Paper I
WILD TYPE P53 IS EXPRESSED AND PHOSPHORYLATED IN LEUKEMIA CELLS WITH FLT3 Y591 AMPLIFICATION AND BCL-2 OVEREXPRESSION

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SUMMARY

In contrast to most solid tumors, loss or mutation of the TP53 tumor suppressor gene or its known regulators is not commonly observed in acute myeloid leukemia (AML). We investigated the hypothesis that Bcl-2 family member overexpression, a common result of altered signaling in AML, suppresses wild type p53 activity in this disease. We identified and characterized several isoforms of wild type p53 protein in primary leukemic blasts from patients with de novo AML using two-dimensional gel electrophoresis (2D-PAGE) and phospho-specific intracellular flow cytometry. In contrast with normal cells, we found that p53 in AML blasts was often expressed at high levels and highly phosphorylated. Surprisingly, DNA damage induced by AML chemotherapy drug idarubicin led to accumulation of phosphorylated p53 isoforms in AML blast cells, as is commonly observed in normal cells undergoing apoptosis. However, overexpression of anti-apoptosis protein Bcl-2 in AML cells was directly correlated with expression and phosphorylation of wild type p53. Increased Bcl-2 expression was specific to AML cells where p53 was phosphorylated at serine residues 15, 46, and 392. No relationship between Bcl-2 expression and phosphorylation of p53 at serines 20 or 37 was observed. The highest levels of Bcl-2 expression were detected in a subset of patients sharing mutations in FLT3 that amplify phosphorylation site Y591. Taken together with the known role of Stat5 as a Bcl-2 transcription factor, these results support a model in which Flt3-mediated Stat5 signaling in AML drives accumulation of Bcl-2 and maintains a downstream block to p53 pathway apoptosis. These results suggest that therapeutic inactivation of Bcl-2, either by inhibition of signaling that maintains Bcl-2 expression or by small molecules that interfere directly with Bcl-2 activity, might improve the efficacy of DNA damage inducing chemotherapies in AML by restoring the p53 apoptosis pathway.
Introduction

We have previously reported that signaling profiles of acute myeloid leukemia (AML) cells identify patients with a poor response to course one of chemotherapy \(^1\). In that study we showed that mutation of fms-like tyrosine kinase 3 (Flt3) was associated with increased activity of signal transduction and activator of transcription (STAT) family members Stat5 and Stat3, but we did not explore how this signaling might contribute to the observed therapy resistance. Here we examine the connection between a target of altered Stat5 signaling in AML – Bcl-2 protein – and the the suppression of normal apoptotic responses to DNA damage in leukemia cells. Evasion of apoptosis is one of the hallmark mechanisms contributing to the formation and continued survival of cancer cells \(^2\), and AML is characterized by increasing resistance to DNA damage and cell death induction therapies. Insight into signaling mechanisms that suppress apoptosis in AML cells could be used to improve the efficacy of existing therapies.

AML is particularly relevant to the study of suppressed apoptosis pathways in cancer, as it is widely reported that p53, a central effector of apoptosis protein and guardian of the genomic integrity, is not frequently lost or mutated in AML \(^3,4\). p53 is a sequence specific transcription factor that can halt progression through the cell cycle or initiate apoptosis \(^5\), and is a key tumor suppressor protein often lost in human cancers. In some solid tumors, mutation of the TP53 gene has been detected in more than 70% of patients \(^6\). Given the widespread loss of p53 in most cancers, it was initially striking when TP53 was reported to be mutant in only 7% (8/112 patients) of AML cases \(^3\). Subsequent research has shown that downstream p53 effector genes, such as p21, are rarely lost in AML, and p53 protein expression can be detected in more than 90% of AML derived cell lines \(^7\).

Here we measure several properties of p53 in primary AML cells, including TP53 gene sequence, per-cell abundance of p53 protein, p53 phosphorylation at five residues, and transcriptional activity. These assays are based on the known molecular biology of p53, which is extensive \(^8\). Following DNA damage and cell stresses, p53 protein is rapidly phosphorylated at several residues \(^9\), including those we measure here (serines 15, 20, 37, 46, and 392). Phosphorylation of p53 is thought to regulate p53 localization, conformation, and activity \(^10\), but the precise contribution of each phosphorylation site is controversial. Phosphorylation of p53 can block interactions with negative regulator mdm2 (stabilizing the protein and increasing abundance) \(^11\) and promote conformations which are suitable for DNA binding and promoting gene transcription \(^9\). Here we will treat each of these five p53 phosphorylations as independent signaling events in order to measure which phosphorylations are associated with each other and with other properties of AML cells, such as mutational status of Flt3 and expression of apoptosis protein Bcl-2.

The Bcl-2 anti-apoptotic protein was first discovered in B cell lymphoma \(^12,13\) and has been widely studied as both a possible prognostic factor for AML and for its potential to inhibit apoptosis in cells reviewed in \(^14\). Bcl-2 is a potent oncogene, and mouse models have demonstrated that Bcl-2 family proteins can induce leukemia \(^15,16\). Bcl-2 has been shown to be heterogeneously expressed in AML in several studies \(^17,18\), and was associated with a poor response to chemotherapy \(^17\). In AML originating from immature, CD34\(^+\) myeloid cells, Bcl-2 expression indicated lowered rates of complete remission \(^19\), and the ratio of Bax to Bcl-2 has been reported to be highly predictive of long term survival in AML \(^20,21\). Despite these reports,
expression of Bcl-2 has not previously been found to correlate significantly with other prognostic indicators in AML, such as differentiation markers (French American British type; FAB), specific cytogenetic alterations \(^{18}\), or Flt3 mutation. However, Bcl-2 family members are transcriptional targets of STAT protein transcription factors Stat5 and Stat3 in AML \(^{22}\), which suggests that altered leukemic cell signaling might be the cause of Bcl-2 expression and allow AML blasts to evade apoptosis.

