The interactive transcript abundance index [c-*myc**p73α]/[p21*Bcl-2] correlates with baseline level of apoptosis and response to CPT-11 in human bronchogenic carcinoma cell lines

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Abstract. Currently available cytotoxic chemotherapy is ineffective for treating bronchogenic carcinoma, and this is partly due to unpredictable inter-tumor variation in resistance. For example, tumors with inactivating p53 mutations or deletions are less likely to respond to certain chemotherapeutics. However, even if p53 is intact, a tumor may be unresponsive if defects in other p53 pathway genes compromise apoptosis. In an effort to identify biomarkers that better predict response to camptothecin, we investigated the association of CPT-11 (irinotecan)-induced cytotoxicity (IC_{50}) with apoptosis or expression of genes upstream or downstream of p53 in cell lines that retain wild-type p53. CPT-11 response was greater in cell lines with higher baseline apoptosis ($p<0.05$). In addition, the interactive transcript abundance index (ITAI) [c-*myc**p73α]/[p21*Bcl-2] was directly correlated with baseline apoptosis ($p<0.01$) and CPT-11 response ($p<0.05$). The ITAI was also correlated with CPT-11 response among cell lines derived from a variety of tissues that had inactivating p53 mutations or deletions, supporting its applicability for predicting response to camptothecins in other tissues regardless of p53 status.

Introduction

The tumor suppressor gene p53 is inactivated by mutation or deletion in approximately 50% of human malignancies. In those that retain wild-type p53, it is activated after the onset of DNA damage and then functions as a transcription factor to induce genes that are responsible for controlling cell cycle regulation and/or apoptosis (1,2). In general, lung tumors that retain wild-type p53 are more responsive to cytotoxic chemotherapeutic agents while those that carry mutant p53 are more resistant (3). However, there is variation in response among cell populations that retain functional p53 protein and this may result from variation in the responsiveness of genes regulated by p53 or functionality of genes that act upstream or downstream from p53 (4). For example, the breast cancer cell line MCF7, while retaining wild-type p53, is resistant to both camptothecin and cisplatin-induced apoptosis, and this may be explained by the following: i) elevated expression of the p53 inhibitor protein MDM2; ii) elevated expression of the anti-apoptotic protein Bcl-2; and/or iii) a deletion in the caspase-3 gene (5,6). Based on results such as these, it is clear that analysis of p53 by DNA sequencing and/or immunohistochemistry is not sufficient for predicting response to chemotherapeutics. Recently, expression signatures that better classify functionality of the p53 pathway than either of these approaches have been identified through gene expression assessment with microarrays (7).

Camptothecin causes DNA damage by forming a complex with topoisomerase I and the DNA replication machinery (8). Topoisomerase I normally functions to relieve the torsional stress associated with DNA replication by inducing single-stranded breaks in DNA (9,10). Camptothecin reversibly stabilizes this complex between topoisomerase I and DNA (11), however when the camptothecin-DNA complex interacts with the replication machinery, it induces irreversible double-stranded breaks enabling caspase activation and apoptosis (9,12,13).

CPT-11 (irinotecan) is a water soluble derivative of the natural form of camptothecin, initially developed in Japan where it was used to treat NSCLC, SCLC, and colon cancer (14-16). It was approved by the FDA in 1996 to treat metastatic colorectal cancer (14) and has since shown promising results for treatment of both NSCLC and SCLC when combined with cisplatin therapy (17,18). CTP-11 is a pro-drug that becomes activated when hydrolyzed by carboxylesterase, an enzyme that is expressed primarily in the liver and GI tract, but also is active in bronchial epithelial cells (9,19). Chemoresistance to CPT-11 is common and several factors responsible for its resistance have been described including inter-tumor variation in carboxylesterase activity, topoisomerase I expression, and drug efflux and transport (14,20). Further, based on knowledge

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Table I. Summary of baseline apoptosis, CPT-11 response, and interactive transcript abundance indices (ITAI) in cell lines with known genetic defects.

Cell line	Tissue type	p53	$p14^{ARF}$	Rb	p73α	c-myc	Baseline apoptosis	CPT-11 IC_{50} (10^{-6} M)	[c-*myc**p73α]/[p21*Bcl-2]
A549	NSCLC	WT[a]	Del[a]	Pos[a]	WT[a]		5.1	117	5.0E-02
H838	NSCLC	WT[b]					3.3	104	6.9E-01
H460	NSCLC	WT[c]	Del[c]	Pos[c]			5.0	63.0	9.3E-02
H322	NSCLC	M[d]	Neg[e]	Neg[e]			NA	25.1[i]	3.4E+00
H522	NSCLC	M[d]					NA	15.8[i]	9.0E+01
H226	NSCLC	M[d]					NA	7.9[i]	6.2E-01
H23	NSCLC	M[d]					NA	7.9[i]	8.8E+00
H146	SCLC	WT[a]	WT[a]	Neg[a]	WT[a]		41.0	43.0	8.4E+00
H446	SCLC	WT[a]	WT[a]	Neg[a]	WT[a]	Amp[f]	54.9	8.0	8.0E+01
N417	SCLC	M[a]	WT[a]	Neg[a]	WT[a]	Amp[f]	NA	0.45[j]	1.7E+04
SKOV3	Ovarian	M[d]	Del[a]	Pos[a]	WT[a]		NA	15.8[i]	3.6E-01
OVCAR3	Ovarian	M[d]					NA	15.1[i]	5.7E+01
MCF7	Breast	WT[a]	WT[a]	Pos[a]	WT[a]		9.9	54	7.3E-01
T47D	Breast	M[d]	WT[a]	Pos[g]	WT[a]		NA	10[i]	4.3E+02
HT29	Colon	M[d]	WT[a]	Pos[a]	WT[a]	Amp[h]	NA	12.6[i]	1.2E+04

