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The interactive transcript abundance index \([c\text{-}myc^*p73\alpha]/[p21*Bcl-2]\) C correlates with spontaneous apoptosis and response to CPT-11: implications for predicting chemoresistance and cytotoxicity to DNA damaging agents

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The Interactive Transcript Abundance Index [c-myc*p73α]/[p21*Bcl-2] Correlates With Spontaneous Apoptosis and Response to CPT-11: Implications for Predicting Chemoresistance and Cytotoxicity to DNA Damaging Agents

Submitted by

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The University of Toledo

2007
DEDICATION

In memory of my loving grandfather, who always encouraged me to be a pharmacist.
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TABLE OF CONTENTS

Dedication..................................................................................................................ii
Acknowledgements.................................................................................................iii
Table of Contents.....................................................................................................iv
Introduction..............................................................................................................1
Literature..................................................................................................................5
Manuscript “Variation in Transcriptional Regulation of Cyclin Dependent Kinase
Inhibitor p21waft/cip1 Among Human Bronchogenic Carcinomas”.........................32
Manuscript “The Interactive Transcript Abundance Index [c-myc*p73α]/[p21*Bcl-2]
Correlates with Spontaneous Apoptosis and Response to CPT-11 in Human
Bronchogenic Carcinoma Cell Lines”.....................................................................55
Materials and Methods...........................................................................................83
Results....................................................................................................................92
Discussion...............................................................................................................102
Conclusions.............................................................................................................111
Summary.................................................................................................................113
References..............................................................................................................117
Abstract...............................................................................................................172
INTRODUCTION

Bronchogenic carcinoma is the leading cause of cancer-related deaths in most industrialized countries. Its diagnosis and classification are made by histo- and cytomorphologic criteria, in which the two primary types of morphology are non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). The classification currently employed by the World Health Organization further divides bronchogenic carcinoma into four subtypes including adenocarcinoma (A), squamous cell carcinoma (SCC), large cell carcinoma (LC), and small cell carcinoma (SC) (Travis, 1999). However, the characteristics of each NSCLC subtype overlap considerably, and thus for therapeutic purposes, lung tumors are classified as NSCLC or SCLC (Stancu, 2001a).

Historically, SCLC has been more responsive to chemotherapeutics and radiation therapy than NSCLC. Although SCLC tends to acquire resistance to many first line treatments, and the molecular mechanisms that enable SCLC to be initially responsive are largely unknown. On the other hand, while surgery is frequently used to treat NSCLC, its use for SCLC has shown little benefit in the absence of systemic chemotherapy. Classical agents used for treatment of SCLC include cisplatin, carboplatin, etoposide, and doxorubicin. Newer agents include topotecan, paclitaxel, and gemcitabine (Rathore and Weitberg, 2001a). Cytotoxic drugs of this sort have shown promising results when administered in combination. For example, the platinum-based therapeutic, cisplatin, administered with the topoisomerase II inhibitor, etoposide, has increased efficacy compared to either drug administered as a single agent (Rathore and Weitberg, 2001b).
Although lung tumors are not yet classified at the molecular level, there are several genetic abnormalities consistent among primary lung malignancies. The tumor suppressor gene, p53 is mutant in approximately 50% of NSCLC and up to 80% of SCLC (Stancu, 2001b). Other common alterations in cell cycling genes include p16 (de Vos et al., 1995), p14ARF (Vonlanthen et al., 1998), overexpression of E2F1 (Eymin et al., 2001; Gorgoulis et al., 2002), and loss of the retinoblastoma gene, Rb (Xu et al., 1996). Additionally, mutations in the oncogene Ras are prevalent in NSCLC but not SCLC (Slebos and Rodenhuis, 1992; Sugio et al., 1992), and genomic amplification and overexpression of c-myc are common in SCLC (Levin et al., 1994; Richardson and Johnson, 1993).

In light of the fact that chemotherapeutic treatment for NSCLC is generally ineffective and acquired resistance occurs in SCLC patients, it would be beneficial to utilize genetic information to predict which patients are responsive to specific treatments. Development of a therapeutic biomarker would not only alleviate the toxic side effects associated with chemotherapy in patients unlikely to respond, but would increase the efficacy of existing cytotoxic agents if restricted to the appropriate population.

In an effort to develop diagnostic and theranostic clinical tests, our laboratory has assessed the expression of multiple genes associated with cell proliferation control, DNA repair, apoptosis, and drug transport using standardized reverse transcriptase-polymerase chain reaction (StaRT-PCR) in normal and malignant bronchial epithelial cells (DeMuth et al., 1998; Weaver et al., 2005; Willey et al., 1998). Our methodology is advantageous because it uses a standardized mixture of internal standards (SMIS), where one internal standard (competitive template) is co-amplified at a known concentration with its native gene product. In this way, the concentration of the target gene is determined by
calculating the ratio of internal standard molecules to target gene molecules while using a reference gene (e.g., β-actin) for normalization (Willey et al., 2004). Because an internal standard is included in every PCR reaction, the data are interactive and comparable between multiple experiments (Crawford et al., 2001).

Much of our work in theranostics was facilitated by preliminary efforts to understand how genes controlling cell proliferation are regulated at the transcriptional level. We found substantial inter-individual variation in the expression of cyclin dependent kinase inhibitor p21 (Waf1/Cip1) in normal and malignant bronchial epithelial cells (Harr et al., 2005). We provided an explanation for its variability in malignant cells after measuring the expression of upstream transcription factors E2F1 and p73. These experiments paved the way for additional studies addressing apoptosis pathways in bronchogenic carcinoma tissues retaining wild type p53. In these studies, we hypothesized that cells expressing high levels of E2F1 and p73, and retaining wild type p53, would be resistant to apoptosis because of a downstream defect in the p53 pathway. In fact, our hypothesis was incorrect because cells that expressed higher levels of E2F1 and p73 manifested biochemical characteristics of apoptosis, even in the absence of DNA damage.

Although partly incorrect, these observations lead to the identification of an interactive transcript abundance index (ITAI), a discriminator that relies on the interaction of multiple genes that associate with a common phenotype. In these studies, the ITAI, in the form of the ratio \([c\text{-}\text{myc}\ast\text{p73}\alpha]/[\text{p21}\ast\text{Bcl}-2]\), was correlated with spontaneous apoptosis and sensitivity to the camptothecin derivative, irinotecan (CPT-11). Thus, genes that were associated with spontaneous apoptosis and response to CPT-
11 were represented in the numerator while genes associated with its resistance were represented in the denominator.

While testing this hypothesis, we observed that several p53-responsive genes could be induced by as much as three orders of magnitude. Therefore, we tested an additional hypothesis, that these genes could serve as biomarkers for cytotoxicity (e.g., lymphopenia, myelosuppression) when induced by *in vivo* chemotherapy by collecting peripheral blood leukocytes from cancer patients before and after their first cycle of treatment. Although further experimentation is needed for a conclusive result, if our hypothesis is correct, those individuals that experience a depletion in peripheral blood leukocytes will have higher expression of pro-apoptotic genes resulting from enhanced p53-mediated transcription. Conversely, individuals who do not experience leukocyte depletion will have higher expression of anti-apoptosis (cell survival) genes.

The overall objective of these studies is to use genetic and molecular criteria to better determine the appropriate course of treatment for individuals diagnosed with cancer. Although initial studies were specific to bronchogenic carcinoma, we have established a putative test for CPT-11 (irinotecan) sensitivity that may have relevance to lung, ovarian, colon, and breast malignancies. Our investigation of peripheral blood leukocytes, while ongoing, attempts to predict which patients will be predisposed to the adverse side effects normally associated with chemotherapy. This type of biomarker would provide physicians with better guidelines for determining the appropriate medication and dose for each individual, while effectively treating the disease and minimizing toxicity to normal tissues and organs.
An Overview of the p53 Pathway

p53 is a tumor suppressor gene that is inactivated by mutation or deletion in approximately 50% of human malignancies (Olivier et al., 2004). In normal cells, it is negatively regulated by the mouse double minute-2 (MDM2) protein (HDM2 in humans) which, in turn, is inhibited by p14ARF (Harris and Levine, 2005; Oliner et al., 1992, 1993; Zindy et al., 1998). The stability of p53 is regulated at the post-translational level and its expression is maintained at a low level under normal physiological conditions (Ashcroft and Vousden, 1999). Upon exposure to a cellular stress, such as DNA damage or oncogene activation, p53 is induced by N-terminal serine phosphorylation by cell cycle proteins Chk1, Chk2, or ATM (Appella and Anderson, 2001; Canman et al., 1998; Hofmann et al., 2002; Shieh et al., 2000). Phosphorylation of p53 alters its confirmation, and consequently, its affinity for MDM2 binding (Robles et al., 2002). The active p53 complex, in the form of a tetramer, may then function as a transcription factor to transcriptionally activate or repress genes associated with cell cycle arrest, DNA repair, or apoptosis (Bates and Vousden, 1999; Harms et al., 2004). A schematic of this pathway is shown in Figure 1.

In malignancies that retain wild type p53, there is often one or more genetic alterations in genes comprising the p53 pathway (Vousden and Lu, 2002). These alterations can occur by mutation, deletion, amplification, or aberrant expression resulting in overall inactivation of the pathway (Woods and Vousden, 2001), even in the presence of functional p53 protein. For example, when downstream effector genes such as Apaf-1 and Casp-9 are knocked-out in mouse embryonic fibroblasts, these cells are no
longer susceptible to p53-dependent apoptosis (Soengas et al., 1999). In tumors that have a low frequency of p53 mutations, such as acute myelogenous leukemia and malignant melanoma, overexpression of MDM2 and loss of Apaf-1 are common and correlate with resistance to p53 activating drugs (Soengas et al., 2001). Moreover, DNA sequencing analysis from multiple NSCLC cell lines show that mutations in p53 and p14\textsuperscript{ARF} rarely occur in the same tumor (Nicholson et al., 2001). These observations are consistent with Bert Vogelstein’s “exclusivity principle” which states that mutations are mutually exclusive among groups of genes that perform similar biochemical functions (Vogelstein and Kinzler, 2004).

The p53 Transcriptional Response

As previously described, p53 functions, in part, as a transcription factor by regulating genes involved in tumor suppression. It has an amino terminal transactivation domain that interacts with basal level transcription factors such as the TFIID complex.
(Lu and Levine, 1995). It has a DNA binding domain between amino acids 102 and 292, allowing direct contact to its responsive promoters in a sequence specific manner (Liu and Chen, 2006; Vousden and Lu, 2002). The majority of missense mutations are found in this region which alter protein conformation and impair its ability to bind DNA (Sengupta and Harris, 2005).

Recently the number of known p53 responsive-genes has increased exponentially. In 1997, there were six or seven genes experimentally confirmed to be regulated by p53 (Levine, 1997). There are now over 4,000 putative p53 binding elements within the human genome (Vousden and Lu, 2002). Although many of these have not been experimentally confirmed, over 50 genes have been identified as p53 transcriptional targets that affect apoptosis, such as Bcl-2 family members (Miyashita et al., 1994a, b; Miyashita and Reed, 1995), genes encoding oxidation/reduction enzymes (Bensaad and Vousden, 2005; Tan et al., 1999), and caspases (Gupta et al., 2001; MacLachlan and El-Deiry, 2002). Additionally, p53 may transcriptionally activate or repress genes involved in cell proliferation control such as p21waf1/cip1, Rb, cyclin D, and cyclin E or DNA damage repair, such as XPC, XPE, and MSH2 (Harms et al., 2004). What has remains to be determined is how p53 determines which effector pathway is induced.

p53 is activated in both normal and malignant cells in response to cellular stress, including but not limited to, DNA damage, oncogene activation, hypoxia, and nucleotide depletion (Robles et al., 2002). Yet, in response to any of these signals, the downstream pathway subsequent to p53 activation could mediate cell survival (cell cycle arrest or DNA repair) or cell death via apoptosis (Figure 1). This has implications for chemotherapeutics because a tumor’s response to therapy may be determined by which
effector pathway is induced (Vousden and Lu, 2002). Thus, before treatment regimens are employed that activate wild type p53 or re-activate mutant p53, it will be helpful to predict how a functionally active protein will respond.

One potential determinant of p53 response may be a single nucleotide polymorphism (SNP) located at codon 72 within the p53 coding region. First described by Matlashewsk and colleagues, allelic variants show differences in their ability to respond to DNA damaging agents such as cisplatin, etoposide, and doxorubicin (Santos et al., 2006; Sullivan et al., 2004; Vikhanskaya et al., 2005). The SNP results in an amino acid change where one variant has a proline and another an arginine at codon 72 (72P and 72R, respectively), causing a conformational change in protein structure (Matlashewski et al., 1987). Both alleles encode a functional wild type protein. The biological difference is that 72P is associated with enhanced transcriptional activity (Thomas et al., 1999), which results in cell cycle arrest (Pim and Banks, 2004), while 72R is associated with enhanced apoptotic activity (Dumont et al., 2003). In addition, point mutations in p53 are more common in individuals with the 72R genotype, implicating its importance for maintaining balance between cell proliferation and apoptosis.

A second determinant of p53 response is its expression level. In null cells transfected with inducible wild type p53, a low level of expression was associated with the induction of p21 followed by cell cycle arrest, and a high level of expression was associated with apoptosis (Chen et al., 1996). It has been hypothesized that these differences reflect p53’s binding affinity to promoter elements upstream of genes with distinct functions (Vousden and Lu, 2002). This is supported by the observation that p53 mutants retain the ability to transcriptionally activate p21, but not Bax (Rowan et al.,
Chromatin immunoprecipitation experiments have shown p53 to bind the p21 promoter with high affinity after DNA damage (Szak et al., 2001). A similar study by Kaeser et al., showed p53 bound to promoter regions p21, MDM2, and p53-upregulated mediator of apoptosis (PUMA) with high affinity after DNA damage, while its affinity for Bax, p53AIP1, and PIG3 promoters was weaker (Kaeser and Iggo, 2002). In these experiments, p21, MDM2, and Bax expression at the RNA level was correlated with the percentage of p53 protein bound to each respective promoter. Thus, consistent with previous findings, a low level of p53 was sufficient for the transcriptional activation of p21 but not Bax, suggesting that increased levels of p53 may be a requirement for apoptosis. However, this hypothesis is clearly not supported by the observation that p53 binds strongly to the PUMA promoter.

The p53 homolog, p73

Cell cycle arrest and apoptosis also may be induced independently of p53 by its structural homolog, p73 (Melino et al., 2002). Both proteins are transcription factors that share homology in DNA binding, N-terminal, and C-terminal domains (Kaghad et al., 1997). However, unlike p53, p73 undergoes RNA splicing between exons 10-14 allowing for at least nine distinct isoforms designated α–θ (De Laurenzi and Melino, 2000; Kaghad et al., 1997; Romani et al., 2003). In addition, p73 contains a sterile alpha motif (SAM) at its far C-terminus which may be involved in protein-protein interactions (Thanos and Bowie, 1999).

