p53 protein biosignatures in acute myeloid leukemia

by

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Scientific environment

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Bergen, October 2005

Nina Ånensen

Man må holde fast ved troen på at det ubegripelige kan begripes.
JW Goethe

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Abbreviations

2DI	Two-Dimenisonal Electrophoresis and immunoblot
AML	Acute myeloid leukemia
AML1-ETO	acute myeloid leukemia-1/-eight-twenty-one
AraC	Cytosine arabinoside
ATRA	All-trans retinoic acid
Bax	bcl 2 associated x protein
Bcl-2	B-cell lymphoma gene 2
BH	Bcl-2 like homology
BMT	Bone morrow transplantation
CBP	CREB-binding protein
Cdk	Cyclin dependent kinase
Flt3	Fms-like tyrosine kinase 3
LFS	Li-Fraumeni syndrome
MAPK	Mitogen activated protein kinase
Mdm2	Murine double minute 2
Nedd8	Neural precursor cell expressed, developmentally down-regulated 8
NES	Nuclear export signal
NLS	Nuclear localization signal
PCAF	p300/CBP-associated factor
PML-RAR	Promyelocytic leukemia/ -Retinoic acid receptor
SH2/SH3	Src homology 2/3
STAT	Signal transducer and activator of transcription
SUMO-1	Small ubiquitin-related modifier-1

Summary

p53 is a tumor suppressor protein often regarded as the guardian of the genome. It is a highly connected protein involved in many signaling processes in the cell. The inactivation of p53 through genetic mutations in TP53 is common in human cancers and can be detected in more than 50% of malignant tumors. In acute leukemia however, p53 inactivation is not normally a part of leukemogenesis and TP53 is found to be wild-type in >90% of the cases. This thesis has sought to elucidate the nature of the p53 protein regulation in acute myeloid leukemia to increase our understanding of disease development. The experiments have proposed that p53 is wild type, expressed and capable of transactivation of target genes. However, p53 phosphorylation could be correlated to expression of the anti-apoptotic protein Bcl-2 suggesting that Bcl-2 can function as a downstream block to p53-mediated apoptosis. Furthermore, Bcl-2 levels could be associated to a specific mutation in the receptor tyrosine kinase Flt3. Flt3 mutation has been found to be a strong predictor of disease relapse in AML, and driving Bcl-2 expression, thereby inhibiting the p53 pathway, may propose a new important event in leukemogenesis.

This thesis has further shown that p53 exists as two main isoforms in patient material from acute myeloid leukemia. The expression of these was influenced by chemotherapy *in vivo* and induction of one specific form correlated to induction of known p53 target genes.

The p53 protein has many known sites for post-translational modifications and serves as a substrate for many enzymes. The p53 protein may thus be a central node in a large network of proteins whose activities are critical for cell life and death. This may suggest that specific p53 signatures could serve as a ‘read-out’ for the p53 network. The expression of the described p53 isoforms were, using a novel correlation algorithm, found to be correlated to several clinical parameters including survival, remission and Flt3 mutation. This could imply that p53 may be used as a possible biomarker for clinical stratification of leukemia patients.

List of papers

Paper I

Wild type p53 is expressed and phosphorylated in leukemia cells with Flt3 Y591 amplification and Bcl-2 overexpression
Irish JM*, Ånensen N*, Hovland R, Børresen-Dale AL, Bruserud Ø, Nolan GP, and Gjertsen BT.
Manuscript.

Paper II

A distinct p53 protein isoform signature reflects the onset of induction chemotherapy for acute myeloid leukemia
Ånensen N, Øyan AM, Abrahamsen JF, Kalland KH, Bruserud Ø, and Gjertsen BT.
Manuscript submitted.

Paper III

Correlation analysis of two-dimensional gel electrophoretic protein patterns and biological parameters: p53 biosignatures reflect origin of cancer and stage of differentiation
Van Belle W, Ånensen N, Haaland I, Bruserud Ø, and Gjertsen BT.
Manuscript.

Paper IV

p53 protein biosignatures correlate with chemotherapy response and survival in acute leukemia
Ånensen N, Van Belle W, Bruserud Ø, and Gjertsen BT.
Manuscript.

Paper V

Acute myelogenous leukemia in a patient with Li-Fraumeni syndrome treated with valproic acid, theophyllamine and all-trans retinoic acid: A case report
Ånensen N, Skavland J, Stapnes C, Ryningen A, Gjertsen BT, and Bruserud Ø.
Manuscript.

*Jonathan Irish and Nina Ånensen are equal first authors.

Preface

The p53 protein, which was discovered in 1979 (1,2) was originally described as a cellular protein bound to the large T antigen of simian virus 40 (SV40). It was suggested that this protein was responsible for SV40 induced cell transformation. Quickly after its discovery it became clear that the p53 protein was highly involved in the development of cancer and an extensive effort was initiated to elucidate its functions. The p53 encoding gene, TP53, was characterized in 1986 (3) demonstrating a 20 kb gene with 11 exons located on the short arm of chromosome 17 (4). Allelic deletions of this chromosome region were early associated with colorectal carcinomas (5) and it was established that these deletions could be related to TP53 (6). Sequencing of TP53 mutations in human cancers later demonstrated that 98% of mutations fall within a 600 base-pair region of the gene which encompasses exons 5 through 8 (7).

For a long time it was assumed that TP53 was an oncogene, but several studies in the late 1980's suggested that the true effect was in fact tumor suppression, summarized by Baker and co-authors (6). Tumor suppressor genes are genes that control unlimited cellular growth either by a repressive effect on cell cycle regulation or promotion of apoptosis. During the 1990's the TP53 protein product, p53, was found to execute its regulatory functions by controlling both of these cellular processes.

Since its discovery in 1979 there has been an overwhelming amount of research in the p53 field with more than 36000 published papers – almost 3000 in 2005 alone. This emphasizes the importance of this protein and the impressive network of cell regulation signals it controls. Fully understanding the biology behind this cellular gatekeeper would therefore be one of the most important scientific revelations in the history of cancer research, aiding the development of novel strategies for treatment including restoration of p53 function in tumors with mutant TP53.

Introduction

Acute myeloid leukemia

Acute myeloid leukemia (AML) is a malignant disease of the myeloid lineage of hematopoietic cells which can develop at any stage of the maturation process. The AML cells have a differentiation block that results in an accumulation of immature myeloblasts ultimately leading to suppression of normal bone marrow function. The clinical signs of AML are diverse and nonspecific including fatigue, hemorrhage, infections and fever, all symptoms resulting from leukemic infiltration of the bone marrow with resultant cytopenia (8). Acute leukemia is diagnosed upon the presence of more than 30% leukemic blasts in the bone marrow and cell morphology according to a system suggested by the French-American-British (FAB) cooperative group (9,10). A new classification for the diagnosis of myeloid malignancies was recently proposed by the world health organization (WHO) (11,12) in which the blast percentage required in the bone marrow for a disease to be characterized as AML has been reduced to 20%. Furthermore, this system recognizes four subgroups of AML; 1) AML with recurrent genetic abnormalities, 2) AML with multilineage dysplasia, 3) therapy related AML, and 4) AML not otherwise categorized (12). Recurring genetic abnormalities combined with morphology are thus used as clinical criteria upon diagnosis.

AML is characterized by a number of non-random genetic defects including several chromosomal translocations (13). These cytogenetic aberrations are used to determine the prognosis of disease outcome (14-16). To date, approximately 200 different chromosomal changes have been detected in AML, some occurring more frequently than others. The more common translocations include t(8;21)(q22;q22) resulting in the AML1-ETO fusion protein often associated with AML-M2 and t(15;17)(q22;q21) leading to the fusion protein PML-RARα associated with promyelocytic AML-M3. These are both prognostically favorable changes. Aberrations found to give adverse prognosis includes e.g. changes involving deletions of chromosomes 5 or 7 (-5/-7) (cytogenetics in acute leukemia is excellently reviewed by 16). In the later years gene expression profiling has refined risk stratification of AML and it has been shown that particular gene expression signatures can correlate to clinical outcome. This is a new methodology that in the future will increase the ability to classify leukemia on the molecular level (reviewed in 17).

Aberrations in oncogenes and transcription factors are also important in determination of AML prognosis. One of the most important of these changes is a length mutation found in the juxtamembrane region of the receptor tyrosine kinase Fms-like tyrosine kinase 3 (Flt3) (18). This mutation is always in-frame but varies in the length of the duplicated area. Recently, a second Flt3 mutation, a point mutation in exon 20, was reported which leads to an amino acid substitution in residue 835 (D835Y/D835H/D835del) (19). Mutation of Flt3 results in constitutive activation of the receptor and has recently been found to be the strongest separate marker for disease relapse (20).

Further prognostic indicators for AML can be found in the Bcl-2 family of proteins. Over-expression of the antiapoptotic Bcl-2 protein leads to prolonged survival of malignant cells and is associated with chemoresistance (21). Recently, the ratio of Bcl-2 to the proapoptotic family member Bax was established to predict disease outcome in AML (22). This ratio will control the choice between cell survival and death and greatly influence disease progression.

TP53 in AML

Loss of p53 function due to mutations in the TP53 gene is very common in human cancers. TP53 mutation frequencies vary among different tumor types but have in some solid tumors been detected in more than 50% of patients (7,23,24). In AML however, TP53 mutations are less common with an occurrence below 10% (25-27). TP53 mutations in AML are associated with cytogenetic aberrations involving chromosome 17p monosomy (28) as well as secondary leukemia (29) and have been known to correspond with resistance to chemotherapy and ultimately lower complete remission rates (30).