Signaling pathways upstream of Bcl-2 in AML can be activated by a number of different mechanisms, including genetic changes that result in improper activation of signaling pathways \(^{23}\), changes in STAT gene sequence and expression \(^{24}\) and abnormalities of upstream activators of signaling (e.g. Flt3, c-kit, platelet derived growth factor receptor) \(^{25}\). Flt3 mutations are especially common in AML, and are observed in approximately 30% of adult AML patients \(^{26}\). Flt3 mutations increase signaling activity of STAT proteins Stat3 and Stat5 in primary AML cells \(^{1}\) and are an accurate, independent predictor of disease relapse after chemotherapy \(^{27}\). The majority of mutations in Flt3 are situated in the juxtamembrane region as internal tandem duplications. In this juxtamembrane region there are 4 potential tyrosine phosphorylation sites (Y589, Y591, Y597, Y599). The region duplicated is highly variable between different patients, but nearly always involves at least one of these four phosphorylation sites. Approximately half of our patients with Flt3 length mutations (LM) have a duplication of Y591. Changes to signaling \(^{1}\) and poor response to chemoptherapy \(^{27}\) associated with Flt3-LM suggest that a key outcome of Flt3 signaling in AML may be evasion of apoptosis mediated by Bcl-2 expression.

Chemotherapy for AML frequently employs an anthracycline, such as idarubicin, in combination with cytarabine \(^{28}\). Despite intensive chemotherapy, only half of treated AML patients show an initial response and overall long term survival without bone marrow transplantation rarely exceeds 50\% \(^{29}\). Clearly, therapy induced cell death of AML blasts is far from complete. Here we examined the in vitro response of AML blast cells to idarubicin in an effort to better understand where the normal p53 mediated induction of apoptosis or arrest fails during AML chemotherapy with idarubicin. Therapies based on inhibiting mechanisms of suppressed apoptosis in AML could increase treatment efficacy and lower associated toxicity of chemotherapy. However, such therapies require intimate knowledge of oncogenic changes to signal transduction and gene regulation, so it is of crucial importance to understand molecular changes contributing to AML progression.

Given the importance of p53 in mediating cell death following DNA damage, we wanted to determine whether wild type p53 protein was expressed and functional in AML and, if so, understand why DNA damage induction therapies are still resisted by AML blast cells. Here we determine whether genetically wild type TP53 is expressed in primary AML blasts and show evidence that p53 DNA damage responses are intact, but blocked downstream by overexpression of Bcl-2 protein. Because of the known role of STAT proteins in transcription of anti-apoptosis Bcl-2 family members, we further examine connections between Flt3 mutations, Bcl-2 protein expression, and levels of phosphorylated p53. We show that AML patients bearing Flt3-LM that amplify Y591 express very high levels of Bcl-2 associated with accumulation of phosphorylated p53. The results presented here suggest that changes to signaling in AML cells, such as
increased Stat5 signaling mediated by Flt3, drive overexpression of anti-apoptosis Bcl-2 family members and enable AML blasts to resist induction of cell death by chemotherapy without loss or mutation of p53.

RESULTS

**P53 IS WILD TYPE AND EXPRESSED AT HIGH LEVELS IN AML**

We were initially interested in the p53 status of primary AML blast cells, which have been reported to contain two wild type TP53 alleles. We sequenced TP53 from blast cells from 18/30 AML patients (Table 1). All 18 sequenced patients had wild type TP53.

We further asked whether wild type p53 was expressed in AML blast cells. Normal cells express high levels of p53 in response to cell stress (e.g. DNA damage), but only express low levels of p53 in the resting state. In order to obtain normal, primary cells with wild type p53, peripheral blood leukocytes (PBL) were isolated from the blood of healthy, normal donors by the same method as for AML blasts. It is especially important to examine p53 in primary cells, as perturbation of p53 pathway function is likely required for generation of a cell line. We used 2D-PAGE immunoblotting with the BP53-12 α-p53 antibody to examine expression level, molecular weight (m.w.), and isoelectric point (measured using pH) of proteins from cell extracts of normal PBL and AML blasts (Figure 1). The BP53-12 antibody recognizes many forms of p53, including wild type, mutant, N-phosphorylated, and cytoplasmic p53 (Table 2). Three primary isoform groups reactive with BP53-12 were detected in both AML blasts from 6 patients (Figure 1A) and normal PBL (Figure 1B). The molecular mass of each isoform group was 63 kDa, 53 kDa, and 47 kDa. The level and presence of individual spots within these isoform groups varied significantly among AML patients, especially within the 53 kDa band corresponding to full length p53. In contrast, isoform groups varied little among different donors of normal PBL (Figure 1B). In normal cells full length p53 isoforms were expressed at much lower levels than in many AML blast samples. The 47 kDa isoform group termed Δp53 was expressed at high levels in all resting cells.

The ratio of full length p53 to Δp53 varied significantly in AML patients. Δp53 was abundant in all AML patient samples tested and was generally restricted to a smaller range of pH than full length p53 (pH range of 0.5, from 4.8 to 5.3). In contrast, full length p53 expression and isoform status varied widely in AML patients (Figure 1A), and the pl of full length p53 was wider (pH range of 1.48, from 6.48 to 5.0). In resting normal cells, Δp53 was expressed at approximately a 10-fold higher level than that of full length p53 (Figure 1B).

**P63, PHOSPHORYLATED P53, FULL LENGTH P53, AND ΔP53 ISOFORMS ARE EXPRESSED IN AML CELLS**

We asked whether phosphorylated p53 was present within the identified isoform groups of p53. We performed 2D-PAGE immunoblotting for total p53 or p53 phosphorylated at serines 37, 46, or 392 from unstimulated or irradiated cells, including Molm-13 (AML derived) and LNCaP (prostate cancer derived) which both express wild type p53. Equivalent results were obtained for each cell type. LNCaP are
shown in Figure 2. In resting cells we detected the full length p53 and ∆p53 isoforms at an equivalent isoelectric point as in normal PBL and AML blast cells. Phosphorylation of p53 at all residues was low or not detected in either of these isoform groups in unstimulated cells. At 2h following irradiation (IR; 25 Gray), phosphorylation of full length p53 was detected. Phosphorylation of serines 37 and 392 was marked and present within more than 5 spots of the 53 kDa band, while phosphorylation of serine 46 was less robust (Figure 2A). Phosphorylation of ∆p53 was not observed at 2h following IR at any of the residues tested.

