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

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Short title: Epigenetic targeting in AML/Li-Fraumeni syndrome

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Li-Fraumeni syndrome is a hereditary predisposition to cancer usually caused by mutations in the TP53 gene or genes encoding p53 regulatory enzymes. Acute myelogenous leukemia (AML) is relatively uncommon in these patients. We describe a patient with Li-Fraumeni syndrome who developed AML with multiple cytogenetic abnormalities. She was treated with the combination of all-trans retinoic acid (ATRA), the histone deacetylase (HDAC) inhibitor valproic acid and the cyclic AMP-increasing agent theophyllamine. The AML cells' p53 level was low/undetectable before and during treatment; this is similar to other malignancies in these patients. Expression of p21 and p63 was undetectable before and during treatment, whereas p73 expression decreased during treatment. The therapy increased chromatin condensation and altered membrane molecule expression in the AML cells, but the most striking difference was altered phosphorylation status/responsiveness of Stat1, Stat3, Stat5 and CREB. We conclude that combined targeted therapy including HDAC inhibitors has important p53-independent cellular effects and should be further investigated even in malignancies with frequent mutations in TP53.

INTRODUCTION

Li-Fraumeni syndrome (LFS) is a genetic predisposition to cancer development at young age (1-7). The underlying genetic defect in many LFS-families is a germline mutation in the TP53 gene; alternatively the patients may have mutations in genes encoding p53-regulatory enzymes (5). Common clinical characteristics of these patients include: (i) childhood malignancies; (ii) typical malignancies like soft tissue sarcomas, osteosarcomas, brain tumors and adrenocortical tumors; and (iii) several family members with typical malignancies or other malignancies diagnosed before the age of 60 years.

Somatic mutations in the TP53 gene are uncommon in acute myelogenous leukemia (AML) (8) and AML is an uncommon malignancy in LFS (3-7). Here we report a patient with LFS and adult AML. The patient had a germ-line TP53 mutation and the AML cells showed multiple cytogenetic abnormalities. She was treated with the histone deacetylase (HDAC) inhibitor valproic acid in combination with all-trans retinoic acid (ATRA) and theophyllamine. The biological effects of this treatment suggest that therapeutic approaches including HDAC inhibition should be further investigated even for malignancies with mutational or functional p53 inactivation.

MATERIAL AND METHODS

The patient was included in a clinical study after written informed consent. The study was approved by the local Ethics Committee. AML cells were isolated by density gradient separation from the peripheral blood (Ficoll-Hypaque; NyCoMed, Oslo, Norway; specific density 1.077) and showed 95% purity. Cells were stored in liquid nitrogen (9).

For protein analysis AML cells were lysed with 7% trichloroacetic acid (TCA) and protein dissolved in sample buffer compatible with two-dimensional electrophoresis (7 M urea, 2 M thiourea, 100 mM dithiotreitol, 1.5% Ampholyte 3-10, 0.5% Ampholyte 5-6, 0.5% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate). p53 protein patterns were detected using two-dimensional electrophoresis (ZOOM® IPGRunner™ system from Invitrogen Corp.,

Carlsbad, CA) and immunoblotting. The Bp53-12 antibody (Santa Cruz Biotechnology, Santa Cruz CA) recognizing amino acids 20-25 was used for visualization of p53 protein.

Antibodies against CD11b, CD14, CD33, CD34, CD64, CD71 and CD117 and their corresponding isotype controls (Becton Dickinson; Erembodegem, Belgium) were used for analysis of membrane molecule expression. Analysis of intracellular phosphorylation of Stat1, Stat3, Stat5, Stat6, CREB, Erk1/2, Akt and p38 was performed as described previously (10). We investigated the phosphorylation status in unstimulated cells and cells stimulated cells with granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, Stromal derived factor 1 (SDF-1), Interferon (IFN)γ and Flt3-ligand (Flt3L). AML cell proliferation in response to exogenous GM-CSF, G-CSF, M-CSF, Interleukin 3 (IL3), Stem cell factor (SCF), Flt3-ligand (Flt3L), thrombopoietin (Tpo) and erythropoietin (Epo) was assayed by ^{3}H-thymidine incorporation (11). All cytokines were purchased from Peprotech (London, UK) and used at 50 ng/ml.