Signaling pathways in AML

AML is a disease characterized by numerous genetic defects including improper activation of signaling pathways leading to inappropriate regulation of cell division and apoptosis (13). Two crucial signal transduction networks known to be active in AML progenitors are the signal transducer and activator of transcription protein (STAT) pathway (31,32) and the Ras/mitogen activated protein kinase (MAPK) pathway (33). One of the most important negative prognostic factors in AML, the receptor tyrosine kinase Flt3, is thought to act upstream of both the STAT and Ras/MAPK pathways. It has been shown that Flt3 activation through a length mutation in the juxtamembrane region will lead to constitutive activation of STAT5 and MAPK (34). A recent study demonstrated that these signaling pathways display distinct network profiles in malignant cells and these profiles can be related to Flt3 and disease outcome in AML (35).

Treatment

Induction therapy for AML has for decades consisted of a combination of an anthracycline (daunorubicin, idarubicin) and cytosine arabinoside (AraC). AraC is given by continuous infusion at 200 mg/m^2 for seven days, while anthracyclines are given as an intravenous infusion for 30 min a day at 45 mg/m^2 for the three first days of AraC treatment (36). Remission is achieved when the bone marrow contains <5% leukemic blasts (8). After remission is achieved, consolidation treatment consisting of high dose AraC (3000 mg/m^2) administered in a 3-hour infusion every 12 hours (twice daily) on days 1, 3 and 5 is initiated. This course of treatment is repeated 3 times for a total of 4 consolidations (36). Bone marrow transplantation (BMT) is often offered as a consolidation treatment to younger patients (<60 years) after first remission. Patients may be offered this treatment based on their previous risk assessment and both allogenic and autologous BMT can be considered. Patients with good-risk cytogenetics are often treated with chemotherapy alone, while intermediate or bad-risk patients are offered BMT as consolidation (reviewed in 37).

Over the years many new treatment options for AML have been proposed. One of the greatest successes has been the introduction of all-trans retinoic acid (ATRA) in the management of promyelocytic leukemia (AML-M3) (38,39). Other molecular targets for treatment involve Flt3 and several inhibitors of this particular kinase have been developed and tested in early clinical trials (40-42). It has been suggested that the use of such inhibitors in combination with additional therapy will significantly improve clinical outcome in AML (43). Regulation of gene expression through the use of histone deacetylase inhibitors (HDACi) has also been suggested for AML therapy. This is still experimental treatment but has demonstrated biological effects in several clinical trials (reviewed in 44). Another experimental treatment was recently reported where it was shown that direct targeting of p53 by Nutlin, a small molecule inhibitor of the p53-Mdm2 interaction (45) exhibited a promising anti-leukemic effect (45). Nutlin is only effective in cells with wild-type p53 and could

provide a great improvement in the management of a disease where the TP53 gene is only occasionally mutated.

The p53 protein

The p53 protein is a 393 amino acid protein (46) composed of five main structural and functional domains (Figure 1). The N-terminal domain consists of residues 1 through 42 and is the main regulatory sequence for activation of p53 as a transcription factor (47). The domain contains several amino acids available for modification (Table 1) and also contains binding sites for proteins of the transcription machinery (48,49) and for other protein partners, of which the most important is the negative regulator Mdm2 (50,51).

The amino acids localized from residue 61 through 94 constitute a proline rich domain that contains five repeats of the sequence PXXP (52). The PXXP motif creates a binding site for SH3 (src homology 3) domains (53) which are common domains found in many signaling proteins. This p53 domain thus functions as a unique signaling component and has been shown to be very important for efficient growth suppression (52).

Activation of p53 as a transcription factor involves direct binding of the protein to DNA. This contact involves a DNA-binding domain located between amino acids 102 and 292 (54). The p53 protein binds to DNA in a sequence specific interaction and mapping of binding sequences revealed a consensus binding site consisting of two copies of a ten base pair motif; 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 base pairs (55). Wild type p53 binds to this sequence as a tetramere with each subunit recognizing five nucleotides of the 20 base pair motif (56). Binding of p53 to DNA is also dependent on the binding of zinc, defining p53 as a zinc metalloprotein (54). The DNA binding domain sequence coincides with the major mutation hot spots found in exons 5 through 8 (7). This explains why tumor derived p53 mutants are defective in DNA binding.

Binding of p53 to DNA requires oligomerization of the protein as it binds only as a tetramere (56). The protein domain responsible for tetramerization corresponds to amino acids 319 through 360 (57) and consists of an α helix and a β strand (58). This domain ensures the formation of a symmetric tetramere consisting of a dimer of dimers where the interface is mediated by helix-helix contacts (58). The tetramerization domain also contains a nuclear export signal (NES) between amino acids 340 and 351 and the formation of a protein tetramer masks this NES, thereby ensuring nuclear retention of the active p53 transcription factor (59). A nuclear localization signal (NLS) also maps to this domain at positions 316 – 322 and mediates the migration of the protein into the cell nucleus in cooperation with two separate NLS's located further downstream in the sequence in the regions 369-375 and 379-384 (60).

The specific DNA binding function of p53 requires rigid control of its function. This control is provided by the C-terminal regulatory domain consisting of amino acids 364 through 393 (61). Regulation of DNA binding is thought to be achieved through conformational changes where the molecule adapts either a low- or high-affinity DNA binding state (62). Modification of the C terminal domain through post-translational modifications like phosphorylation or acetylation will change the conformation from low- to high-affinity (63). A second negative regulatory sequence has been identified between amino acids 80 and 93 and it has been suggested that the two separate domains contribute cooperatively in maintaining p53 in the low-affinity DNA binding conformation (64).

Posttranslational modifications of p53

The p53 protein is activated by a number of stress signals (Figure 1). Stress such as ionizing and UV radiation, chemotherapeutics, hypoxia and other signals are known to influence p53 activity by affecting the activity of kinases, acetyl transferases and other modifying enzymes. Specific residues are known to be modified rapidly following exposure to certain signals (reviewed in 65). Modification is accomplished through the addition of small proteins or chemical groups to the p53 protein. Already when p53 first was described in 1979 it was identified as a phosphoprotein (2). After more than 25 years of research it has become clear that there are a number of different modifications (Table 1) with unique functions that contribute to the sophisticated regulation of this complex protein.

Phosphorylation

Stress induced activation of p53 is to a great extent controlled through phosphorylation. Phosphorylation is the most common signal for protein activation in a cell and p53 has a number of serines and threonines available for accepting phosphate groups (Table 1). The phosphate functional group is a chemical group that increases the acidity of the p53 protein and may be used to distinguish differences in the level of modifications of p53 protein isoforms.

Phosphorylation seems to be a highly controlled event with the N-terminus of p53 as the main target for initiation of activating signals. Serines 15, 20, 33 and 37 are the main initiation sites and phosphorylation of these residues is detected rapidly after stress induction (66) as a result of the activity of a number of kinases. Phosphorylation of the N-terminus activates p53 partly by attenuation of the p53-Mdm2 complex (described in more detail below). Modification of serine 15 and serine 37 will specifically disturb this interaction (67). This interference inhibits Mdm2 induced ubiquitination and subsequent degradation, thereby contributing to p53 stabilization. Inhibition of p53-Mdm2 interaction is also followed by increased recruitment of transcriptional coactivators such as p300 and the p300/CBP-associated factor (PCAF) (68) and this is followed by increased acetylation of the C-terminus as discussed below.

Table 1. Overview of p53-residues reported to be modified post-translationally. (A), Acetylation; (M), Methylation; (N), Neddylation; (P), Phosphorylation; (U), Ubiquitination.

Residue	Modification	Modifying Enzyme	Reference
Ser6	Phosphorylation	Unknown kinase	(69)
Ser9	Phosphorylation	Casein Kinase 1 (P)	(69)
Ser15	Phosphorylation	ATM (P), ATR (P), Chk2 (P), DNAPK (P), ERK (P), p38 (P)	(70), (67), (71), (72), (73), (74)
Thr18	Phosphorylation	Casein Kinase 1 (P), Chk2 (P)	(75), (71)
Ser20	Phosphorylation	Chk1 (P), Chk2 (P), JNK (P)	(71), (76)
Ser33	Phosphorylation	CAK (P), p38 (P), GSK-3β	(77), (78), (79)
Ser37	Phosphorylation	ATR (P), Chk1 (P), Chk2 (P), DNAPK (P)	(67), (71), (72), (74)
Ser46	Phosphorylation	ATM (P), p38 (P)	(70), (77)
Thr55	Phosphorylation	ERK (P)	(80)
Thr81	Phosphorylation	JNK (P)	(81)
Ser149	Phosphorylation	COP9 Signalosome (P)	(82)
Thr150	Phosphorylation	COP9 Signalosome (P)	(82)
Thr155	Phosphorylation	COP9 Signalosome (P)	(82)
Lys305	Acetylation	p300/CBP (A)	(83)
Ser315	Phosphorylation	CDK2 (P)	(84)
Lys320	Acetylation	PCAF (A)	(85)
Lys370	Acetylation, Ubiquitination, Neddylation	p300/CBP (A), Mdm2 (N, U), p300 (U)	(86), (87), (88), (89)
Ser371	Phosphorylation	PKC (P)	(90)
Lys372	Acetylation, Ubiquitination, Methylation, Neddylation	p300/CBP (A), Set9 (M), Mdm2 (N, U), p300 (U)	(91), (86), (87), (88), (89)
Lys373	Acetylation, Ubiquitination, Neddylation	p300/CBP (A), Mdm2 (N, U), p300 (U)	(86), (87), (88), (89)
Ser376	Phosphorylation	PKC (P)	(92), (93)
Ser378	Phosphorylation	PKC (P)	(92), (93), (94)
Lys381	Acetylation, Ubiquitination	p300/CBP (A), Mdm2 (U), p300 (U)	(87), (88), (89)
Lys382	Acetylation, Ubiquitination	p300/CBP (A), Mdm2 (U), p300 (U)	(87), (88), (89)
Lys386	Sumoylation, Ubiquitination	Ubc9 (S), p300 (U)	(87), (95), (96), (88)
Ser392	Phosphorylation	p38 (P), PKR (P)	(97), (98)