2D-PAGE analysis of cell extracts from normal PBL using antibodies against p63 indicated the isoform group detected by BP53-12 at 63 kDa is likely p63 protein (Figure 2B). An additional p63 isoform group of low molecular weight (triplet of approximately 35 kDa molecular mass) was specifically detected by α-p63. Based on these results we have indicated the three major isoform groups using an example 2D-PAGE immunoblot of normal PBL (Figure 2C); isoform groups were designated p63 (63 kDa), p53 full length (53 kDa), and ∆p53 (47 kDa).

**WILD TYPE P53 IN AML BLAST CELLS IS PHOSPHORYLATED AND ACCUMULATES FOLLOWING DNA DAMAGE**

Following cell stress, wild type p53 in normal cells is rapidly phosphorylated, binds DNA, and activates expression or repression of target genes. To examine the transcriptional activity of p53 in leukemia cells, we transfected primary AML blasts or normal PBL with p53RE.GFP, a p53 reporter construct where the p53 binding site of the p21 gene drives GFP expression, alone or in combination with pC53.SN, a plasmid overexpressing wild type p53. Transfection efficiency for these AML samples was 48.5% +/- 15.8% and was gauged by parallel transfection of a control pCMX.GFP construct constitutively expressing GFP (Figure 3). Transfection of p53RE.GFP alone into normal PBL or primary AML blasts revealed rare activity of p53 in some primary AML cells (see subset of highly GFP positive cells in AML-P0 2). Co-transfection of p53RE.GFP and pC53.SN led to activation of p53 reporter gene expression in more than 33% of transfected cells in four AML patient samples (AML-P02, P03, P05, P06). More modest p53 reporter gene expression (less than 19%) was seen in the remaining two AML patient samples (AML-P01, P04) and normal PBL (Figure 3).

We next asked how p53 was regulated following DNA damage in AML using an *in vitro* model of idarubicin chemotherapy. AML blast cells from six patients were treated with idarubicin for 0h, 2h, or 4h prior to 2D-PAGE mapping of p53 (representative immunoblots shown in Figure 4A). Following idarubicin treatment, full length p53 accumulated and shifted to a more acidic pH. We quantitated changes in the pH of full length p53 detected in treated AML blasts as the acidic movement, a measure of the shift of full length p53 towards a lower, more acidic pH (N = 6; Figure 4B). The density of full length p53 increased following DNA damage and the density of ∆p53 decreased following DNA damage (Figure 4B).

We measured the phosphorylation of p53 at serines 15, 20, 37, 46, and 392 using phospho-specific flow cytometry in primary AML blast cells treated for 0h or 4h with idarubicin (Figure 4C). The degree of p53 phosphorylation was quantitated as the log$_{10}$ ratio of 4h idarubicin treatment compared with control AML patient samples exposed to idarubicin and then immediately fixed (0h treatment). Levels of total p53
protein were also detected in these cells (Figure 4C) using the DO7 α-p53 antibody (Table 2). Results were quantitated as log_{10} ratio of the mean fluorescence intensity (MFI) of the flow cytometry histogram peak at 4h, as compared to the MFI at 0h. For the purpose of quality control, cell size and autofluorescence were also measured and showed no change over time following idarubicin (Figure 4C). Following idarubicin treatment, increases in p53 phosphorylation varied among AML patients and followed a general pattern of increased phosphorylation and accumulation of p53. In no patient did p53 phosphorylation decrease, however the level of phosphorylation was low for some residues in specific samples (e.g. serine 392 in AML-P01, P02, and P05).

**HIGH LEVELS OF BCL-2 PROTEIN EXPRESSION ARE DETECTED IN AML PATIENTS EXPRESSING PHOSPHORYLATED p53**

Phospho-specific flow cytometry was used to detect p53 phosphorylation in resting AML blasts from 30 patients. We calculated the MFI for each of the measured parameters (Bcl-2, p53, p-p53-S15, p-p53-S20, p-p53-S37, p-p53-S46, and p-p53-S392) for AML blasts from each patient (Figure 5A). In order to normalize the fluorescent scale, the MFI of each marker for each sample was compared to the median for the group. For example, Bcl-2 expression in a particular patient sample that was above the median for AML was colored yellow, whereas decreasing Bcl-2 expression below the median was colored darker blue.

AML patients were clustered according to p53 phosphorylation at serines 15, 20, 37, 46, and 392 and characterized as having one of three patterns of p53 phosphorylation, designated low phospho-p53, phosphorylation at serine 20 or 37, or phosphorylation at serine 15, 46, or 392 (Figure 5A). Patients with a pattern of high p53 phosphorylation at serines 15, 46, and 392 tended to express a high amount of Bcl-2 (Figure 5A). Patients with a pattern of p53 phosphorylation at serines 20 or 37 (and lacking other p53 phosphorylations) did not have a particularly high or low level of Bcl-2 expression (this pattern was not correlated with Bcl-2 expression). AML patients where a pattern of low p53 phosphorylation was detected typically expressed lower than the median amount of Bcl-2 (Figure 5A). Total p53 protein expression was also very high in patients with a pattern of p53 phosphorylation at serines 15, 46, and 392 (Figure 5A).

By plotting two factors and determining the correlation coefficient one can gauge whether the association has a specific threshold for association or is the result of a scalable relationship (e.g. increasing amounts of Bcl-2 are associated with increasing amounts of p53 phosphorylation). The observed correlations between p53 phosphorylation and Bcl-2 protein expression were analyzed by linear regression (Figure 5B), and no threshold of Bcl-2 or p53 phosphorylation was observed. No correlation of Bcl-2 expression with serines 20 and 37 was observed (Figure 5B). A scalable correlation between Bcl-2 and serines 15, 46, and 392 was observed over a wide range of Bcl-2 expression and each phosphorylation (Figure 5B).