NSCLC, non-small cell lung carcinoma; SCLC, small-cell lung carcinoma; WT, wild-type; M, mutant; Del, deleted; Neg, negative; Pos, positive; Amp, amplification; NA, not assessed. The percentage of apoptosis was determined by Annexin-V assay. CPT-11 IC_{50} was determined by MTS cell proliferation assay, or was obtained from the NCI developmental therapeutics program or van Ark-Otte *et al* (23). ITAI values were determined by StaRT-PCR, where each gene was measured in triplicate. Genes with no detectable transcript were assigned a value of 1. Individual transcript measurements are in units of molecules/10^6 ß-actin molecules. ITAI values are unitless. [a]Nickolson *et al* (27); [b]You *et al* (30); [c]Gao *et al* (32); [d]Oliver *et al* (28); [e]Yu *et al* (35); [f]Bernasconi *et al* (31); [g]Wang *et al* (34); [h]Augenlicht *et al* (33); [i]NCI; Development Therapeutics Program; [j]van Ark-Otte *et al* (23).

of these resistance mechanisms, some biomarkers for resistance have been identified (21-23). In spite of this progress, the mechanisms of drug response are still not completely understood (24,25). For example, conflicting results are prevalent in the literature, and markers that accurately predict response may be tissue specific (6,23).

In this study we assessed the association of CPT-11 response with apoptosis or expression of genes upstream or downstream of p53 among bronchogenic carcinoma cell lines that retain wild-type p53. These cell lines provide optimal models for analysis because they have been extensively characterized at the genetic level, including with respect to p53, $p14^{ARF}$, Rb, c-*myc*, and p73 (26-35).

Materials and methods

Cell culture and camptothecin treatment. A list of cell lines used in this study and their respective genetic alterations are summarized in Table I. All cell lines were cultured in RPMI supplemented with 10% fetal bovine serum (FBS). Adherent cell lines (A549, H838, H460, and MCF7) were grown to confluence in a T-75 culture flask. Cells were washed in PBS buffer, trypsin dissociated, resuspended in medium, and introduced into T-25 culture flasks at a concentration of $2.5x10^5$ cells/ml. After 12-16 h of incubation at 37˚C, cells were treated with fresh medium containing 30 μM camptothecin for up to 24 h. Cells in suspension (H146, H446) were collected by centrifugation and $2.5x10^5$ cells/ml were resuspended in media containing 30 μM camptothecin and incubated for up to 24 h. H446 can grow either in suspension or as a monolayer. To avoid accumulation of dead cells across passages prior to treatment, only cells growing in monolayer were collected for passage. Following incubation with 30 μM camptothecin, both monolayer and suspended cells were collected for analysis.

Annexin-V assay. Cells were incubated for 24 h with 30 μM camptothecin. Adherent and suspended cells were collected as described above. For adherent cells, the cell culture media which may contain floating (dead) cells, was combined with the trypsin dissociated cell population. Cells were centrifuged and resuspended in Annexin binding buffer solution. Cells were subsequently stained with propidium iodide and Annexin-V protein conjugated to Alexa-488 (Phoenix Flow Systems) and were detected and quantified by flow cytometry. Cells that grow only in suspension were collected by centrifugation and stained as described above. Dot-plots and contour-plots shown in Fig. 1 were created using FlowJo 6.3.3 for Macintosh (Treestar).

TUNEL assay. Adherent and suspended cells were collected as described above, resuspended in cell culture medium containing 30 μM camptothecin, and incubated for 24 h. After treatment, cells were collected from flasks (as described

