Paper II

A DISTINCT p53 PROTEIN ISOFORM SIGNATURE REFLECTS THE ONSET OF INDUCTION CHEMOTHERAPY FOR ACUTE MYELOID LEUKEMIA

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Abstract

Purpose: The anti-oncogene protein product p53 has not been studied in cancer patients under actual chemotherapy. This study examined the early p53 protein and gene expression during induction chemotherapy in acute myeloid leukemia (AML).

Experimental design: Leukemic cells were collected from five AML patients during their first 18 hours of induction chemotherapy, and examined for p53 protein and gene expression by two-dimensional gel immunoblot and high-density gene expression arrays. The p53 isoform signature *in vivo* was compared to *in vitro* treated AML cells, peripheral blood mononuclear cells (PBMC) and mobilized $CD34^+$ peripheral blood progenitor cells.

Results: Up-regulation of p53 protein expression post-treatment was accompanied by increased transcription of putative p53 target genes and subsequently cytopenia in the patients. Two-dimensional gel immunoblots demonstrated two main forms of p53, denominated αp53 and Δp53, both recognized by various amino-terminal directed antibodies. In untreated patient leukemic cells and normal cells Δp53 was dominating, while leukemia cells isolated post-induction treatment expressed higher levels of the full-length corresponding αp53. PBMC and $CD34^+$ cells demonstrated a less intense shift towards αp53. The α/Δ ratio was significantly and similarly altered from control to 4 h treatment in AML cells *in vivo* and *in vitro*, while no significant change in α/Δ ratio was found in PBMC or $CD34^+$ cells *in vitro*. The p53 isoform shift could contribute to the understanding of the mechanisms behind frequent incomplete remission in AML.

Conclusion: The p53 α/Δ isoform shift is associated with leukemic cell debulkment in patients undergoing chemotherapy.

Introduction

Acute myeloid leukemia (AML) is a rapidly developing malignancy characterized by proliferation of immature cells which suppress normal bone marrow function (1-3). The overall long term disease-free survival rarely exceeds 50%, even for younger patients who can receive the most intensive treatment (4, 5). Current therapy of AML often consists of an induction regimen with anthracycline (e.g. daunorubicine, idarubicine) and cytarabine followed by consolidation therapies determined by prognostic classification (5). However, most patients are over the age of 60 years and therefore unable to undergo this intensive chemotherapy due to high risk of severe treatment-related toxicity. A constant challenge is therefore to offer effective therapy to the older patients where low-toxicity therapy is needed to obtain disease control (6-8). The development of molecular targeted therapy with attenuated toxicity may be the most effective way of obtaining increased overall survival for AML patients (9, 10).

The p53 protein is a sequence specific transcription factor that can halt progression through the cell cycle or initiate apoptosis upon activation by genotoxic stimuli (11). In contrast to other malignancies, where as many as 70% of cases may comprise mutations in TP53 (12), more than 90% of AML cases have the wild type gene (13, 14). Development of AML is a result of combined genetic defects, often including constitutive kinase activation together with alterations of transcription factors (15, 16). Gene expression profiling has demonstrated varying kinase and phosphatase expression (17), resulting in altered responsiveness in signaling pathways (18). This may be a possible mechanism for AML blast survival even in the presence of wild type p53.

The p53 protein is regulated by multiple post-translational modifications in response to chemotherapy as well as other cellular stress inducers (19). Post-translational modifications will

contribute to p53 stabilization and accumulation, leading to nuclear localization of the protein and specific gene transactivation (19). As many as 1500 genes have been reported to respond to p53 activation (20), including the extensively studied genes p21/cyclin-dependent kinase inhibitor-1A (21), BAX (22), MDM2 (23), and GADD45(24).

The p53 protein pattern has previously been examined by two-dimensional gel electrophoresis resolving p53 into multiple isoforms (25, 26), including protease truncated forms (27, 28). These forms have been postulated to be fundamentally important in the decision between growth arrest or apoptosis as the p53 response to genotoxic stress (27). In addition, alternative splicing of p53 mRNA can encode an amino-terminally deleted isoform of p53 termed p47 (29, 30). This isoform will suppress p53 transcriptional activity and impair p53-mediated growth suppression (29). Based on these observations, one would expect the presence of multiple isoforms of p53 protein that could disclose nuances in the cellular response *in vivo*.

We explored p53 protein modulation *in vivo* within the first 18 hours after start of chemotherapy. The p53 response was analyzed by using information dense methodology like two-dimensional p53 protein analysis and gene arrays with high number of probes (31), disclosing that the p53 protein responded to standard chemotherapy by protein accumulation and was accompanied by putative p53 target gene activation. A panel of p53 and p53-family directed antibodies was tested, demonstrating that the amino-terminal directed antibodies visualized the most dynamic protein response, with only limited responses in p63 and p73. The p53 isoform-analysis demonstrated a shift in p53 distribution from a hypothesized suppressive truncated protein form to the full-length protein as a response to *in vivo* chemotherapy in primary AML.

Materials and Methods

Patients

The study was approved by the local Ethics Committee and samples collected after informed consent. We collected peripheral blood AML blasts from five patients during chemotherapy. The patients were treated with intravenous infusion of idarubicine (12 mg/m^2 during 30 min on days 1-3) and cytarabine (190 mg/m^2 daily as a continuous infusion days 1-7). The clinical and biological characteristics of the patients are presented in Table 1. Analysis of 20 AML patients demonstrated that the p53 protein pattern shown in the present study was associated with wild type TP53 (N.A. B.T.G., manuscript in preparation).

