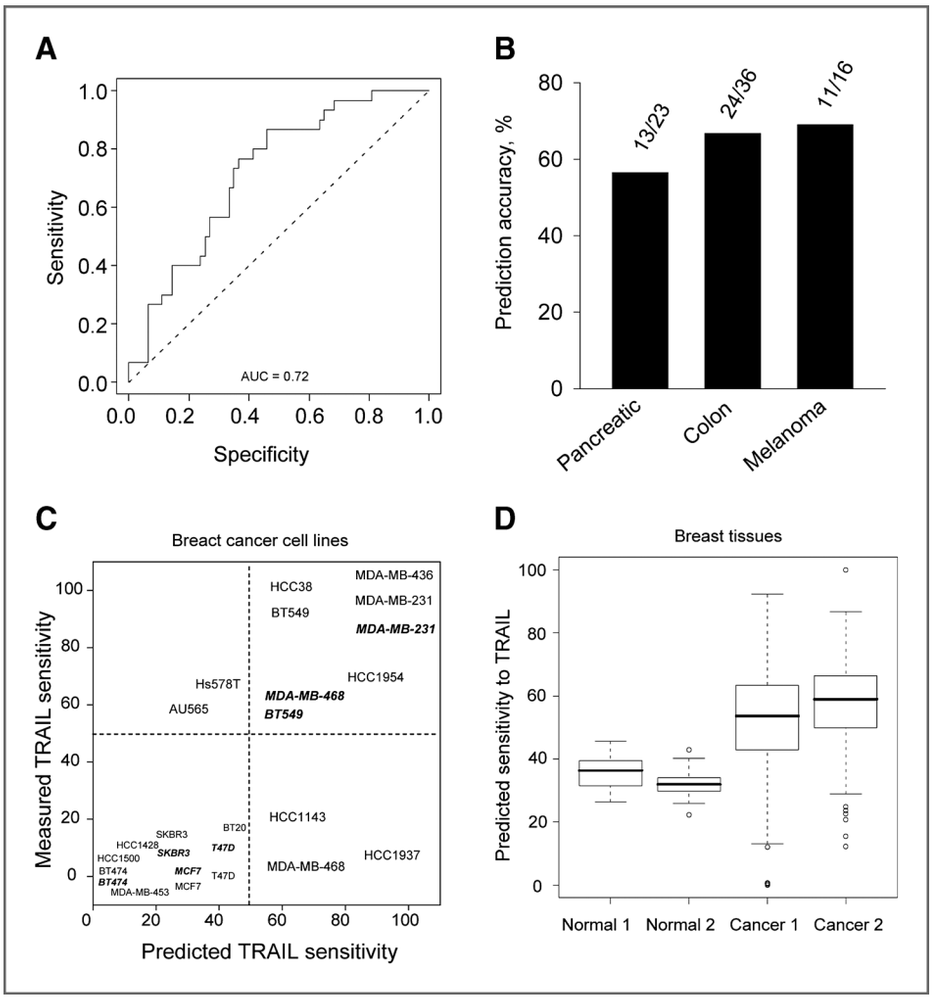

Figure 3. Evaluation of predictor on independent test sets. A, prediction of TRAIL sensitivity in a panel of 77 cancer cell lines, including cell lines derived from pancreatic, melanoma, and colorectal carcinoma whose sensitivities to TRAIL were tested by Wagner and colleagues (9). ROC shows a dependence of prediction sensitivity and specificity on the cutoff chosen to separate TRAIL-sensitive from TRAIL-resistant cell lines. The AUC is 0.72, where an area of 0.5 (indicated by the dotted line) corresponds to tossing a coin to predict sensitivity to TRAIL. B, prediction accuracy (%) for the 77 cancer cell lines was determined by the number of matches between the predicted and measured TRAIL sensitivity (Supplementary Data SI) for each cancer type. C, comparison of the predicted TRAIL sensitivity with the measured TRAIL sensitivity in an independent test set of 18 breast cancer cell lines. The X-scale (0–100) represents the average values of the 71 genes expression in each cell line relative to the difference between the maximum (Max) value in MDA-MB-436 and the minimum (Min) value in HCC1500 cell line of the NCI60 panel. The mean value (Min + Max)/2 or 50% is used as a cutoff in determining a cell line to be TRAIL sensitive or resistant. The measured TRAIL sensitivity data were obtained from Rahman and colleagues (22) and our previous work (bold italic; ref. 8), where 7 cell lines were tested by the 2 groups. The CC between prediction and measurement is 0.73 ($P = 0.00003$). D, predicted sensitivity to TRAIL in 4 independent clinical data sets consisting of 71 normal breast tissue samples (Normal 1: 42 patients; Normal 2: 29 patients) and 414 breast tumors (Tumor 1: 278 patients; Tumor 2: 136 patients), based on the expression data of the 71 genes (http://wwww.ncbi.nlm.nih.gov/geo). All normal tissues were found to express lower levels of the 71 genes than tumor tissues, with average values below cutoff of 50 on a 0 to 100 scale and thus predicted to be resistant to TRAIL-induced cytotoxicity.