Another unique aspect of p73 is its internal promoter (P2) found in a region denoted exon 3’ (Grob et al., 2001). Transcription from this promoter results in a
truncated protein, ΔNp73, that lacks the N terminal transactivation domain (Ishimoto et al., 2002). Like their full length counterpart, ΔN transcripts also undergo splicing at their 3’ end (Melino et al., 2002). ΔNp73 proteins function as dominant negative inhibitors by antagonizing full-length p73 responsive promoters or by sequestering p73 or p73 in complex with p53 (Ishimoto et al., 2002; Melino et al., 2002). Thus, ΔNp73 seems to function as an oncogene by promoting cell proliferation and inhibiting apoptosis (Pozniak et al., 2002; Zaika et al., 2002), however, this mechanism remains controversial. A recent study provides evidence that ΔNp73β promotes tumor suppression, although its transcriptional activation was less profound than that of full-length p73β (Liu et al., 2004). Since ΔNp73 is a competitive inhibitor, the relative concentration of both proteins may be an important determinant for maintaining cell proliferation control.

Also like p53, p73 is activated in response to DNA damage by phosphorylation, albeit by different upstream kinases. This mechanism involves signaling from MLH1 to c-Abl and then to p73, and does not occur in cells that are deficient in DNA mismatch repair (Agami et al., 1999; Catani et al., 2002; Gong et al., 1999; Yuan et al., 1999). Further, p73 upregulates genes involved in cell cycle arrest and apoptosis (Jost et al., 1997; Zhu et al., 1998). Both p73α and p73β upregulate p21 (De Laurenzi et al., 1998; Nozell et al., 2003), although, the preference for either isoform may be cell type specific. Acetylated p73 has binding affinity for the p53AIP1 promoter but not for p21 indicating that post-translational modifications contribute to p73’s role in transcriptional activation (Costanzo et al., 2002). Other studies have shown that synergism between p53, p63, and p73 is required for Bax transcription and apoptosis (Flores et al., 2002). Although less information is known about p73 transcriptional activation, it upregulates many of the
same genes as p53, but with less efficiency (Melino et al., 2002). Nevertheless, the two proteins share many biochemical characteristics and may have co-evolved in such a way to compensate for each other’s deficiencies. For example, it is speculated that p73 facilitates sensitivity to DNA damage in tumors with mutant or inactivated p53 (Melino, 2003).

**Upstream Oncogenic Transcription Factors**

As shown in Figure 1, p53 can be activated by oncogenic signals (Bates et al., 1998; Hsieh et al., 2002; Zindy et al., 1998) which frequently arise from alterations in the Rb/E2F pathway. In order to understand how the p53 pathway can become inactivated in a tumor that retains wild type p53, it is necessary to consider the regulation of upstream transcription factors such as E2F1 and c-myc.

Under normal physiological conditions, E2F proteins are securely regulated by the retinoblastoma gene product (Rb). During cell cycle progression from G1 to S phase, Rb is phosphorylated by a cyclin and cyclin-dependent kinase complex (Zetterberg et al., 1995). Phosphorylation of Rb alters its conformation and results in the release of free E2F proteins (Weinberg, 1995) whereby E2Fs (E2F1-3) function to transactivate genes required for entry into S phase. Among these are E2F1, cyclin E, and c-myc (Stevaux and Dyson, 2002; Trimarchi and Lees, 2002), where c-myc can directly induce transcription of cyclin E and cdk4 (Hermeking et al., 2000; Lee et al., 1988), which in conjunction with E2F1 autoregulation, increases Rb phosphorylation.

Many human cancers have aberrations in these cell cycle regulatory genes. The Rb gene frequently undergoes chromosomal deletion and its ectopic expression can
suppress cell cycle progression and tumorigenicity (Huang et al., 1988). Consequently, tumors that have inactive Rb are likely to have deregulated expression of E2F1 (Imai et al., 2004; Yamasaki et al., 1998) and tumors that have intact Rb may circumvent repression of E2Fs by overexpressing cyclin D (Sherr, 1996). In addition, amplification, mutation, and translocation of c-myc are common to many tumors including breast, lung, neuroblastoma, and lymphomas, and thus, contribute to its overexpression (Hermeking, 2003; Levin et al., 1994). However, these classically defined oncogenes also have tumor-suppressor like characteristics (Yamasaki et al., 1996) in that they induce cell cycle arrest (Lindstrom and Wiman, 2003) or apoptosis (Field et al., 1996; Matsumura et al., 2003; Pediconi et al., 2003) by various mechanisms.

One way in which both c-myc and E2F1 induce p53 activation is through the transactivation of p14ARF (Bates et al., 1998; DeGregori et al., 1997; Zindy et al., 1998). p14ARF is a tumor suppressor gene that functions to inhibit MDM2 through direct association causing its degradation, and therefore, stabilizing p53 (Pomerantz et al., 1998; Zhang et al., 1998). Apoptosis is attenuated in cell lines that overexpress c-myc but lack functional p53 (Hermeking and Eick, 1994; Wagner et al., 1994) or p14ARF (Zindy et al., 1998) demonstrating that this mechanism is p53-dependent. However, E2F1 still induces apoptosis in the absence of p53 (Rodicker et al., 2001; Stiewe and Putzer, 2000) or p14ARF (Tsai et al., 2002) suggesting that E2F1 can also mediate apoptosis through a p53-independent pathway.

It has now become clear that E2F1 overexpression can result in the transcriptional activation of p73 (Rodicker et al., 2001; Stiewe and Putzer, 2000; Zhu et al., 1998) which can result in either cell cycle arrest or apoptosis depending on the downstream effector
There is also evidence that c-myc can transcriptionally activate p73 in a similar manner as E2F1, leading to apoptosis (Zaika et al., 2002). However, this pathway is controversial because of data suggesting that c-myc has an inhibitory effect on p73 transactivation (Watanabe et al., 2002). Figure 2 illustrates how E2F1 and c-myc can induce cell cycle arrest or apoptosis and summarizes the key signaling events discussed thus far.

**Downstream Effectors and Mediators of Apoptosis**

Apoptosis is defined as programmed cell death initiated by caspase activation leading to the proteolytic degradation of cellular components (Blagosklonny, 2001; Reed,
Apoptosis can occur by the intrinsic mitochondrial pathway or extrinsic death receptor pathway (Sprick and Walczak, 2004), both of which may be p53-dependent. This discussion will focus on the intrinsic pathway because it is the primary mechanism by which apoptosis occurs in response to chemotherapeutic agents.

The intrinsic pathway is initiated at the outer mitochondrial membrane by Bcl-2 family members (Adams and Cory, 1998). Pro-apoptotic proteins Bax and Bak are multi-domain family members (BH1-3), and are distinct from Bid, Bad, PUMA, and NOXA which contain only one Bcl-2 homology domain (BH3) (Donovan and Cotter, 2004; Sorenson, 2004). The interaction of these proteins enables oligomerization of Bax and Bid which causes the release of cytochrome c from the outer mitochondrial membrane (Donovan and Cotter, 2004). Upon release of cytochrome c, Apaf-1 is recruited to the site where it initiates activation of pro-caspase 9 with the help of a caspase recruitment domain (CARD) to form the apoptosome (Chen and Wang, 2002; Lindholm and Arumae, 2004). Once active, caspase 9 can activate the executioner caspase 3 (Lindholm and Arumae, 2004). Anti-apoptotic family members such as Bcl-2 and Bcl-XL (BH1-4) inhibit the release of cytochrome c by directly interacting with Bax and Bid (Donovan and Cotter, 2004). Altered homeostasis of these proteins, such as overexpression of Bcl-2, may result in the inhibition of apoptosis by keeping pro-caspase 9 inactive (Blagosklonny, 2001; Green and Amarante-Mendes, 1998). In addition, caspase 3 has
proteolytic activity against Bcl-2 (Blanc et al., 2000), thus Bcl-2 maintains its integrity by inhibiting the activation of caspases 9 and 3.

Although p53 functions as a transcription factor in response to DNA damage, it is not limited to this role because it also can induce apoptosis at the mitochondria (Marchenko et al., 2000) by interacting with Bcl-2 family members (Chipuk et al., 2005). This transcription-independent function of p53 was first elucidated by experiments showing that apoptosis occurred in the presence of actinomycin D, an inhibitor of transcription, and also transcriptionally inactive p53 mutants (Caelles et al., 1994; Chipuk and Green, 2003). Further, in vitro evidence suggested that p53 mediated apoptosis by working directly at the outer mitochondrial membrane and facilitating the release of cytochrome c (Schuler et al., 2000). Others have shown that its association with Bax and Bak resulted in mitochondrial membrane permeabilization (Moll et al., 2005). Recently, Green and colleagues uncovered a transcription dependent mechanism that supplements p53’s activity at the mitochondria. In their model, cytoplasmic p53 is rendered inactive at the outer mitochondrial membrane by Bcl-X_L. In response to DNA damage, nuclear p53 compensates by upregulating transcription of PUMA, which in turn translocates to the outer mitochondrial membrane where it binds Bcl-X_L, thus displacing p53 to cause membrane permeabilization and apoptosis (Chipuk et al., 2005).

It is becoming clear that nuclear and cytoplasmic p53 function synergistically to mediate apoptosis in tumor cells. Importantly, the latter process can be abrogated by mutations in Bcl-X_L (Chipuk et al., 2005), just as the former (transcription-dependent) process can be abrogated by mutations in MDM2, p14, or Apaf-1 (as previously
discussed), further illustrating that wild type p53 may become functionally inactivated by selecting for mutations in other genes.

Because these pathways are only responsive to cellular stress, they can be investigated in the context of specific DNA damaging agents. In our studies we used the topoisomerase I inhibitor, camptothecin, as an effective tool for measuring the p53 transcriptional response to DNA damage. The following section will discuss the history and pharmacology of camptothecin molecules as chemotherapeutic agents, and their applicability to the signaling pathways reviewed in previous sections.

**Camptothecins: History and Pharmacology**

Camptothecin is a naturally occurring topoisomerase I inhibitor originally isolated in 1958 from *camptotheca acuminata* (Wall, 1966), a tree native to China and Tibet. It was identified as an anti-tumor agent as early as 1966, but its high level of cytotoxicity made it a poor candidate for chemotherapy (Gottlieb et al., 1970; Moertel et al., 1972). The chemistry behind camptothecin activation is complex. It is a pentacyclic alkaloid whose activity is dependent upon the reversible hydrolysis of its lactone ring (Fassberg and Stella, 1992; Wall, 1966). Early studies were primarily conducted using the sodium salt form which is more water soluble (Thomas et al., 2004), but favors the open (inactive) conformation (Garcia-Carbonero and Supko, 2002). Thus, high concentrations of drug were required for cytotoxicity, and reversal to the active form was unpredictable (Pizzolato and Saltz, 2003). It wasn’t until 1985 that camptothecin was identified as a target of topoisomerase I (Hsiang et al., 1985).
Topoisomerases are evolutionarily conserved enzymes that function to relieve the torsional stress associated with DNA replication. In mammals there are two classes of topoisomerases. Topoisomerase I produces a single stranded break in DNA downstream of the replication fork, while topoisomerase II produces a double stranded break, both of which are reversible by re-ligation. Camptothecin interacts covalently with topoisomerase I, which inhibits the re-ligation of the cleaved DNA by stabilizing the enzyme-DNA complex (Champoux, 2000; Hsiang et al., 1985). When the camptothecin bound enzyme-DNA complex contacts the replication machinery, an irreversible double stranded break is formed, resulting in apoptosis (Hsiang et al., 1989). Caspase inhibition by zVAD-fmk causes cells to arrest in G1 until death occurs by necrosis (Sane and Bertrand, 1999).

The idea that camptothecin was a specific inhibitor of topoisomerase I provoked an interest in synthesizing derivatives that were more soluble and produced fewer side effects. The first of these to be approved by the FDA was topotecan (Takimoto and Arbuck, 1997). It was made more soluble by addition of a hydroxyl group and a basic side chain on the A ring (Kollmannsberger et al., 1999; Pizzolato and Saltz, 2003). Data from clinical trials indicated that topotecan had increased response rates over paclitaxel when used to treat ovarian cancer (ten Bokkel Huinink et al., 1997) but showed no significant advantage for treatment of other cancers, such as breast and NSCLC (Mainwaring et al., 1997). Topotecan is currently approved in the United States to treat ovarian carcinoma and advanced SCLC.

A second camptothecin derivative that is widely used is irinotecan, or CPT-11. It was developed in Japan where it was approved to treat NSCLC, SCLC, and colon cancer (Saijo, 1996, 2000), then later sold to Pfizer and marketed as Camptosar. In 1996 it was
approved in the United States as a second line defense for treating colon cancer (Rothenberg, 1996, 2001).

Irinotecan (CPT-11) is a water soluble semi-synthetic camptothecin derivative with the addition of a piperidino side chain and ester linkage to the A ring (see comparison of camptothecin and its derivatives in Figure 3) (Xu and Villalona-Calero, 2002). However, it is structurally inactive and must be converted to its active form, SN-38, by *in vivo* carboxylesterase (CE) mediated hydrolysis (Kawato et al., 1991). Conversion of CPT-11 into SN-38 results in a molecule resembling the natural form of camptothecin, with exception of a hydroxyl group at position 10 of the A ring and an ethyl group at position 7 of the B ring (Figure 3) (Toffoli et al., 2003).

**Figure 3. Structure of Camptothecin and Synthetically Derived Irinotecan (CPT-11) and SN-38.**

Adapted from Toffoli et al. A) camptothecin; B) irinotecan (CPT-11), the piperidino side chain is attached by ester linkage at position 10 of the A ring. An ethyl group is added to position 7 of the B ring; C) SN-38, *in vivo* hydrolysis by carboxylesterase (CE) cleaves the piperidino side chain shown in B, leaving a hydroxyl at position 10.
Because CPT-11 is far less toxic than SN-38, it can be administered to patients intravenously at higher concentrations (Toffoli et al., 2003). Carboxylesterases are primarily expressed in the liver and intestinal tract, but also have activity in tumor cells (de Jong et al., 2006), and therefore, their activity have been correlated with sensitivity to CPT-11 \textit{in vitro} (van Ark-Otte et al., 1998; Wierdl et al., 2001).

In the liver SN-38 is glucuronidated to form SN-38-glucuronide (SN-38G) (Iyer et al., 1998). This reaction is carried out by uridine diphosphate glucuronosyltransferase isoform 1A1 (UGT1A1) and failure to undergo this detoxification step results in cytotoxicity (Gupta et al., 1994; Wasserman et al., 1997; Xu and Villalona-Calero, 2002). Much attention has been given to UGT1A1 in recent years since it was discovered that a genetic polymorphism in its regulatory region accounted for a significant decline in enzyme activity (Iyer et al., 1999). Individuals with this polymorphism have a TA insertion within the TATA box of the UGT1A1 promoter (designated TA7), which is highly linked to Gilbert’s syndrome, a genetic disorder caused by reduced UGT activity (Monaghan et al., 1996). In addition, patients with Crigler-Najjar syndrome type I, a genetic disease manifested by the complete lack of bilirubin glucuronidation, have little to no UGT1A1 activity (Mathijssen et al., 2001). Consequently, these individuals are highly susceptible to myelosuppression and diarrhea, which are the two primary dose-limiting side effects from CPT-11 cytotoxicity (Iyer et al., 1998; Wasserman et al., 1997). These observations have warranted the use of genotyping for defective UGT alleles in order to identify those individuals at risk for toxicity. A genetic test for CPT-11-induced toxicity has been developed by Genzyme and Third Wave Technologies and was recently approved by the FDA in 2005 (Hasegawa et al., 2004; Marsh and McLeod, 2006a).
On the contrary, high activity of UGT1A1 is unfavorable for treating cancer because it decreases concentrations of active CPT-11 (SN-38) (Takahashi et al., 1997), and other drugs such as dexamethasone and phenobarbital can indirectly inactivate CPT-11 by increasing glucuronidation (Friedman et al., 1999; Gupta et al., 1997; Xu and Villalona-Calero, 2002). Clinical trials in which CPT-11 was used in combination with dexamethasone and/or phenobarbital for treating glioma have shown that patients experience decreased toxicity and have lower area under-the-curve concentrations of CPT-11, SN-38, and SN-38G (Friedman et al., 1999). In a phase I study, glioma patients receiving anti-epileptics were able to tolerate over twice the dosage of CPT-11 than patients not receiving epileptic therapy (Prados et al., 2004). These data indicate a role for UGT1A1 not only as a predictor for increased toxicity, but also decreased drug efficacy as well. Whether or not UGT1A1 activity can be used as an indicator of optimal dosing in patients with a TA6 (major frequency allele) genotype receiving CPT-11 has yet to be determined.