Protein phosphatases counteract kinases and thus provide a switch mechanism through protein dephosphorylation. It has been shown that multiple phosphatases can dephosphorylate both the N- and C-terminus of p53 *in vitro*, including PP1, PP2A, PP5, Wip1 and Cdc14 (99-102). PP1 and PP2A specifically interact with p53 and they both dephosphorylate the C-terminal residue serine 378 (94). PP1 has also recently been reported to dephosphorylate N-terminal serine 15 (103), while PP2A dephosphorylates N-terminal serine 37 (104). The dephosphorylation of p53 by Cdc14 is dependent on protein-protein interaction and upon binding to p53, Cdc14 specifically dephosphorylates serine 315 (102). Furthermore, p53 can

transactivate the phosphatase Wip1 in response to ionizing radiation. Wip1 subsequently dephosphorylates p53, thereby establishing a negative feedback-loop (100).

The ubiquitin family of proteins

The p53 protein is highly modified by a family of small polypeptides known as the ubiquitin protein family. In normal cells, p53 has a short half life with a turnover of about 20 minutes due to polyubiquitination, targeting p53 for degradation through the proteasome pathway. Covalent binding of ubiquitin to p53 is achieved through direct interaction of p53 with the ubiquitin E3 ligase Mdm2 (51,105). Mdm2 mediates monoubiquitination at several C-terminal lysine residues (106,107), but monoubiquitination is not sufficient for degradation and the presence of a polyubiquitin chain is required. Polyubiquitination is achieved through interaction of p53 with the transcriptional co-activator p300 which has an intrinsic ubiquitin ligase activity as well as acetyl transferase activity (88). However, this polyubiquitination is dependent on the previous monoubiquitination by Mdm2 (88). C-terminal ubiquitination of p53 ultimately leads to nuclear export and protein degradation (108).

Two type I ubiquitin-like proteins, SUMO-1 and Nedd8, have been found to modify p53. Conjugation of the small ubiquitin-like protein SUMO-1 was reported by two independent studies in 1999 (95,96). This protein does not appear to target p53 for destruction, like ubiquitin, but rather seems to change the ability of the modified protein to interact with other cellular proteins thereby increasing p53 transactivation ability (95). It has also been shown that the conjugation of SUMO-1 to certain substrates can defend these molecules against modification by ubiquitin and thus prevent protein degradation (109) offering an additional mechanism for the regulation of p53 activity. Nedd8 is another ubiquitin-like molecule reported to be conjugated to p53 by Mdm2 (86). This modification appears to have the same negative regulatory effect as ubiquitin and has been reported to inhibit p53 transcriptional activities.

Acetylation

The C-terminal lysine residues functioning as acceptor sites for ubiquitin are also acceptor sites for acetyl groups (Table 1). Acetylation by the acetyl transferases CBP, p300 and PCAF is, in contrast to ubiquitination, an event that stabilizes and activates the specific DNA binding activities of p53 (85,89) and levels of acetylation are significantly raised in response to almost every type of stress (110). Because acetyl and ubiquitin compete for the same lysine residues, activation and inactivation of p53 function are consequently tightly regulated through differences in the activity of modifying enzymes. Further it has been established that histone deacetylases HDAC1/2/3 interact with p53 and down-regulate its function by deacetylation (111) as also does the oncogenic transcription factor PML-RARα (112). The PML-RARα fusion protein is, as previously described, the result of a translocation involving chromosomes 15 and 17 (t(15;17)(q22;q21)) in AML-M3 and it has been shown that PML-RARα mediated deacetylation is dependent on wild-type PML which acts as a bridge between p53 and PML-RARα. PML is also required for p53 acetylation by stabilizing the interaction between p53 and CBP/p300 (113).

In some cases, previous phosphorylation is required for subsequent acetylation of p53. A study by Sakaguchi et al (114) revealed that prior phosphorylation of serine 33 and/or serine 37 in response to DNA damage enhanced the interaction of p300 and PCAF with p53. A second report concluded with increased p300 binding to p53 as a response to phosphorylation of serine 15 (68). Other reports have also provided evidence for a

phosphorylation-acetylation cascade demonstrating a highly structured and cooperative activating process for p53 functions (115).

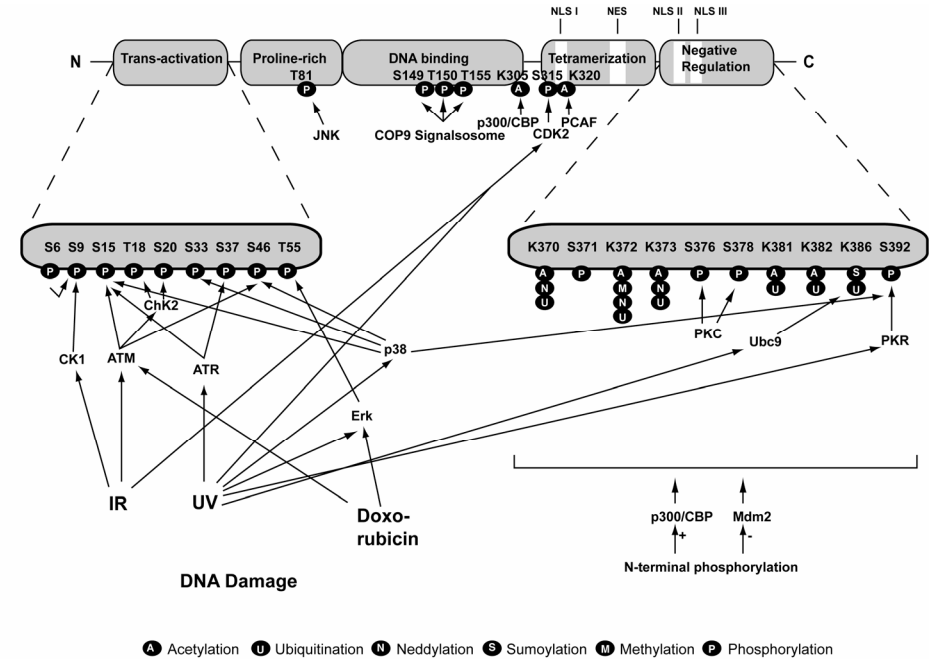

Figure 1. Stress signals induce post-translational modifications on p53. Induction of DNA-damage by signals including gamma-irradiation (IR), Ultra-violet radiation (UV) and chemotherapy (doxorubicin), leads to extensive modification of the p53 protein by the activity of multiple enzymes. Upon modification, p53 is stabilized and activated as a transcription factor. In the absence of stress signals, p53 is ubiquitinated or neddylated by Mdm2 and thereby targeted for destruction.

Other modifications

Recently a novel p53 modification was reported in which the conjugation of a methyl group to the p53 C-terminus was implied. Methylated p53 is restricted to the nucleus and this particular modification positively affects the stability and activity of the protein (91). Methylation is achieved through the activity of the lysine specific methyl-transferase Set9 which adds a methyl group to lysine 372 (91). This lysine is also a residue for Nedd8, ubiquitin and acetyl modifications (Table 1) and serves as an excellent illustration of the complexity of the regulation of the p53 protein.

The function of active p53 protein

In the inactive state p53 is a short lived protein with a turnover of about 20 minutes. Upon activation however, it is stabilized through the previously described post-translational modifications and can contribute to a number of cellular activities by transactivation of specific target genes (Table 2). As many as 1500 genes have been reported to respond to p53 activation, directly or indirectly as a secondary effect (116). Activation of these genes have been reported to regulate multiple functions including cell growth by the induction of cell cycle arrest and apoptosis (reviewed in 24), differentiation (117) and senescence (118). It is

not fully understood how p53 switches between these activities, but it has been proposed that; 1) p53 activity is determined by the cooperation of p53 with additional signals, or 2) p53 activity is determined by the activity of critical kinases and cofactors (119,120). Switching between life and death signals is now a field of considerable interest.

Table 2. Selected p53 target genes of relevance in AML and their cellular function.