**HIGH BCL-2 EXPRESSION IN AML BLASTS IS SIGNIFICANTLY ASSOCIATED WITH LENGTH MUTATION OF FLT3 AND SPECIFIC P53 PHOSPHORYLATIONS**

We next asked whether LM of the receptor tyrosine kinase Flt3, which has been observed to drive transcription of antiapoptotic members of the Bcl-2 family 33, was correlated with observed Bcl-2 protein
expression. 13/30 patients in our material had a Flt3 length mutation. Of these, 11/13 AML patients had been sequenced as part of another study (R. Hovland, unpublished observations) and were classified in two groups depending on type of mutation: 1) Flt3-LM-SPY591, containing a single Y591 phosphorylation site, and 2) Flt3-LM-AmpY591, containing ≥2 Y591 phosphorylations sites. We used phospho-specific flow cytometry to compare p53 phosphorylation with Bcl-2 protein expression at the single cell level (Figure 6A) in these groups. The MFI for Bcl-2 expression was observed to be high in patients with Flt3-LM-AmpY591 Flt3 length mutation sequence compared to patients with Flt3-LM-SPY591 mutation (p<0.001) (Fig. 6B). Primary AML blast cells expressing high amounts of Bcl-2 protein generally expressed higher amounts of p53 phosphorylated at serines 15, 46, and 392 (e.g. AML-P28). No association between p53 phosphorylation at either serine 20 or serine 37 with Bcl-2 was observed at the individual cell level (see Supplementary Information).

Bcl-2 expression, p53 expression, and p53 phosphorylation data are included in full with other patient characteristics (Table 1), including known signaling profiles ¹. A graphical summary of clinical outcome (response to course 1 of chemotherapy) in Flt3 length mutation groups and signaling clusters is shown for comparison (Figure 7).

DISCUSSION

We show here that not only is p53 wild type in AML blast cells, but it is expressed, diversely phosphorylated in resting cells, and able to respond to DNA damage. In malignant cells where high expression and phosphorylation of wild type p53 were observed, very high levels of Bcl-2 protein expression were also detected. In a defined subset of AML patients with extremely high levels of phosphorylated p53, we detected very high expression of Bcl-2 that was associated with a specific mutational sequence group of Flt3 (Flt3-LM-AmpY591). Together, these results suggest that a vital function of altered signaling in AML blast cells is to drive expression of anti-apoptosis proteins, such as Bcl-2, which can block the activity of p53 effector proteins and allow survival of AML blasts expressing wild type p53 (Figure 8).

Primary AML blast cells express wild type p53

TP53 was determined to be wild type and expressed in 18/18 sequenced AML patients in this study (Table 1). Three BP53-12 reactive isoforms were commonly detected in normal cells and AML blasts. We identified these major isoform groups as p63, various phosphorylated forms of full length p53, and an assumed deletion form of p53 termed Δp53 (Figure 2). It is possible this Δp53 is a deletion form of wild type p53. We observed Δp53 and full length p53 isoforms in all examined cell types expressing wild type p53, including normal primary PBL, primary AML blasts, the AML cell line Molm-13, and LNCaP cells.

The pH of the lowest acidity spot for full length p53 was approximately 6.5, which is in agreement with the computationally predicted pH of 6.48 for unphosphorylated, full length p53 protein (Figure 1). Several additional isoforms of full length p53 were detectable at more acidic pH, and specific detection of phosphorylated p53 by immunoblotting indicated that these were phosphorylated full length p53 (Figure
Based on the observed changes in the ratio of Δp53 to full length p53 following DNA damage, we suggest that Δp53 may be an endogenously occurring regulator of full length p53 activity.

It is notable that most of the variation in p53 between AML patients occurs in full length p53. This suggests that differences in p53 levels among AML patients detected by flow cytometry are representative of changes in full length p53. p63 expression was frequently much lower or not detectable in AML blast cells, compared with normal PBL. With the number of patients in this study we did not have the statistical power to compare the expression of p63 or full length p53 with clinical outcomes, and this was not the intended goal of these studies. The aim was instead to understand why AML blast cells survive despite the expression of high levels of wild type p53.

Intact DNA damage responses of wild type p53 in AML

Following DNA damage, p53 protein is normally phosphorylated at several residues. Idarubicin treatment led to accumulation of p53 in a highly phosphorylated form, as seen by 2D-PAGE and flow cytometry (Figure 4). Increased acidity of full length p53 following idarubicin suggested that the normal phosphorylation of p53 in response to DNA damage may be intact in AML blast cells. The use of idarubicin is especially relevant to AML therapy as it is used extensively in the clinic. Phospho-specific 2D-PAGE for p53 indicated that phosphorylation of p53 in response to DNA damage occurs primarily within the full length p53 isoform group (Figure 2A).

We did not examine in detail the question of p53 transcriptional activity in primary AML blast cells here, however we know it is possible for p53 to be transcriptionally active in AML blast cells as co-transfection of wild type p53 and a p53 reporter element driving GFP into AML blasts leads to p53 reporter activity in some cases in more than 33% of transfected cells (Figure 3). Furthermore, expression of p53 target genes, including p21, can be detected in AML blasts during therapy (Anensen et al, submitted).

The finding that wild type p53 in AML blast cells responds to DNA damage caused by a common chemotherapeutic agent is particularly striking (Figure 4). Typically, the p53 response of malignant cells has been disabled, either by loss of heterozygosity of p53 or by loss of p53 pathway effectors. Instead, in AML we see that p53 is wild type and appears to be activated by chemotherapy treatment. These results indicate that idarubicin treatment causes full length p53 to accumulate in several acidic isoforms similar to phosphorylated isoforms seen in cell lines (Figure 2A). This suggests that there exists a downstream block to p53 pathway driven apoptosis and that therapeutic removal of this block could increase the efficacy of induction chemotherapy for AML.

Together, these results showed that wild type p53 was expressed in AML blast cells, was phosphorylated at multiple residues following DNA damage, and could activate target gene expression. The question remained as to why AML blast cells expressing what appeared to be normal, active p53 did not undergo apoptosis. As anti-apoptosis proteins of the Bcl-2 family are known to directly interfere with activity of p53 effectors, such as Bax and Bak, and are known to be expressed at different levels in AML patients, we
asked whether expression of Bcl-2 was higher in AML blast cells expressing high levels of phosphorylated p53.