The metabolism of CPT-11 (SN-38) is further complicated by β-glucuronidation. Commensal bacteria including Escherichia coli and Clostridium perfringens, which are at high cell density in the lower intestinal tract and colon, excrete β-glucuronidase (Skar et al., 1988), which is responsible for the reactivation of SN-38G. This results in the accumulation of active SN-38 in the cecum causing severe diarrhea and tissue damage (Takasuna et al., 1996). Concomitant treatment with antibiotics reduces the potential for β-glucuronidation, and prevents SN-38-induced diarrhea (Takasuna et al., 2006).

A second metabolic product of CPT-11 is aminopentanecarboxylic acid (APC) which is produced by CYP3A4 oxidation of the piperidino side chain (Santos et al.,
APC is not converted to SN-38 and may result in decreased drug activity (Xu and Villalona-Calero, 2002) because APC has little effect on tumor cells (Rivory et al., 1996). Similarly, CYP3A4 also produces a metabolite called NPC [7-ethyl-10-(4-amino-1-piperidino) carbonyloxy camptothecine] which results from cleavage of a different piperidino side chain. The NPC form may be converted back into SN-38, although like APC, it is a weak substrate for topoisomerase I and has little anti-tumor activity (Dodds et al., 1998). The metabolism of CPT-11 is summarized in Figure 4.

**Figure 4. Metabolism of CPT-11 (Irinotecan)**

CPT-11 is administered by intravenous injection. In the liver it is converted from CPT-11 into its active form SN-38 by carboxylesterase (CE)-mediated hydrolysis. CPT-11 can also be metabolized by the cytochrome p450 enzyme, CYP3A4, into APC and NPC which have little to no anti-tumor activity. NPC can be reactivated into SN-38. UGT1A1 catalyzes the conversion of SN-38 to SN-38G which also lacks anti-tumor activity. This process can be reversed in the lower intestinal tract by microflora with β-glucuronidase activity resulting in severe diarrhea and damage to the colon.

**Mechanisms of Resistance to Camptothecins**

Chemoresistance to camptothecin and its derivatives are common, and although some biomarkers have been correlated with sensitivity (Nakajima et al., 2004; Pavillard et al., 2004; van Ark-Otte et al., 1998), the mechanism of drug response is not well understood (Seve and Dumontet, 2005; Sugimoto et al., 1990). Several components to its
resistance have been experimentally confirmed, including the metabolic factors previously discussed, such as inter-individual variation in CE activity (Sanghani et al., 2003; van Ark-Otte et al., 1998), SN-38 glucuronidation (Carlini et al., 2005; Takahashi et al., 1997; Toffoli et al., 2006), and CYP3A4 conversion to inactive metabolites (Mathijssen et al., 2004; Xu and Villalona-Calero, 2002). Second, proteins that modulate drug efflux and transport including MDR1 and MRP1 affect response by controlling intracellular drug concentrations. For example, overexpression of the multidrug resistance protein (MRP4) was correlated with chemoresistance and poor prognosis in a neuroblastoma cell line (Norris et al., 2005). Third, the level of topoisomerase I has been proposed as a key factor for response to CPT-11 (Arakawa et al., 2006; Ohashi et al., 1996), although no correlation was observed between topoisomerase I and CPT-11 response in vivo (Calvet et al., 2004).

Because CPT-11 and other camptothecin molecules induce DNA damage and apoptosis, p53 may itself be a mechanism of resistance if a mutation is present, or resistance may be conferred by pathways downstream of p53 activation. Although there have been several studies supporting this hypothesis, the relationship between apoptotic activity and p53 dependence has not been appropriately clarified and remains inconsistent. Evidence supporting a relationship between p53-dependent apoptosis and sensitivity to camptothecin was provided by Lai et al. in lung cancer cell lines. In this study, cell lines with mutant p53 were more intrinsically resistant to camptothecin and transfection of wild type p53 increased their sensitivity. Conversely, in a wild type p53 cell line transfected with mutant p53, sensitivity was decreased, and p53-dependent apoptosis was lost (Lai et al., 2000). In another study where two p53 mutant lung cancer
cell lines were used, Osaki et al. determined that adenoviral delivery of p53 enhanced sensitivity to CPT-11 and 5FU more effectively than 10 other chemotherapeutic agents (Osaki et al., 2000). Conflicting results have been observed in colon cancers. For example, isogenic HCT116 cell lines with wild type (+/+) or null (-/-) p53 made selectively resistant to CPT-11 had identical IC$_{50}$ concentrations (Boyer et al., 2004). Paradoxically, when p53 was transfected into HT29 cells, sensitivity to CPT-11 was increased relative to the parental cell line (Bras-Goncalves et al., 2000).

Several other genes related to p53 function have been correlated with response to camptothecin. p21 was associated with resistance or attenuated response in several studies (Arango et al., 2003; Han et al., 2002; Zhang et al., 1999b) due to the induction of cell cycle arrest and senescence (Han et al., 2002). Recent evidence shows that p21 impairs DNA damage by inhibiting the formation of camptothecin-induced double stranded breaks during DNA replication (Furuta et al., 2006). Further, p21 is a substrate for caspase 3 cleavage during the onset of DNA damage (Gervais et al., 1998; Zhang et al., 1999a), and a cell line made selectively resistant to camptothecin had increased p21 expression but failed to show caspase-mediated cleavage of p21 (Zhang et al., 1999b). Together, these data strongly indicate that p21 exerts a pro-survival response after treatment with camptothecin, and therefore, functions as an antagonist of apoptosis.

In contrast, camptothecin induced apoptosis in colon cancer cells that overexpressed c-myc and retained wild type p53 (Arango et al., 2003). In addition, the amplification of c-myc and wild type p53 were strong predictors for response to 5FU therapy (Arango et al., 2001), which is frequently administered with CPT-11. In vitro data from the same authors show that inhibition of p53 abrogates the pro-apoptotic
activity of overexpressed c-myc (Arango et al., 2001). This is supported by earlier studies demonstrating that wild type p53 is required to achieve c-myc-induced apoptosis in tumor cells through transactivation of p14ARF (Hermeking and Eick, 1994; Hermeking et al., 1994; Wagner et al., 1994). Moreover, c-myc can physically interact with p21 to block cell cycle arrest (Kitaura et al., 2000), demonstrating that p21 can be inhibited in wild type p53 tumor cells. Of further importance is the transcriptional relationship between both proteins. In fibrosarcoma cells, c-myc can function as a transcriptional repressor for p21, and vice versa, p21 can repress transcription of c-myc (Vigneron et al., 2005). In response to DNA damage, c-myc is recruited to the p21 promoter by an adaptor protein, Miz-1 and specifically inhibits its transcription by p53 (Seoane et al., 2002). Therefore, overexpression of c-myc not only sensitizes cells to apoptosis, but also neutralizes the antagonistic effect of p21.

The inhibition of apoptosis by Bcl-2 in hematopoietic tissues has been studied extensively (Reed and Pellecchia, 2005; Verma et al., 2006), as well as its ability to enhance resistance to chemotherapeutic agents (Chanan-Khan, 2004; Davis et al., 2003; Ohmori et al., 1993). Thus, it is not surprising that Bcl-2 expression has been shown to influence response to camptothecins, and may act synergistically with p21 to inhibit apoptosis (Zhang et al., 1999b). Further, when stably transfected with Bcl-2, the SCLC line SBC-3 became resistant to CPT-11 and failed to show characteristic features of apoptosis after treatment (Ohmori et al., 1993). However, the level of Bcl-2 expression is seldom correlated with response to most drugs, or apoptosis, and studies using solid tumors have been largely inconsistent (Blagosklonny, 2001). For example, high expression of Bcl-2 has been found in chemosensitive tissues such as SCLC (Sirzen et al.,
1998), whereas low expression of Bcl-2 has been found in apoptosis resistant cells (Wu and El-Deiry, 1996).

Such incongruity lead to the hypothesis that apoptosis and drug resistance were controlled, not by the level of Bcl-2 alone, but by the relative abundance of anti-apoptotic and pro-apoptotic Bcl-2-related proteins. Therefore, Bcl-2/Bax ratios were employed in order to predict apoptosis induction or response to treatment (Joseph et al., 2000; Reed, 1999) which may be informative for some tissues. For example, in neuroendocrine lung tumors and SCLC with variable p53 status, a high Bcl-2/Bax ratio (>1) was inversely correlated with apoptosis (Brambilla et al., 1996). Similar results have been reported in leukemia patients (Dabrowska et al., 2003; Del Poeta et al., 2003).

A theory behind the inter-relationship between p53, Bcl-2, and apoptosis has been proposed by Mikhail Blagosklonny (2001) and is summarized here. The theory suggests that there are two kinds of cells, those that are apoptosis prone and those that are apoptosis resistant. Cells that are apoptosis prone have functional apoptotic machinery, consisting of wild type p53 and constitutively low expression of Bcl-2. However, many of these malignancies are able to select for mutations in downstream genes such as Apaf-1, Caspase 9, and Caspase 3. Because these mutations result in apoptotic deficiency, cells can retain wild type p53, low expression of Bcl-2, and continue to proliferate. On the other hand, increased expression of Bcl-2 is suggestive that the apoptosis machinery is in tact and that there are no downstream mutations. This theory offers an explanation for why cells retaining wild type p53 and low expression of Bcl-2 can be resistant to chemotherapy, and in contrast, why tumors such as SCLC have elevated expression of Bcl-2, and yet, are apoptotic prone and chemosensitive (Joseph et al., 2000).
**Predicting Cytotoxicity and Dosimetry of Chemotherapeutic Agents**

Genes that affect drug response in tumor cells also are likely to contribute to cytotoxicity in normal cells. Most chemotherapeutics are not specific to tumor cells, but rather, they have a propensity for targeting rapidly proliferating cells (Perry, 1984) (a characteristic common to all malignant cells). However, many normal cells such as hematopoietic stem cells and cells that line the GI mucosa, also proliferate rapidly and are susceptible to these agents. (Efferth and Volm, 2005).

There has been an extraordinary volume of literature citing the inter-individual variation in drug response that occurs in tumor cells. However, much less has been done to examine the effects of cytotoxic chemotherapy in normal (non-malignant) cells. Because of inter-individual variation in drug metabolism and response, the therapeutic window for these agents is low and cytotoxicity in normal tissues is often unpredictable. The following will discuss newly developed efforts that are underway to predict cytotoxicity and dosimetry using pharmacogenomic approaches.

In pre-clinical studies with animal models, drug dosing is determined by body surface area (BSA) and these data are translated for use in humans by starting with 1/10 the LD$_{10}$ concentration (Felici et al., 2002). The practice of using BSA to identify optimal dosing has been employed for over 50 yr. During this time BSA has shown to be more effective than other measures, for example, body mass index, due to its correlation with glomerular filtration rate, blood volume, and metabolic rate (Baker, 1957; Kleiber, 1932; Smith, 1951). However, more recent findings have shown otherwise (Dooley and Poole, 2000). Felici et al. summarize key studies published in the last 15 yr suggesting BSA is
not associated with drug clearance or pharmacokinetics. Among those drugs in which no correlation was found were cisplatin, doxorubicin, epirubicin, etoposide, CPT-11, SN-38, ifosfamide, methotrexate, topotecan, and ZD9331. Drugs in which BSA was correlated with drug clearance or pharmacokinetics in at least one study were busulfan, cyclophosphamide, eniluracil/5FU, gemcitabine, paclitaxel, and temozolomide, and many of these results conflicted with other studies (Felici et al., 2002). Thus, it has become clear that routine use of BSA for determining dosimetry has become obsolete. Rationale has been provided for use of molecular genotyping assays that would better predict cytotoxicity for drugs in which the pharmacodynamics are known (de Jongh et al., 2001; Loos et al., 2000; Mathijssen et al., 2002).

Since completion of the human genome project, the field of pharmacogenomics has been leading in efforts to predict optimal treatment strategies and dosage for chemotherapy patients. Single nucleotide polymorphisms (SNP) are the primary source of genetic variation in humans and many of the genes involved in drug metabolism have variant alleles that associate with cytotoxicity (Marsh and McLeod, 2006a). The newly available HapMap database has identified many of the common disease alleles, and thus, it is becoming easier to determine which genes are responsible for altering the pharmacodynamics of chemotherapeutic agents (The International HapMap Project, 2003; Marsh and McLeod, 2006b).

Single nucleotide polymorphisms in the cytochrome p450 genes are common examples of how genetic alterations can cause inter-individual variation in drug response (Evans and Relling, 1999), and FDA approved genetic tests are currently available for several cytotoxic drugs (de Leon et al., 2006). Multiple types of chemotherapeutic agents
are substrates for the CYP3 family, and at least 80 SNPs have been identified for CYP3A4 and CYP3A5 that result in decreased protein expression (Lee and Goldstein, 2005). For example, CYP3A4, CYP3A5, CYP3A7 affect cytotoxicity to etoposide (Evans and Relling, 2004), CYP3A4 and CYP3A5 are substrates for CPT-11 as well as doxorubicin, cyclophosphamide, and tamoxifen (Toffoli et al., 2003). CYP3A4*1B is a variant allele of CYP3A4 found in 9.6% of Caucasians and 52% in Africans. It is caused by a single base transition in the regulatory region of the gene and correlates with decreased protein expression as well as inter-individual variation in cytotoxicity (Toffoli et al., 2003). Thus, as the case for UGT1A1, SNPs present in non-coding regions can affect not only protein expression, but also enzymatic activity which may significantly impact an individual’s response to a particular medication (Evans and Relling, 1999).