signature in 2 independent test sets consisting of a total of 94 cancer cell lines.

In studying TRAIL resistance, a classical top–down approach has been widely used to search for mutations and/or alterations in TRAIL signaling pathways. These studies have identified numerous factors contributing to TRAIL resistance, including upregulation of antiapoptotic molecules (e.g., c-FLIP, NF-8B, Bcl-2, Akt, survivin, and XIAP) and downregulation of proapoptotic molecules (e.g., caspase-8 and FADD). Recently, the posttranslational modifications of the DR4 and DR5 receptors, including endocytosis (7, 8, 20) and *O*-glycosylation (9), as well as the ubiquitination of caspase-8 (29) were implicated as mechanisms for affecting TRAIL-induced cell death. However, the top–down approach requires prior knowledge on the mechanisms of action of a drug. Moreover, most of the studies were done in specific cell lines and not surprisingly, none of these mechanisms was generally applicable regarding TRAIL resistance across different cancer types. Bearing

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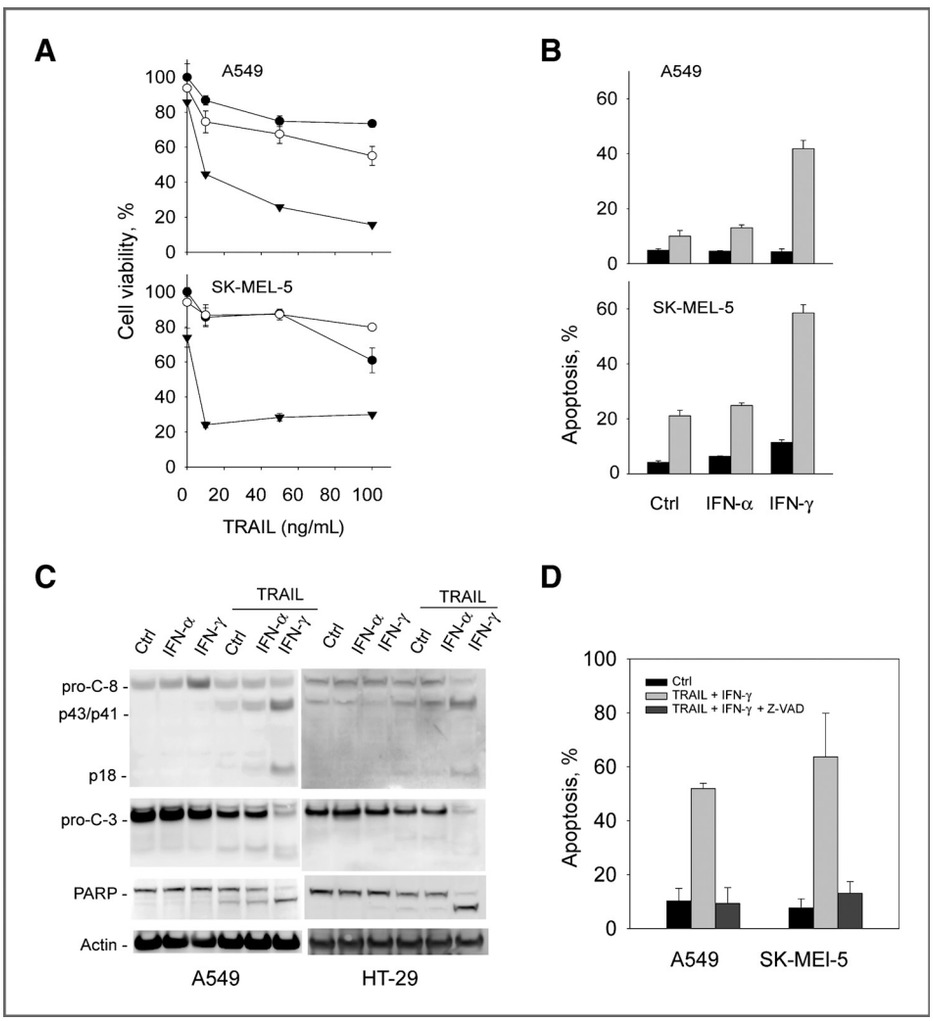