**Bcl-2 is overexpressed in AML blasts where phosphorylated p53 has accumulated**

Bcl-2 is a major anti-apoptosis gene of general interest in the development of malignant diseases. In AML, expression levels are known to be highly heterogeneous and the ratio of Bcl-2 to pro-apoptotic Bax protein is known to be useful in prognostic evaluation of AML patients. Expression of Bcl-2 protein can inhibit the action of pro-apoptotic p53 effectors, such as Bax and Bak, and could allow the survival of cells expressing high levels of p53.

In order to understand how AML blast cells resist p53 mediated apoptosis, we extensively mapped p53 phosphorylation at five residues in 30 AML patients and compared patterns of p53 phosphorylation with expression of Bcl-2. In general, the level of p53 phosphorylation was unimodal and could be well represented as the MFI in a hierarchical clustering analysis. We detected three clusters of p53 phosphorylation (Figure 5A) representing characteristic p-p53 profiles; 1) Low phosphorylation, 2) Increased phosphorylation at residues 20 or 37, and 3) increased phosphorylation at residues 15, 46 or 392 (Figure 5A). Linear regression analysis of p53 phosphorylation vs. Bcl-2 expression indicated that level of Bcl-2 was significantly associated with phosphorylation of p53 at serines 15, 46, and 392, but not at serines 20 and 37 (Figure 5B, Table 3).

In summary, at both the individual cancer cell level and in populations of malignant cells from individual AML patients (Figure 5, Table 3) the level of p53 phosphorylation at serines 15, 46, and 392, but not at serines 20 and 37, were seen to correlate with Bcl-2 protein expression.

**Bcl-2 overexpression in AML blasts with Flt3 Y591 amplification**

Flt3 has been observed to drive transcription of anti-apoptotic members of the Bcl-2 family (e.g. Bcl-2, Bcl-XL). Flt3 length mutation sequence group was known for 11/13 patients with length mutations, and so we examined whether length mutations of a specific form were associated with expression of Bcl-2 and patterns of p53 phosphorylation. Strikingly, those patients with the highest levels of p53 phosphorylation and Bcl-2 expression were nearly all members of the same Flt3 length mutation sequence group (p < 0.001, Figure 6B). Surprisingly, however, we find that AML patients with the worst chemotherapy response are those patients with SP-Y591 (refer to 1). This might indicate that expression of Bcl-2 to block wild type p53 activity is a tenuous balance that is disrupted during therapy. What is striking is that patients where Y591 is not amplified do not exhibit p53 phosphorylation or Bcl-2 overexpression and display a uniformly poor response to course one of chemotherapy (Figures 6 and 7).

**Summary**

These results suggest that wild type p53 is functional in AML blast cells, but that induction of apoptosis by p53 effectors is overridden by expression of anti-apoptosis proteins, such as Bcl-2. The results further indicate that one mechanism for increased expression of Bcl-2 in AML is aberrant signaling driven by
specific length mutations of Flt3. In patients where Flt3 length mutation is of the Flt3-LM-AmpY591 group we observed extremely high levels of Bcl-2 and of wild type, phosphorylated p53. Our model would suggest that treatment of patients using anti-Flt3 or anti-Bcl-2 therapies would reverse the anti-apoptotic effects of Bcl-2 and lead to rapid apoptosis of AML blast cells. In AML patients with a high Bcl-2 to Bax ratio, inhibition of Bcl-2 family members might similarly lead to rapid apoptosis. These results further suggest that direct inhibition of signaling, combined with conventional chemotherapy, might be required to address AML such as those with Flt3 LM that do not amplify Y591.

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EXPERIMENTAL PROCEDURES

PATIENTS AND PREPARATION OF AML BLASTS, NORMAL PBL, AND CELL LINES

The study was approved by the local Ethics Committee and samples collected after informed consent. Samples were selected from a large group of consecutive patients with de novo AML and high peripheral blood blast counts. These patients were admitted to the hospital from April 1999 to August 2003, the median age was 60 and ages ranged from 29 to 84. As these patients were selected for high blast counts, enriched AML cell populations containing >95% tumor cells were prepared using a simple density gradient separation of peripheral blood samples (Ficoll-Hypaque; NyCoMed, Oslo, Norway; specific density 1.077) before safe, standardized cryopreservation according to previously developed techniques. These patients represent the latter portion of a group studied previously for Flt3 signaling and mutation in AML and were previously characterized for signaling profiles associated with chemotherapy response and Flt3 mutation. Peripheral blood leukocytes (PBL) were collected from healthy donors, separated as described for AML cells and were used fresh. The AML cell line Molm-13 was from DSMZ (The German Resource Centre for Biological Material) and the prostate cancer cell line LNCaP was from American Type Culture Collection (www.atcc.org). Both were cultured in RPMI medium (Sigma Aldrich Inc.) + 10% Fetal Calf Serum (HyClone, South Logan, UT, USA).

SEQUENCING OF p53 IN AML PATIENT SAMPLES

Sequencing of TP53 was performed by temporal temperature gradient gel electrophoresis (TTGE) as previously described.
IN VIVO TREATMENT OF PRIMARY AML BLASTS

Peripheral blood containing >95% AML blasts was thawed into 5 mL Stem Span H3000 defined, serum free medium (Stem Cell Technologies, Vancouver, BC, Canada), counted, pelleted, and resuspended at 2 x 10^6 cells per mL. Leukemia samples were transferred to individual wells or FACS tubes (Falcon 2052, BD-Biosciences, San Jose, CA) and allowed to rest at 37 °C for 2h. AML blasts were resuspended gently to prevent aggregation and allowed to rest at 37 °C for another 45 minutes. At this time samples were treated according to the following procedures. For irradiation (IR) induced DNA double strand breaks, samples were irradiated by 25 Gray from a Ce^{137} source and analyzed 2h later by 2D-PAGE. For an in vitro model of course one of AML chemotherapy, samples were cultured in StemSpan H3000 alone or containing 360 nM idarubicin (Pfizer Inc., New York, USA) and either analyzed immediately or at 2h or 4h after treatment by 2D-PAGE or flow cytometry. Following treatment, samples were returned to the 37 °C incubator for indicated times. For 2D-PAGE analysis, cells were lysed and protein extract analyzed as described below. For flow cytometric analysis, 100 µL of 32% para-formaldehyde (PFA, Electron Microscopy Services Fort Washington, PA, USA) was added to each 2 mL tube of cells to a final concentration of 1.6%. Cells were fixed for 15 minutes at room temperature, pelleted, then permeabilized by resuspension in 2 mL ice cold methanol for 10 minutes, and stored at 4 °C until being stained for flow cytometry.