Gene name	Gene product function	Reference
14-3-3σ	Cell cycle arrest	(121)
BAX	Apoptosis	(122)
CDKN1A	Cell cycle arrest	(123)
FAS/CD95	Apoptosis	(124)
FDXR	Apoptosis	(125)
GADD45	Cell cycle arrest	(126), (127)
KILLER/DR5	Apoptosis	(128)
MDM2	Auto-regulation	(129)
NOXA	Apoptosis	(130)
P53CSV	Survival	(131)
p53DINP1	Apoptosis	(132)
PUMA	Apoptosis	(133), (134)
SES2/Hi95	Survival	(135)
SESN1/PA26	Cell cycle arrest	(136)
WIP1	Apoptosis	(100)

Cell cycle regulation

Cell cycle control is dependent on the action of checkpoint proteins and evidence has shown that p53 has a checkpoint function in both G_1/S and G_2/M transition (24,137). The ability of p53 to induce G_1 growth arrest is correlated with its ability to transactivate the cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$ (123,138). p21 functions by binding to a number of cyclin and cyclin dependent kinase (Cdk) complexes including Cdk2/cyclinE, Cdk2/CyclinA, Cdk4/CyclinD2 and Cdk6/CyclinD2 (139) and this interaction inhibits Cdk activity. Inhibition of Cdk activity leads to accumulation of hypophosphorylated Retinoblastoma (Rb) protein which remains associated with transcription factors such as E2F. This results in failure to activate E2F responsive genes, including proliferating cell nuclear antigen (PCNA) which is required to initiate DNA synthesis in cell cycle S-phase. The ultimate consequence of this is G_1 arrest. It has lately been shown that transcription of p21 also may function as a signal for choice of p53 activity. It has been reported that the transcription factor Myc is recruited to the p21 promoter by the DNA-binding protein Miz-1. This interaction blocks p53 transactivation of p21 and leads to a switch from a cytostatic to apoptotic p53 response (140).

Cell cycle arrest in the G_2-M transition has also been reported upon p53 activation (137,141). This involves the transcriptional down-regulation of Cyclin B1 (142) as this cyclin in complex with the Cdc2 (Cdk1) is the major regulatory factor required for entry into mitosis. Cdc2 can also be inhibited in order to achieve G_2-M arrest by transcriptional activation of Gadd45 which can bind directly to Cdc2 to inhibit its activity (126) or transcriptional activation of 14-3-3σ which binds to the Cdc2 regulator Cdc25C (121,143).

Regulation of Apoptosis

Triggering apoptosis is a major function of p53 and it has been suggested that this may be controlled through mechanisms involving both transcriptional activation of target genes and a

nontranscriptional pathway (reviewed in 144). Many p53-regulated genes are known to regulate both the extrinsic apoptotic pathway involving death receptors (e.g. KILLER and FAS), and the intrinsic pathway involving the mitochondria. The principal mechanism has been suggested to be the intrinsic pathway with ultimate mitochondrial release of cytochrome *c* (145). This pathway involves p53 mediated transactivation of proapoptotic Bcl-2 family proteins, described below.

In addition to activation of apoptosis inducers, p53 can also transcriptionally repress anti-apoptotic proteins like Bcl-2, Bcl-X_L and Survivin thereby preventing cell survival (120). Transcriptional repression is, in contrast to transcriptional activation, not fully understood and the precise mechanism for this apoptosis regulation remains to be determined.

Recently, a transcriptional independent role for p53 in the induction of apoptosis has been suggested. This involves a model where activated p53 is redistributed to the mitochondria where it forms complexes with Bcl-2 and Bcl-X_L thus stimulating the release of cytochrome *c* by interfering with the antiapoptotic properties of these proteins (146). This has been implicated to be associated with a polymorphism found at codon 72 in the TP53 gene resulting in a protein expressing either a proline or an arginine in position 72. Expression of the arginine 72 variant has been suggested to be related with a higher apoptotic potential due to increased localization to the mitochondria (147,148).

The Bcl-2 protein family

The Bcl-2 proteins have a great impact on cell survival and death. The family contains both pro-apoptotic and anti-apoptotic proteins that are known to control the intrinsic apoptotic mechanism. By localizing to the mitochondria these proteins control the permeabilization of the outer mitochondrial membrane and thereby regulate the release of cytochrome *c* to the cell cytosol. The Bcl-2 family is classified in three sub-groups; 1) the anti-apoptotic proteins containing BH1-4 domains (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1/Bfl-1), 2) the pro-apoptotic proteins containing BH1-3 domains (Bax, Bak, Bok/Mtd) and 3) Pro-apoptotic BH3-only proteins (Bid, Bim/Bod, Bad, MAP-1, Bmf, Bik/Nbk, Blk, Noxa, Puma, Hrk/DP5) (Bcl-2 proteins are reviewed in 149,150). The p53 protein is able to mediate apoptosis through transcriptional activation of Bax (122), Puma (134) and Noxa (130) which are all pro-apoptotic Bcl-2 proteins. These pro-apoptotic proteins promote apoptosis upon heterodimerization with an anti-apoptotic family member. In many cancers, including AML, the ratio of pro- versus anti-apoptotic proteins is useful in determination of prognosis (22).

Senescence and differentiation

Evidence exists for p53 involvement in cellular senescence, but its precise role is not fully understood. However, it has become clear that p53 elicits its senescence response in part by increasing the expression of p21 thereby slowing or stopping the division rate of the cell (118). This seems to be a response to shortening of telomeres which is sensed by the DNA damage sensor proteins ATM, Chk1 and Chk2 (151,152). These are all p53 modifying enzymes (Table 1) and will trigger p53 activation though it is not known which genes the active protein transactivates. Senescence by p53 does however appear to have a tumor suppressor effect as the process limits the replicative capacity of cells, thus preventing the proliferation of cells that are at different stages of malignancy.

It has previously been suggested that p53 is implicated in the induction of differentiation and that this is associated with G_2-M growth arrest (117). p53 dependent differentiation of acute leukemia cell lines has also been reported together with findings of different p53 protein levels at distinct stages of cell maturation (153,154). Furthermore, a role

for p53 in the differentiation of mouse embryonic stem cells (ESC) was recently reported (155). This specific p53 activity relied on phosphorylation of serine 315 and the authors speculate whether p53 induces differentiation of ESCs into other cell types that undergo efficient p53-dependent cell-cycle arrest and apoptosis as a mechanism for p53 to maintain genetic stability.

Variants of the p53 protein

Major variants of the p53 protein may be formed through alternative splicing of mRNA or protein cleavage. To date, the human p53 gene has been reported to hypothetically encode nine isoforms, resulting from variously spliced mRNA (156). These are the full length protein, three N-terminally truncated isoforms translated from an alternative point of initiation at codon 40, or by alternative splicing of intron 2 (156-158), two C-terminally truncated isoforms produced by alternative splicing of intron 9 (156,159) and three isoforms produced from an internal promoter in intron 4 (156). In addition, truncated p53 protein products are known produced as a result of protease action, most likely through an autoproteolysis mechanism (160,161). In fact, one of the p53 cleavage products, p35, has protease activity and can cleave the full length p53 protein to generate an alternate protein product (160).

The different p53 isoforms are known to have different functions. Most is known about the N-terminally truncated isoform ΔNp53, also termed p47. This isoform lacks the 40 most extreme N-terminal amino acids and thus has impaired transcriptional activation capacity. Also, it does not complex with the negative regulator of p53; Mdm2 (157). Furthermore, this isoform has been shown to have a dominant negative effect on full length p53 (157,158) thus repressing transcriptional activity and growth suppression mediated by full length p53. p47 was also shown to modify p53 localization and inhibit p53 degradation most likely due to deficient ubiquitination resulting from impaired ability to complex with Mdm2 (158).

Bourdon et al. recently reported that all nine p53 isoforms known are expressed in normal human tissue in a tissue-dependent manner (156). They also report differential binding of these isoforms to p53-responsive promoters and alternate apoptotic responses. At least one additional isoform apart from p47 was suggested to be dominant negative toward full length p53 (Δ133p53).

Taken together these studies suggest a major role for p53 isoforms in the regulation of p53-mediated responses to cell stress and further studies are required to elucidate the complex organization of expression of p53 proteins.

The p53 family of proteins

The p53 protein is part of a family of proteins that includes p63 (162-164) and p73 (165). These proteins are also transcription factors and share structural and functional similarities with p53 having a transactivation domain, a DNA binding domain and an oligomerization domain mediating tetramerization. Sequence homology between p53 and its family members enables p63 and p73 to bind to p53 DNA-binding sites, thereby transactivating p53 target genes.

Many different isoforms of the p63 and p73 proteins are known. The p63 gene expresses six mRNA variants that encode six protein isoforms, and the p73 gene expresses at least 35 mRNA variants that theoretically could encode 28 protein isoforms. So far, 14 p73 protein isoforms have been described (156). Both p63 and p73 have been shown to express N-

terminally deleted isoforms that exert dominant negative effects on both themselves and p53 (162,166). It is becoming increasingly clear that most p63 and p73 isoforms have specific and distinct activities, many being able to induce apoptosis (167-169) or cell cycle arrest (167,170) through specific transcriptional activation of target genes.