CASE REPORT

Previous diseases. The patient was a 51 years old female with a history of breast cancer, three soft tissue sarcomas and anal carcinoma. Her first malignancy was a sarcoma diagnosed at the age of 28. Two of the sarcomas were treated with surgery alone, one sarcoma with surgery + chemotherapy and the breast cancer with surgery + irradiation + chemotherapy. Anal carcinoma was diagnosed at the age of 48 and treated with surgery, multiple liver metastasis were later detected and she was treated with repeated cycles of oxilaplatin, 5-fluorouracil and folic acid.

The patient's oldest son had a rhabdomyosarcoma and died from acute leukemia 12 years old. Both the patient and her son had the same TP53 mutation in exon 8, codon 290 (base substitution CGC→CTC, amino acid substitution Arg→ Leu).

Diagnosis and treatment of AML. Leukemia was diagnosed at the age of 51 and was characterized by (i) AML-M0 with membrane molecule phenotype $CD11c^{-}CD13^{+}CD14^{-}CD15^{-}CD33^{+}CD34^{+}CD45^{+}$ $CD117^{+}$; (ii) no genetic Flt3 abnormalities, (iii) multiple cytogenetic abnormalities with karyotype 44-46, XX, del(2)(q2?), del(4)(q21?), del5(5)(q21?), i(17)(q10) +21[cp17]. The patient was treated with ATRA 22.5 mg/m^2 twice daily days 1-14 and from day 3 also valproic acid (serum levels 179-365

μM) and theophyllamine (serum levels 49-64 μM). During this treatment hemoglobin levels and platelet counts (range 178-233 x 10^9/l) were stable. Peripheral blood blast counts showed an initial decline from 30.4 to 23.1 x 10^9/l on day 7. This was accompanied by signs of chromatin condensation in the leukemia cells. However, from day 21 a marked increase in peripheral blood blast counts were observed and valproic acid/theophyllamine were stopped due to progressive disease.

RESULTS AND DISCUSSION

p53 protein analysis. Protein was isolated from enriched AML cells (i) before therapy; (ii) on day 3 following treatment with ATRA alone; and (iii) on days 7 and 11 when she received ATRA in combination with valproic acid and theophyllamine. Wild type p53 is usually detected in two distinct isoforms with alternating expression in native human AML cells (Fig. 1A), and intensive chemotherapy seems to cause a shift towards the full-length isoform (Ånensen, submitted). In contrast, for our patient only minor amounts of p53 could be detected in the leukemia cells before and during therapy (Fig. 1B). Previous studies suggest that the Li-Fraumeni-associated p53 mutants induce a trans-dominant loss of function effect on the wild type protein (6). Our present results demonstrate that this is true also in AML, and the low levels were not significantly altered during treatment. Thus, the present AML disease should be regarded as a functional p53 knockout, suggesting that biological effects of treatment are p53 independent.

We also investigated the expression of the p53-family proteins p63 and p73 before and during treatment (Fig. 2). No p63 was observed before or during therapy, while a minor decrease in p73 was observed. The p21 protein was not expressed before or during treatment.

Membrane molecule phenotype. We investigated the membrane molecule phenotype for AML cells isolated before therapy, after two days of ATRA treatment (day 3) and after combination therapy with ATRA+theophyllamin+valproic acid (days 7 and 10). The leukemia cells showed a gradual decrease in CD71 and an increase in CD11b during treatment (Fig. 1C). In contrast, no major alterations were observed for CD13, CD14, CD15, CD33, CD34, CD64 and CD117 (data not shown). Previous studies in the U-937 cell line suggest that ATRA may contribute to the effects on CD11b and

CD71 (12), but studies in native human AML cells do not support this (13). We conclude that it seems unlikely that these effects are caused by ATRA alone.