TRANSFECTION OF PRIMARY PBL AND AML BLASTS

Transfection of primary cells was performed using the AMAXA nucleofector system (AMAXA Biosystems, Germany). PBL were transfected using the AMAXA nucleofector kit for human T-cells (catalog number VPA-1002) and AML blasts using the kit for human CD34^+ cells (catalog number VPA-1003) according to the manufacturers instructions. Cells were transfected with a p53 reporter construct, p53RE.GFP (a kind gift from Dr. M. Laiho, University of Helsinki), alone or co-transfected with a plasmid over-expressing wild type p53, pC53.SN (a kind gift from Dr. G. Lozano, MD Anderson Cancer Center). As control of transfection efficiency, the cells were transfected with a control plasmid expressing green fluorescent protein (GFP), pCMX.GFP_{37}. After transfection the cells were left to incubate for 6 hours at 37°C before analysis using flow cytometry.

SAMPLE PREPARATION AND TWO-DIMENSIONAL (2D) SDS-PAGE

Cells were washed twice in 100 vol 0.9% NaCl, the cells were lysed in 7% trichloroacetic acid, desalted with three washes in water saturated ether (1 ml, 14 000xg for 20 min, 4°C) and resolved in 2D-sample buffer (7M urea, 2M thiourea, 100 mM DTT, 1.5% Ampholyte 3-10, 0.5% Ampholyte 5-6, 0.5%CHAPS). 2D was performed using 7 cm pH 3-10 (Zoom Strip, Invitrogen Corp., Carlsbad, CA, USA) isoelectric focusing gel strips, following the manufacturers instructions. The focusing strips were incubated with the Sample Rehydration Buffer (8M urea, 1% CHAPS, 20 mM DTT, 1.5% Ampholyte 3-10, 0.5% Ampholyte 5-6, Brom-phenol Blue) containing the protein sample to rehydrate the strips. Rehydration was performed over night at room temperature. IEF was performed at 200 V for 40 minutes, 450 V for 30 minutes, 750 V for 30 minutes and 2000 V for 60 minutes. Following IEF, the strips were either stored at −80°C until further use or equilibrated directly for 15 min in LDS sample buffer (Invitrogen Corp., Carlsbad, CA, USA) containing 100
mM DTT and then 15 min in LDS sample buffer containing 125 mM iodoacetamide. For the second dimension the ZOOM strip was aligned in a 0.5% agarose solution added into the IEF well of a NuPAGE Novex 4 to 12 % Bis-Tris ZOOM Gel (Invitrogen Corp., Carlsbad, CA, USA). Electrophoresis was performed at 200 V for 60 minutes, after which the proteins were transferred to polyvinylidene fluoride (PVDF) membrane by standard electroblotting.

**IMMUNOBLOTTING**

p53 protein was detected using primary BP53-12 antibody (Santa Cruz Biotechnology, CA, USA) or phosphospecific antibodies (Cell Signaling Technology, Beverly, MA, USA) directed towards phosphorylated S37, S46 or S392. Primary antibodies were used in combination with secondary alkaline phosphatase conjugated mouse antibody (Applied Biosystems, Foster City, CA, USA) visualized using the Western-star™ Immunodetection system (Applied Biosystems - Tropix, Foster City, CA, USA) or secondary horseradish peroxidase conjugated mouse or rabbit antibody (Jackson ImmunoResearch) visualized using the Supersignal west Pico system (PIERCE). Additional description of antibodies is included (Table 2).

**INTRACELLULAR PHOSPHO-SPECIFIC FLOW CYTOMETRY**

Performed as previously described for AML patient samples 1. Briefly, PFA fixed, methanol permeabilized AML blasts were rehydrated by addition of 2 mL phosphate buffered saline (PBS), gentle resuspension, and then centrifugation. The cell pellet was washed once with 2 mL PBS and then resuspended in 50 µL PBS + 0.1% BSA (Sigma). 50 µL of an antibody mix containing 0.13 µg primary conjugated phospho-specific antibody per sample was added to each tube of AML blasts and staining proceeded for 20 minutes at room temperature. Primary conjugated antibodies (Cell Signaling Technology, Beverly, MA, USA) included monoclonal α-phospho-p53-S15 clone 16G8, and polyclonal α-phospho-p53-S20, α-phospho-p53-S37, α-phospho-p53-S46, and α-phospho-p53-S392. These antibodies were conjugated to biotin, bound to the streptavidin (SA) conjugated fluorophores SA-Alexa488, SA-PE, and SA-Alexa647 (Molecular Probes, Eugene, OR, USA), and then used in combination with α-Bcl-2-FITC, or α-p53-FITC clone DO-7 (both from BD-Pharmingen, San Diego, CA, USA). Stained AML blasts were then washed by adding 2 mL PBS + 0.1% BSA and resuspended in a final volume of 200 µL PBS. At least 30,000 ungated live cell events were collected for each sample on a benchtop FACSCalibur dual-laser cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). Additional description of antibodies is included (Table 2).