Isolation and culture of cells

Cells were isolated from peripheral venous blood and collected before treatment at 4 and 18 hours after start of chemotherapy. Cells were prepared by density gradient separation (Ficoll-Hypaque; NyCoMed, Oslo, Norway; specific density 1.077). The percentage of blasts exceeded 95% for all samples.

Cryopreserved cells (32) sampled at diagnosis were also exposed to idarubicin (Pfizer Inc., New York, USA) *in vitro*. Cells were then incubated (37°C, 5% CO_2, humified atmosphere) for 2 and 4 hours in the presence of 360 nM idarubicin. The serum-free culture medium was Stemspan H3000 (StemCell Technologies, Vancouver, BC, Canada).

Peripheral blood mononuclear cells (PBMC) were collected from healthy donors, separated as described for AML cells, and were used fresh. CD34$^+$ peripheral blood progenitor cells were collected by apheresis from five patients with multiple myeloma or non-Hodgkin's lymphoma after mobilization with disease specific chemotherapy followed by granulocyte colony

stimulating factor (G-CSF). The cells were cryopreserved with 10% DMSO and stored for 6-24 months in liquid nitrogen (33). After thawing and washing with the CytoMateTM device (Baxter Oncology, Chicago, IL), isolation of $CD34^+$ cells was performed using the Isolex (3000 i) magnetic cell separation system (Baxter Cellular Therapies, Newbury, UK) following the manufacturers instructions. More than 90% of the isolated cells were $CD34^+$ and these cells were subsequently cryopreserved.

The human AML cell line HL-60 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunsweig, Germany; www.gbf.de/dsmz) and cultured in RPMI medium (Sigma-Aldrich Corp., St. Louis, MO, USA) with 10% fetal bovine serum.

Sample preparation for protein and gene expression analysis

Material for protein analysis was collected as previously described in detail (34, 35). Briefly, cells were washed in NaCl (9 mg/ml) and then lysed in 7% trichloroacetic acid (TCA). Protein precipitate was then washed in 5% TCA and water saturated ether to remove salts. The protein pellet was suspended in sample buffer for two-dimensional gel electrophoresis (7 M urea, 2 M thiourea, 100 mM dithiothreitol, 1.5% Ampholyte 3 – 10, 0.5% Ampholyte 5 – 6, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate). RNA was isolated from AML blasts as described previously (17). Aminoallyl-U (aminoallyl-UTP from Ambion) was incorporated into cRNA followed by cross-coupling of Cy5- and Cy3 by means of reactive Cy-NHS compounds (Amersham Biosciences AB, Uppsala, Sweden) in order to generate fluorochrome labeled targets for DNA microarray analysis.

Gene expression analysis

The Agilent Human Whole Genome Oligo Microarray (44K; Agilent Technologies, Inc., Palo Alto, CA, USA) was used for gene expression analysis of patient 1, 4 and 5, and the Human 1 cDNA microarray (Agilent) containing 12.814 cDNA clones was used for validation purposes (17). The procedure was performed according to the manufacturer's protocols, except for a more stringent wash (0.1xSSC at 35°C for 10 minutes for the Oligo Array). The oligonucleotide microarrays were scanned and features automatically extracted, recorded and analysed using the Agilent Microarray Scanner Bundle. Normalization, flooring or filtration of data were done according to previously described in-house bioinformatic procedures (17, 36). Rank Product analysis (37) was used to detect differentially expressed genes in the oligonucleotide microarray experiments. The data were formatted in a J-Express-file suitable for additional data mining (http://www.molmine.com/). Selected genes and samples were clustered by hierarchical clustering using average linkage and Pearson correlation as similarity metrics.

For further confirmation of array data, mRNA level of p21, BAX, PUMA, GADD45 and MDM2 was analyzed using TaqMan Low Density Arrays (LDA), 384-well customizable microfluidic cards for real-time PCR (Applied Biosystems, Foster City, CA, USA). Each LDA card was configured for genes in duplicates including Celera gene ID. Hexamer-primed single stranded cDNA corresponding to 5 ng of AML blast total RNA was diluted in TaqMan Universal buffer (Applied Biosystems) and added to each loading well. The samples were distributed to the microwells by centrifugation for 1 min at 343xg. The cards were sealed and placed in the ABI 7900 Sequence Detection System using the following cycling parameters: 2 min at 50°C, 10 min at 95°C, and 40 alternate cycles of 15 s at 95°C and 60 s at 60°C. The SDS2.2 software was used for qualitative analysis and data were exported to Excel spreadsheet (Microsoft, Inc.) for further exploration and visualization.

Gel electrophoresis and immunoblotting

Protein separation was performed by SDS-PAGE following standard procedures. Two-dimensional gel electrophoresis (2DE) 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 sample rehydration buffer (8 M urea, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 20 mM dithiothreitol, 1.5% Ampholyte 3 – 10, 0.5% Ampholyte 5 – 6, traces of bromphenol blue) over night at room temperature to rehydrate the strips. Protein was added directly to the rehydration buffer. Isoelectric focusing (IEF) was performed at 200 V for 40 min, 450 V for 30 min, 750 V for 30 min and 2000 V for 60 min. 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 dithiothreitol and then 15 min in LDS sample buffer containing 125 mM iodacetamide. 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 min, after which the proteins were transferred to polyvinylidene fluoride membrane by standard electroblotting.