Mouse models to elucidate p53 biology

The generation of knock-out and knock-in mouse models has provided profound insights into the role and importance of upstream and downstream signals of the p53 protein, elucidating the importance of p53 functionality (reviewed by 171,172). It was early shown that $p53^{-/-}$ mice were highly prone to tumor development (173-175), thereby establishing the critical tumor suppressor functions of this protein. Analysis of such mouse models, where p53 loss-of-function promotes tumorigenesis, has helped to elucidate the role of p53 as a cell cycle checkpoint and regulator of apoptosis. To increase knowledge of the mechanisms of cancer initiation and progression, knock-in mouse models have now been established to mimic p53 mutations known from human tumors. A hot spot mutation commonly observed in human cancers, $p53^{R175H}$ (murine $p53^{R172H}$), was recently shown to increase tumor frequency in mice. These mice developed tumors with the same latency as $p53^{+/-}$ mice but more often developed carcinomas with metastasis (176). This indicated that mutant p53 protein was dominant in activity, either through a dominant-negative effect or a dominant gain-of-function effect. The same results were also obtained in two new studies were mouse models of the Li- Fraumeni syndrome were established. This confirmed that mutant p53 expressed under physiological control has enhanced oncogenic potential beyond the simple loss of p53 function (177,178). It was also suggested that functional inactivation of p63 and p73 through binding to mutant p53 may increase tumor and metastasis potential (178). This has also been proposed in a recent publication and suggests a more significant role for the p53 family (179).

To assess the role of p53 post-translational modifications *in vivo,* investigators have begun to generate knock-in mice with single phosphorylation sites altered. Analysis of the serine 18 (equal to human serine 15) phosphorylation site by a $p53^{S18A}$ mutant, has shown that this site specifically regulates the apoptotic function of p53 and is not required for p53-mediated tumor suppression (180). A $p53^{S389A}$ (human serine 392) mutant revealed that phosphorylation of this site is an important response following UV irradiation, but not for γ-radiation. Mutant mice were compromised in transcriptional activation of p53 target genes and apoptosis after exposure to UV (181). Further studies of such mouse models will presumably aid in elucidating the specific roles of single modifications and the ways in which these different modifications are interrelated.

Li-Fraumeni syndrome

In human disease, the classical example of the consequences of a p53 loss-of function is offered by the cancer syndrome Li-Fraumeni (LFS). This is a familial cancer syndrome first described by F. Li and J. Fraumeni in 1969 (182). Generally, LFS is caused by a germline TP53 mutation (183,184) and 70% of LFS families have been found to carry this genetic alteration (185). In contrast to other inherited cancer syndromes, which are predominantly characterized by site-specific cancers, LFS presents with a variety of tumor types. The patients with this syndrome share clinical criteria involving (i) childhood malignancies, (ii) common malignancies such as soft tissue sarcomas osteosarcomas, breast cancer, brain tumors and adrenocortical carcinoma (186), and (iii) relatives diagnosed with the typical

malignancies before the age of 60. In later years LFS families with no TP53 mutation have been found to carry mutations in p53 regulating kinases like Chk2 (187), thereby disturbing the p53 pathway and perturbing p53-dependent cell cycle control and apoptosis. This family syndrome is characterized as Li-Fraumeni-like syndrome (LFL).

Recently it has been suggested that the p53 family members, p63 and p73, may play a role in general tumorigenesis and LFS (178,179,188). This suggests interplay between these proteins and should be elucidated further in order to improve our understanding of the biology behind this disease.

Aims of the study

The main aim of this thesis has been to describe the regulation of wild type p53 protein in acute myeloid leukemia. The study has focused on the following topics:

1) Study of the relationship between AML signaling networks and p53 phosphorylation to elucidate the role of important signaling molecules in the development of AML.

2) Study of the regulation of p53 isoform expression and p53 target gene activation during *in vivo* therapy in AML patients to determine a role for p53 protein in the treatment response pathways.

3) Examination of the correlation between p53 isoform expression and clinical parameters in AML patients in order to investigate the implications of a diverse and heterogenic expression of p53 protein in AML

Methodological considerations

Two dimensional electrophoresis and immunoblotting (2DI)

Much of this thesis is based on the visualization of p53 protein using two-dimensional electrophoresis (189). This is undoubtedly the best way to portray the complexity of p53 constituted by differential isoform expression and differing modifications at the post-translational level. The use of two-dimensional electrophoresis enables the detection of isoforms with differences in both isoelectric point (pI) and molecular weight (MW). Since p53 is highly regulated by post-translational modifications, many isoforms will have detectable differences only in pI and not in mass. These isoforms can not be distinguished using standard gel electrophoresis and their separation is therefore dependent on the two-dimensional electrophoresis methodology.

Unmodified p53 has a pI of 6.4, but different modifications will change the protein properties (Table 3). For example, both phosphorylation and acetylation confer negative charges to the protein and cause an acidic shift of the isoforms; the higher number of modifications, the more acidic the protein. Other modifications like ubiquitin, Sumo-1 and Nedd-8 are larger protein entities with sizes of approximately 10 kDa. These modifications will not affect the isoelectric point to a great extent, but rather cause the appearance of larger forms of the p53 protein that appears as a typical laddering pattern as previously described for ubiquitination (190). Taken together, the existence of p53 isoforms varying in molecular mass or electrical charge makes two-dimensional electrophoresis an absolute requirement to have the best picture of isoform expression.

Table 3. Properties of p53 post-translational modifications.

Functional group	Number of Amino acids	Size	pI	Shift
Phosphate		80 Da		Acidic
Acetyl		42 Da		Acidic
Methyl		14 Da		-
Ubiquitin	76	8.57 kDa	6.56	Ladder
Nedd-8	81	9.07 kDa	7.99	Ladder
SUMO-1	101	11.56 kDa	5.34	Ladder

The 2DI technique also offers a reliable and reproducible method for detection of p53 protein isoforms in our material. Fixed protocols with precast gels are used in all experiments, for both primary cells and cell lines, and little variation is detected between untreated samples of the same origin (Figure 2).

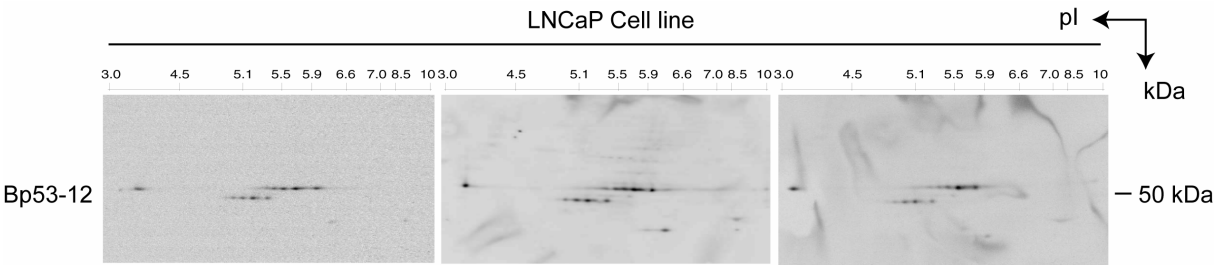

Figure 2. The 2DI method offers reproducible results with little variation due to technical issues. This figure represents three independent experiments and p53 isoforms are detected in the cell line LNCaP with no prior stimulation of the cells.

Correlation of two-dimensional gel protein patterns with biological parameters

The expression of p53 protein detected by 2DI is highly heterogeneous in AML patient material. Correlation of distinct isoform distribution patterns to specific subsets of patient characteristics was an important and challenging task in the analysis of our patient data. Several techniques to distinguish groups of patients with similar protein patterns were attempted:

- Manual alignment and signal quantification using gel imaging equipment
- Manual gridding of area of interest
- Software for two-dimensional gels
- Computed correlation algorithm

Rather than relying on software, manual alignment of the gels and quantification of areas of interest based on the definition of a grid was initially attempted. This gave a measure of the intensity of the signal in one defined area on the gel (Figure 3). The intention was for this to be used in order to identify patients with increased intensity in specific areas of the gel and see whether these signals coincided with specific clinical parameters. However, in many cases background noise was measured as significant signal, making it impossible to distinguish the real protein signal from signal due to noise. This made it difficult to reliably identify patient subgroups without manually removing false signals. This method also chose to focus on the region of the gel images expressing p53. By selecting only one region important areas of the gel may have been missed and the approach was therefore changed into reading the entire image, to ensure that no information was lost in the processing.

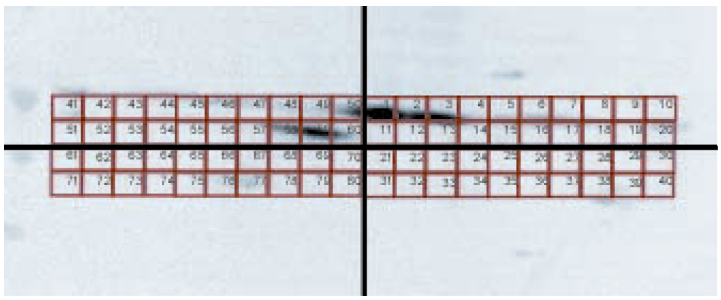

Figure 3. Patient gels were manually aligned based on the location of the p53 spots. Intensity of expression was measured in arbitrary units and detected for regions 1 – 80, defined as areas of interest.

Current proteomics software like PDQuest (BioRad) can be used to detect spots and changes in spot expression, but they are not able to distinguish changes in the shape of a spot because they use Gaussian modeling to create a synthetic spot image. This synthetic image is processed to enable distinction between overlapping spots, but makes it impossible to detect whole distribution patterns in a gel because every spot is singled out. Commercial software also detects noise as protein signal, again forcing the manual removal of false signal. The correlation of signal to clinical parameter also has to be done separately as the software can not handle this.

Due to the described technical problems, manual analysis and commercial software based analysis failed in retrieving p53 immunoblot features that were significantly correlated with clinical parameters. To enable reliable correlations between protein distribution and patient characteristics a correlation algorithm was written. After manual alignment of images the algorithm generated a composite image containing the statistical correlation of clinical features. Location and volume of the protein signal was detected and related to a biological characteristic based on several parameters.