**STATISTICAL ANALYSIS AND RELATIONSHIP MAPPING**

Normalization of flow cytometry was performed by comparing MFI values to either an unstimulated control (Figure 4) or the median in AML (Figures 5 and 6) using the log_{10} ratio of a sample divided by the control. This MFI normalization allowed comparison of p53 phosphorylation, p53 expression, and Bcl-2 on comparable scales. Significance of differences in averages for two populations (e.g. expression of Bcl-2 in Flt3 length mutation groups) was determined using a Students t-test. Linear regression analysis of correlation between graded parameters was determined using the square of the Pearson product momentum correlation coefficient ($r^2$). Those relationships that are above a significance threshold ($r^2 > 0.5$)
were included in a map of relationships between detected parameters (Figure 5B). Each related parameter was placed at approximately $1 - r^2$ units from the nodes to which it was related. For example, the correlation between Bcl-2 expression and p53 phosphorylation at serine 46 across 30 AML patients was $r^2 = 0.64$, so the nodes “Bcl-2” and “ser46” were placed 0.46 units apart on the map. Therefore, the more closely related two parameters were in AML, the closer they were placed on the map.

REFERENCES


32. Matsuo, Y. et al. Two acute monocytic leukemia (AML-M5a) cell lines (MOLM-13 and MOLM-14) with interclonal phenotypic heterogeneity showing MLL-AF9 fusion resulting from an occult chromosome insertion, ins(11;9)(q23;p22p23). *Leukemia* 11, 1469-77 (1997).


**FIGURE 1 - EXPRESSED, WILD TYPE P53 IN PRIMARY AML BLASTS EXISTS IN SEVERAL ACIDIC ISOFORMS NOT SEEN IN RESTING NORMAL CELLS**

A) Expression of wild type p53 was detected at high levels in many AML blast samples, and the acidity and expression of different p53 isoforms was highly variable across AML patients.

B) Among samples of PBL from normal, healthy donors, full length wild type p53 is expressed at low levels (53 kDa band).
FIGURE 2 - CHARACTERIZATION OF P53 ISOFORMS BY 2D-PAGE IMMUNOBLOTTING

A) Phosphorylated p53 was detected by 2D-PAGE in resting or irradiated LNCaP cells expressing wild type p53. Following IR, expression of total full length p53 increases and a characteristic laddering is detected. Induction of p53 phosphorylation following IR occurs at serines 37, 46, and 392 and is detected primarily in the full length p53 isoform group. Following IR, no change in reactivity with phospho-specific antibodies is observed for p63 or Δp53 isoforms.

B) Detection of p63 in normal PBL identifies the 63kDa isoform reactive with the BP53-12 antibody as p63. Also seen is a triplet of low molecular weight α-p63-reactive spots which were not detected by BP53-12.

C) p63, full length p53, and a putative p53 truncation designated Δp53, were identified as the three major isoform groups reactive with BP53-12.
**FIGURE 3 - WILD TYPE p53 CAN ACTIVATE TRANSCRIPTION IN AML BLAST CELLS**

A) Normal PBL or primary AML blast cells were transfected using the AMAXA nucleofection system. Transfection efficiency was gauged by co-transfection of pCMX.GFP (a positive control for GFP) and pCMX (empty vector). The empty vector allowed for an equal amount of DNA to be transfected in all experiments.

B) p53 reporter activity was measured at 6h following transfection as the percentage of GFP positive cells following transfection of p53RE.GFP (a p53 reporter driving GFP) and pCMX into normal PBL or AML blast cells. Cells from some AML patient samples showed low levels of endogenous p53 activity.

C) Co-transfection of pC53.SN (a wild type p53 expression construct) and p53RE.GFP into normal PBL or AML blast cells led to robust induction of p53 reporter gene expression (GFP) in primary cells from AML patient samples.
FIGURE 4 - INDUCTION OF P53 BY DNA DAMAGE IN AN IN VITRO CHEMOTHERAPY MODEL

A) Idarubicin treatment of AML blasts led to rapid accumulation of numerous full length p53 isoforms of high acidity. The blue line indicates the resting pH of the most acidic full length p53 isoform for this representative AML patient sample (AML-P18).

B) The change in pH of full length p53 following idarubicin treatment was measured in AML blast samples from six patients and indicated that the acidic movement of full length p53 was statistically significant ($N = 6$). Similar results were obtained for normal PBL.

C) Phosphorylation of p53 at five residues (serines 15, 20, 37, 46, and 392) and total per-cell level of p53 were detected by phospho-specific flow cytometry in AML blast samples from six patients. Phosphorylation and accumulation of p53 in AML blast samples following DNA damage was measured as the $\log_{10}$ fold increase in mean fluorescence intensity (MFI) at 4h following idarubicin, as compared to cells treated with idarubicin and fixed immediately (0h). Black indicates a comparable MFI was observed at 4h and 0h.
A) B) Expression of Bcl-2, p53, p-p53-ser15, p-p53-ser20, p-p53-ser37, p-p53-ser46 and p-p53-ser392 was measured for all 30 patients. Three clusters of p-p53 patterns were detected; low-phospho-p53, phosphorylation at serine 20 or 37, phosphorylation at serine 15, 46 or 392.

B) Correlations between p53 phosphorylation and Bcl-2 expression were analyzed by linear regression. Bcl-2 was found to significantly correlate with phosphorylation at serine 15, 46 and 392.
FIGURE 6 – FLT3 Y591 AMPLIFICATION IS ASSOCIATED WITH P53 PHOSPHORYLATION AND BCL-2 OVEREXPRESSION

A) Phosphorylation of p53 at serine 15 and Bcl-2 expression were measured in peripheral blood cells from normal healthy donors (PBL) or patients with AML. Four samples with Flt3 mutations including amplification of Y591 were known (AmpY591). Seven samples with Flt3 mutations that did not amplify Y591 were also known (SPY591; six representatives shown). The remaining samples displayed other Flt3 mutations or considered wild type (six representatives shown).