The p53 protein was detected using Bp53-12 monoclonal antibody, recognizing an aminoterminal epitope of p53 (epitope mapping aminoacids 20-25). Bp53-12 visualized p53 identical to the antibody DO-1 (epitope mapping aminoacids 21-25) and Ab-2/ PAb1801 in two-dimensional immunblot. Bax protein was detected using primary 2D2 antibody, p63 protein was detected using primary D-9 antibody and p73 was detected using primary E-4 antibody (all purchased from Santa Cruz Biotechnology, CA, USA). Actin was detected using primary AC-15 antibody from Abcam, Cambridge, UK. Phosphospecific p53 antibodies against serine residues 37, 46 and 392 were purchased from Cell Signaling Technology, MA, USA. Secondary horse

radish peroxidase conjugated mouse or rabbit antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were visualized using the Supersignal West Pico or Supersignal West Femto Chemoluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). The immunoblots were imaged using a Kodak Image Station 2000R (Eastman Kodak Company, Lake Avenue, Rochester, NY, USA), and spots were quantified using the Kodak analysis software. Data were exported to Excel spreadsheet, and a Students two-tailed t-test were used for statistics of the ratio between αp53 and Δp53.

Results

p53 and gene expression in human AML cells during *in vivo* exposure to chemotherapy

In cell protein extract from five AML patients we found an expected up-regulation of p53 protein in response to induction chemotherapy (Fig. 1A, Fig. 4) followed by induction of the p53 inducible protein Bax (Fig. 1A). This observation indicated p53 functionality. To determine if the p53 response also involved additional p53 targeted genes we randomly selected three patients (Table 1, Patients 1, 4 and 5) for gene expression analysis (Fig.1B; see Fig. 4 for *in vivo* p53 protein response of these patients). mRNA isolated from AML cells before and after 4 hours of chemotherapy was analyzed by high density oligonucleotide microarray (Fig. 1B), and the ten most up-regulated mRNA signals were all putative p53 inducible genes: p53-inducible cell survival factor (38); BAX (22); sestrin 2/Hypoxia induced gene 95 (39, 40); HDM2 (41); GADD45A (24); Ferredoxin reductase variant 2 (42); BBC3/PUMA (43); TP53INP1/p53 inducible nuclear protein 1 (44); p21/cyclin-dependent kinase inhibitor-1A (21, 45); and sestrin 1/p53 regulated PA26 nuclear protein (46). The increased expression of p53-induced genes was reproduced by mRNA expression analysis of GADD45, PUMA, p21, and sestrin 1 using cDNA

microarray (data not shown). The false discovery rate of the presented genes varied from <0.05% to 0.1%, and the correlation between the gene expression data obtained from oligonucleotide microarray and cDNA microarray analysis ranged between 85% and 97%. The strong chemotherapy-induced expression of BAX, HDM2, GADD45, PUMA, p21, and sestrin 1 was further confirmed using TaqMan Low Density Arrays (data not shown). Taken together, our protein- and gene expression analysis in AML cells from patients undergoing chemotherapy suggest that p53 is functional and causes appropriate induction of p53-regulated genes (Fig. 1A, B).

Mapping of p53 protein patterns by two-dimensional immunoblots (2DI)

Our experimental models were further characterized by examining normal PBMC and the p53-deficient AML cell line HL-60. This method revealed a number of p53 reactive spots (Fig. 2A), including an approximately 63 kDa spot (pI 5.2), the putative full-length p53 (approx. 53 kDa, pI 5.0-6.5) denominated αp53, and a group of spots below 50 kDa (approx. 45 kDa, pI 4.8-5.3) denominated Δp53. The αp53 and Δp53 were not present in extract from the p53 deficient HL-60 cell line (data not shown). The observed pattern was detected using the Bp53-12 antibody directed towards the extreme amino-terminus of p53 (aminoacids 20-25). This part of the protein contains several phosphorylation sites modified by stress-related kinases like Chk1, Chk2, ATM and p38 and is a highly important domain for p53 activity. Using an antibody directed towards this part of the protein ensures the detection of transcription capable p53 and might also detect changes in isoform distribution caused by changes in expression of p53 kinases. Other amino-terminal antibodies (DO-1 and PAb1801) revealed the same isoform distribution (data not shown). The 63 kDa spot could be visualized using a p63 specific antibody, suggesting that our

p53-specific antibody recognized this distinct member of the p53 family (Fig. 2B). The p63 specific antibody was also reactive with other spots showing inter-patient variance (Fig. 2B). The p53 family member p73 protein was visualized in the same PBMC extract, but appeared non-reactive with the Bp53-12 antibody (Fig. 2B). Phosphorylated p53 (phospho-Ser37, phospho-Ser46 and phospho-Ser392) was not detected in unstimulated PBMC or HL-60 (Fig. 2C).