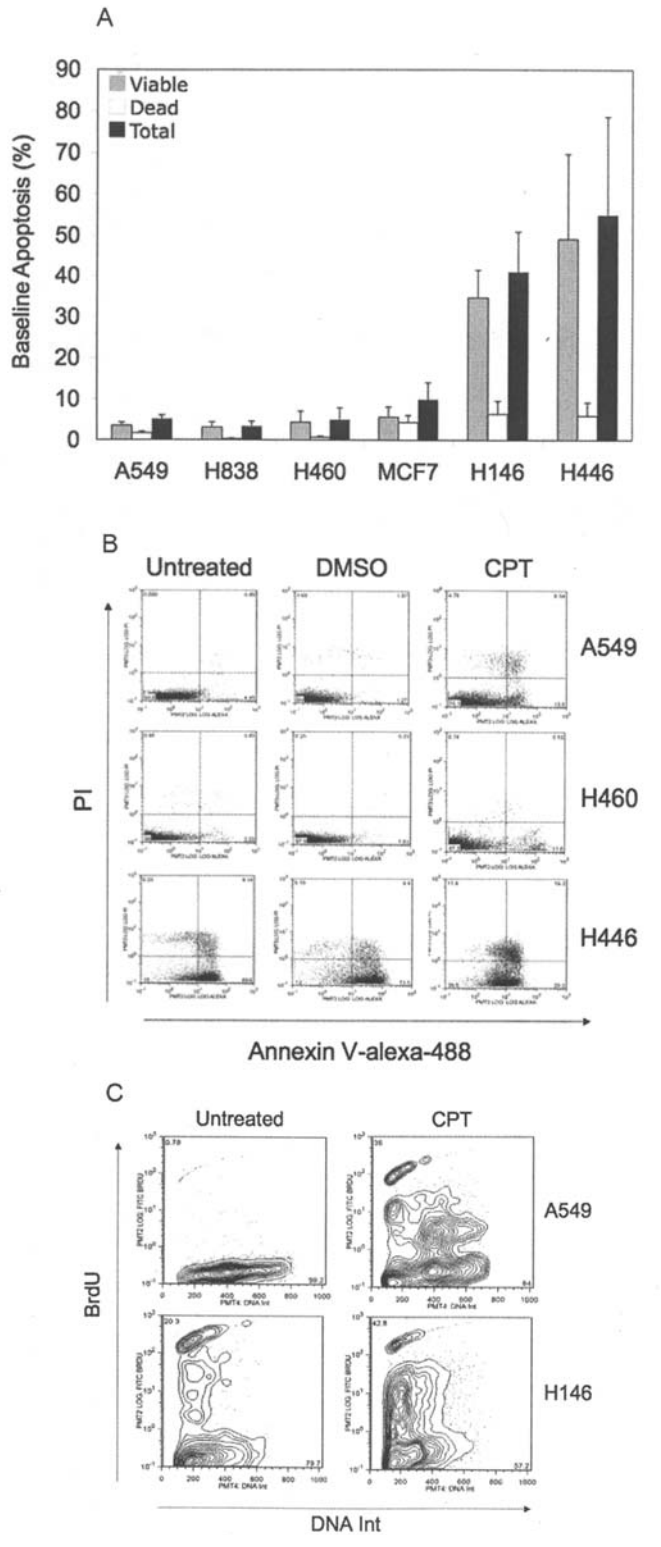

Figure 1. Inter-variation in baseline level and camptothecin-induced apoptosis. (A) Baseline apoptosis is higher in cell lines H146 and H446. The percentage of apoptosis was determined by Annexin-V assay as decribed in Materials and methods. Grey bars represent apoptotic viable cells [Annexin-V positive, propidium iodide (PI) negative], white bars represent apoptotic dead cells (Annexin-V positive, PI positive), black bars represent the total percentage of apoptotic cells (Annexin-V positive). At least 5000 cells were assessed for all cell lines in all experiments. Results represent the mean from three or more independent experiments (2 independent experiments for H446). Error bars represent the SEM. (B) Camptothecin-induced apoptosis is high in A549 (5-fold), moderate in H460 (1.8-fold) and low in H446 (1.1-fold). The percentage of apoptosis was determined by Annexin-V assay after treating each cell line with 30 μM camptothecin (CPT) for 24 h. (C) Cells were treated with 30 μM camptothecin (CPT) for 24 h and apoptosis was measured by TUNEL assay to confirm results in A and B.

above), centrifuged, resuspended in 0.1% paraformaldehyde solution, and fixed in 70% ethanol for a minimum of 12 h. Following fixation, cells were washed in PBS and BrdU-labeled nucleotides were added to the 3'-OH end of the fragmented DNA using a terminal transferase enzyme (Promega). BrdU-labeled nucleotides were detected using an anti-BrdU antibody conjugated to FITC (BD Bioscience) and were detected and quantified by flow cytometry.

MTS cell proliferation assay. A549, H838, H460, and MCF7 cells were incubated with RPMI supplemented with 10% FBS and transferred to a 96-well plate in triplicate at a density of $2.5x10^4$ cells/ml in a 100-μl volume for a minimum of 12 h. Once adhered, cells were incubated with media or media containing CPT-11 at concentrations ranging from 5-500 μM (0, 5, 10, 30, 50, 100, 300, 500). H146 and H446 were plated in triplicate at a density of $2.5x10^4$ cells/ml in media supplemented with 10% FBS. Cells were treated by adding the appropriate volume of CPT-11 (for above concentrations) to each well. Twenty-four hours following treatment, cells were exposed to 30 μl of CellTiter 96 AQ_{ueous} One Solution (Promega) and incubated at 37˚C for approximately 4 h. Viability was assessed by measuring the absorbance of each well at 490 nm.

RNA extraction and reverse transcription. Total RNA was extracted by phenol/chloroform methods using TRI-reagent (Molecular Research Center). The aqueous phase from each lysate (containing total RNA) was precipitated in isopropanol, washed in ethanol, and resuspended in 30 μl of RNase-free H_2O. The RNA was then subjected to DNase I (Ambion) for 30 min at 37˚C in order to rule out genomic contamination. Approximately 1 μg of denatured RNA was combined with 500 μg/ml Oligo dT primer (Promega), 10 mM dNTPs, 25 units/μl RNasin (Promega), and 200 units/μl MMLV-Reverse Transcriptase (Invitrogen) and incubated for 1 h at 37˚C.

Standardized RT-PCR (StaRT-PCR). Transcript abundance data were obtained by StaRT-PCR. In the StaRT-PCR method, each gene is co-amplified with an internal standard within a standardized mixture of internal standards (SMIS) at a known concentration ranging from 10^{-12} M to 10^{-17} M. Each internal standard is 10-20% shorter in length than the target gene PCR product. However, both the internal standard and target gene are amplified by the same pair of forward and reverse primers at the same efficiency. Internal standards for genes, p53, *c-myc*, p21, E2F1, Bax, Bcl-2, Bcl-X, Casp-1, Gpx-1, and Cox-2 were commercially prepared (Gene Express, Inc.) while internal standards for p73 were prepared in this laboratory. Bcl-X primers amplify both the long and short transcripts designated Bcl-X_L and Bcl-X_S, respectively, and p73 primers amplify at least four distinct isoforms (α-δ), but do not discriminate between TA and ΔN forms. For each PCR reaction, the amount of cDNA in balance with 10^{-13} M ß-actin internal standard was used, with the exception of H446 in which the cDNA concentration was too low and the amount of cDNA in balance with 10^{-14} M ß-actin internal standard was used in each reaction. The appropriate dilution of cDNA was determined by calibrating the native ß-actin in

Table II. Transcript abundance of p53-related genes following treatment with camptothecin.