Although significant advancements have been made in the field of pharmacogenomics, there are several limitations to this approach. For example, there are an abundance of cytochrome p450 genes. Even among the CYP3A family, each gene encodes a distinct enzyme with a wide range of substrate specificity (Lee and Goldstein, 2005). The SNPs in many of these genes may not be associated with any changes in expression, or enzymatic activity, the biologically significant endpoint (Baker et al., 2004; Garcia-Martin et al., 2002). A lack of association could be due to other polymorphisms unaccounted for as well as regulation at the transcription, translation, or post-translational levels (George et al., 1995). Second, SNPs in multiple CYP encoding enzymes may have synergistic effects on drug metabolism (Evans and Relling, 2004), or alternatively, a CYP SNP may neutralize the effect of a polymorphic p-glycoprotein (MDR1) allele, common in Caucasians (Evans et al., 2001). Further, inter-individual
variation in enzymatic activity may not be related to variant CYP alleles, but the transcription factors that regulate them, such as PXR receptor (Wolbold et al., 2003) and HNF4α (Tirona et al., 2003). Other variables that affect CYP enzymatic activity include sex (Bebia et al., 2004; Wolbold et al., 2003), age (Gurley et al., 2005), diet (Lee and Goldstein, 2005) and liver function (Baker et al., 2004). Nevertheless, pharmacogenomic approaches have been deemed successful by the FDA, and future availability of high-throughput genetic screening may be advantageous for discovering new SNPs and developing algorithms that facilitate individualized medicine.

In addition to drug metabolizing genes, genes that regulate programmed cell death in the normal epithelium and hematopoietic tissues are altered in response to cytotoxic drugs. Pifithrin α is a chemical inhibitor of p53 that was initially synthesized in order to inhibit its activity in proliferating cells after \textit{in vivo} chemotherapeutic treatment (Komarov et al., 1999). Thus, a role for p53 and its transcriptional response to DNA damage has been implicated normal cells.

\textit{p53 in Hematopoietic Cells}

p53 is generally not expressed at high levels in normal non-stressed cells, although its mRNA is abundantly expressed in splenic tissue as well as embryonic stem cells (Rogel et al., 1985). In addition, low expression of p53 protein was found in mature and non-proliferating cells of lymphoid, granulocytic, and monocytic origin, but its expression was high in proliferating CD34+ precursors (Kastan et al., 1991). Hematopoietic progenitor cells from p53 knock-out mice show deficiencies in apoptosis in response to DNA damage by irradiation (Lotem and Sachs, 1993). In another study,
the repopulation of hematopoietic stem cells was markedly increased in p53 knock outs after treatment with 5-FU, further supporting the observation of an apoptosis defect during hematopoiesis (Wlodarski et al., 1998). Thus, like tumor cells, proliferating hematopoietic cells activate p53 in response to DNA damage. This is best exemplified by Komarova et al., in which transgenic mice were engineered to express a LacZ reporter construct regulated by the p53 promoter. When mice were treated with the topoisomerase II inhibitor, doxorubicin (adriamycin), LacZ expression was induced in the spleen, thymus, and intestine which was associated with p53 accumulation followed by apoptosis (Komarova et al., 1997). This mechanism of p53 activation is, at least in part, transcriptionally dependent, which is indicated by increased mRNA in these tissues (Komarova and Gudkov, 1998).

One important question is whether p53-dependent apoptosis occurs in peripheral blood leukocytes or it is limited to hematopoietic cells of the bone marrow. In a follow-up study Teofili et al. measured the p53 response in peripheral blood and bone marrow of five Non-Hodgkin’s lymphoma patients treated with cyclophosphamide. They found p53 was significantly induced in CD34+ cells from bone marrow but not peripheral blood cells. In addition, Bcl-2 decreased while Bax and apoptosis increased in marrow derived cells, and neither of these genes were altered in peripheral cells (Teofili, 1998). Further, when apoptosis was measured ex vivo in peripheral blood lymphocytes from pediatric cancer patients, it was induced within 12 h after exposure to chemotherapy and correlated with lymphocyte depletion. Increased protein expression of Bax and activation of caspases 3 and 8 also were noted. However, p53 remained undetectable in peripheral blood lymphocytes and was only activated by doxorubicin from cells cultured in vitro.
(Stahnke et al., 2001). However, an additional study found that normal mature B cells express higher levels of p53 than those from B-Chronic Lymphocytic Leukemia (CLL) patients and treatment of normal cells with fludarabine induces p53 accumulation (Secchiero et al., 2006).

From these studies it is inconclusive as to whether p53 accumulation occurs in mature peripheral blood lymphocytes. It is possible that a low level of p53 (or lack thereof) is required for survival during hematopoiesis. An alternative explanation is that p53 accumulation occurs only in proliferating lymphocytes, whether in the bone marrow or periphery and that its activation is cell type or drug specific.
Variation in transcriptional regulation of cyclin dependent kinase inhibitor p21waft/cipl among human bronchogenic carcinomas

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Abstract

Background

Cell proliferation control depends in part on the carefully ordered regulation of transcription factors. The p53 homolog p73, contributes to this control by directly upregulating the cyclin dependent kinase inhibitor, \( p21^{\text{waf1/cip1}} \). E2F1, an inducer of cell proliferation, directly upregulates p73 and in some systems upregulates p21 directly. Because of its central role in controlling cell proliferation, upregulation of p21 has been explored as a modality for treating bronchogenic carcinoma (BC). Improved understanding of p21 transcriptional regulation will facilitate identification of BC tissues that are responsive to p21-directed therapies. Toward this goal, we investigated the role that E2F1 and p73 each play in the transcriptional regulation of p21.

Results

Among BC samples (\( N=21 \)) p21 transcript abundance (TA) levels varied over two orders of magnitude with values ranging from 400 to 120,000 (in units of molecules/\( 10^6 \) molecules \( \beta \)-actin). The p21 values in many BC were high compared to those observed in normal bronchial epithelial cells (BEC) (\( N=18 \)). Among all BC samples, there was no correlation between E2F1 and p21 TA but there was positive correlation between E2F1 and p73\( \alpha \) (\( p<0.001 \)) TA. Among BC cell lines with inactivated p53 and wild type p73 (\( N=7 \)) there was positive correlation between p73\( \alpha \) and p21 TA (\( p<0.05 \)). Additionally, in a BC cell line in which both p53 and p73 were inactivated (H1155), E2F1 TA level was high (50,000), but p21 TA level was low (470). Transiently expressed exogenous p73\( \alpha \) in the BC cell line Calu-1, was associated with a significant (\( p<0.05 \)) 90% increase
in p21 TA and a 20% reduction in E2F1 TA. siRNA mediated reduction of p73 TA in the N417 BC cell line was associated with a significant reduction in p21 TA level (p<0.01).

**Conclusion**

p21 TA levels vary considerably among BC patients which may be attributable to 1) genetic alterations in Rb and p53 and 2) variation in TA levels of upstream transcription factors E2F1 and p73. Here we provide evidence that p73 upregulates p21 TA in BC tissues and upregulated p21 TA may result from E2F1 upregulation of p73 but not from E2F1 directly.

**Background**

Cell cycle homeostasis in normal human bronchial epithelial cells (BEC) is highly regulated at the G1/S transition. In G1 phase of the cell cycle, formation of a heterodimeric complex between cyclin D and cyclin dependent kinases 4 or 6 (cdk 4,6) leads to the phosphorylation of the tumor suppressor retinoblastoma gene product (pRb) [1-3]. Phosphorylation causes conformational change of the pRb/E2F complex, followed by release, and activation of the E2F1, 2, and 3 transcription factors [4-6]. Free E2F proteins bind strongly to DNA and were first identified by their ability to transactivate the adenoviral E2 promoter [7]. E2F1 functions to upregulate transcription of genes required for entry into S phase, including cyclin E, c-myc and itself [8-10]. In turn, c-myc directly upregulates transcription of cyclin E and cdk4 [11, 12]. Thus, phosphorylation of pRb by one or more cyclin/cdk complexes causes activation and upregulation of E2F1, upregulation of c-myc transcription by E2F1, and upregulation of cdk4 transcription by c-
*myc*. These interactions are initiated at the restriction point of G1/S, which is associated with independence of the cell from extracellular growth factors [4, 13]. The events described above contribute to a cell proliferation signal amplification cycle that would be uncontrolled in the absence of compensatory negative feedback.

Compensatory feedback signals, including the activation of p53 and transcriptional upregulation of p73 and p21<sup>wafl/cip1</sup> (p21 hereafter) act to slow cell proliferation [14-16]. Unlike p53, p73 is not frequently mutated in human cancers [17], and thus it is not considered a classical tumor suppressor gene, as defined by Knudson’s two hit hypothesis [18, 19]. However, it functions to promote cell cycle arrest, DNA repair, and apoptosis much like p53 [18, 20]. E2F1 (and c-*myc*) transactivation of p14<sup>ARF</sup> leads to stabilization of p53 [21, 22] which slows cell cycling through the upregulation of p21 [23, 24], and also induces apoptosis [25]. E2F1 also upregulates p73 [15, 26, 27] and p73 upregulates p21[15], which in turn acts to inhibit the release of E2F1 from pRb, resulting in compensatory feedback for the loss of cell proliferation control. In some systems, E2F1 also upregulates p21 directly [28].

In previous studies, we determined that p21 transcript abundance (TA) levels vary considerably among bronchogenic carcinoma (BC) primary tissues and cell lines, and in some of these samples the p21 TA level is higher than the level observed in normal BEC [29, 30]. Because p21 normally slows cell proliferation, this observation was unexpected, and counter-intuitive. If validated, these preliminary findings would have important implications for the many efforts presently underway to design gene specific cancer treatments that function to achieve cell proliferation control [31].
The purpose of this study was to better understand inter-tumor variation in the mechanisms responsible for loss of proliferation control and to better define the role of E2F1, p73 and p21 regulatory pathways as they relate to cell proliferation control in human BC. Our approach included additional descriptive studies in BC cell lines, as well as primary BC tissues and normal BEC to better quantify inter-tumor variation of p21 TA levels. With respect to the fourteen BC cell lines used in this study, because each has been extensively characterized at the genetic level [17, 25, 32-35], we were better able to define the regulatory relationship between E2F1, p73, and p21 after considering the known alterations in each individual cell line. Our descriptive approach was followed by experimental testing of the hypothesis posed to explain these observations.

**Results**

Transcript abundance (TA) levels of E2F1, p73α, and p21 in normal bronchial epithelial cells (BEC) (N=18) and bronchogenic carcinoma (BC) tissues (N=21) were measured by StaRT-PCR. Cultured cells and primary tissues are shown in Tables 1 and 2, respectively. For the BC cell lines, known alterations in p53, p16, p14ARF, Rb, p73, and c-myc also are presented, along with the TA values. Bivariate analysis of the TA data from these cultured human BEC samples provided data consistent with observations in other tissues that E2F1 regulates p73 transcription [15, 26, 27] and that p73 regulates p21 transcription [15]. However, in contrast to other cell types, they suggested that E2F1 does not directly regulate p21 transcription. To further investigate the significance of this finding, seven primary BC tissues (Table 2) were also assessed.
The mean, median, and quartile values for each gene in normal (N=18) compared to malignant samples (N=21) are shown in Table 3. E2F1 TA levels were over 30-fold higher in BC relative to normal BEC (p<0.0001). Conversely, mean p73α TA was higher in normal BEC by over 2-fold, but this difference was not statistically significant (p=0.07), although the median p73 TA value was higher in normal BEC by nearly 9-fold. With respect to p21 TA level, there was no statistical difference in the mean value between the two groups.

Median and quartile values were used to determine if TA levels were high or low for a given sample. For example, the median value for E2F1 in BC was 19,000. Therefore, a sample with an E2F1 TA level greater than 19,000 would be considered high and a TA level less than 19,000 would be considered low. These criteria were used to identify cell lines with low (Calu-1) or high (N417) p73α TA for use in the exogenous p73 and p73 siRNA transfection experiments.

**Bivariate analysis of E2F1 and p21**

There was no significant correlation between E2F1 and p21 TA levels among BC cell lines and primary tissues (N=21) (Figure 1).

**Bivariate analysis of E2F1 and p73α TA levels**

E2F1 and p73α TA values were positively correlated (p<0.001) among BC cell lines and primary tissues (N=21) (Figure 2).
Bivariate analysis of p73α and p21 TA levels

Among BC cell lines in which p53 is known to be completely inactivated by mutation or deletion (N=7), p73α and p21 were significantly correlated (p<0.05), as shown in Figure 3. In contrast, there was a borderline but insignificant (p=0.06) positive correlation between p73α and p21 TA among all BC cell lines and primary tissues (N=21). This result may be explained by p53 regulation of p21 transcription as a confounding variable in those cell lines with wild type p53.

E2F1 and p21 TA analysis in BC cell line with inactivated p53 and p73

In the H1155 cell line in which p53 and p73 are both inactivated, E2F1 TA level was high, yet p21 TA level was low (Table 1). Consistent with our observation that E2F1 and p73α are correlated in BC tissues (Figure 2), E2F1 and p73α TA levels were both high in H1155. However, the missense mutation in the DNA binding domain of p73 inhibits its transactivational function [35]. Thus, H1155 is a cell line with a naturally occurring p73 mutation that directly supports our hypothesis that p21 is not upregulated by E2F1 directly.

Expression of exogenous p73α is associated with increased p21 and decreased E2F1 TA in Calu-1

To test the hypothesis that p21 transcription is regulated by p73 and not by E2F1 directly, p73α was transiently expressed in the squamous carcinoma cell line Calu-1. This line expresses low levels of endogenous p73α TA (28), low levels of p21 TA (2,000), and
low levels of E2F1 TA (8,500). p73α TA was induced over 1,000-fold relative to the mock, 24 hours post-transfection (Figure 4A). Exogenous p73 protein expression was confirmed by Western analysis using an anti-HA antibody specific for an amino terminal tag on p73 (Figure 4B). p21 and E2F1 TA levels were quantified 24 hours post-transfection. While p21 TA was upregulated 90% (p<0.001) relative to mock-transfected cells, there was a 20% downregulation (p<0.05) of E2F1 TA (Figure 4C).

**Gene specific silencing of p73 associated with decreased p21 TA level**

According to bivariate analysis shown in Figure 3, p73α and p21 were significantly correlated in cell lines that have inactivated p53. Therefore, we anticipated that gene specific silencing of p73 in one of these lines would directly reduce p21 TA. We used a pool of siRNAs to target all isoforms of p73 in the null-p53 small cell carcinoma N417. This cell line is an appropriate model because it expresses high p21 (34,000), E2F1 (19,000), and endogenous p73α (5,200) TA levels. p73α TA decreased by 80% relative to the non-specific siRNA control (p<0.05), while p21 TA decreased by 70% (p<0.01).

**Discussion**

Relative to normal bronchial epithelial cells (BEC), p21 is upregulated in some bronchogenic carcinoma (BC) tissues and downregulated in others (Tables 1, 2, and 3). Elevation of p21 TA in malignant cells may seem counter-intuitive because it would be expected to slow cell proliferation. However, in some tumors pRb is dysfunctional and feedback signals such as p73 upregulation by E2F1 may increase p21 transcription in an ineffective attempt to prevent phosphorylation of pRb and release of activated E2F1. It is
likely that such tumors would be poor candidates for therapy intended to control cell proliferation through specific upregulation of p21 transcription. However, in other tumors, such as those with a genetic profile similar to that of A549 or Calu-1 (Table 1), pRb is intact, and TA levels of E2F1, p73, and p21 are all low. In a tumor such as this, there is reason to believe that the upregulation of p21 transcription would be an effective treatment. However, in order to develop effective p21 gene-specific therapeutics, and biomarkers that predict which tumor will respond, it is necessary to better understand p21 transcriptional regulation.