Modulation of p53 protein patterns by chemotherapy

For examination of p53 modulation after chemotherapy, AML cells derived from patients 1 and 2 were treated with idarubicin *in vitro* and p53 protein was thereafter characterized using 2DI. After two hours, we observed a marked increase in αp53 and the αp53/Δp53 ratio (Fig. 3A). In untreated cells the ratio usually is Δp53> αp53, but after cell stress this balance was disturbed and a shift towards αp53 was observed. For comparison with non-transformed primary cells we used $CD34^+$ mobilized progenitor cells and normal leukocytes (PBMC), where a similar but slightly attenuated response was observed (Fig. 3B, C). The p53 family members p63 and p73 did not respond significantly to *in vitro* idarubicine treatment, and freshly isolated PBMC appeared and responded similar to cryopreserved PBMC (data not shown).

p53 protein from five patients sampled *in vivo* was then analyzed using 2DI and compared to the *in vitro* treated cells. In most patients we detected the same shift in αp53/Δp53 ratio as observed when patient cells were treated *in vitro* (Fig. 4). This was an early response (4 hours) to treatment. The increase in αp53 most likely reflects stabilization of the wild type p53 protein. Later during treatment the increase in αp53 was halted, possibly through HDM2 feedback down-regulation of p53 (47).

The ratio of αp53/ Δp53 was determined for the five *in vivo* treated patients (Fig. 4) and compared with five different in vitro treated cryopreserved AML patients. The αp53/ Δp53 ratios in pre-chemotherapy samples (mean 0.96, standard deviation 0.06, n=5) and control *in vitro* samples (2-4 h incubation with vehicle; mean 0.63, standard deviation 0.17, n=5) were similarly and significantly increased after 4 h chemotherapy (*in vivo*: mean 1.72, standard deviation 0.65, n=5, p=0.01; *in vitro*: mean 1.26, standard deviation 0.48, n=5, p=0.01). The αp53/Δp53 ratio in control incubated PBMC (mean 0.63, standard deviation 0.10, n=5) or CD34+ progenitor cells (mean 0.57, standard deviation 0.23) demonstrated no significant shift in αp53/Δp53 ratio compared to 4 h incubation with idarubicin (PBMC: mean 0.77, standard deviation 0.17, n=5, p=0.10; CD34+: mean 0.93, standard deviation 0.28, n=3, p=0.08). Patients and normal donors were randomly selected.

For one patient (Pt 1), an increase in αp53/ Δp53 ratio was observed only *in vitro* but not *in vivo* (Fig. 4), and the αp53 immediately before chemotherapy was up-regulated as during cell stress. The apparent difference in αp53 signal strength between *in vitro* and *in vivo* control cells (Figs. 3A, 4) can probably be explained by collection of samples at different time points. Crypreserved cells were collected at diagnosis, whereas two days later at the start of chemotherapy the patient had developed progressing disseminated intravascular coagulation followed later by multiorgan failure and early death (Table 1).

Discussion

In our experiments we observed p53 and Bax protein induction in response to chemotherapy as would be expected from previous *in vitro* studies (48) (Fig. 1A). Up-regulation of putative p53 target genes was also observed after chemotherapy (Fig. 1B). During AML induction therapy

apoptosis was not seen in the peripheral circulation up to 18 hours (B.T.G., unpublished data), and previous studies could only detect apoptotic AML cells in bone marrow after 24-48 hours (49, 50). At the same time, cytopenia developed within 3-6 days in our patients (Table 1) with significant decline in peripheral leukemic cells during the first 18 hours. The lack of apoptotic cells in the circulation during induction chemotherapy is probably due to the organism's high capacity of apoptotic cell eradication from peripheral blood by the spleen and liver (51). We therefore suggest that the gene induction observed in this study represents an early chemotherapy-induced event and exhibits the importance of p53 inducible genes *in vivo*. The majority of the most intensely induced genes, e.g. sestrin 1, sestrin 2, BAX, PUMA, TP53INP1, and cyclin-dependent kinase inhibitor-1A, are reported to induce apoptosis if their expression is enforced one by one *in vitro* (39, 40, 43, 44, 46, 52). On the other hand, p53-inducible cell survival factor (p53CSV), HDM2 and ferredoxin reductase variant 2 are known to protect against cell death (38, 41), suggesting that this p53 transcriptional induction comprises genes that may improve the efficiency of therapy if targeted. A question that calls for more extensive studies is whether the p53 response in AML may mirror a pro-apoptotic gene expression bias compared to normal myeloid progenitor cells.

The p53 protein is extensively regulated post-translationally by phosphorylation, acetylation, sumoylation and ubiquitination (11). Many signaling enzymes known to modify p53 are themselves mutated in cancers, thereby mimicking disease with p53 mutation (53). One of the enzymes of particular interest over the recent years has been Chk2 which has been reported to be mutated in a Li-Fraumeni syndrome family with normal p53 (54). As p53 mutations are rare in AML, kinase mutations may contribute to the AML pathogenesis and be a contributing factor causing incomplete remission. In this study we used a p53 antibody directed towards the extreme amino-terminus of p53 (aminoacids 20-25), a region that contains phosphorylation sites modified

by Chk2 as well as other cell stress-related kinases (19, 55). The p53 protein isoform distribution could reflect altered expression and/or activity of these kinases in AML, suggestive for a study of the p53 protein in a more extensive AML-material. In AML, another example of mutations affecting p53 function is the increased deacetylation of p53 promoted by the oncogenic fusion protein PML-RAR, resulting in p53 degradation and repressed p53 activity (56). In our study the 2DI methodology failed to map the distribution of selected phosphorylated residues in p53 isoforms in resting cells (Fig. 2C). However, it has previously been shown that most of the p53 forms visualized on a two-dimensional map are phosphorylated (25), and future efforts are planned to elucidate the nature of post-translational modifications behind the p53 isoform map.