	A549			H838			H460		
CPT	-	+	p-value	-	+	p-value	-	+	p-value
p53	7.2E+03	2.9E+04	0.057	**8.8E+01**	**1.6E+03**	**0.043**	**1.9E+03**	**3.5E+04**	**0.014**
p73α	1.0E+00	1.0E+00		**3.7E+01**	**1.0E+00**	**0.003**	1.0E+00	2.0E+01	0.185
p73ß	1.0E+00	1.0E+00		1.0E+00	1.0E+00		1.0E+00	1.0E+00	
p73γ	1.0E+00	1.0E+00		1.0E+00	1.0E+00		1.0E+00	1.0E+00	
p73δ	1.0E+00	1.0E+00		1.0E+00	1.0E+00		1.0E+00	1.0E+00	
c-myc	1.5E+04	2.5E+04	0.110	**1.7E+03**	**2.4E+04**	**0.006**	**5.2E+04**	**1.6E+05**	**0.008**
p21[a]	**1.5E+04**	**3.4E+05**	**0.005**	**1.2E+03**	**4.5E+04**	**0.008**	**3.9E+03**	**3.9E+05**	**0.001**
E2F1	**1.4E+04**	**3.0E+03**	**0.025**	**8.1E+03**	**5.9E+04**	**0.005**	**5.6E+03**	**5.5E+04**	**0.013**
Bax[a]	**1.5E+04**	**5.2E+04**	**0.018**	**2.1E+03**	**8.4E+03**	**0.021**	**2.3E+03**	**1.8E+04**	**0.001**
Bcl-2[a]	**2.0E+01**	**1.0E+00**	**0.012**	7.6E+01	2.2E+01		1.4E+02	1.1E+02	
Bcl-X_L[a]	9.3E+03	9.6E+03	0.642	1.8E+03	2.7E+03	0.108	1.8E+03	7.0E+03	0.094
Bcl-X_S[a]	**5.5E+03**	**1.2E+04**	**0.036**	6.9E+02	1.6E+03	0.141	**3.4E+02**	**7.4E+03**	**0.031**
Casp-1[a]	**5.0E+00**	**3.1E+03**	**0.009**	4.0E+01	3.9E+02		**2.2E+01**	**1.7E+03**	**0.040**
Gpx-1[a]	**7.4E+04**	**1.2E+06**	**0.004**	**5.0E+04**	**4.0E+05**	**0.002**	**1.1E+05**	**1.7E+06**	**0.001**
Cox-2[a]	**2.0E+02**	**1.6E+03**	**0.022**	1.0E+00	2.1E+01		2.7E+00	1.9E+02	

	MCF7			H146			H446		
CPT	-	+	p-value	-	+	p-value	-	+	p-value
p53	**3.5E+04**	**1.5E+04**	**0.003**	**1.1E+02**	**4.2E+02**	**0.005**	**6.1E+03**	**8.2E+04**	**0.007**
p73α	**5.6E+02**	**1.0E+00**	**0.000**	5.9E+02	1.4E+02	0.173	1.9E+03	NA	
p73ß	1.1E+02	1.9E+01	0.158	**6.4E+02**	**1.9E+03**	**0.029**	NA	NA	
p73γ	**1.1E+02**	**1.0E+00**	**0.005**	7.7E+02	1.6E+02	0.310	NA	NA	
p73δ	9.9E+01	1.0E+00		6.0E+02	3.3E+03	0.235	NA	NA	
c-myc	**5.6E+04**	**1.3E+04**	**0.013**	2.2E+04	1.5E+04	0.410	**9.3E+04**	**9.5E+05**	**0.024**
p21[a]	**5.8E+03**	**1.1E+05**	**0.000**	**1.4E+03**	**7.9E+03**	**0.004**	**2.6E+04**	**4.6E+04**	**0.001**
E2F1	6.5E+03	5.6E+03	0.747	2.3E+04	4.5E+04	0.054	**2.6E+04**	**2.9E+05**	**0.005**
Bax[a]	**9.6E+03**	**3.0E+03**	**0.011**	3.2E+03	5.8E+03	0.263	**5.2E+03**	**1.7E+04**	**0.007**
Bcl-2[a]	**7.4E+03**	**1.0E+02**	**0.001**	1.1E+03	3.7E+02	0.084	1.7E+02	4.0E+02	
Bcl-X_L[a]	**1.4E+04**	**8.7E+02**	**0.000**	6.2E+03	9.3E+03	0.273	4.8E+03	7.7E+03	
Bcl-X_S[a]	**4.1E+03**	**5.1E+02**	**0.017**	**1.0E+03**	**4.6E+03**	**0.032**	1.3E+03	1.3E+04	0.104
Casp-1[a]	1.0E+00	1.0E+00		1.6E+01	1.0E+00	0.185	NA	NA	
Gpx-1[a]	NA	6.9E+02		**6.3E+04**	**1.6E+05**	**0.035**	**1.5E+05**	**1.4E+06**	**0.009**
Cox-2[a]	8.3E+01	5.3E+01		2.5E+01	2.0E+00		NA	1.3E+03	