In some cell types, E2F1 directly regulates p21 [28], however, the data presented here support the hypothesis that E2F1 does not upregulate p21 directly in human BC, but rather, elevated p21 TA results from E2F1 upregulation of p73. This hypothesis, generated initially from empirical observation, is supported by experimental data. There were four supportive empirical observations. First, there was lack of correlation between E2F1 and p21 TA among BC tissues. Second, there was positive correlation among BC tissues between E2F1 and p73 TA. Third, there was positive correlation between p73 and p21 TA among BC cell lines with inactivated p53. Fourth, in the H1155 BC cell line, in which p53 and p73 are inactivated, E2F1 and p73 TA levels were high, but p21 TA level was low. If E2F1 upregulated p21 directly, it would be reasonable to expect that p21 would be upregulated in this cell line, not downregulated.

In experiments designed to directly test this hypothesis, transient exogenous expression of p73α was associated with increased p21 and decreased E2F1 TA levels and siRNA mediated silencing of p73 was associated with decreased p21 TA levels. Although the siRNA experiments support our hypothesis, they are not as supportive as the transient
transfection experiments because E2F1 TA was reduced along with p73 and p21. We speculate that this decrease was due to a non-specific effect of the siRNAs. A non-specific effect of the siRNA method has been previously reported for cell cycling genes, including p53 and p21 [36].

**Conclusions**

In this study, we provide strong empirical and experimental evidence that in human bronchogenic carcinoma, p21 transcription is regulated by p73 but, in contrast to other cell types, not directly by E2F1. This knowledge will facilitate a) development of p21 gene-specific therapeutics necessary for individualized treatment strategies, and b) discovery of biomarkers that will predict which tumors will respond to p21 gene-specific therapeutics.

**Methods**

**Normal cell populations**

Normal BEC stock populations (lot numbers: 17378, 6F0333, 6F0395) were obtained from Clonetics (San Diego, CA) and incubated in BEGM medium.

**Carcinoma cell lines**

Fourteen BC cell lines (Table 1) were obtained from American Type Culture Collection (Rockville, MD) and incubated in RPMI with 10% FBS.
Culture conditions

Normal BEC and BC cells proliferate optimally under different conditions [37]. The medium that is optimal for BC cell lines, RPMI with 10% fetal bovine serum (FBS), induces terminal squamous differentiation in normal BEC [38]. In contrast, BC cell lines do not divide in serum-free media that are optimal for proliferation of normal BEC. BC cell lines were incubated in RPMI with 10% FBS and normal BEC from three individuals were incubated in bronchial epithelial growth medium (BEGM). In order to directly compare with carcinoma cell lines under the same optimal conditions, normal BEC cell populations (17378, 6F0333, 6F0395) were also incubated for 24 hours in RPMI with 10% FBS.

Primary tissue samples

Primary normal BEC and primary BC samples (Table 2) were obtained under IRB approved protocols as previously described [29, 39, 40]. In each case informed consent was obtained from the patient.

RNA extraction and reverse transcription

Total RNA was extracted by phenol/chloroform methods using TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH). Approximately 1 µg of total RNA was reverse transcribed using oligo dTs and MMLV-reverse transcriptase (Invitrogen, Inc., Carlsbad, CA).
Transcript abundance measurement

Transcript abundance (TA) was measured by Standardized RT (StaRT)-PCR [30, 41, 42]. With this method there is an internal standard, within a standardized mixture of internal standards (SMIS) for each gene amplified in the PCR reaction. This enables regular assessment of performance characteristics as recommended by recent FDA guidelines [43]. Among these performance characteristics are reproducibility, lower detection threshold, linear dynamic range, signal to analyte response, false negatives, and false positives. For each gene measured in this study, the lower detection threshold was less than 10 molecules and the linear dynamic range was less than 10 to greater than $10^7$ molecules. False negatives are eliminated due to the presence of an internal standard and false positives are eliminated by using a water control to ensure that there is no contamination within the PCR reaction.

The reagents for analysis of E2F1 and p21 were commercially prepared (Gene Express, Inc., Toledo, OH). To analyze p73, a SMIS containing internal standards for only p73 and β-actin were prepared in this laboratory, because p73 is not in a commercially available SMIS. p73 forward and reverse primers amplify at least four distinct isoforms including α-δ, but do not distinguish between the full-length and ΔN transcripts. A separate pair of primers published by Kartasheva, et al. [44] were used to determine the presence of ΔNp73. The internal standard for p73 was prepared using the forward primer and a competitive template (CT) primer. The CT primer hybridizes upstream of the reverse primer but retains its sequence at the 5’ end [45]. This enables the simultaneous amplification of the internal standard and endogenous native template (NT) using only the forward and reverse primers. p73 forward and reverse primer sequences
are as follows: p73 F, 5’ ACT TTG AGA TCC TGA TGA AG 3’ R, 5’ CAG ATG GTC ATG CGG TAC TG 3’. Primer sequences for p21 and E2F1 were previously reported [30].

Six SMISs (A-F) were used for all TA measurements, with p73 CT at concentrations ranging from $10^{-12}$ (SMIS A) to $10^{-17}$ M (SMIS F) and β-actin CT constant at $10^{-13}$ M in all six SMISs. The dilution of each cDNA sample that contained 60,000 molecules of β-actin cDNA was determined through calibration to 1 µL of SMIS F. Such calibrated samples were then used in all StaRT-PCR experiments. In some experiments, if the amount of cDNA sample available was low, the cDNA and the SMIS were both diluted 10-fold. Equal volumes of cDNA and SMIS were combined in a master mix along with 30 mM MgCl$_2$ (Idaho Technology, Inc., Idaho Falls, ID), 2 mM dNTPs, Taq Polymerase (Promega, Madison, WI), and RNase-free water. For each TA measurement, a 10 µL reaction volume was PCR amplified in a Rapidcycler (Idaho Technology, Inc., Idaho Falls, ID) for 35 cycles. PCR reactions were denatured for 5 seconds at 94ºC, annealed for 10 seconds at 58ºC, and elongated for 15 seconds at 72ºC.

**Plasmid DNA**

A pcDNA 3.0 (Invitrogen Inc., Carlsbad, CA) expression vector was kindly provided from the laboratory of Vincenzo DeLaurenzi (University of Rome, Italy). The p73 gene contains a hemeagglutinin tag sequence and is regulated by a CMV promoter sequence. An expression vector encoding a CMV regulated green fluorescent protein was obtained from (Gene Therapy Systems, San Diego, CA), and used as a negative control (mock) and determinant for transfection efficiency.
Transient transfection assays

Calu-1 cells were incubated in RPMI supplemented with 10% FBS and grown to confluence. Twenty-four hours prior to transfection, cells were trypsinized and transferred to 60 mm dishes. For transfections, 5 µg of plasmid DNA was diluted in 0.5 mL of serum-free medium with the appropriate concentration of Lipofectamine 2000 transfection reagent (Invitrogen, Inc., Carlsbad, CA). Cells were incubated with DNA-lipid complexes in serum-containing medium for 4-8 hours and subsequently treated with fresh medium. RNA was isolated 24 hours post-transfection and analyzed by StaRT-PCR. 60 molecules of p73 internal standard were sufficient to quantify endogenous p73α TA and 6,000 molecules were required to quantify the combined endogenous and exogenous transcript. To exclude the possibility that the high level measured was partly due to amplification of contaminating plasmid DNA, RNA from p73α transfected Calu-1 cells was PCR-amplified with p73 specific primers. No PCR product was observed.

Western blot analysis

Calu-1 cells were lysed 24 hours post-transfection by three consecutive freeze-thaws in a 0.25 M Tris lysis buffer (Invitrogen, Inc., Carlsbad, CA). Total protein concentration was determined colorimetrically using the bicinchoninic acid (BCA) assay (Pierce, Inc., Rockford, IL). 20 µg of total protein from Calu-1 cells were loaded on a 7% SDS Tris Acetate NuPage gel (Invitrogen, Inc., Carlsbad, CA). Proteins were transferred to a PVDF membrane and incubated with an anti-HA primary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). The PVDF was
then incubated with chemiluminescent substrates (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by autoradiography.

**siRNA (RNAi)**

Approximately 1 million N417 cells were incubated in a six-well plate with RPMI supplemented with 10% FBS. Five siRNA oligonucleotides specific for the p73 gene or a non-specific pooled duplex control (Dharmacon, Inc., Lafayette, CO) were diluted in serum-free media and added to the appropriate concentration of TKO transfection reagent (Mirus Corp., Madison, WI). Untransfected cells were treated with transfection reagent but not siRNA. Transfected cells were incubated continuously with siRNA complexes at a final concentration of 100 nM. RNA was isolated 24 to 48 hours post-transfection and analyzed by StaRT-PCR.

**Bivariate and statistical analysis**

Pearson correlation and paired-sample and independent T-tests were performed using SPSS 11.5.1 for Windows (SPSS, Chicago, IL) Due to inter-sample variation it was necessary to normalize the data by logarithmic transformation. For each test, a p-value of less than 0.05 was considered statistically significant. Bivariate graphs were created using Microsoft Excel 2000 (Microsoft Corp, Redmond, WA).

**Authors’ Contributions**

MWH was responsible for TA measurement of p73 in all primary samples, TA measurement of E2F1 and p21 in primary samples, siRNA and transient transfection
experiments, statistical analysis, and was the primary author of this manuscript. TGG was responsible for the preparation of the p73 SMIS, TA measurement of p73, E2F1, and p21 in all cultured cell lines, and contributed to the research design of this study. ELC, KAW, and CAMR were responsible for TA measurement of E2F1 and p21 and were involved in the acquisition and preparation of primary samples. JCW coordinated and obtained funding for this study and drafted and revised this manuscript. MWH, TGG, and JCW jointly conceived the experiments.

**Competing Interests**

ELC, KAW, and JCW each have significant equity interest in Gene Express, Inc., which produces and markets StaRT-PCR reagents used in these studies.

**Acknowledgements**

We would like to thank Dr. Vincenzo DeLaurenzi for providing the p73 plasmid DNA. We would also like to thank Drs. Dawn-Alita Hernandez, Yongsook Yoon, Jeffrey Hammersley, Ragheb A. Assaly, and Stacie L. Roshong-Denk for helping us acquire primary samples, Dr. Sakik Khuder for helping with statistical analysis, and Charles Knight, Bradley Austermiller, and D’Anna Mullins for their technical assistance.

**References**


**Figures**

**Figure 1 - Lack of correlation between E2F1 and p21 TA**

There was no correlation between E2F1 and p21 TA among BC cell lines (N=14) and primary BC tissues (N=7). Each point represents the mean value from triplicate measurements of E2F1 and p21 as shown in Tables 1 and 2 (except where indicated).

**Figure 2 - Bivariate analysis of E2F1 and p73α TA**

E2F1 and p73α TA were positively correlated among BC cell lines (N=14) and primary BC tissues (N=7). Each point represents the mean value from triplicate measurements of E2F1 and p73α as shown in Tables 1 and 2 (except where indicated).

**Figure 3 - Bivariate analysis of p73α and p21 TA**

p73α and p21 TA were positively correlated (p<0.05) among BC cell lines (N=7) where p53 was known to be inactivated by mutation or deletion and p73 was wild type. Each point represents the mean value from triplicate measurements of p73α and p21 as shown in Table 1.
Figure 4 - Effect of p73α transient expression on p73α, p21, and E2F1 TA in Calu-1 cells

A) p73α TA was induced by over 3 orders of magnitude relative to mock transfected cells. Calu-1 cells were transfected with 5 µg of GFP control plasmid or HA-p73α. B) Total HA-p73 protein was analyzed in mock or p73α transfected cells. 20 µg of lysate were blotted on a PDVF membrane and incubated with an anti-hemagglutinin primary antibody conjugated to HRP. C) p21 was upregulated by 90% and E2F1 was reduced by 20% in p73α transfected cells. Results represent the mean value from triplicate measurements from three independent experiments. Error bars represent the S.E.M. RNA was extracted 24 hours post-transfection and treated with DNaseI. RNA was PCR amplified to rule out the possibility of plasmid contamination. No PCR products were detected.

Tables

Table 1 - Transcript abundance measurements for cultured normal BEC and BC cell lines

N, normal; Sq, squamous carcinoma; LC, large cell carcinoma; SC, small cell carcinoma; A, adenocarcinoma; BA, bronchoalveolar carcinoma; B, BEGM medium; R, RPMI with 10% FBS; I, inactivated; A, amplification. Non-detectable TA were entered as 1 to allow plotting on a logarithmic scale. Cell lines are grouped by increasing E2F1 TA. All values are in units of molecules/10⁶ molecules β-actin and represent the mean from three independent measurements. *H1155 has a missense mutation in the transactivation
domain of p73. **A427 has a deletion in the p73 coding sequence but the protein product still retains its transactivational function [35].

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**Table 2 - Transcript abundance measurements for primary normal BEC and BC tissues**

NC, non-cancer; C, cancer; N, normal; Sq, squamous carcinoma; A, adenocarcinoma; BA, bronchoalveolar carcinoma; SC, small cell carcinoma. Non-detectable TA were entered as 1 to allow plotting on a logarithmic scale. Normal BEC are grouped by diagnosis and cell type. BC tissues are grouped by increasing E2F1 TA. All values are in units of molecules/10<sup>6</sup> molecules β-actin and represent the mean from three independent measurements. *This value represents the mean from two measurements due to insufficient sample.
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Table 3 - Descriptive statistics for E2F1, p73α, and p21 transcript abundance measurements

Min, minimum; LQ, lower quartile; UQ, upper quartile; max, maximum. Values were derived from statistical analysis of samples presented in Tables 1 and 2.
Figure 1

The figure shows a scatter plot with the x-axis labeled as "E2F1 Molecules/10^5 β-actin" and the y-axis labeled as "p21 Molecules/10^6 β-actin". The data points are represented by triangles, indicating a correlation between the two variables.
Figure 2

The figure shows a scatter plot with the x-axis labeled as "E2F1 Molecules/10^5 β-actin" and the y-axis labeled as "p73α Molecules/10^6 β-actin". The data points are scattered across the graph, with a trend line indicating a positive correlation between the two variables. The y-axis ranges from 1 to 10,000, while the x-axis ranges from 1 to 1,000,000.
Figure 3
The interactive transcript abundance index \([c-my\text{c}^*p73\alpha]/[p21*Bcl-2]\) correlates with baseline level of apoptosis and response to CPT-11 in human bronchogenic carcinoma cell lines

Michael W. Harr\textsuperscript{1} and James C. Willey\textsuperscript{1,2,3}

RUNNING TITLE: Apoptosis and response to CPT-11 in bronchogenic carcinoma

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\textsuperscript{3}JCW has significant equity interest in Gene Express, Inc. which commercially manufactures and markets reagents for StaRT-PCR.