In vitro experiments have suggested that certain cleavage forms of p53 influence the apoptotic response after chemotherapy (27) and a frequently occurring shorter splice form of p53 has been detected in normal tissue, obeying a dominant negative effect on the full length protein (29). Based on this one could speculate if the shorter Δp53 observed here will hold qualities that to some degree control the activity of full-length αp53. A possible interpretation of the p53 modulation seen *in vivo* (Fig. 4) is that the chemotherapy releases the cancer cell from the suppressive Δp53. Therefore, a cancer cell's ability to modulate αp53 and Δp53 may influence on chemoresitance.

$CD34^+$ progenitor cells seemed to have an attenuated p53 induction (Fig. 3), an observation that could be in line with reports that normal progenitors are more chemoresistant than AML cells (57-59). The question that rises is if analysis of p53 protein isoform signatures could provide additional information about therapy response and risk for relapse even when the TP53 gene is wild type. A more extensive study including more patients, compared with carefully selected progenitor cells, will be needed to address this question.

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Table 1. Clinical and biological characteristics of acute myeloid leukemia patients[1]

Patients	Sex	Age	FAB	Membrane molecules					Karyotype	Flt3[3]	WBC before therapy	Time to Cytopenia	Remission after 1th induction (3)	Survival (months)
				CD13	CD14	CD15	CD33	CD34						
1	M	30	M3	+	-	-	+	+	t(15;17)	ITD	29	3	n.d.	0
2	F	58	M1	+	-	-	+	+	Normal	WT	18	6	-	5
3	M	46	M4e	+	-	-	+	+	inv16	WT	50	4	+	>8
4	M	60	M4/5	+	-	+	+	-	Normal	ITD	71	6	+	>23
5	M	55	Atypical	+	-	+	+	-	Multiple	WT	6	5	+	>23

Note: The five patients were randomly selected between a total of 15 patients admitted to the hospital during a one-year period. Patient 1 had platelets <5 x10^9/L, LDH 815 U/L, fragment D-dimer >20 mg/L before start of chemotherapy, and developed sepsis and multi organ failure within two weeks. Patient 1 died before determination of haematological remission (n.d.). Patient 4 had previously diagnosed prostate cancer not treated with chemotherapy. The class III receptor tyrosine kinase Flt3 was analysed for juxtamembranous length mutation (ITD) as previously described (18). Time to cytopenia is defined as days before <0,5x10^9/L white blood cells (WBC) in peripheral blood.

Figure legends

Figure 1. p53 response in de novo AML patients treated with standard chemotherapy. (A) A representative p53 protein response after chemotherapy induction with daunorubicine and cytarabin illustrated in patient 2 (see Table 1). Cells were collected as described in Materials and methods before start of induction and at three different time points (h; hours). Note the delay of Bax protein expression. (B) Hierarchical cluster analysis showing the ten highest ranked p53-induceable genes activated by *in vivo* chemotherapy examined in patients P1, P4 and P5 by 44k oligonucleotide microarray. p53 induceable genes were selected and ranked according to t-scores. The genes and patients were clustered by hierarchical clustering using average linkage and Pearsons correlation as similarity metrics. The heat diagram illustrates changes in mRNA expression in AML blasts from three patients before treatment (before chemo) and 4 hours post-induction chemotherapy. Red fields indicate higher values than standard, green fields indicate lower values than standard and black fields indicate values that are equal to the standard. False discovery rates for the ten genes indicated in the dendrogram were 0.0% to 0.1%, and p-values ranged from $3.9x10^{-11}$ to $4.1x10^{-8}$. Abbreviations used: P53CSV, p53-inducible cell survival factor; BAX, BCL2-associated X protein variant beta; SES2, Hypoxia induced gene 95; MDM2, Homo sapiens MDM2 (transformed 3T3 cell double minute 2); GADD45A, growth arrest and DNA damage-inducible alpha; FDXR, ferredoxin reductase variant 2; BBC3/PUMA, BCL2 binding component 3; TP53INP1, p53 inducible nuclear protein 1; CDKN1A, p21/Cip with mutation Ser31→Arg and p21/Cip transcript variant 1; SESN1, p53 regulated PA26 nuclear protein.

Figure 2. Mapping of the p53 protein in normal PBMC by two-dimensional gel electrophoresis and immunoblot (2DI). Our analysis facilitated mapping of total p53 in AML cells detected by antibody Bp53-12 (A), identifying putative p63 (circled upper spot), a

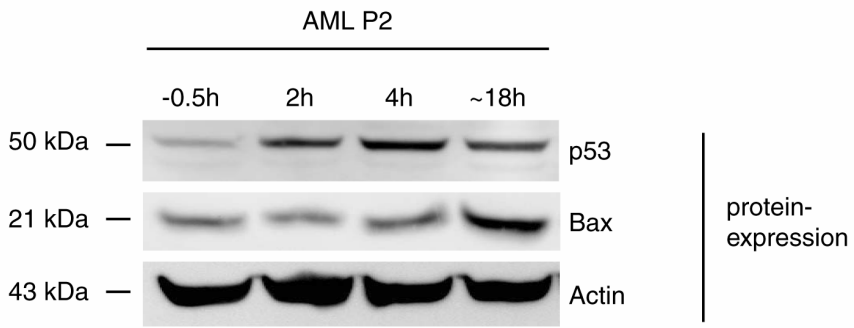
non-identified putative p53 cleavage product or short form (lower dotted spot), and a spot group corresponding to full length p53 (upper dotted spot). Reproducibility of 2DI was explored by examining three healthy donors of peripheral blood mononuclear cells (PBMC) in three separate runs of 2DI, examining total p53 (Bp53-12), p63 (D-9) and p73 (E-4) (B). The estimation of molecular weight (kDa) and isoelectric point (pI) of different p53 family members are described in Materials and methods. Note that p73 is not detected by Bp53-12. Phosphospecific antibodies did not detect phosphorylated p53 at three serines (pSer37, pSer46, pSer392) in unstimulated PBMC and the HL-60 AML cell line (C).