[a]Genes known to be regulated by p53. p21, Bax, Casp-1, and Gpx-1 are transcriptionally activated. Bcl-2 and Bcl-X are transcriptionally repressed. Cox-2 can be transcriptionally activated or repressed. For a complete review see Harms *et al* (38). NA, not assessed. Cell lines were incubated with 30 μM camptothecin (CPT) for 24 h. Transcript abundance was measured by StaRT-PCR and mean values are shown in units of molecules/10^6 ß-actin molecules. Genes with no detectable transcript were assigned a value of 1. Statistical significance was determined by a paired Student's t-test. p-values were obtained from genes that had detectable transcript abundance and at least three or more independent measurements from treated and untreated samples. p-values <0.05 are in bold.

1 μl of cDNA to 10^{-13} M ß-actin (60000 molecules). Calibrated cDNA samples were used in all PCR reactions in which the same SMIS was used. Prior to amplification, equal volumes of cDNA and internal standard were combined into a master mix along with the appropriate volume of RNase free H_2O, 30 mM $MgCl_2$, 2 mM dNTPs, and a minimum of 0.1 unit of taq polymerase (Promega, Inc.). All PCR reactions were performed in a rapidcycler (Idaho Technologies, Inc.) for 35 cycles. All reactions were denatured for 5 sec at 95˚C, annealed for 10 sec at 58˚C, and elongated for 15 sec at 72˚C.

Western blot analysis. Cells were lysed in 0.25 M Tris-buffer by three consecutive freeze-thaws. Total protein was quantified colorimetrically by the bicinchoninic acid (BCA) assay (Pierce). Equivalent concentrations of protein were reduced, denatured, and subjected to SDS-PAGE using 14% tris-

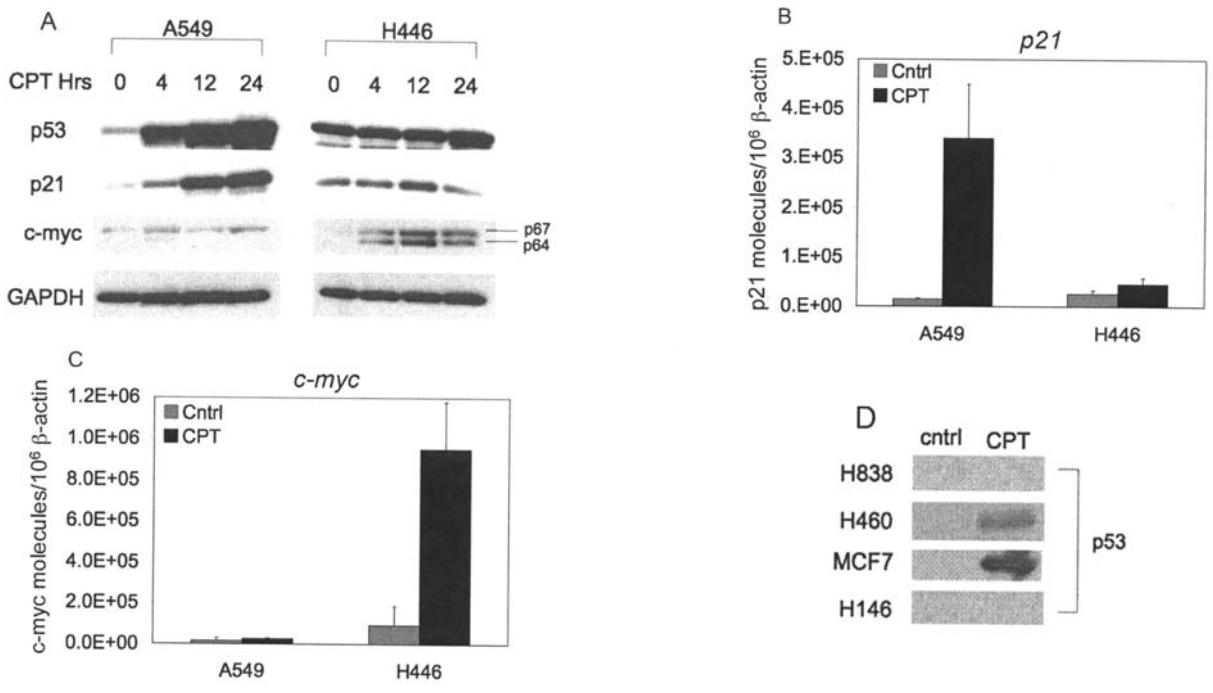

Figure 2. Camptothecin induces p53 and p21 in A549 but not in H446. (A) Camptothecin induces p53 and p21 accumulation in A549 but not H446 cells and c-*myc* in H446 but not A549 cells. Cells were treated with 30 μM camptothecin (CPT) for 0-24 h and expression of p53, p21, and c-*myc* was assessed by Western blot analysis. GAPDH was used as a loading control. Results are representative of three independent experiments. (B and C) Camptothecin (CPT) induces p21 transcript abundance in A549 (22.9-fold) relative to H446 (1.9-fold) and c-*myc* in H446 (10.2-fold) relative to A549 (1.7-fold). Cells were treated with 30 μM camptothecin (CPT) relative to an untreated control (Cntrl) for 24 h and transcript abundance was measured by StaRT-PCR as described in Materials and methods. Results represent mean values from triplicate measurements. Error bars represent the SEM. (D) p53 is induced in H460 and MCF7 but not H838 or H146. Cells were treated with 30 μM camptothecin (CPT) relative to an untreated control (Cntrl) for 24 h and p53 expression was assessed by Western blot analysis.