Figure 4. Involvement of IFN-γ in TRAIL sensitivity. The indicated cell lines were left untreated (solid circle) or pretreated with 25 ng/mL of IFN-γ (triangle) or 250 units/mL of IFN-α (open circle) for 24 hours, and then incubated with rhTRAIL at increasing doses for an additional 24 hours (A) or with 10 ng/mL TRAIL (B) and analyzed for cell viability (A) and apoptosis (B), and caspase cleavage by Western blotting (C). The predicted TRAIL resistance (Table 1) was confirmed in all cell lines tested, which was overcome by combination with IFN-γ, but not IFN-α. D, IFN-γ–enhanced TRAIL cytotoxicity was blocked by caspase inhibition. Cells were pretreated with a general caspase inhibitor Z-VAD at 20 μmol/L for 1 hour, followed by treatment with 25 ng/mL of IFN-γ for 48 hours and 10 ng/mL of TRAIL for 24 hours. Shown are representative of 3 independent experiments. Ctrl, control.

this in mind, we took a genomic approach that explores the systems biology of cells in response to TRAIL treatment. This approach allowed us to identify a 71-gene expression signature that predicts TRAIL sensitivity with high accuracy across a wide range of cancer cell lines (Fig. 3B and C) as well as primary breast tumors (Fig. 3D). In the latter, the expression pattern of the 71 genes predicted normal breast tissues to be significantly less sensitive than breast tumors to TRAIL cytotoxicity. The 71 genes are expressed at a significantly higher level in TRAIL-sensitive cells than resistant cells, suggesting that selecting patients whose tumor has this gene expression profile is likely to improve the odds of obtaining clinical benefit from this agent. A similar approach has been successfully used for targeted therapies such as Herceptin and Avastin, where patients are screened for Her2 or Ras mutant status (30). In relation to TRAIL therapy, Wagner and colleagues (9) reported that the mRNA expression of the peptidyl *O*-glycosyltransferase GALNT14 was associated with a higher TRAIL sensitivity in certain cancer cell lines. We examined the mRNA expression of GALNT14 in the NCI60 panel and found no statistically significant difference between the most sensitive and most resistant cell lines in the NCI60 panel (data not shown). Araki and colleagues (31) recently proposed a 4-gene expression predictor (*STK17B*, *SP140L*, *CASP8*, and *AIM1*) for cellular sensitivity to a monoclonal antibody against TRAIL-R1 (DR4 mAb) and TRAIL using a training set of 6 colon cancer cell lines. CASP8 was also differentially expressed between TRAIL-resistant and TRAIL-sensitive cell lines in the NCI60 training set. However, the performance of prediction is unaffected by including CASP8 to the 71 genes (CC = 0.73 without CASP8 and CC = 0.73 with CASP8). The other genes are not differentially expressed in our data set. We speculate that the differences in outcome may be due largely to the difference in mechanisms of action between TRAIL and TRAIL-R1 mAb. Although both agents share a downstream signaling pathway, it is generally agreed that TRAIL induces apoptosis through DR4 and/or DR5, whereas DR4 mAb only targets DR4. We have

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shown that the 2 death receptors are differentially regulated in cancer cells, which directly impacts on TRAIL sensitivity. Another possibility lies in the difference in the origin tissues and number of the cell lines used, as we used the NCI60 panel instead of only 6 colon cancer cell lines by Araki and colleagues. These results suggest that predictive biomarkers should be validated specifically to individual therapies even if/when they share the similar mechanisms of action (or signaling pathways). To our knowledge, this is the first report of gene expression indicator of tumor cell sensitivity to TRAIL-induced apoptosis that has been evaluated in independent test sets consisting of a wide range of cancer types.