Figure 3. p53 protein response in AML , $CD34^+$ progenitor cells, and normal PBMC after treatment with idarubicin *in vitro*. Patient 1 and 2 were incubated with 360 nM idarubicin for the time indicated (h; hours) (A). In parallel, peripheral blood progenitor cells ($CD34^+$) and peripheral blood mononuclear cells (PBMC) were treated similarly (B,C). The spot intensities in spots representing full-length p53 (αp53) and Δp53, were measured. The diagram indicates the ratio of αp53/Δp53 in arbitrary units. Note the slightly delayed response in full-length p53 in peripheral blood progenitor cells ($CD34^+$) and PBMC compared to the patients samples. Storage, thawing and experimental procedure was identical for AML and $CD34^+$ cells, as described in Material and methods. The blots shown are representative for three independent experiments using primary AML, $CD34^+$ cells or PBMC from healthy donors.

Figure 4. p53 isoform modulation during chemotherapy *in vivo*. AML cells from patients treated with chemotherapy *in vivo* are analyzed by 2DI. Note the shift in p53 from the Δp53 form towards the full length αp53 protein. Control cells were collected 0.5 hour before start of treatment (-0.5h). Right side plots demonstrate changes in α/Δ p53 protein ratio in

arbitrary units. The blots shown are representative for two-three independently performed immunoblots.

A



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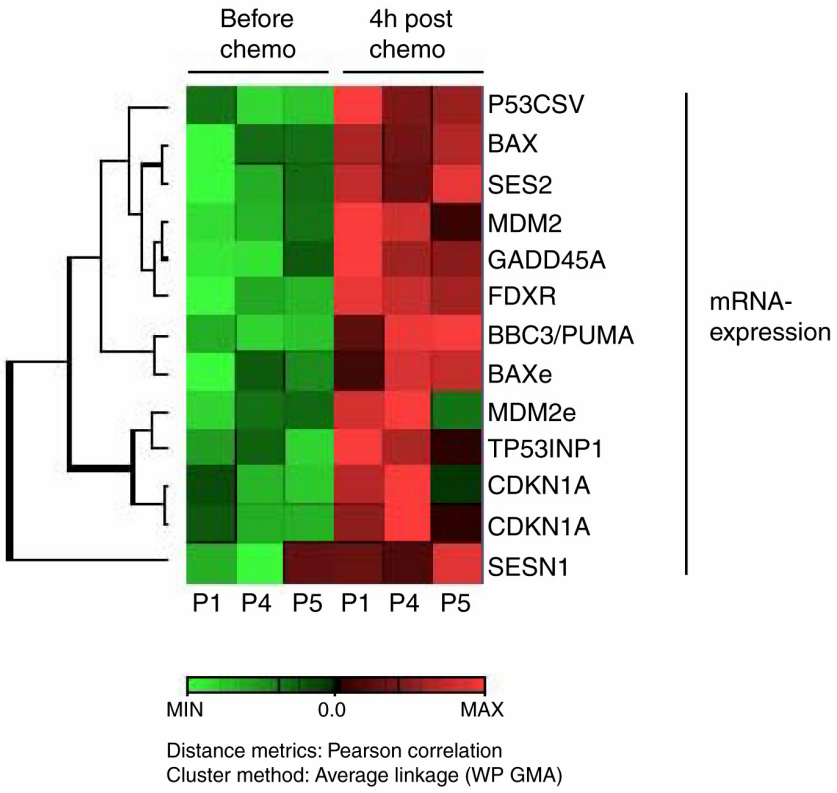

Figure 1. p53 response in de novo AML patients treated with standard chemotherapy.