Key Words: apoptosis, bronchogenic carcinoma, p53, CPT-11, irinotecan
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 (IC₅₀) 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 1) elevated expression of the p53 inhibitor protein MDM2, 2) elevated expression of the anti-apoptotic protein Bcl-2, and/or 3) 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 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 incompletely 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, p14ARF, 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 1. 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 2.5x 10⁵ cells/mL. After 12-16 hours of incubation at 37 degrees, cells
were treated with fresh medium containing 30 µM camptothecin for up to 24 hours. Cells
in suspension (H146, H446) were collected by centrifugation and 2.5 x10⁵ cells/mL were
resuspended in media containing 30 µM camptothecin and incubated for up to 24 hours.
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 hours 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
Figure 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 hours. After treatment, cells were collected from flasks (as described above), centrifuged, resuspended in 0.1% paraformaldehyde solution, and fixed in 70% ethanol for a minimum of 12 hours. 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 2.5 x10⁴ cells/mL in a 100 µL volume for a minimum of 12 hours. 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 2.5x10⁴ 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. 24 hours following treatment, cells were exposed to 30 µL of CellTiter 96 AQueous One Solution (Promega) and incubated at 37°C for approximately 4 hours. 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$_2$O. The RNA was then subjected to DNase I (Ambion) for 30 minutes 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 hour 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 β-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 β-actin internal standard was used in each reaction. The appropriate dilution of cDNA was determined by calibrating the native β-actin in 1 µL of cDNA to $10^{13}$ M β-actin (60,000 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$_2$O, 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 seconds at 95°C, annealed for 10 seconds at 58°C, and elongated for 15 seconds 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-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) for 1 hour, blocked, and re-probed with the appropriate antibody.
Statistical analysis

StatRT-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 done 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 Figure 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 (Figure 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 hours. 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 Figure 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 molecules/10^6 β-actin molecules are summarized in Table 2. There was inter-cell line variation in p53 and p21 protein expression in response to camptothecin (Figure 2). Camptothecin induced p53 and p21 protein accumulation in A549 cells in a time dependent manner but c-myc was relatively unchanged (Figure 2A). Conversely, H446 incubation with camptothecin was associated with a 10-fold increase in abundance of c-myc transcript as well protein. Further, p53 was increased at the RNA level but not the protein level (Table 2, Figure 2) and for p21 neither protein nor RNA was increased to the extent of A549 (Table 2, Figure 2). Interestingly, in H838 and H146 p53 was low at the RNA level and non-detectable at the protein level (Table 2, Figure 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 2). H460 showed an even greater transcriptional response, with p21 being induced over 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 (Figure 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 be correlated 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 2). 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 (Figure 3B). Because single gene values were poorly diagnostic, we evaluated each of these genes in the form of interactive transcript abundance indices 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 \([c\text{-}myc*p73\alpha]/[p21*Bcl-2]\) (p<0.01 and p<0.05, respectively) (Figure 3C and 3D).

**Evaluation of the interactive transcript abundance index \([c\text{-}myc*p73\alpha]/[p21*Bcl-2]\)**

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 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, 2 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 \([\text{c-myc}*\text{p73}\alpha]/[\text{p21*Bcl-2}]\) and response to CPT-11 (p=0.01) (Figure 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) \([\text{c-myc}*\text{p73}\alpha]/[\text{p21*Bcl-2}]\) was 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 p14ARF 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 is 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 p14ARF 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.

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**References**


Figure legends

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 described 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 5,000 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 S.E.M. 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 hours. C) Cells were treated with 30 μM camptothecin (CPT) for 24 hours and apoptosis was measured by TUNEL assay to confirm results in A and B.

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 hours 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 hours 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 S.E.M. 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 hours and p53 expression was assessed by western blot analysis.

Figure 3 – The interactive transcript abundance index \([c-my^c*p^73\alpha]/[p21*Bcl-2]\) is correlated with baseline apoptosis and CPT-11 (irinotecan) IC\(_{50}\)

A) Cells were treated with CPT-11 (irinotecan) for 24 hours and IC\(_{50}\) concentrations were measured by MTS cell proliferation assay and correlated with baseline apoptosis (as reported in Figure 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 \(\beta\)-actin) and baseline apoptosis. \(R^2 = 0.855\) and \(p<0.05\) by Pearson correlation. C) Bivariate plot of \([c-my^c*p^73\alpha]/[p21*Bcl-2]\) interactive transcript abundance index and baseline apoptosis. \(R^2 = 0.919\) and \(p<0.01\) by Pearson correlation. D) Bivariate plot of \([c-my^c*p^73\alpha]/[p21*Bcl-2]\) interactive transcript abundance index and CPT-11 IC\(_{50}\). \(R^2 = -0.812\) and \(p<0.05\) by Pearson correlation.

Figure 4 – Evaluation of the interactive transcript abundance index \([c-my^c*p^73\alpha]/[p21*Bcl-2]\) in p53 mutant cell lines

The \([c-my^c*p^73\alpha]/[p21*Bcl-2]\) interactive transcript abundance index was inversely correlated with CPT-11 (irinotecan) IC\(_{50}\) concentration in the six cell lines retaining wild
type p53 (Group 1) and in an additional nine cell lines with mutant p53 (Group 2). 5 of which were derived from lung tissue, 2 breast, and 1 colonic. $R^2 = -0.640$ and $p = 0.01$ by Pearson correlation. Potential outliers SKOV3 (ovarian) and H226 (lung) are indicated, but were not excluded from statistical analysis.
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a Nicholson et al. (ref 27)  
b You et al. (ref 30)  
c Gao et al. (ref 32)  
d Olivier et al. (ref 28)  
e Yu et al. (ref 35)  
f Bernasconi et al. (ref 31)  
g Wang et al. (ref 34)  
h Augenlicht et al. (ref 33)  
i NCI Developmental Therapeutics Program  
j van Ark-Otte et al. (ref 23)
Table I – Summary of baseline apoptosis, CPT-11 response, and interactive transcript abundance indices (ITAI) in cell lines with known genetic defects

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\textsubscript{50} was determined by MTS cell proliferation assay, or was obtained from the NCI developmental therapeutics program or van Ark-Otte et al. 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\textsuperscript{6} β-actin molecules. ITAI values are unitless.
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Table II – Transcript abundance of p53-related genes following treatment with camptothecin
*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. (ref 38). NA, not assessed. Cell lines were incubated with 30 μM camptothecin (CPT) for 24 hours. Transcript abundance was measured by StaRT-PCR and mean values are shown in units of molecules/10^6 β-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 less than 0.05 are in bold.
The graph shows a scatter plot with data points representing different cell lines (SKOV3 and H226) on the x-axis and CPT-11 IC₅₀ (10⁻⁶ M) on the y-axis. The line of best fit indicates a negative correlation, with an R² value of 0.637 and p-value less than 0.01.
MATERIALS AND METHODS

Acquisition of Peripheral Blood Samples

Cancer patients of the University Medical Center receiving standard of care chemotherapy for treatment of one or more solid tumors, (including lung, breast, colon, ovarian, uterine, prostate, GI, liver, and testicular cancers) were asked to participate in this study. Informed consent was obtained by IRB approved protocols. For those patients being treated every 7 d, peripheral blood was collected 1) prior to the first treatment, 2) approximately 1 h after the first treatment, 3) approximately 24 h after the first treatment 4) and 1 wk after the first treatment. Patients who had been previously treated with chemotherapy were still asked to participate, however, blood only was collected during the first cycle of chemotherapy. For those patients being treated every third wk, peripheral blood was collected 1) prior to the first treatment, 2) approximately 1 h after the first treatment, and 3) approximately 24 h after the first treatment. Samples from these patients were not obtained after the first or before the third week of treatment.

Prior to treatment, 2.5 mL of blood were drawn into two PAXgene tubes (PreAnalytiX, GmbH), 3 mL of blood were drawn into two lithium heparin tubes, and 3 mL of blood were drawn into one EDTA tube. Blood drawn into the PAXgene tubes were incubated at room temperature for a minimum of 24 h. Blood drawn into the first lithium heparin tube was subjected to RNA extraction using conventional phenol/chloroform methods. Blood drawn into the second lithium heparin tube was used for ex vivo treatment of leukocytes with varying concentrations of topotecan. Blood drawn into the EDTA tube was used for complete blood count (CBC) with differential.
Approximately 1 h, 24 h, and 1 wk (if applicable) after treatment, 2.5 mL of blood were drawn into two PAXgene tubes (as described above), 3 mL of blood were drawn into one lithium heparin tube for RNA extraction, and 3 mL of blood were drawn into an EDTA tube for CBC with differential.

A schematic of our experimental design is shown in Figure 5.

**Figure 5. Experimental Design**

Cancer patients were consented by University of Toledo IRB approved protocols. Prior to the first cycle of chemotherapy, peripheral blood was collected for CBC and differential, gene expression analysis by StaRT-PCR, and *ex vivo* incubation with topotecan. One hour, 24 h, and 1 wk (depending on the course of treatment) post-treatment, peripheral blood was collected for CBC and differential and gene expression analysis by StaRT-PCR.

*Ex Vivo Treatment of Leukocytes with Topotecan*

Blood drawn in a lithium heparin tube prior to the first treatment was centrifuged at 1300 x g to pellet cells. The plasma was removed by aspiration and the buffy coat was transferred to a fresh conical tube and resuspended in a low concentration of phosphate
buffered saline to lyse contaminating red blood cells. The cell suspension was centrifuged a second time at 1300 x g and the pellet containing a heterogeneous population of leukocytes was resuspended in Hank’s Buffered Salt Solution (HBSS). Cells were counted using a hemocytometer and plated at concentrations ranging from 3-5x10^5 cells/mL in RPMI (without serum) depending on the total number of cells recovered. Cells were treated with a low dose (30 nM), moderate dose (300 nM), or high dose (1 µM) of topotecan, dissolved in sterile water, for 4 h at 37°C. After incubation, cells were lysed in TRI-reagent and RNA was extracted by phenol/chloroform methods as described in the following section.

**RNA Extraction**

RNA from peripheral blood drawn into PAXgene tubes was extracted using the PAXgene Blood RNA Kit (PreAnalytiX, GmbH). A proprietary reagent is stored inside the PAXgene tube for immediate cell lysis when the blood contacts the tube. In addition, a preservative protects the RNA molecules from degradation, and according to the manufacturer, inhibits false changes in gene expression.

2.5 mL aliquots of blood drawn into PAXgene tubes were incubated for a minimum of 24 h. After incubation, total cell lysate was pelleted by centrifugation at 1300 x g. The lysate was washed and incubated with proteinase K and homogenized through a column to purify nucleic acids which then were incubated with DNase, washed, and eluted.

RNA from peripheral blood drawn into lithium heparin tubes was extracted by conventional phenol/chloroform methods. Blood was centrifuged at 1300 x g to pellet
cellular material. The plasma was removed by aspiration and cells were resuspended in 1.5 mL TRI-Reagent (Molecular Research Center, Inc.) for whole blood preparations and supplemented with acetic acid and chloroform. RNA from the aqueous phase was precipitated in isopropanol, washed in ethanol, and eluted in RNase-free water. RNA was subsequently treated with 1 unit of recombinant DNase I (Ambion, Inc.).

RNA preparations from both PAXgene tubes and lithium heparin tubes were quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

Reverse Transcription

1 µg of total RNA (collected from PAXgene and lithium heparin tubes) was denatured and reverse transcribed by incubating with a mixture of oligo dT primer (500 µg/mL) (Promega, Inc.), 10 mM dNTPs, RNase inhibitor (25 U/µL, and moloney murine leukemia virus (MMLV) reverse transcriptase (200 U/µL) (Invitrogen, Inc.) for 1 h at 37°C.

Standardized Reverse Transcriptase-Polymerase Chain Reaction

Gene expression was measured by StaRT-PCR in the Standardized Expression Measurement Center at the University of Toledo.

In the StaRT-PCR procedure, a standardized mixture of internal standards (SMIS) is used to measure the concentration of target gene expression after PCR amplification is completed (35 cycles). Each internal standard is designed as a competitive template that co-amplifies with the target amplicon at the same efficiency. The target gene is amplified using a pair of forward and reverse primers (F and R). The internal standard is amplified
using the same forward primer, but uses a second reverse primer (F and R’) that hybridizes upstream of the reverse primer (R) used for amplification of the target gene. In addition, R’ retains the sequence of R at the 5’ end. Therefore, amplification of the internal standard produces a PCR product 20% shorter in length than the target gene product and both products can be amplified using the same pair of forward and reverse primers (F and R) and separated by electrophoresis.

In order to determine the appropriate dilution of cDNA, 1 µL is calibrated to a standardized mixture of internal standards (SMIS), where the internal standard for the reference gene, β-actin, is held constant at a concentration of $10^{-13}$ M. The appropriate dilution of cDNA is that which produces a 1:1 ratio between the native β-actin PCR product and its internal standard. This dilution of cDNA can be used for subsequent PCR reactions, because it produces the same ratio of target gene β-actin molecules to internal standard β-actin molecules.

When assaying a specific gene, a process of range finding is necessary to determine the appropriate concentration of SMIS. The concentration of SMIS must produce a ratio of target gene PCR product to internal standard PCR product that is within 0.1-10. If this ratio exceeds 10, the concentration of SMIS is increased until it is less than 10, and ideally, close to 1. On the other hand, if this ratio is less than 0.1, the concentration of SMIS is decreased by 10-fold until the ratio is greater than 0.1 and in close proximity to 1. The most efficient way to determine SMIS concentration is to screen all genes at a SMIS concentration of $10^{-15}$ M, with β-actin constant at $10^{-13}$ M. If this concentration does not produce a desired ratio, the SMIS concentration should be increased or decreased accordingly.
PCR Reaction and Conditions

Each PCR reaction was carried out in 96-well plates using a robotic liquid handling system. 1 µL of 0.1 µM forward and reverse primers were transferred to each well. A master mix containing 30 mM MgCl$_2$ (Idaho Technologies, Inc.), 2 mM dNTPs (Promega, Inc.), cDNA, SMIS (Gene Express, Inc.), and 0.1 units of taq polymerase (Invitrogen, Inc.) were added to the appropriate primers in a 10 µL volume. The PCR reactions were transferred to a block thermalcycler and subjected to denaturation for 5 sec at 94°C, annealing for 10 sec at 58°C, and elongation for 15 sec at 72°C. Following amplification, the reactions were quantified by capillary electrophoresis using a Caliper AMS 90 (Caliper, Inc.) or Agilent 2100 Bioanalyzer (Agilent, Inc). The primer sequences used in this study are shown in Table I.

Statistical Analysis

To determine if genes were differentially expressed prior to treatment we used an independent samples Student’s T-test. To determine if gene expression was altered after in vivo chemotherapeutic treatment we used a one-way ANOVA. p values were adjusted by the Bonferroni method, whereby the p value is divided by the number of genes analyzed in the study. Thus, for the data presented here, a significant p value was less than 0.0018.
### Table I. Gene Nomenclature and Primer Sequences

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<th>Primer Sequence</th>
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<td>ataxia telangiectasia mutated</td>
<td>Forward 5' CAGCGAACATCCAGCTC 3' Reverse 5' GAACCACGGAGAATACCC 3' IS 5' GAACCACGGAGAATACCC 3'</td>
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<td>Bcl-2-associated X protein</td>
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<td>B-cell CLL/lymphoma-2</td>
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<td>Bcl-2-like-1</td>
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<td>catalase</td>
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<td>X-ray repair complementing defective repair in Chinese hamster cells 1</td>
<td>5' CCCCTGAAGGACCAAGACCA 3'</td>
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IS, internal standard. Symbols, descriptions, and primer sequences for the 28 genes (excluding β-actin) assessed in this study.
RESULTS

Our first objective was to compare gene expression data derived from blood collected in PAXgene tubes and lithium heparin tubes. We found variability in expression between RNA samples collected from the same individual at the same time points. RNA samples from blood collected in PAXgene tubes showed less variation in expression kinetics after treatment, and had smaller amounts of genomic contamination. On the contrary, RNA samples from blood collected in lithium heparin tubes showed profound variation in expression kinetics and significant amounts of genomic DNA were found in some samples. For example, p21 (CDKN1A) was increased over 10-fold relative to pre-treatment, but there was relatively no change in p21 kinetics from samples collected in PAXgene tubes. In addition, there was over 1000-fold difference in UGT1A1 between the two sample collection methods. A summary of these results are shown in Tables II and III, and as a result, replicate measurements and statistical testing was done only on samples collected in PAXgene tubes.