The 71-gene signature produced a better prediction for TRAIL sensitivity in breast, leukemia, melanoma, and CNS (Figs. 2B and 3B) than in other cancer types such as NSCLC (not shown) and pancreatic (Fig. 3B). This might be related to the following factors: (i) the difference in genetic background between cancer types; (ii) the size of samples for each cancer type included in a test data set; and (iii) the discrepancy of TRAIL sensitivity for a specific cell line measured by different laboratories. For example, MDA-MB-468, MCF7, T47D breast cancer cell lines were shown to be TRAIL-resistant by several groups; however, they were shown to be TRAIL-sensitive by others (6, 8, 22). This could be a result of cell line variation and/or a difference in the preparation of rhTRAIL protein used in the assays. Despite the same tissue origin, a cell line used in the gene expression analysis may not be identical to the one used for TRAIL sensitivity assay which could cause mismatch and a lowered prediction accuracy. Regardless, the performance of the 71-gene predictor could be improved by increasing the number of samples in a test data set.

Strikingly, the IFN and MHC-related genes dominate the 71-gene predictor. The expression of 13 IFN-induced genes was found to be systemically higher in TRAIL-sensitive cell lines than resistant cells, suggesting a role for IFN pathways in promoting apoptosis by TRAIL. In support of this notion, combination treatment with IFN-γ and TRAIL significantly enhanced cell killing of TRAIL-resistant cell lines, including randomly selected A549, SK-MEL-5, and HT-29 cell lines. Similar observations have been made with colon (25), bladder (26), renal (27), and gastric carcinomas (28). IFN-γ is likely to augment TRAIL-induced apoptosis through upregulation of caspase activity (Fig. 4). It is not clear how MHC genes might be involved in the regulation of TRAIL signaling; however, several MHC genes are directly regulated by IFN induction (see Supplementary Data SIII). Additional studies are required to assess the functional relevance of individual genes that could lead to a better understanding of the 71-gene predictor in the context of systems biology that views cells as a network of nonredundant interactions. Nonetheless, our findings suggest that concurrently targeting IFN pathways may increase TRAILefficacy and reduce the probability of developing resistance.

In summary, the current studies suggest that the 71 genes are an important set of biomarkers for predicting the sensitivity of cancer cells to TRAIL cytotoxicity. The results could also justify an approach targeting TRAIL death receptors in combination with IFN-γ in treating cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. Targeting death-inducing receptors in cancer therapy. Oncogene 2007;26:3745–57.
2. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305–8.
3. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155–62.
4. Ashkenazi A, Holland P, Eckhardt SG. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol 2008;26:3621–30.
5. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 1999;59:734–41.
6. Singh TR, Shankar S, Chen X, Asim M, Srivastava RK. Synergistic interactions of chemotherapeutic drugs and tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand on apoptosis and on regression of breast carcinoma *in vivo*. Cancer Res 2003;63:5390–400.
7. Yoshida T, Zhang Y, Rivera Rosado LA, Zhang B. Repeated treatment with subtoxic doses of TRAIL induces resistance to apoptosis through its death receptors in MDA-MB-231 breast cancer cells. Mol Cancer Res 2009;7:1835–44.
8. Zhang Y, Zhang B. TRAIL resistance of breast cancer cells is associated with constitutive endocytosis of death receptors 4 and 5. Mol Cancer Res 2008;6:1861–71.
9. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med 2007;13:1070–7.
10. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, et al. Inhibition of death receptor signals by cellular FLIP. Nature 1997;388:190–5.