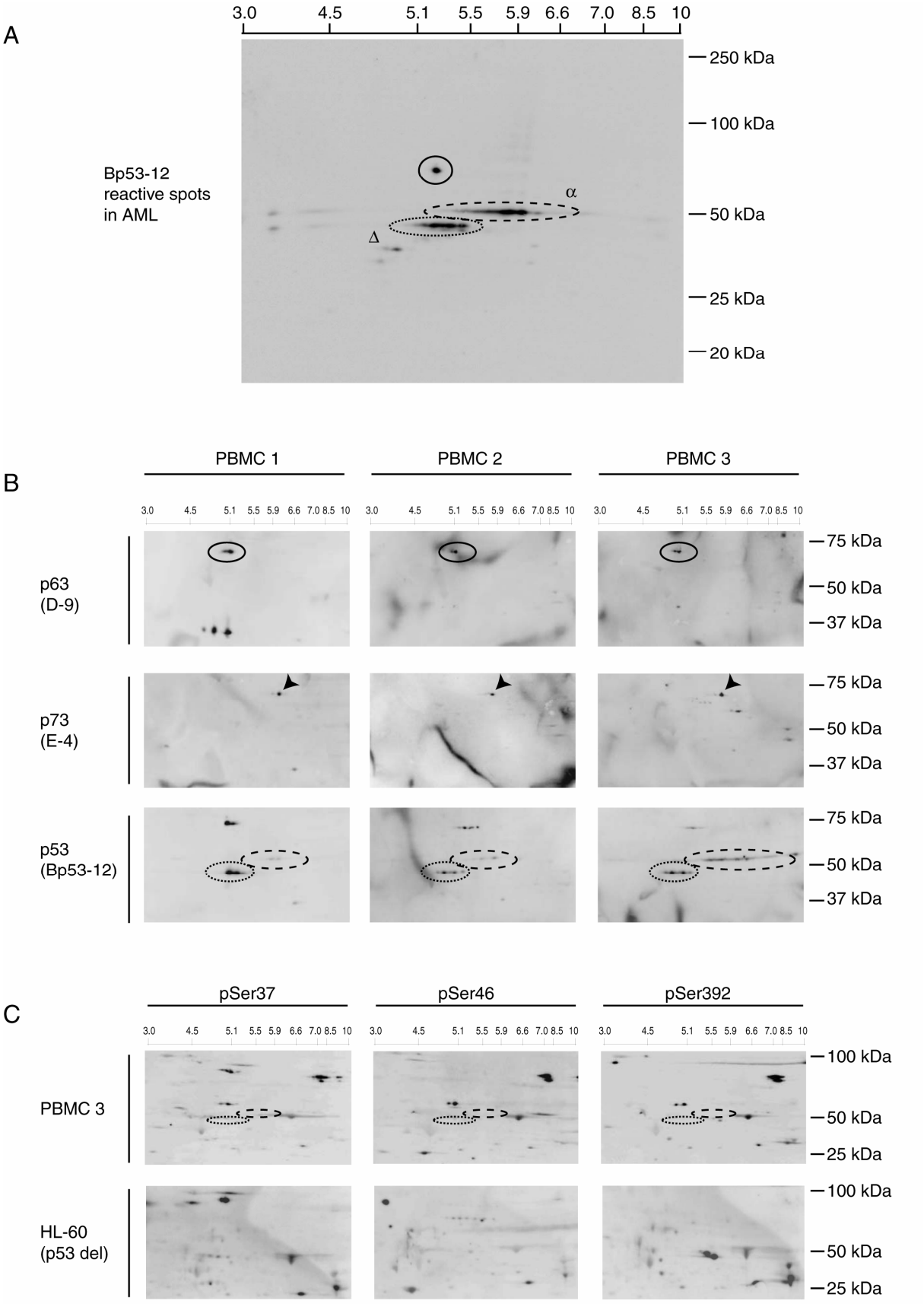

Figure 2. Mapping of the p53 protein in normal PBMC by two-dimensional gel electrophoresis and immunoblot (2DI).