Basis for algorithm:

a. The gels are first aligned by detection of a specific spot at a defined pixel location in every gel. All gels are rotated to fit this location horizontally. The Y-axis is thereafter normalized by determining a scaling factor based on the vertical distance between the main p53 isoforms found in the patient material; αp53 and Δp53 (see paper I for explanation of isoforms). In this way, image differences resulting from gel preparations are eliminated.
b. After alignment, the protein expression levels are normalized to allow better visualization of the morphology and pattern of the protein detected in different areas of the gel. The first step of normalization is the removal of background. This is achieved by subtracting or dividing the median background value of a gel from the image to give a background intensity of zero. Secondly, expression of the spots is normalized by dividing the gel image by the maximum grey value. This makes sure that all spots are expressed equally strong. After these steps all background values are zero and the maximum protein expression is one.
c. The correlation image is generated by determining one value for the expression signal and one value for a clinical parameter for every pixel in a gel. All values are replaced by a specific rank according to the Spearman rank order correlation thereby making a ‘normalized’ dataset and a correlation of these normalized data is calculated from a standard linear Pearsons correlation. One correlation test is performed for every pixel position on the gel based on these datasets.
d. The correlation test detects the location and the volume of a signal on a gel and whether these change or not as a function of the associated biological feature. Spots that correlate positively with the biological feature are given one color (green) and spots that correlate negatively (anti-correlation) are given a second color (brown). Spots that do not change at all are left out of the correlation image.
e. Gel areas with no signal have a constant intensity and either strongly correlate or strongly anti-correlate. This is mathematically correct but gives no information because it is constant, and can be removed by applying a filter. This is achieved by calculating the standard variance measured on the relative, non-ranked gel intensities. This will give a large number where the gel expression varies, but in cases of constant expression (empty areas) the number will be zero.

f. Correlations using this algorithm may occur by coincidence and therefore a second filter is applied to ensure the detection of genuine correlations only. This is done by using a significance test typically associated with the Spearman correlation. A calculated value close to one means that there is a low probability of the correlation having occurred by chance. However, a value close to zero means that it is likely that the correlation is coincidental and this signal can be filtered out to produce a masked correlation image.

Using this correlation algorithm also eliminated the problem with gel differences in background noise because noise was represented as white color. By adding noise to a test image, it was actually shown that the correlation image was 'cleaner' because the background detection of empty areas was reduced.

The new algorithm provided a good solution for the analysis of our gel images. The technique did not require perfect gels as background noise did not influence the analysis. Furthermore, the analysis detected total protein distribution and took into account spot shapes, tails and total areas without detecting or selecting single spots. Unfortunately, proper alignment of the gels was still a time-consuming task and needs to be further improved. Also, the comparison of spot volumes is not yet possible but may be allowed by newer versions of the method in the future.

Intracellular flow cytometry

Intracellular visualization of phospho-proteins by flow cytometry is a technique that has aided in providing information about a cell's immediate functional responses to stimuli. It is a powerful technique that now allows the simultaneous analysis of 13 parameters or more (191) and has many advantages over western blotting or ELISA (reviewed in 192). The most important advantage for its use in the analysis of AML material is that it allows multiparameter analysis of small numbers of cells thereby decreasing the need of large protein concentrations often required in the application of other immunological methods. It also enables monitoring of signaling events in an environment close to the *in vivo* situation, thus eliminating the risk of changes to protein status due to cell purification and protein extraction. As such, the technique is invaluable for the successful detection of intracellular phospho-signaling in limited patient material.

Summary of papers

Paper I

The TP53 gene is normally wild type in AML as opposed to other human cancers. We investigated whether there is a down-stream block of the p53 pathway that may contribute to leukemogenesis in AML. We found that p53 protein was expressed and responded to chemotherapy with increased phosphorylation and protein accumulation as is commonly observed in normal cells. Subsequently, phosphorylation of specific residues in resting cells from 30 AML patients was detected. We found three different subsets of patients differing in the level of phosphorylation at serines 15, 20, 37, 46 and 392. One group of patients had low levels of p53 phosphorylation. A second group had increased phosphorylation of serines 20 and 37 and the third group had increased phosphorylation of serines 15, 46 and 392. Alterations in the levels of anti-apoptosis protein Bcl-2 is commonly observed in AML. We therefore investigated the expression of Bcl-2 in the three identified patient subsets. We found increasing levels of Bcl-2 in the patients expressing high levels of phosphorylation at serines 15, 46 and 392. We further found the high levels of Bcl-2 to be associated with a specific mutation in the receptor tyrosine kinase Flt3. This suggests that specific Flt3 signaling may lead to accumulation of Bcl-2 which ultimately maintains a downstream block of p53-mediated apoptosis.

Paper II

Having found in paper I that TP53 is wild type in our patients, we sought to investigate whether p53 protein responded to chemotherapy in AML patients. We sampled 5 patients during the first 18 hours of *in vivo* induction chemotherapy. Using two-dimensional electrophoresis and immunoblots we identified two main isoforms of p53. These isoforms were denoted αp53 and Δp53. After treatment an induction of αp53 was observed in the patients. Up-regulation of αp53 post treatment was also followed by increased transcription of p53 target genes, suggesting a functional protein response. In resting patient cells, Δp53 was the dominating form, but following up-regulation of αp53, Δp53 was down-regulated. The shift in α/Δ ratio was more predominant in malignant than normal cells suggesting that malignant cells may have alternate mechanisms for the regulation of p53 isoform expression and activity.

Paper III and IV

The p53 protein is heterogeneously expressed in AML patient material. This may be a result of alterations in signaling pathways due to individual differences in leukemogenesis. Distinctions in signaling may be related to prognosis and clinical outcome of malignant diseases and we investigated whether the patterns of p53 protein could be used in prognostic evaluation of AML patients. We wrote a correlation algorithm to test whether alternate p53 protein patterns could predict clinical outcome in AML. Using this method, we found that specific biosignatures of p53 correlated with several clinical parameters. The p53 signature could be related to origin of cancer and stage of cell differentiation. Furthermore, expression of Δp53 was correlated with long survival. This isoform was also correlated to remission after first course of chemotherapy, supporting the survival correlation. Expression of mutated Flt3 was correlated to expression of αp53. This suggests that the specific regulation of the p53

protein can be used to reflect perturbations in cell signaling networks an may offer a new tool in stratification of AML patients.

Paper V

Li-Fraumeni syndrome is a cancer syndrome caused by germline mutations in the TP53 gene or in genes encoding p53 regulatory enzymes. We studied the effect of a new combination treatment including ATRA, valproic acid and theophyllamin in an AML patient with LFS. We found only miniscule amounts of p53 expressed in the leukemic cells of this patient suggesting that all effects of treatment were p53-independent. Furthermore, we detected changes in the levels of two membrane molecules, CD11b and CD71, during treatment. We also found changes in the activities of signal transducers Stat1, Stat3 and Stat5 after stimulation with exogenous cytokines. This could be due to effects of the new combination treatment and encourages a further study of the clinical use of these anti-leukemic drugs.

General discussion

Leukemia has been described for centuries and treatment of this patient-group has been a constant challenge. The first successful treatment of AML was described in the 1930's when a man was reported to have entered remission after receiving radiation, arsenic and thorium-X (193,194). Since this early stage of therapy the availability and diversity of therapeutics have gone through a revolution and median survival for AML patients is constantly increasing. Since the introduction of anthracyclines in combination with AraC in the early 1970's the treatment response rates have improved dramatically. Bone marrow transplants were also introduced in the mid 1970's, further improving overall survival for this patient group. However, even though anti-leukemic treatment is improving, the overall disease-free survival still does not exceed 50%. A major concern is the large number of older patients (>60 years) who can not get the most intensive treatment because of therapy-related toxicity. It is therefore necessary to find new molecular targets to improve treatment specificity, thereby increasing efficiency and lowering toxicity with the ultimate goal of increased survival rates.

The p53 network is a good candidate in the search of new targets for treatment. The p53 protein is a highly connected node in the cell, so even though the protein it self is wild type in sequence in AML, other changes in the p53 network can change the specificity of its action. In a heterogeneous disease like AML which is characterized by such a myriad of genetic defects, it is highly likely that such alterations might take place. This thesis has therefore aimed to describe the nature of the p53 network by focusing on the regulation of the protein itself in the leukemic cell.

Signal transduction in AML

In general, cancers are derived from a single abnormal cell where changes in the cell's DNA have occurred. Normally one DNA mutation is not enough for a cell to loose growth control and for AML a 'two-hit' model of leukemogenesis has been proposed (195). This hypothesis implies that two mutations with different consequences are needed in collaboration for AML to develop. The first type of mutation is a class I mutation that confers a proliferative and/or survival advantage to cells, the second is a class II mutation that primarily will impair hematopoietic differentiation and subsequent apoptosis (Table 4). In collaboration these two types of mutations will lead to rapidly dividing immature cells invading the blood stream. This causes loss of the functions of normal blood cells and ultimately leads to the symptoms of leukemia.