glycine gels. Protein was transferred to a PVDF membrane, blocked in 5% milk, and incubated with primary antibodies specific for p53 (Santa Cruz Biotechnology), p21 (Oncogene Science), c-*myc* (Santa Cruz Biotechnology), or GAPDH (Abcam). The PVDF was then incubated with a secondary antibody conjugated to horseradish peroxidase and visualized by exposing to chemiluminescent substrates. For analysis of other proteins, the membrane was stripped in 50 mM glycine and 0.1% SDS buffer (pH 3.0) for 1 h, blocked, and re-probed with the appropriate antibody.

Statistical analysis. StaRT-PCR data were normalized by logarithmic transformation. A paired Student's t-test was used to determine if each gene was significantly altered by camptothecin treatment. A one-way ANOVA was used to determine which genes varied statistically among untreated control samples. Pearson correlations were used to assess bivariate analyses. All statistical tests were performed using SPSS 11 for Macintosh.

Results

Inter-cell line variation in baseline and camptothecin-induced apoptosis. The baseline level of apoptosis as measured by the Annexin-V binding assay is presented in Fig. 1A. For H146 and H446 cell lines, a relatively high fraction of cells were undergoing apoptosis even though over 90% of the cells remained viable based on negative stain for propidium iodide. These results were further validated by TUNEL assay (Fig. 1C), in which baseline level of apoptosis was high in H146 relative to A549.

H446 cells grow either in suspension or attached to substrate. The high baseline level of apoptosis continued to be observed even when only adherent H446 cells were collected and passed. This confirmed that the high baseline level of apoptosis in H446 was not due to the accumulation of dead cells that grow in suspension.

To determine the effect of camptothecin on apoptotic response, cell lines were incubated with 30 μM camptothecin for 24 h. A549 and H838 had the highest response (5.0- and 4.8-fold over baseline level, respectively) as determined by the increase in fraction of Annexin-V-positive cells relative to untreated controls. MCF7 and H460 showed a lower response (2.4- and 1.8-fold, respectively). H146 and H446 showed no overall change in the total fraction of apoptotic cells (1.0- and 1.1-fold, respectively), but importantly, the fraction of dead apoptotic cells based on propidium iodide stain was markedly increased in H446. Representative scatter plots for three cell lines (A549, H460, H446) with a varying level of camptothecin-induced apoptosis are shown in Fig. 1B.

Inter-cell line variation in the effects of camptothecin on expression of genes regulated by p53. In order to determine the gene expression changes associated with camptothecin treatment, we measured the transcript levels of fifteen p53 pathway-related genes. Mean values presented in units of