Published OnlineFirst October 25, 2011; DOI: 10.1158/1535-7163.MCT-11-0620

11. Liston P, Fong WG, Korneluk RG. The inhibitors of apoptosis: there is more to life than Bcl2. Oncogene 2003;22:8568–80.
12. Salter KH, Acharya CR, Walters KS, Redman R, Anguiano A, Garman KS, et al. An integrated approach to the prediction of chemotherapeutic response in patients with breast cancer. PLoS One 2008;3: e1908.
13. Williams PD, Cheon S, Havaleshko DM, Jeong H, Cheng F, Theodorescu D, et al. Concordant gene expression signatures predict clinical outcomes of cancer patients undergoing systemic therapy. Cancer Res 2009;69:8302–9.
14. Lee JK, Havaleshko DM, Cho H, Weinstein JN, Kaldjian EP, Karpovich J, et al. A strategy for predicting the chemosensitivity of human cancers and its application to drug discovery. Proc Natl Acad Sci U S A 2007;104:13086–91.
15. Staunton JE, Slonim DK, Coller HA, Tamayo P, Angelo MJ, Park J, et al. Chemosensitivity prediction by transcriptional profiling. Proc Natl Acad Sci U S A 2001;98:10787–92.
16. Holbeck SL, Collins JM, Doroshow JH. Analysis of Food and Drug Administration-approved anticancer agents in the NCI60 panel of human tumor cell lines. Mol Cancer Ther 2010;9: 1451–60.
17. Kummar S, Chen HX, Wright J, Holbeck S, Millin MD, Tomaszewski J, et al. Utilizing targeted cancer therapeutic agents in combination: novel approaches and urgent requirements. Nat Rev Drug Discov 2010; 9:843–56.
18. Shankavaram UT, Reinhold WC, Nishizuka S, Major S, Morita D, Chary KK, et al. Transcript and protein expression profiles of the NCI-60 cancer cell panel: an integromic microarray study. Mol Cancer Ther 2007;6:820–32.
19. Hoeflich KP, O'Brien C, Boyd Z, Cavet G, Guerrero S, Jung K, et al. *In vivo* antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. Clin Cancer Res 2009; 15:4649–64.
20. Zhang Y, Yoshida T, Zhang B. TRAIL induces endocytosis of its death receptors in MDA-MB-231 breast cancer cells. Cancer Biol Ther 2009; 8:917–22.
21. Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, et al. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci U S A 2000;97:1754–9.
22. Rahman M, Davis SR, Pumphrey JG, Bao J, Nau MM, Meltzer PS, et al. TRAIL induces apoptosis in triple-negative breast cancer cells with a mesenchymal phenotype. Breast Cancer Res Treat 2009;113:217–30.
23. Morrow AN, Schmeisser H, Tsuno T, Zoon KC. A novel role for IFN-stimulated gene factor 3II in IFN-gamma signaling and induction of antiviral activity in human cells. J Immunol 2011;186:1685–93.
24. Sanda C, Weitzel P, Tsukahara T, Schaley J, Edenberg HJ, Stephens MA, et al. Differential gene induction by type I and type II interferons and their combination. J Interferon Cytokine Res 2006;26:462–72.
25. Langaas V, Shahzidi S, Johnsen JI, Smedsrod B, Sveinbjornsson B. Interferon-gamma modulates TRAIL-mediated apoptosis in human colon carcinoma cells. Anticancer Res 2001;21:3733–8.
26. Papageorgiou A, Dinney CP, McConkey DJ. Interferon-alpha induces TRAIL expression and cell death via an IRF-1-dependent mechanism in human bladder cancer cells. Cancer Biol Ther 2007;6:872–9.
27. Clark PE, Polosukhina DA, Gyabaah K, Moses HL, Thorburn A, Zent R. TRAIL and interferon-alpha act synergistically to induce renal cell carcinoma apoptosis. J Urol 2010;184:1166–74.
28. Qu J, Zhao M, Teng Y, Zhang Y, Hou K, Jiang Y, et al. Interferon-alpha sensitizes human gastric cancer cells to TRAIL-induced apoptosis via activation of the c-CBL-dependent MAPK/ERK pathway. Cancer Biol Ther 2011;12:494–502.
29. Kang Z, Chen J, Yu Y, Li B, Sun SY, Zhang B, et al. Drozitumab, a human antibody to death receptor 5, has potent anti-tumor activity against rhabdomyosarcoma with the expression of caspase-8 predictive of response. Clin Cancer Res 2011;17:3181–92.
30. Abramson V, Arteaga CL. New strategies in HER2-overexpressing breast cancer: many combinations of targeted drugs available. Clin Cancer Res 2011;17:952–8.
31. Araki S, Nakayama Y, Hori A, Yoshimura K. Biomarkers for predicting the sensitivity of cancer cells to TRAIL-R1 agonistic monoclonal antibody. Cancer Lett 2010;292:269–79.