In this study, we evaluated the expression of 28 genes involved in cell proliferation control, DNA repair, apoptosis, oxidation-reduction, and drug metabolism (see Table I for complete list of genes) in two individuals that differed in respect to their leukocyte and lymphocyte counts before and after receiving chemotherapy. Many of these genes are involved in p53 signaling pathways, and are transcriptional targets for the p53 family of transcription factors. Our rationale for choosing these genes was based on results from other studies using bronchogenic carcinoma cell lines, where we observed significant inter-cell line variation in the expression of p53-responsive genes after treatment with camptothecin. A summary of these findings is shown below in Table IV.
Table II. StaRT-PCR Data from Blood Samples Collected in Lithium Heparin Tubes

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Blood from subject 667 was collected in lithium heparin tubes at the above time points. RNA was extracted and reverse transcribed as described in Materials and Methods. The data shown represents a single StaRT-PCR measurement. Genomic DNA contamination was assessed by amplifying an intronic region of CC10.
Table III. StaRT-PCR Data from Blood Samples Collected in PAXgene Tubes

<table>
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</table>

Blood from subject 667 was collected in PAXgene tubes at the above time points. RNA was extracted and reverse transcribed as described in Materials and Methods. The data shown represents a single StaRT-PCR measurement. Genomic DNA contamination was assessed by amplifying an intronic region of CC10.
Leukocyte and Lymphocyte Counts

The two individuals enrolled in this study were designated as subjects 667 and 668, respectively, and their total leukocyte and lymphocyte counts are shown in Figure 6A and 6B. Subject 667 was diagnosed with NSCLC and was treated with paclitaxel and carboplatin. Subject 668 was diagnosed with colon cancer and treated with oxaliplatin and 5FU.

Table IV. Inter-Cell Line Variation in p53 Transcriptional Response to DNA Damage

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Malignant cell lines were treated with 30 µM camptothecin for 24 h, and expression of p53-responsive genes were measured by StaRT-PCR. Numbers represent fold change in gene expression (treated/control).

Figure 6. Leukocyte and Lymphocyte Counts in 2 Chemotherapy Patients

A) Total leukocyte counts and B) Lymphocyte counts from subjects 667 and 668. Subject 667 was diagnosed with lung cancer (NSCLC) and treated with paclitaxel and carboplatin. Subject 668 was diagnosed with colon cancer and treated with oxaliplatin and 5FU.
Subject 668 was diagnosed with colon cancer and treated with oxaliplatin and 5-fluorouracil (5FU). The total leukocyte count for both individuals was relatively unchanged up to 1 wk after the first cycle of chemotherapy. However, subject 667, with an abnormally high leukocyte count, experienced a decline in lymphocytes from 1,500 to 580 cells/µL 1 h post-treatment which continued to decline to 370 cells/µL 1 wk post-treatment. In contrast, subject 668 did not experience a reduction in lymphocyte count, but an increase from 1,100 to nearly 2,100 cells/µL. Unfortunately, we were not able to receive a lymphocyte count from subject 668 prior to treatment, thus it is not clear whether a change in lymphocyte count (in either direction) occurred immediately following treatment. However, there was a substantial difference in lymphocyte count between subjects 1 wk post-treatment (370 and 2,100 cells/µL, respectively). In subject 667, neutrophil counts were elevated but did not change over the course of treatment (mean = 1.1x10^4), thus accounting for the high and consistent leukocyte counts in Figure 6A. Monocytes were in normal range at all collection times except at 1 h post-treatment in which they decreased from 530 to 120 cells/µL. In subject 668, neutrophils were in normal range 24 h and 1 wk post-treatment (undetermined prior to 24 h post-treatment). Consistent with the increase in lymphocytes, monocytes increased from 120 cells/mL at 1 h to 740 cells/mL post-1 wk. Red blood cell counts, hemoglobin, and hematocrit were low for both individuals and did not change over the course of treatment.
Inter-individual Variation in Constitutive Expression

Because these two individuals had markedly different changes in lymphocyte counts up to 1 wk post-chemotherapy, we first asked if there were individual differences in constitutive expression prior to treatment. Subject 668 had elevated levels of four genes relative to subject 667, including ATM, Bcl-2, TRAF4, and SOD1 (Figure 7A-D). There was not a significant difference in general transcription factor IIH (GTF2H2) (Figure 7E), indicating that not all genes measured showed the same pattern of variation. Although not statistically significant due to a high standard deviation, UGT1A1 was non-detectable in subject 667 but was 130 ± 150 molecules/10^6 β-actin molecules in subject 668 (Figure 7F).

Alterations in Gene Expression Following Chemotherapeutic Treatment

In order to determine if either chemotherapeutic regimen (e.g., carboplatin and paclitaxel for subject 667, oxaliplatin and 5FU for subject 668) was associated with alterations in gene expression, we assessed each gene at 1 h, 24 h, and 1 wk after chemotherapeutic treatment. No significant alterations were found for subject 667 (data not shown), while several genes were altered in subject 668 at various time points (Figure 8A and 8B).
Figure 7. Inter-individual Variation in Constitutive Gene Expression Between Subject 667 and 668.

A-D) ATM, Bcl-2, SOD1, and TRAF4 were significantly elevated in subject 668 relative to 667. Statistical analysis was performed by an Independent Student’s T-test and significance was determined by Bonferroni adjustment (p<0.0018). E) UGT1A1 was non-detectable in subject 667 but expressed in subject 668 F) There was no statistical difference in expression of GTF2H2 between both individuals. Black bars represent the mean from triplicate StaRT-PCR measurements. Error bars represent the standard deviation.
A) Moderately expressed cell cycle and apoptosis genes (c-myc, Bax, and Bcl-2) were decreased after 1 h of treatment. p16 was increased after 1 wk. B) Highly expressed antioxidant genes GSTP1, Gpx-1, and Bcl-XL were altered after 1 h of treatment. p values were determined by one-way ANOVA relative to pre-treatment (significance threshold = 0.0018 after Bonferroni adjustment). Bars represent the mean from triplicate measurements. Error bars represent standard deviation.
To determine if peripheral blood leukocytes would respond to a DNA damaging agent in culture, we extracted the total heterogeneous leukocyte population from peripheral blood obtained prior to treatment and incubated them with the camptothecin derivative, topotecan, for 4 h. Consistent with our results from \textit{in vivo} chemotherapeutic treatment, alterations in expression were observed for subject 668 in a dose dependent manner, whereas alterations for subject 667 were not as suggestive. In subject 668, p21 decreased by nearly 10-fold after treatment with 1 $\mu$M topotecan, whereas c-myc was increased over 6-fold after treatment with 300 nM topotecan (Figure 9A and 9B). Alterations of this magnitude were not observed in subject 667, and due to the low concentration of RNA recovered, we were not able to obtain triplicate measurements to determine if these smaller changes were statistically significant. However, a dose-dependent increase in catalase (CAT) expression was observed in subject 667, and conversely, a dose-dependent decrease in subject 668 (Figure 9C). Lastly, a small decrease in Bcl-X\textsubscript{L} was observed in subject 668 (Figure 9D).
Transcript Abundance Alterations by *ex vivo* Incubation with topotecan for 4 h.

A) p21 decreases in subject 668 in response to increasing concentrations of topotecan. B) c-myc increases in subject 668 in response to 300 nM topotecan. C) catalase increases in subject 667 in response to 1 µM topotecan, but decreases in subject 668 in response to increasing concentrations of topotecan. D) Bcl-X\(_L\) decreases in subject 668 after incubation with increasing concentrations of topotecan. Total leukocytes were extracted from peripheral blood from subjects 667 and 668 prior to treatment and incubated with topotecan for 4 h. Bars represent single gene measurements.
DISCUSSION

The hypothesis to be tested in these studies is that inter-individual variation in gene expression, whether constitutive or drug-induced, is a predictor for cytotoxicity. Further, because of inter-variation in expression of p53-responsive genes in bronchogenic carcinoma cell lines, and other studies implicating a role for p53 in cytotoxicity to chemo- and radiation therapy (Botchkarev et al., 2000; Komarova and Gudkov, 2000; Strom et al., 2006), we primarily assessed genes that were known to be regulated by p53 or were involved in p53 signaling pathways (See Harms, et al. for a review of these genes). If our hypothesis is correct, we would expect that an elevated p53 response would be associated with enhanced cytotoxicity to chemotherapy. The objective is to develop a clinical test, based on transcript abundance, that accurately defines the proper dose of treatment so that side effects such as neutropenia, lymphopenia, and myelosuppression can be minimized without compromising the efficacy of treatment.

Changes in gene expression caused by extraneous variables, for example, time between blood collection and processing leading to RNA degradation and blood coagulation, can have a confounding effect when assaying for inter-individual variation. Thus, to eliminate alterations in expression due to time between blood collection and processing we used PAXgene tubes, which immediately cause cell lysis when blood is drawn into the tube and contain a reagent that inhibits RNA degradation. We compared data obtained from blood collected in PAXgene tubes to data obtained from blood collected in lithium heparin tubes in 1 individual at 4 times. Blood collected from PAXgene tubes was processed using a proprietary RNA extraction kit. RNA from blood collected in lithium heparin tubes was extracted by conventional phenol/chloroform
methods. Although RNA yields were similar for both methods, we observed extensive variation in samples collected in lithium heparin tubes relative to those collected by PAXgene tubes. This is best illustrated by the >1000-fold increase in UGT1A1 expression 24 h and 1 wk post-treatment from blood collected in lithium heparin tubes. False positives such as these may be due to actual changes in expression that occur after blood has been drawn, or may be due to genomic DNA contamination. When testing for genomic contamination, we found an unacceptable amount of DNA in RNA harvested from lithium heparin tubes but not PAXgene tubes. For these reasons, all further measurements were taken only from blood collected in PAXgene tubes.

In these preliminary studies we have evaluated a panel of 28 genes related to cell cycle and proliferation control, apoptosis, DNA repair, oxidation/reduction, and drug metabolism in 2 cancer patients receiving chemotherapeutic treatment. Subject 667 was diagnosed with NSCLC and received carboplatin and paclitaxel. Subject 668 was diagnosed with colon cancer and received oxaliplatin and 5FU. Peripheral blood was obtained from both patients before and after treatment and gene expression data were collected and compared to complete blood counts at various times. Interestingly, these patients showed remarkably different phenotypes, in which subject 667 had an elevated but stable leukocyte count and a 4-fold decline in lymphocytes up to 1 wk after treatment. In contrast, subject 668 had a stable leukocyte count in normal range, but experienced an increase in lymphocyte count 1 wk after treatment. Because peripheral lymphocytes undergo alterations in apoptosis-related genes after DNA damage (Stahnke et al., 2001), we primarily focused on the difference in lymphocyte counts between both individuals.
Inter-individual Variation in Constitutive Expression

In an effort to identify inter-individual variation in constitutive gene expression, we tested each of the 28 genes measured prior to treatment by an independent samples T-test. Genes that were differentially expressed between subject 667 and 668 were ATM (ataxia telangiectasia mutant), Bcl-2, SOD1 (superoxide dismutase-1), and TRAF4 (TNF receptor associated factor-4). Each of these genes were elevated in subject 668 relative to 667, and were statistically significant after Bonferroni adjustment. It is possible that these genes were elevated because this individual had a higher percentage of lymphocytes, however, only 4 of 28 genes were significantly higher. Although these data are preliminary, these genes are particularly important because of their role in cell survival and apoptosis, and will be discussed here.

First, the anti-apoptotic role of Bcl-2 has been documented extensively (Wickremasinghe and Hoffbrand, 1999), and has important implications for promoting cell survival in lymphocytes in order to maintain homeostasis (Khaled and Durum, 2002). During hematopoiesis, Bcl-2 expression is low in T-cells which enables negative selection, however, during positive selection and maturation, Bcl-2 levels are elevated in both CD4 and CD8 positive cells (Davis and Distelhorst, 2006). Similarly, Bcl-2 levels are low in immature B-cells, but high in precursors and mature B-cells (Merino et al., 1994). Therefore, because lymphoid homeostasis is primarily regulated by apoptosis (Blagosklonny, 2001), we speculate that elevated Bcl-2 would be associated with protection from cytotoxicity induced by chemotherapy. Thus, Bcl-2 expression, in terms of transcript abundance, may be a strong predictor for not only response to chemotherapeutics but also cytotoxicity to lymphoid cells.
Second, ATM is a kinase that acts upstream of p53. It is phosphorylated in response to DNA damage (Khanna et al., 1998), and in turn, phosphorylates p53 directly, thereby inhibiting its negative regulation by MDM2 (see Figure 2). Defects in ATM are commonly found in leukemias and lymphomas, and germ line mutations are linked to ataxia telangiectasia (Lavin and Shiloh, 1997), a cerebellar disorder, characterized by lymphopenia and immunosuppression (Bredemeyer et al., 2006). Studies from patients with ataxia telangiectasia have shown low percentages of naive peripheral blood lymphocytes with decreased expression of Bcl-2, and in culture, ATM mutant cells have a higher rate of spontaneous apoptosis relative to control patients (Schubert et al., 2000).

Third, tumor necrosis factor (TNF) associated factor 4 (TRAF4) belongs to a family of adaptor proteins that interact with the TNF receptor superfamily and interleukin-1 receptor/toll like receptor-1 families (Lee and Lee, 2002), however, there is not in vivo evidence that TRAF4 physically interacts with any of these TNF receptors (Sax and El-Deiry, 2003). Other TRAF proteins including TRAF2 and TRAF5 are recognized for their ability to activate NFκB and MAPK signaling pathways, thereby contributing to anti-apoptosis or cell survival (Aggarwal, 2003). However, we chose TRAF4 in these studies because it was shown to be regulated by p53, and when overexpressed, induced apoptosis in response to DNA damage (Sax and El-Deiry, 2003). Originally cloned from the lymph node of a metastatic breast tumor (Regnier et al., 1995), a recent study identifies TRAF4 as a putative oncogene provided by evidence of its amplification and overexpression in breast, lung, ovarian, bladder, and colon carcinoma tissue (Camilleri-Broet et al., 2006). It also has been shown to protect cells from apoptosis after induction by the tumor promoter, phorbol 12-myristate 13-acetate
(PMA) (Busuttil et al., 2002). Thus, the role of TRAF4 in immune cells has not been well established, although there is clearly evidence that it is involved in the regulation of apoptosis and survival. Because other TRAF family members are linked to NFκB activation, and TRAF4 associates with MEK kinase 4 to stimulate Jnk activation (Abell and Johnson, 2005), it would be advantageous to measure NFκB and MAPK genes in subsequent samples.