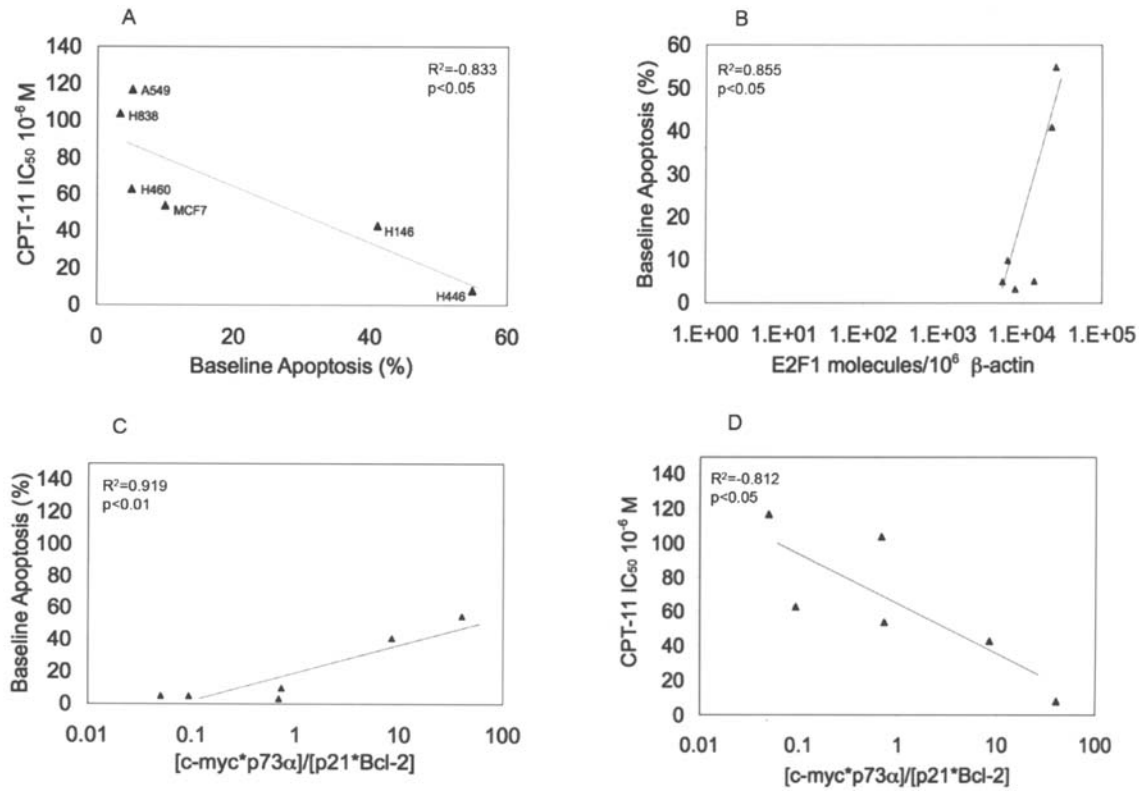

Figure 3. The interactive transcript abundance index [c-myc*p73α]/[p21*Bcl-2] is correlated with baseline apoptosis and CPT-11 (irinotecan) IC_{50}. (A) Cells were treated with CPT-11 (irinotecan) for 24 h and IC_{50} concentrations were measured by MTS cell proliferation assay and correlated with baseline apoptosis (as reported in Fig. 1). R^2=-0.833 and p<0.05 by Pearson correlation. (B) Bivariate plot of E2F1 transcript abundance (in units of molecules/10^6 molecules ß-actin) and baseline apoptosis. R^2=0.855 and p<0.05 by Pearson correlation. (C) Bivariate plot of [c-myc*p73α]/[p21*Bcl-2] interactive transcript abundance index and baseline apoptosis. R^2=0.919 and p<0.01 by Pearson correlation. (D) Bivariate plot of [c-myc*p73α]/[p21*Bcl-2] interactive transcript abundance index and CPT-11 IC_{50}. R^2=-0.812 and p<0.05 by Pearson correlation.

molecules/10^6 ß-actin molecules are summarized in Table II. There was inter-cell line variation in p53 and p21 protein expression in response to camptothecin (Fig. 2). Camptothecin induced p53 and p21 protein accumulation in A549 cells in a time-dependent manner but *c-myc* was relatively unchanged (Fig. 2A). Conversely, H446 incubation with camptothecin was associated with a 10-fold increase in abundance of c-*myc* transcript as well as protein. Further, p53 was increased at the RNA level but not the protein level (Table II, Fig. 2) and for p21 neither protein nor RNA was increased to the extent of A549 (Table II, Fig. 2). Interestingly, in H838 and H146 p53 was low at the RNA level and non-detectable at the protein level (Table II, Fig. 2D), further highlighting the extreme inter-cell line variation in p53 response to camptothecin in tumors that retain wild-type p53. There was also inter-cell line variation in expression of other genes known to be p53-responsive including Bax, glutathione peroxidase-1 (Gpx-1), caspase-1 (Casp-1), and cyclooxygenase-2 (Cox-2). A549 and H460 had the greatest amount of increase in the expression of these genes and H146 and MCF7 had the least amount of increase.

In the case of A549, which had the highest fold-change in camptothecin-induced apoptosis and substantial p53 protein accumulation, all five p53-responsive genes were increased by large fold levels (Table II). H460 showed an even greater transcriptional response, with p21 being induced 100-fold. H146 and H446, each with a high level of baseline apoptosis, did not exhibit the same changes. More strikingly, in MCF7 cells there was a consistent down-regulation of these p53 responsive genes with the exception of p21.

Baseline apoptotic fraction is directly correlated with response to CPT-11. We determined response to CPT-11 by measuring cell viability following treatment with concentrations ranging from 1-500 μM (Fig. 3A). As shown, CPT-11 IC_{50} concentrations were inversely correlated with baseline level of apoptosis (p<0.05).

Identification of interactive transcript abundance indices. Because response to CPT-11 was correlated with baseline level of apoptosis, we hypothesized that the expression of apoptosis-related genes would correlate with both baseline level of apoptosis and response to CPT-11. We evaluated E2F1, c-*myc*, Bcl-2, p21, and p73α because they are mechanistically associated with apoptosis and there was large inter-cell line variation in expression among untreated samples (Table II). For example, inter-cell line variation in expression of *c-myc*, p73α, p21, and Bcl-2 was significant (p<0.0001) and varied from 20 to 100-fold. Genes showing less or insignificant variation were excluded from the analysis, with the exception of E2F1 which was the only single gene that correlated with baseline level of apoptosis (Fig. 3B). Because single gene values were poorly diagnostic, we evaluated each of these genes in the form of interactive transcript abundance indices

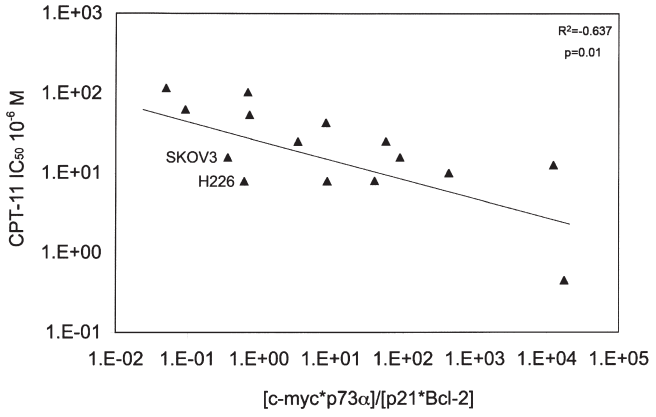

Figure 4. Evaluation of the interactive transcript abundance index [c-myc*p73α]/[p21*Bcl-2] in p53 mutant cell lines. The [c-myc*p73α]/[p21*Bcl-2] interactive transcript abundance index was inversely correlated with CPT-11 (irinotecan) IC_{50} concentration in the 6 cell lines retaining wild-type p53 (group 1) and in an additional nine cell lines with mutant p53 (group 2). Five of which were derived from lung tissue, one breast, two ovarian, and one colonic. R^2=-0.637 and p=0.01 by Pearson correlation. Potential outliers SKOV3 (ovarian) and H226 (lung) are indicated, but were not excluded from statistical analysis.

comprising ratios of two, three, four, or five genes. Genes that were positively associated with apoptosis (e.g., c-*myc*, E2F1, and p73α) were assessed as factors in the numerator and genes that were positively associated with cell survival or anti-apoptosis (Bcl-2 and p21) were assessed as factors in the denominator. The predictive model that best correlated with both baseline level of apoptosis and response to CPT-11 was [c-*myc**p73α]/[p21*Bcl-2] ($p<0.01$ and $p<0.05$, respectively) (Fig. 3C and D).