B) Bcl-2 expression, relative to the median in AML, is shown for normal PBL or AML patient samples with different Flt3 mutational status. Significantly greater Bcl-2 expression was observed in AML patients with Flt3-LM that amplified Y591 than in Flt3 LM that did not (AmpY591 vs. SPY591; p < 0.001). Wild type Flt3 AML patients displayed a spread of Bcl-2 values centered at the median observed for all 30 AML patients (a value of zero on the log_{10} scale). The median Bcl-2 expression for each group is marked with a black line.
A graphical summary of chemotherapy response, AML signaling cluster, and Flt3 mutational status based on Table 1. No AML patient with Flt3-LM-SPY591 responded to course 1 of chemotherapy, and these patients primarily displayed a potentiated type 2 signaling profile (SC-P2), as was commonly observed in patients with Flt3-LM. SC-P2 is characterized primarily by potentiated Stat5 and Stat3 signaling responses and an attenuated Stat1 response to IFN-γ.
Idarubicin, given in standard course one of AML chemotherapy, causes DNA damage and leads to accumulation of phosphorylated wild type p53 in AML blast cells. High levels of p53 activity normally lead to a program of arrest or apoptosis driven by p53 effectors, such as Bax and Bak. However, in AML blast cells we previously observed patterns of aberrant STAT and MAPK family signaling transduction and show here that signaling associated with Flt3 mutation drives overexpression of Bcl-2, blocking apoptosis. Based on these results, we suggest that standard AML chemotherapy could be improved to a three tiered strategy: 1) inhibition of the signaling mechanisms known to drive proliferation and Bcl-2 family member expression (kinase inhibitors; KI), 2) inhibition of Bcl-2 family member activity (BH3 mimic HA14-1), and 3) combining these with standard anthracycline based chemotherapy to induce DNA damage and activate wild type p53 (Idarubicin; Ida).
Table 1 - AML patient characteristics

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*Patients are ordered first by type of FLT3 length mutation (C) and then by Bcl-2 expression (A).

A) Mean fluorescence intensity (MFI) of Bcl-2 protein expression detected by flow cytometry (average of three measurements) and normalized MFI of Bcl-2 for each patient, calculated as the log_{10} ratio of a given MFI divided by the median MFI across all 30 AML patients (median Bcl-2 MFI = 20.39).

B) MFI values for expression of p53 protein and phosphorylation of p53 at serines 15, 20, 37, 46, and 392, as detected by flow cytometry.

C) For FLT3, AML patient samples were either considered wild type (wt) or tested positive for length mutation (LM), loss of a wild type allele (wt), or Asp835 point mutations in the kinase domain (KD). Patients with a sequenced LM were scored as belonging to two different groups, 1 (AmpY591) or 2 (SPY591). Mutation of c-kit was noted for one wt FLT3 patient.

D) N-RAS was identified to have point mutations in exon 1 (Gly12 or Gly13) in two patients.

E) c-kit was identified to have a deletion as well as a point mutation in exon 8 of AML-P09.

F) Not all patients were treated with course 1 chemotherapy (C1). For those that received C1 therapy, remission is scored as a "-" and relapse is scored as a "+". For some parameters no data (n.d.) were available.

G) Signaling cluster groups from prior analysis of signaling profiles are listed. 1

H) For cytogenetics, the karyotypes are listed and were scored according to Wheatley.
## Table 2 - Antibodies used in this manuscript

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SUPPLEMENTARY INFORMATION – CORRELATIONS BETWEEN BCL-2, P53, AND P53 PHOSPHORYLATION ACROSS 30 AML PATIENTS

Table 3 – Correlations between Bcl-2, p53, and p53 phosphorylation across 30 AML patients*

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</table>

* The degree to which two parameters tended to be directly related in AML biopsies was determined using linear regression analysis. For example, total Bcl-2 expression and serine 46 phosphorylation ($r^2 = 0.64$) were closely related, indicating that a patient sample with high levels of p53 serine 46 phosphorylation usually expressed high levels of Bcl-2. Correlations were drawn in Figure 5B.
Phosphorylation of p53 at serine 20 and Bcl-2 expression were measured in peripheral blood cells from normal healthy donors (PBL-D01, D02) or patients with AML (AML-P01 – P30). Four samples with Flt3 mutations including amplification of Y591 were known (P05, P11, P12, P28). Six samples with Flt3 mutations that did not amplify Y591 were also known (P04, P20, P21, P22, P29, P30). The remaining samples displayed other Flt3 mutations or were wild type for Flt3 length mutation or changes in the kinase domain. The first six AML patients with wild type Flt3 are shown (P01, P02, P07, P08, P10, P15).
Phosphorylation of p53 at serine 37 and Bcl-2 expression were measured in peripheral blood cells from normal healthy donors (PBL-D01, D02) or patients with AML (AML-P01 – P30). Four samples with Flt3 mutations including amplification of Y591 were known (P05, P11, P12, P28). Six samples with Flt3 mutations that did not amplify Y591 were also known (P04, P20, P21, P22, P29, P30). The remaining samples displayed other Flt3 mutations or were wild type for Flt3 length mutation or changes in the kinase domain. The first six AML patients with wild type Flt3 are shown (P01, P02, P07, P08, P10, P15).
Phosphorylation of p53 at serine 46 and Bcl-2 expression were measured in peripheral blood cells from normal healthy donors (PBL-D01, D02) or patients with AML (AML-P01 – P30). Four samples with Flt3 mutations including amplification of Y591 were known (P05, P11, P12, P28). Six samples with Flt3 mutations that did not amplify Y591 were also known (P04, P20, P21, P22, P29, P30). The remaining samples displayed other Flt3 mutations or were wild type for Flt3 length mutation or changes in the kinase domain. The first six AML patients with wild type Flt3 are shown (P01, P02, P07, P08, P10, P15).
**Supplementary Information – p-p53-S392 vs Bcl-2**

Phosphorylation of p53 at serine 392 and Bcl-2 expression were measured in peripheral blood cells from normal healthy donors (PBL-D01, D02) or patients with AML (AML-P01 – P30). Four samples with Flt3 mutations including amplification of Y591 were known (P05, P11, P12, P28). Six samples with Flt3 mutations that did not amplify Y591 were also known (P04, P20, P21, P22, P29, P30). The remaining samples displayed other Flt3 mutations or were wild type for Flt3 length mutation or changes in the kinase domain. The first six AML patients with wild type Flt3 are shown (P01, P02, P07, P08, P10, P15).