The class I mutation known to be most prominent in AML to date is a length mutation (LM) in the receptor tyrosine kinase Flt3 (18). Normally, signal transduction pathways involving this receptor (and other receptor tyrosine kinases) are activated through the stimulation of external binding of a ligand which leads to receptor dimerization, auto-phosphorylation and ultimately phosphorylation and activation of Src homology 2 (SH2) or phosphotyrosine binding (PTB) domain containing proteins. These activated proteins then recruit signaling molecules which cause activation of several downstream pathways, including the Ras pathway, effecting cell proliferation, differentiation and apoptosis (reviewed in 196) (Figure 4). When mutated, the Flt3 receptor is constitutively active and the signaling control provided by ligand binding is therefore lost leading to constant signaling that drives the downstream cell regulatory pathways (reviewed in 197).

Table 4. (Table adapted from 198). Class I and II mutations associated with development of AML according to the 'two-hit' model. Class I provides a growth advantage and class II impairs hematopoietic differentiation. AML1-COPINE VIII could be viewed as a unique mutation since the resultant fusion protein may possess both class I and class II activities.

Class I mutations	Class II mutations
BCR-ABL	CBFβ-MYH11
N-RAS	AML1-ETO
K-RAS	TEL-AML1
c-KIT (exon 8)	PML-RARα
c-KIT (Asp816)	NUP98-HOXA9
FLT3 (ITD)	PU.1
FLT3 (Asp 835)	C/CEPα
PTPN11	AML1
NF1	AML1-AMP19
TEL-PDGFRβ	
? AML1-COPINE VIII	

Other studies have shown the presence of two separate Flt3 length mutations in our patient material (R. Hovland, unpublished observations). The mutations are distinguished by the presence of a single Y591 phosphorylation site (Flt3-LM-SPY591) or an amplified Y591 phosphorylation site (Flt3-LM-AmpY591). In paper I we found that the Flt3-LM-AmpY591 mutant correlated to increased levels of Bcl-2. A speculative mechanistic explanation for this can be provided by the STAT proteins. Mutated Flt3 has previously been described to drive signaling of Stat5 in AML (199). The STAT proteins control cell proliferation and differentiation and induction of Stat5 through Flt3 signaling increases the transforming potential of hematopoietic cells. Furthermore, Stat5 has been reported to induce the transcription of the anti-apoptotic Bcl-2-family proteins Bcl-2 and Bcl-X_L (200) thereby increasing cell survival signaling. Different potential in driving Stat5 signaling may explain the differences in Bcl-2 expression between the specific Flt3 mutations.

Many cancers develop due to mutations in the tumor suppressor p53. This will permit uncontrolled cell growth that would normally lead to cell cycle arrest or early apoptosis. In AML however, p53 seems to be wild type. How do the malignant AML cells overcome this obstacle? We report in paper I that the different Flt3 mutations cause differential expression of Bcl-2. A possible hypothesis relating to the increased survival signals conferred by mutant Flt3-LM-AmpY591 suggests that this might block signaling downstream of wild type p53 thus overriding the normal p53 response. It has recently been shown that activation of receptor tyrosine kinase ErbB can override DNA damage induced growth arrest in breast cancer (201) suggesting that p53 cell cycle arrest or apoptosis could be potentially blocked through increased signaling by mutant Flt3. Furthermore, several of the enzymes known to modify p53 can be controlled by signaling through Flt3, including ERK which is also found to phosphorylate Bcl-2 and in some cases increases Bcl-2 anti-apoptotic activity (202). This may lead one to imagine a situation where the malignant cell is committed to survival and chooses to focus signal transduction towards proliferation and survival molecules.

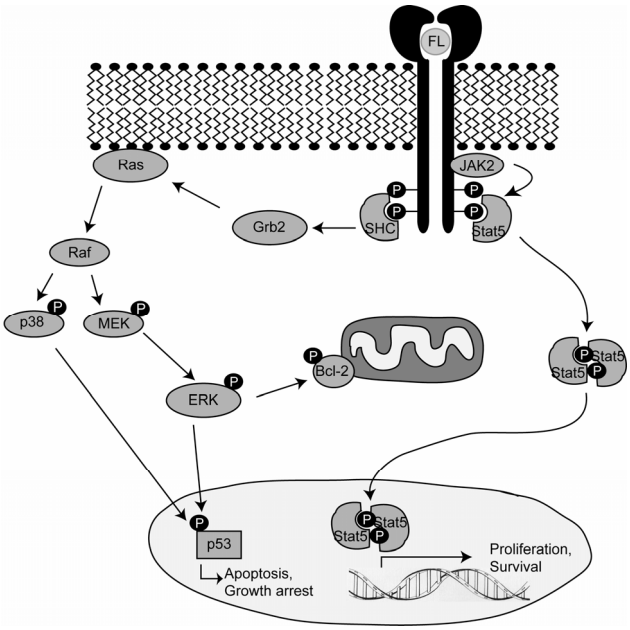

Figure 4. Upon binding of Flt3 ligand to the receptor, the receptor dimerizes and auto-phosphorylates. This causes binding of proteins with SH2 domains (SHC/Stat5) to the receptor. SHC activates Ras which further causes activation of several kinases that can stimulate survival by phosphorylation of anti-apoptotic protein Bcl-2 or cell death by phosphorylation of p53. The active Flt3 receptor also recruits the kinase JAK2 and Stat5 will bind to the active Flt3-JAK2 complex through its SH2 domain. Upon binding, Stat5 is phosphorylated by JAK2 and is released from the receptor. It thereafter dimerizes and the dimer is translocated to the nucleus where it stimulates transcription of genes related to cell proliferation and survival.

Expression of p53 protein isoforms in AML

Many proteins are expressed as several unique protein isoforms due to alternative splicing of their genes. It has been estimated that ~70-80% of human genes are alternatively spliced (203,204) and this generates a highly complex proteome. A random sample of 50 alternatively spliced genes revealed that nearly 75% were involved in signal transduction (reviewed in 205). The different isoforms of all these signal transducers may have unique properties due to specific tissue of expression, cellular location or unique post-translational modifications.

The TP53 gene has recently been found to transcribe nine distinct protein isoforms (156). These p53 variants are expressed in a tissue-dependent manner suggesting that their expression can be selectively regulated and they also seem to have distinct transcriptional activities thereby regulating alternate cellular functions (156). We have detected two main isoforms of p53 and find these expressed in all cell lines and primary cells tested. The isoforms vary in size and distribution. The αp53 variant has a molecular weight of about 53 kDa and is assumed to correspond to full length p53. Δp53 is around 47 kDa and is possibly a truncated version of the protein. Both isoforms are detected by antibodies directed towards the N-terminal domain of p53 (Bp53-12 and DO-1; epitopes at residues 20-25) and Δp53 can thus not be the previously reported p47 because this variant lacks the 40 most extreme N-terminal residues (157). It is unlikely that the size difference between these two main forms is due to post-translational modifications like neddylation, sumoylation or ubiquitination as these protein entities are too large to cause a 6 kDa change (Table 3). Phosphorylation and acetylation are assumed to cause the horizontal spread of the isoforms as these are known to change the pI of a protein. The αp53 isoform seems to be much more predisposed to these modifications than does Δp53. This suggests that Δp53 lacks sites known to be modified, although we can not conclude which sites these would include.

In paper II we found an up-regulation of αp53 combined with down-regulation of Δp53 in response to chemotherapy. This was a much stronger event in leukemic than in normal cells and could be due to the nature of cancer cells versus fully matured normal leukocytes. Knowing that the expression of p53 isoforms varies with cell type, it is very likely that p53 isoforms detected in malignant cells will hold unique qualities as opposed to the normal cell counterpart. The leukemic and normal cells are at very different stages in the differentiation process and will presumably respond differently to certain stimuli, including chemotherapy. We observed a less efficient regulation of the p53 isoforms in normal cells than in AML blasts. This was most noticeable for the Δp53 variant. As the αp53 variant is assumed to be the full-length p53 protein, the variable Δp53 regulation could suggest an important distinction in the p53 pathway regulation in normal versus malignant blood cells. An explanation could be that Δp53 holds qualities that may control the activity of αp53, possibly by repression of activity. This repression might be reversed in malignant cells, meaning that the ability of a cancer cell to modulate αp53 and Δp53 activity may have an influence on chemoresistance.

Characterization of the main p53 isoforms expressed in AML would strengthen the findings in paper II. This would allow us to learn, in more detail, what the specific effect of αp53 is as opposed to Δp53 and whether this has specific consequences for transcription of target genes in AML patient material.

Activities of different p53 protein isoforms

p53 isoforms are subject to a high level of post-translational modifications, and dysregulation or alternate expression of the modifying enzymes could lead to distinct regulation of p53 proteins in AML blasts versus normal blood cells. We also know that several of the reported p53 isoforms lack important parts of regulatory domains making them less available for modification, including parts of the N- and C-terminal domains (156). Post-translational modifications are, to a great extent, the key to regulation of p53 activity and differences in a cell's ability to apply these regulatory modifications could have great impact on protein signaling and transactivation of target genes.

We observed in paper II an up-regulation of known p53 target genes in cells from AML patients undergoing chemotherapy *in vivo*, including both inducers of apoptosis and cell survival factors. The question is whether this bias is due to a unique feature of the malignant cell's response to DNA damage induction. Paper I identified an increased expression of the pro-survival protein Bcl-2 in a subset of patients, and this unexpectedly correlated to increased p53 phosphorylation. We therefore suggested that the p53 pathway is inactivated downstream of p53 signaling and that this is related to cancer cell survival commitment. This could offer an explanation for the gene expression bias as it would allow the transcription of apoptosis inducers but that these would be inactivated on protein level to allow survival signaling. The specific expression of p53 isoforms in AML cells could contribute to specific gene activation as it has been proposed that regulation of RNA splicing can be a factor that may contribute to the development of malignant diseases by controlling the specific expression of protein isoforms with distinct activities. The regulation of splicing is therefore now emerging as an intriguing new field in cancer research.