*Evaluation of the interactive transcript abundance index [c-myc*p73α]/[p21*Bcl-2] in p53 mutant cell lines.* As described above, among the cell lines in which p53 DNA sequence was wild-type there was variation in both the baseline level of apoptosis and response to CPT-11. It is likely that this variation was due to defects upstream or downstream of the p53 pathway. For example, the baseline level of apoptosis in H146 was high even though p53 protein was undetectable. This indicates that baseline level of apoptosis is, in part, independent of p53 activity. To test this hypothesis, we evaluated an additional nine cancer cell lines (5 lung, 1 breast, 2 ovarian, 1 colon) known to have inactivating p53 mutations. IC_{50} data for these cell lines were obtained from the NCI developmental therapeutics program, except for N417 which was obtained from van Ark-Otte *et al* (23). When the data from these cell lines with inactivated p53 (N=9) were combined with the data from cell lines with wild-type p53 (N=6) there was significant correlation between [c-*myc**p73α]/[p21*Bcl-2] and response to CPT-11 (p=0.01) (Fig. 4).

Discussion

The purpose of this study was to use recently available knowledge regarding the p53-related DNA damage response in order to develop a biomarker that predicts which bronchogenic carcinomas will respond to camptothecin-based therapy. Here we demonstrate that a subset of bronchogenic carcinoma cell lines, each retaining wild-type p53, have measurable variation in baseline fraction of cells undergoing apoptosis and that this correlates with variation in CPT-11 IC_{50}. We hypothesized that inter-tumor variation in expression of genes upstream and/or downstream of p53 could account for variation in both baseline apoptosis rate and sensitivity to CPT-11. The interactive transcript abundance index (ITAI) [c-*myc**p73α]/[p21*Bcl-2] significantly correlated with both baseline apoptosis rate and sensitivity to CPT-11 suggesting that these genes function cooperatively and that variation in their expression is responsible, in part, for variation in rate of baseline apoptosis.

Of particular interest was the robust apoptotic process in H446. Presumably, H446 is immortal and malignant in spite of the high apoptosis rate because cell loss from apoptosis is more than compensated for by rapid cell proliferation resulting in net population growth. Consistent with this, although the baseline apoptotic fraction is high, the baseline dead cell fraction is low. Further, although the percentage of total apoptotic cells was not substantially increased by camptothecin, the fraction of dead cells was markedly increased. This is what would be expected if the apoptotic process remained the same and the rate of proliferation decreased. This is in contrast to A549 and H460, for which the same concentration of camptothecin did not induce this extent of cell death.

There was profound inter-cell line variation in camptothecin-induced changes in p53-regulated gene expression. For example, in A549 cells camptothecin treatment was associated with rapid increase in p53 and p21 protein. In A549, because $p14^{ARF}$ is inactivated by deletion and p53 is bound by MDM2, p53 protein is unstable and present at a low level at baseline even though E2F1 and c-*myc* expression are low. However, DNA damage mediates increased phosphorylation of p53 protein causing its stabilization and increase in function (1). Thus, it is likely that in the experiments reported here, camptothecin-induced DNA damage mediated activation of p53 and this then up-regulated p21 transcription (36), resulting in the induction of cell cycle arrest, senescence, and cell survival (37) in the large fraction of cells that escaped apoptotic cell death.

In contrast to these findings, neither p53 nor p21 protein was induced by camptothecin in H446. One reasonable explanation for this is that p53 is maximally up-regulated at baseline due to the high expression of E2F1 and c-*myc* in these cells. Both E2F1 and c-*myc* upregulate $p14^{ARF}$ which acts to stabilize p53 by inhibiting MDM2. Thus, in H446 even in the absence of DNA damage, p53 protein is stabilized and its function is up-regulated at baseline.

The [c-*myc**p73α]/[p21*Bcl-2] biomarker was also assessed for applicability to carcinoma tissues with mutant p53 status. To do this, the ITAI was evaluated in a second group of cell lines in which p53 was inactivated by mutation or deletion. The ITAI remained positively correlated with CPT-11 IC_{50}, suggesting that factors other than p53 contribute significantly to the cell death induced by CPT-11. Further, five of the cell lines were derived from tissues other than lung, including breast, ovarian, and colonic tissue, indicating that the ITAI may have value when applied for use in other malignancies. The potential clinical implications are particularly important

for colon and ovarian carcinomas considering that they are commonly treated with camptothecin derivatives.

In conclusion, it is likely that the best biomarker for CPT-11 resistance will include the [c-*myc**p73α]/[p21*Bcl-2] biomarker reported here and one or more previously reported biomarkers, such as those related to carboxylesterase activity, topoisomerase I expression, and drug efflux and transport (38).

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