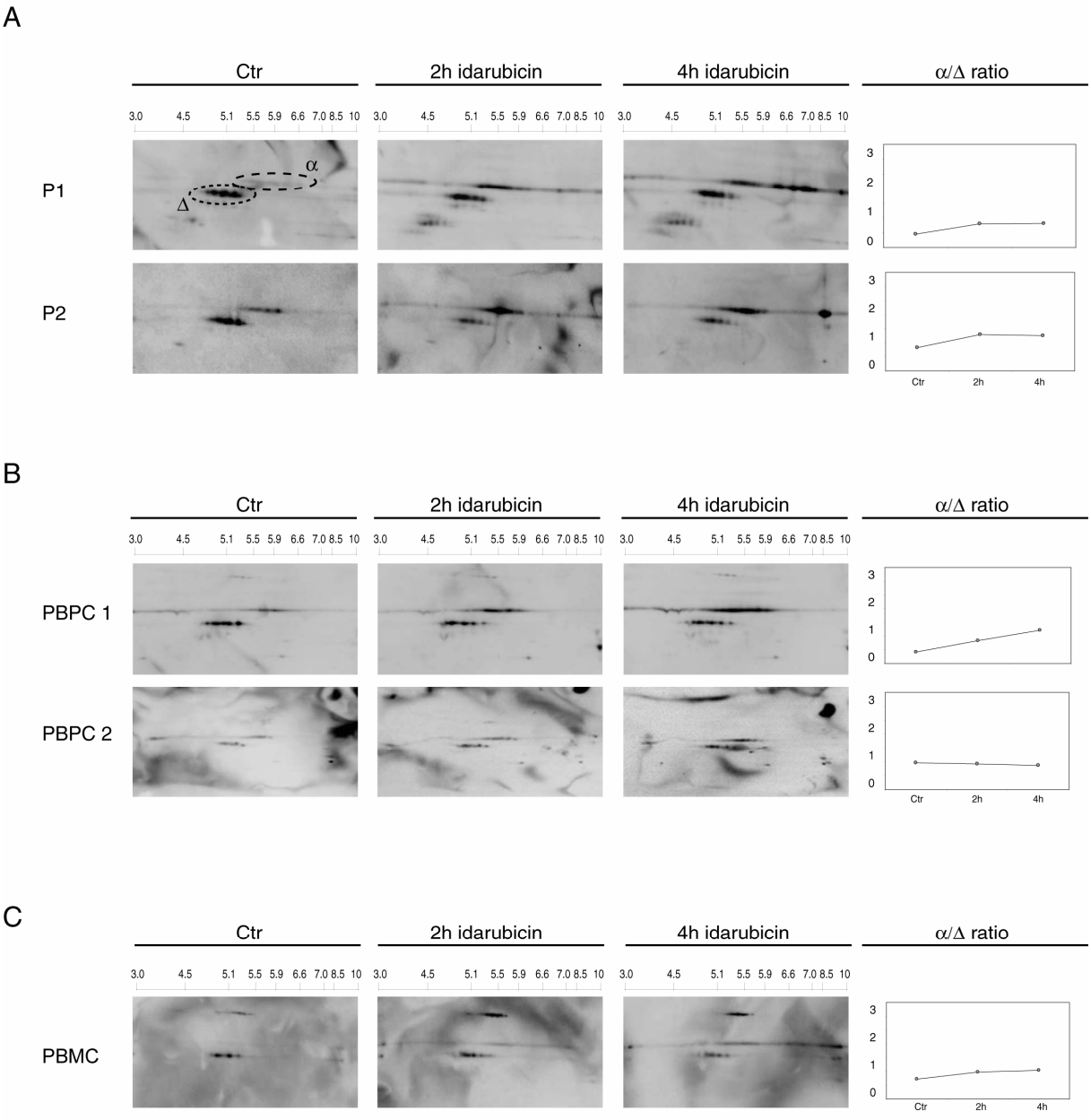

Figure 3. p53 protein response in AML , CD34+ progenitor cells, and normal PBMC after treatment with idarubicin in vitro.

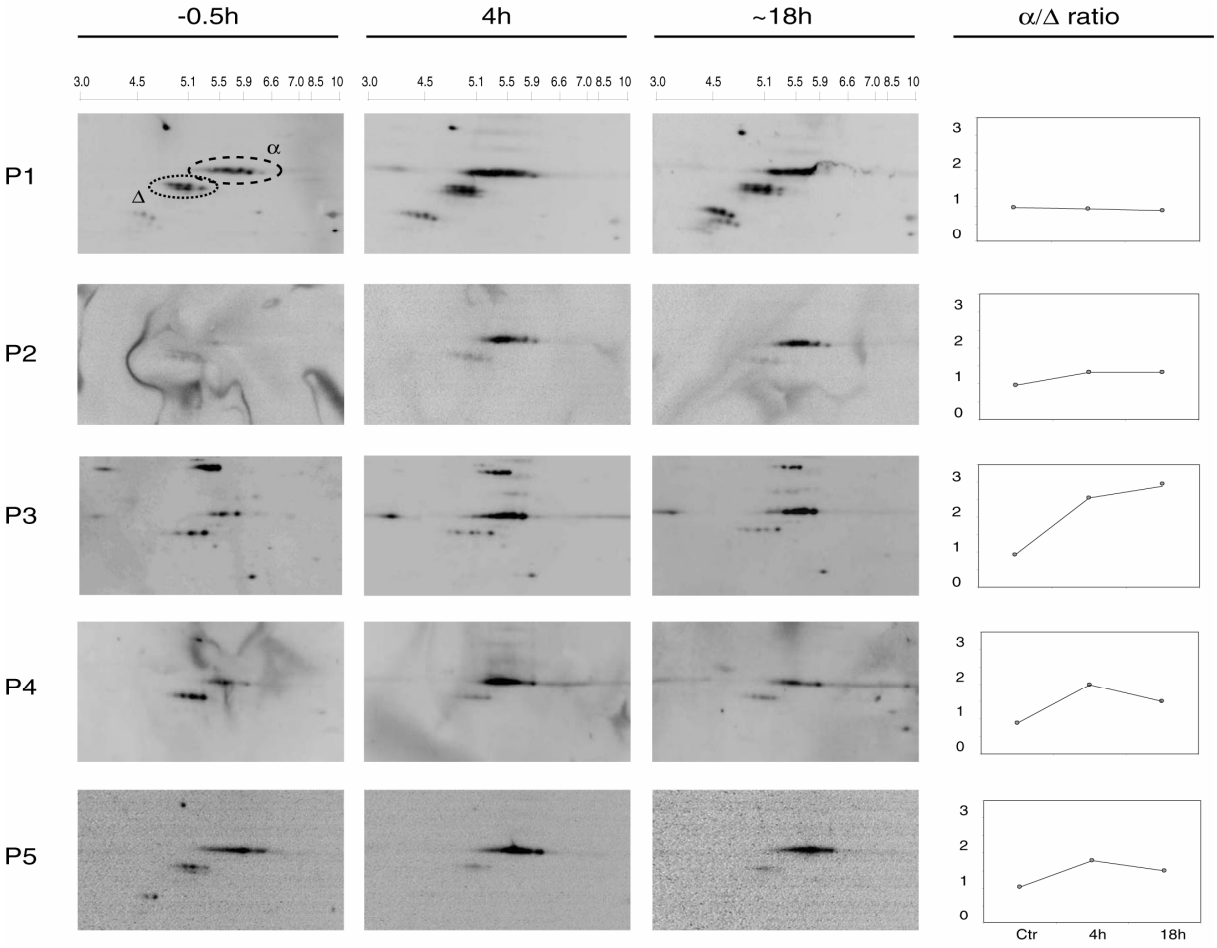

Figure 4. p53 isoform modulation during chemotherapy in vivo.