The p53 family members p63 and p73

Neither p63 nor p73 are targets of inactivating mutations in human cancers, however they may still play a central role in tumorigenesis. For both p63 and p73, N-terminally deleted dominant-negative proteins (ΔNp63, ΔNp73) that inhibit all three family members have been

described. These isoforms might play an active oncogenic role in some human cancers. In AML there is increasing evidence that p73 may be implicated in leukemogenesis. It has been reported that ΔNp73 is ubiquitously expressed in AML, with the exception of AML-M3 (206). It was also recently reported that mutations in the p73 gene cause the expression of mutant protein which may play a role in the development of AML (207). Furthermore, it has been shown that p73 is induced by a wide variety of chemotherapeutic drugs and blocking p73 activity can lead to chemoresistance (207). Both p63 and p73 protein can be detected in our material. p63 can be observed using the p53 specific antibody and is found to be expressed in nearly half of the patients, often in more than one isoform. Unfortunately, we have not looked at the regulation of the p63 and p73 proteins during chemotherapy, but this may be an important supplement to the study of p53 in AML as the expression of these family members now have been reported to seemingly associate with the prognosis of other malignant diseases (208,209). Furthermore, in an AML patient with LFS, p63 expression was not detected while p73 was found to be down-regulated during treatment *in vivo*, as described in paper V. Previous studies have suggested that mutant p53 may inactivate p63 and p73 in LFS (178,188) thus additionally suggesting the importance of p53 family evaluation.

Prognostic markers in cancer – a role for p53 protein analysis?

Risk stratification of patients in AML and other malignant diseases is achieved using prognostic markers to identify patients with different probabilities of clinical outcome. Prognostic markers are therefore important instruments in the evaluation and management of diseases. For AML, cytogenetic abnormalities and disease relapse after induction chemotherapy have been recognized as the most important prognostic markers (14). However, close to 50% of patients diagnosed with de novo AML have a normal karyotype (210) and new molecular markers are therefore warranted to improve prognostic stratification. Recently several independent markers have been reported, such as the previously discussed Flt3 abnormalities, partial tandem duplication of the mixed lineage leukemia (MLL) gene (211), mutations of nucleophosmin (212), mutations of transcription factor C/EBPα and expression of the BAALC (brain and acute leukemia, cytoplasmic) protein (210).

Gene arrays have previously made it possible to study the genetic signature of a tumor and in breast cancer it has been shown that each tumor displays a distinct gene expression portrait (213). We are now however, moving into a new era were the application of proteomics will be increasingly important in terms of identifying proteins that are over-expressed or altered in cancer cells since the gene expression patterns do not always correlate with changes at the functional protein level (214). Using proteomic techniques enables the detection of post-translational modifications attributed to proteins and thereby makes it possible to discover functional differences that can not be determined from genomic information. As such, the protein signature of a highly connected signaling molecule might provide an extensive insight into the regulation of the AML proteome based on protein partners and the activity of enzymes conferring post-translational modifications.

It has been suggested that signaling pathways involving p53 cannot be understood by looking at isolated components of the network and that it is essential to consider the entire network in order to comprehend the full potential of this highly connected protein (215). This means that mutation of the TP53 gene is not the only marker that should be considered in prognostic evaluation of a patient. Aberrations in a p53 modifying enzyme, protein partner or any other member of the p53 network could potentially have dramatic effects on the control of cell life and death.

Recently it has been reported that p53 mutational status is associated with a gene expression signature in breast cancer (216). The authors report that the transcriptional p53

fingerprint can be used to predict prognosis and therapeutic response. In paper III and IV the p53 protein signature was used to evaluate the same characteristics. It was found that survival and chemoresistance could be correlated to the expression of specific p53 isoforms in AML. This may indicate that the p53 protein signature, serving as a 'read-out' for the p53 network, could be used as a novel biomarker for clinical outcome in this particular disease.

Li-Fraumeni syndrome in AML

LFS is not usually associated with AML and presentation of a patient with this diagnosis in our clinic offered a unique opportunity to study the effects of a functional knock-out of the p53 protein. Paper II described the regulation of p53 isoforms in patients undergoing chemotherapy and an up-regulation of the full-length protein followed by a decrease of the truncated Δp53 and induction of p53 target genes was shown. The LFS patient had only miniscule amounts of p53 and no protein induction as a response to therapy was observed. We did however detect changes in membrane molecule expression and in phosphorylation of important signaling molecules. The up-regulation of CD11b and down-regulation of CD71 can both be explained by the effect of ATRA treatment (217,218). Stat1, Stat3 and Stat5 responses were more surprising. Stat3 and Stat5 are both implicated in growth factor regulated control of myelopoiesis (219) and can be constitutively activated in response to altered signaling in leukemic blasts (220). However, ATRA has previously been reported to induce Interferon (IFN) γ responsive signaling in AML (221,222) and the opposite effect was observed in cells from the LFS patient examined, as described in paper V. Stat-activity was reduced upon treatment and this was a p53-independent effect as no p53 protein could be detected. The patient was treated with ATRA in combination with the histone deacetylase inhibitor valproic acid and theophyllamine. The combination treatment may account for the unexpected signaling effect and should be further investigated in the treatment of AML.

The normal cell counterpart of AML

A technical problem faced upon preparation of this thesis has been finding an appropriate control-system for AML. AML blasts are not fully matured blood cells. They are released into the blood stream at early differentiation stages and are as such, not completely comparable to mature peripheral blood leukocytes. We therefore tried to include CD34+ stem cells as a control for undifferentiated cells. However, these cells are not perfect controls either because they are sampled from patients with multiple myeloma or non-Hodgkin's lymphoma after mobilization with disease specific chemotherapy and granulocyte colony stimulating factor (G-CSF). Using stem cells from non-AML patients who have undergone pre-treatment is of course suboptimal and not entirely comparable to leukemic cells. A second concern regarding the CD34+ cells is that chemosensitivity might be changed as a result of previous exposure to this particular class of agents. Therefore, to achieve fully comparable results, these concerns must be considered.

The desired control for AML cells would be bone marrow from healthy individuals. Unfortunately, the supply of such material is limited and alternatives have therefore been considered necessary for our experiments.

Conclusions

Previous studies have shown that the TP53 gene is mainly wild type in AML and the results gained in our material correlate with these studies. The work in this thesis has also shown that p53 is accumulated and phosphorylated in response to DNA damage induced by chemotherapy and that known p53 target genes are transcribed after induction chemotherapy *in vivo* suggesting that p53 is a functional protein in AML. Furthermore, it has been shown that specific Flt3 mutations may lead to abrogation of p53-mediated apoptosis by maintaining a downstream block of p53 signaling through driving increased expression of Bcl-2. In patients undergoing treatment, it was found that the regulation of p53 isoforms post chemotherapy is a distinct event in malignant compared to normal cells. However, in cells from a Li-Fraumeni patient where p53 was not detected, several p53-independent effects of treatment were also detected. This included changes in membrane molecule expression and activity of signal transducers.

The use of a novel correlation technique, suggests that the biosignature of p53 protein can be used as a marker for clinical outcome in AML. Specific isoforms of p53 were found to correlate to survival, remission and Flt3 mutational status suggesting that changes in signaling pathways may contribute to distinct regulation of this highly connected protein.

Future perspectives

As discussed, differential regulation of p53 protein isoforms in AML patients has been described. To date, it has not been possible to identify the exact nature of these isoforms and their cellular functions. Describing the physical properties of these isoforms would be of great importance towards elucidating the mechanisms of AML development. Many p53 isoforms that may harbor distinct activities have been previously described in the literature. These might have potential significance for chemosensitivity and AML disease course and it would therefore be interesting to determine the functions of the isoforms found expressed in our patient material and to associate isoform expression to disease characteristics like; AML subtypes, treatment response and survival. The two main isoforms presented in this thesis, αp53 and Δp53, may represent differently spliced mRNA encoded by p53. Analysis of p53 splice forms in the clinical material may elucidate the representation and regulation of p53 mRNA in AML. This could also be extended to include p63 and p73 variants that have been shown to regulate p53 functionality.

Furthermore, it is necessary to perform a more complete mapping of all the post-translational modifications found on the p53 protein isoforms. The optimal technology has to be carefully determined, particularly if multiple modifications on the same p53 molecule have to be determined in order to predict biological impact. Multiple labeling using intracellular flow cytometry will not determine whether these are located on the same molecule or in different pools of p53 in the cell. Mass spectrometric analysis of immunoprecipitated proteins might offer a valuable method to perform such experiments. However, this technique may not provide the complete information about combinations of modifications if the protein is trypsinated before analysis, and an intact protein analysis may be necessary.

The indication of p53 as a biomarker for prognostic purposes is based on a new correlation technique. Further testing of this algorithm on cell lines and clinical material is needed to determine its importance. A new and extended set of AML patients should be tested. Furthermore, cell model systems with mutagenized p53 could elucidate the roles of specific residues on the bioprofile as could also modulation of signal transduction pathways by small interfering RNA and small molecule inhibitors.

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