The gene encoding the Cu/Zn superoxide dismutase enzyme (SOD1), although normally involved in a diversity of oxidation/reduction reactions, protects cells from toxicity induced by the accumulation of reactive oxygen species. Moreover, mutant SOD1 accounts for 20% of inherited amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder characterized by the loss of motor neurons in the central nervous system (Broom et al., 2006; Rosen et al., 1993). One study showed that mutant SOD1 was attributable to DNA damage in motor neurons associated with an increase in Fas, however, cell death occurred by necrosis and not apoptosis (Martin et al., 2005). Additionally, epithelial cells from SOD1 knockouts have an increase in double stranded breaks induced by oxidants such as hydrogen peroxide (Reddy et al., 2004). It was further shown that neurons from SOD1 transgenic mice are protected from apoptosis induced from the PKA inhibitor H89, specifically by preventing interaction between Bad and Bcl-Xl (Saito et al., 2003). Since most of these experiments have been conducted in neurological systems, it remains to be determined whether SOD1 has a protective function in lymphoid cells.
Altered in Gene Expression Following Chemotherapeutic Treatment

To investigate the effects of chemotherapy in vivo, we measured the same panel of 28 genes at 1 h, 24 h, and 1 wk after treatment. Peripheral blood from subject 667 did not show any significant changes in gene expression, however, subject 668 had significant alterations in 1) cell cycling genes p16 (CDKN2A) and c-myc, 2) apoptosis genes Bax, Bcl-2, and Bcl-XL, and 3) antioxidant genes GSTP1 and Gpx-1. At this time, it is unclear how the direction and magnitude of these changes could affect phenotypic variation. Notably, regardless of magnitude or direction, each of these genes (with the exception of p16) was altered after 1 h of exposure to chemotherapy, thus indicating the importance of this time point in the pharmacokinetics of oxaliplatin/5FU based therapy. Yet, it is unknown if lymphocyte count increased or decreased after 1 h of chemotherapy. Given that the lymphocyte count continued to increase from 1 h to 1 wk post-treatment, changes in expression occurring at 24 h and 1 wk time points may be as equally important.

Based on our hypothesis, we would reason that any changes in gene expression would be associated with the upregulation of cell survival genes and downregulation of pro-apoptotic genes. This is supported by a decrease in Bax but not supported by a concomitant decrease in Bcl-2. It is unclear why both genes would be downregulated immediately following treatment and rise thereafter. One possibility is that Bcl-XL, expressed at higher levels than both Bax and Bcl-2, is increased 1 h after treatment to compensate for downregulated Bcl-2.

Second, changes in cell cycling genes p16 and c-myc also may be contributing factors. In bronchogenic carcinoma cell lines, c-myc was positively associated with
spontaneous apoptosis and induced in an SCLC cell line after DNA damage. Although, c-myc is low in normal cells, and therefore, unlikely contributing to spontaneous apoptosis in peripheral blood lymphocytes. Whether c-myc induces or protects cells from apoptosis in normal cells after DNA damage has yet to be determined. In contrast with our findings, p16 may promote apoptosis in T-cell lymphocytes. p16 knock out mice have an enlarged thymus due to increased proliferation rates and are resistant to apoptosis induced by reactive oxygen species and irradiation (Bianchi et al., 2006).

Third, we anticipate an increase in the expression of gene-encoding antioxidant enzymes because Gpx-1 and GSTP1 interfere with apoptosis induction by scavenging reactive oxygen species. For example, overexpression of Gpx-1 has been shown to decrease the expression of Bax in vitro (Faucher et al., 2005), and conversely, mice deficient in Gpx-1 have increased levels of Bax and accumulation of apoptotic cells in the brain (Flentjar et al., 2002). Thus, increased expression of Gpx-1 at 1 h and 1 wk post-treatment are consistent with these findings, although the decrease in GSTP1 is not, given that its overexpression attenuates apoptosis in lymphoma cells (Zhou et al., 2005). But regardless of kinetics, the expression of Gpx-1 and GSTP1 are elevated and 1-2 orders of magnitude higher than the expression of p16, c-myc, Bax, or Bcl-2, which may be, in part, responsible for an increase in lymphocyte production.

*Alterations in Gene Expression Following Ex Vivo Treatment with Topotecan*

One of the primary limitations of this study is that multiple forms of chemotherapy are being used, and it is difficult to understand how a particular agent contributes to DNA damage-induced cytotoxicity. The ideal test for cytotoxicity would
be one that could be administered to patients before their initial treatment with chemotherapy. However, this is only achievable by identifying correlative genes that show high inter-individual variability. In order to determine if there was inter-individual variation in gene expression following DNA damage, we isolated each subject’s peripheral blood leukocytes and cultured them for 4 h with varying concentrations of topotecan. In this way, each patient is evaluated before treatment and only one drug is used to measure their DNA damage response.

Our results from cultured peripheral blood leukocytes were consistent with those obtained after *in vivo* chemotherapeutic treatment in the sense that alterations in subject 668 were more profound than in subject 667. The higher level of expression relative to PBLs collected from *in vivo* treatments likely reflects changes in gene expression that occurred after these cells were extracted and put into culture. Unfortunately, we were unable to obtain replicate measurement due to the low yield of cDNA obtained from these experiments. However, it is clear that there is a dose-dependent effect of topotecan on c-myc and p21 in subject 668, and the relationship between p21 and c-myc in PBLs is similar to what was observed in bronchogenic carcinoma cell lines after treatment with camptothecin. Of interest is the observation that PBLs from subject 668 respond in a dose-dependent manner to each of the four genes, whereas these genes are either unchanged or inversely related (e.g., catalase) in PBLs from subject 667. Further experiments on cells extracted from additional individuals will be necessary to determine if these changes are significant, and if so, how they are associated with each individual’s blood count.
CONCLUSIONS

1) Bronchogenic carcinomas have high inter-individual variation in p21 expression which can be explained by mutations in p53 and Rb as well as expression in upstream transcription factors E2F1 and p73α. We provide evidence that in bronchogenic carcinomas, high expression of p21 results from E2F1 upregulation of p73α, but not from E2F1 directly.

2) Lower levels of apoptosis induced by camptothecin were associated with p53 protein accumulation and upregulation of p53 responsive genes in cell lines retaining wild type p53. However, these findings are not indicative of sensitivity to irinotecan.

3) Sensitivity to irinotecan is best predicted by spontaneous apoptosis which is associated with high expression of c-myc and p73 and low expression of p21 and Bcl-2. Therefore, the interactive transcript abundance index (ITAI) \([c\text{-}myc*p73\alpha]/[p21*Bcl\text{-}2]\) is predictive of both spontaneous apoptosis and response to irinotecan in bronchogenic carcinomas and may also predict response in ovarian, breast, and colon derived malignancies with inactivated p53.

4) Collection of peripheral blood leukocytes has important implications for the development of transcriptomics and expression profiling. PAXgene tubes are advantageous for collecting peripheral blood over conventional methods because they eliminate the time between blood collection and processing, inhibit RNA degradation, and minimize genomic DNA contamination.

5) Increased lymphocyte production after \textit{in vivo} treatment with oxaliplatin and 5FU is associated with 1) elevated constitutive gene expression, 2) changes in
expression from 1 h post-treatment, and 3) dose-dependent changes in expression after *ex-vivo* treatment with topotecan in peripheral blood leukocytes. Further experimentation is necessary to determine if these observations statistically correlate with lymphocyte production or depletion in cancer patients.
SUMMARY

Bronchogenic carcinoma is the leading cause of cancer related deaths in the United States and most industrialized countries. Treatment is often ineffective due to intrinsic or acquired resistance of tumor cells to chemotherapeutic agents, and the molecular mechanisms that regulate these events are poorly understood. Many types of cytotoxic and targeted therapies function by interfering with proteins that control cell proliferation.

In bronchial epithelial cells, the cyclin dependent kinase inhibitor p21 is a key component of cell proliferation control. It inhibits cell proliferation by preventing the phosphorylation of Rb, a physiological signal for cells to progress from G1 into S phase of the cell cycle. Thus, it would seem counter-intuitive for malignant bronchial epithelial cells to express a high level of p21. However, we found that p21 expression was high in some bronchogenic carcinoma samples and low in others, with a range approaching 4 orders of magnitude. Because p21 is a transcriptional target for the tumor suppressor p53, and p53 is mutant in over 50% of lung malignancies, we hypothesized that p21 transcription results from E2F1 mediated transcription of p73, but not from E2F1 directly. This hypothesis was supported by several observations, including 1) lack of correlation between E2F1 and p21, 2) positive correlation between E2F1 and p73, 3) positive correlation between p73 and p21 in p53 mutant cell lines, and 4) alteration in p21 expression after targeted overexpression and RNAi mediated silencing of p73. Importantly, this relationship seems to be specific for tumor cells, because in normal bronchial epithelial cells E2F1 expression is low, but p73 expression is high suggesting
that p73 is regulated by one or more additional transcription factors. There is an ongoing effort in our laboratory to better understand how p73 is regulated in normal cells.

Bivariate analysis of 21 bronchogenic carcinomas with varying p53 status revealed two groups of samples. Those maintaining low levels of E2F1 and p73 and those expressing high levels. Based on these data, we hypothesized that bronchogenic carcinomas with high levels of E2F1 and p73 and wild type p53, would have a defective apoptosis program due to alterations in downstream genes. Likewise, we anticipated that cell lines with low levels of E2F1 and p73 and wild type p53 would have an intact apoptosis pathway and p53 response after DNA damage. To test this hypothesis, we treated five bronchogenic carcinoma cell lines with the topoisomerase I inhibitor, camptothecin, a strong inducer of apoptosis. Contrary to our hypothesis, we observed a high percentage of apoptotic cells in two SCLC cell lines, with high expression of E2F1 and p73, in the absence of camptothecin (spontaneous apoptosis). On the other hand, spontaneous apoptosis was low in the other cell lines, but camptothecin induced apoptosis up to five-fold (25% of the total cell population). Camptothecin-induced apoptosis was associated with p53 accumulation and upregulation of p53 responsive genes. However, upregulation of p53 responsive genes in SCLC cell lines occurred to a lesser extent and p53 protein was non-detectable or stabilized.

To determine if these characteristics were associated with chemoresistance, we measured each cell line’s response to the camptothecin derivative, irinotecan (CPT-11, Camptosar). Our results indicated that response to irinotecan, as determined by IC$_{50}$ concentration, was highest in SCLC lines and inversely correlated with spontaneous apoptosis. We then chose five genes that showed high variability in constitutive
expression and tested them for correlation to spontaneous apoptosis and response to irinotecan. Only E2F1 was correlated with spontaneous apoptosis, however, the ratio [c-myc*p73α]/[p21*Bcl-2], in the form of an interactive transcript abundance index (ITAI), was significantly correlated with spontaneous apoptosis and response to irinotecan. These results may have partly explained our previous findings that p21 was variably expressed in bronchogenic carcinoma. In fact, a high level of p21 may not be counter-intuitive for a tumor if it serves to inhibit spontaneous apoptosis and decrease drug sensitivity.

Since the ITAI was correlated in five bronchogenic carcinoma cell lines (in addition to one breast cancer cell line), we tested an additional set of nine cell lines (five lung, two ovarian, two breast, and one colon) with a p53 inactivation using data obtained from the NCI developmental therapeutics program. In the combined data set (N=15), the ITAI remained correlated with response to irinotecan.

When conducting these experiments, we observed that there was inter-variation in each cell line’s response to camptothecin, in terms of apoptosis induction and transcription of p53 responsive genes. This lead us to the hypothesis that inter-individual variation in expression of p53-related genes could predict cytotoxicity to chemotherapy in peripheral blood leukocytes of cancer patients diagnosed with solid tumors.

We have since collected and analyzed blood from two individuals, subjects 667 and 668, respectively. Subject 667 was diagnosed with NSCLC and treated with carboplatin and paclitaxel. Subject 668 was diagnosed with colon cancer and treated with oxaliplatin and 5FU. While subject 667 had elevated levels of leukocytes beyond normal range, their lymphocytes had declined from 1,500 to less than 600 cells/µL 1 wk after
their first cycle of chemotherapy. In contrast, the lymphocyte count from subject 668 increased from 1 h to 1 wk after receiving chemotherapy.

To test our hypothesis, we collected blood in PAXgene tubes and measured 28 genes associated with p53 signaling as well as UGT1A1, an enzyme involved in the metabolism of CPT-11, by StaRT-PCR. We found several genes to be significantly elevated in subject 668 before receiving chemotherapy, including ATM, Bcl-2, TRAF4, and SOD1. We found an additional 7 genes (p16, c-myc, Bcl-2, Bax, Bcl-X₁, GSTP1, and Gpx-1) to be significantly altered from 1 h to 1 wk after chemotherapy in subject 668, while there was no gene significantly altered in PBLs from subject 667. Further, there was a dose-dependent response in four genes (c-myc, p21, catalase, and Bcl-X₁) in subject 668 after ex vivo treatment with topotecan, which was not observed for cultured PBLs from subject 667. It is possible that subject 668 was protected from the cytotoxic effects of chemotherapy by expressing higher levels of cell survival genes or by altering lymphoid homeostasis in favor of cell proliferation after exposure to chemotherapy. However, further experimentation will be necessary to determine if these genes are truly associated with increased lymphocyte production.

In summary, these data encompass three inter-connected projects that attempt to define cell proliferation control and the balance between cell survival and death by correlating gene expression data with phenotypic characteristics. This has enabled us to identify putative biomarkers while better understanding the molecular basis of cancer biology.


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ABSTRACT

Cytotoxic and targeted therapies are ineffective in the majority of individuals diagnosed with bronchogenic carcinoma, due in part, to alterations in genes that regulate cell proliferation control and apoptosis. In order to develop more effective therapies, and better utilize existing therapies, it is necessary to understand how inter-individual variation in gene expression contributes to therapeutic response, so that each individual receives the appropriate medication at the appropriate dose. First, we found extensive inter-individual variation in the expression of the cyclin-dependent kinase inhibitor, p21, which was attributable to upstream transcription factors E2F1 and p73 in bronchogenic carcinoma cells, but not normal bronchial epithelial cells. Second, we hypothesized that bronchogenic carcinoma cell lines with high expression of E2F1 and p73, and wild type p53, would have a defect in their apoptosis pathway, and therefore, would be resistant to chemotherapy. However, high expression of E2F1 and p73 as well as c-myc, was associated with spontaneous apoptosis and chemosensitivity, but induction of p53 and p21 was associated with small increases in apoptosis and chemoresistance. From these results we identified an interactive transcript abundance index (ITAI) in the form of a ratio, \([c\text{-}\text{myc}*p73\alpha]/[p21*Bcl\text{-}2]\) which was correlated with response to CPT-11 in bronchogenic carcinomas and other tissues, regardless of p53 status. These studies lead to an additional hypothesis, that inter-individual variation in expression of p53 and related genes in peripheral blood leukocytes (PBLs) could predict cytotoxicity to chemotherapy. In these studies, variation in gene expression was observed between two individuals that showed vast differences in lymphocyte counts following chemotherapy. In summary, we have identified a set of genes that contribute to cell proliferation control and apoptosis in...
normal and malignant cells, and may collectively predict chemoresistance or cytotoxicity. The use of expression profiling in this context will increase the efficacy of currently available mediations, while minimizing adverse side effects, and will facilitate the development of newer therapies that target specific genetic defects.