Phosphorylation status of intracellular mediators. We first compared the phosphorylation status of intracellular mediators (Stat1, Stat3, Stat5, Stat6, Erk1/2, Akt, p38, CREB) for cells isolated before and after 10 days of treatment. The most striking effects were decreased mean fluorescence intensity (MFI) especially of Stat1 and Stat6, and increased MFI of CREB (data not shown). We then investigated the effect of combination therapy on mediator phosphorylation after exposure to exogenous cytokines. Dual AML cell populations were detected when we examined the phosphoresponse of Stat1 to IFNγ, the Stat3 response to G-CSF and the Stat5 response to IFNγ, and GM-CSF (Fig. 1E). For all these responses we observed a gradual reduction/disappearance of the low-fluorescent subset during treatment. The responses to SFD-1 and Flt3L were not altered for Stat1/3/5. The phosphoresponses of Stat6, Erk1/2, Akt, CREB and p38 were not altered for any of the 5 cytokines during therapy. Thus, the combination therapy has p53-independent effects both on the basal phosphorylation level as well as the phosphoresponsiveness of several intracellular mediators in native human AML cells. Previous studies in the U-937 cell line suggest that ATRA can increase Stat1 phosphorylation (14), but the more complex effects observed in our patient are unlikely to be caused by ATRA alone. Furthermore, the increased basal phosphorylation of CREB is probably caused by theophyllamine/cAMP (15).

AML cell proliferation. The AML cells showed no spontaneous in vitro proliferation. Detectable proliferation was observed in response to IL3, Flt3 and GM-CSF and this was not altered during treatment. However, detectable proliferation in response to lineage-associated growth factors (G-CSF, M-CSF, Tpo, Epo) was not observed for any sample.

Concluding comments. The use of HDAC inhibitors in AML therapy is now considered, and the first clinical studies suggest that this therapeutic strategy can improve peripheral blood cell counts and even induce hematological remission (16). HDACs constitute a large group of enzymes that deacetylate not only histones but also many other nuclear and cytoplasmic proteins including p53, heat shock protein 90, and several transcription factors and structural proteins. Thus, other mechanisms than altered histone acetylation may contribute to the effects of HDAC inhibitors on malignant cells, and p53-mediated effects may then be important.

Valproic acid has been used for HDAC inhibition in several studies. The biological effects of HDAC inhibitors have been suggested to affect p53 through at least two different mechanisms: (i) altered histone acetylation with altered TP53 gene expression; and (ii) altered acetylation of the p53 protein (16). In the present patient we combined valproic acid with ATRA and theophyllamine. ATRA is used for differentiation induction in acute promyelocytic leukemia (17) and can also induce biological alterations in other forms of AML (18). Theophyllamine seems to inhibit AML cell proliferation and increase their sensitivity to ATRA through the increase of intracellular cAMP levels (19, 20). Based on our present results we conclude that combination of targeted therapy has p53 independent effects on signal transduction and membrane molecule expression of malignant cells, and these therapeutic approaches should be further investigated in AML as well as in other malignancies with mutational or functional p53 inactivation.

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LEGENDS

Figure 1.

AML cell characteristics before and during treatment with ATRA+theophyllamine+valproic acid. A. p53 isoforms in native human AML cells derived from a representative patient; the p53 protein has a characteristic pattern visualized using two-dimensional gel electrophoresis and immunoblotting. The observed isoforms are presumed to be the full-length protein (upper dotted circle), a truncated isoform at ~47 kDa (lower dotted circle) and in addition a ~63 kDa spot presumed to be p63. B. p53 protein isoform pattern in our Li Fraumeni syndrome patient. The patient did not display the isoforms normally observed. We detected a minor signal possibly corresponding to the truncated ~47 kDa form but observed no expression of full length p53 protein and no induction of p53 during chemotherapy. C. Membrane molecule expression of native human AML cells. Cells were sampled before therapy and on days 3, 7 and 10 during therapy. Molecule expression was analysed (X-axis) by flow cytometry. The results are presented as the percentage of CD11b and CD71 positive cells compared with an isotypic control. D. Stat1/3/5 and CREB phosphorylation of native human AML cells derived before therapy and after 10 days of treatment. The results are presented as the mean fluorescence intensity (MFI). E. Treatment effects on Stat phosphoresponses in native human AML cells. Cells were investigated before therapy and on days 3, 7 and 10 during therapy. We present the results for day 0 and day 10. We observed a gradual alteration in phosphoresponsiveness during the treatment period when investigating Stat1 phosphorylation in response to IFNγ (pStat1, IFNg; left part), pStat3 in response to G-CSF (middle left), pStat5 in response to GM-CSF (middle right) and IFNγ (right).

Figure 2.

The expression of p21 and the p53-family members p53, p63 and p73 in AML cells derived from a patient with Li-Fraumeni's syndrome and AML. The patient was treated with ATRA+theophyllamine+valproic acid and cells were assayed before (day 0) and during treatment (day3, 7 and 10).

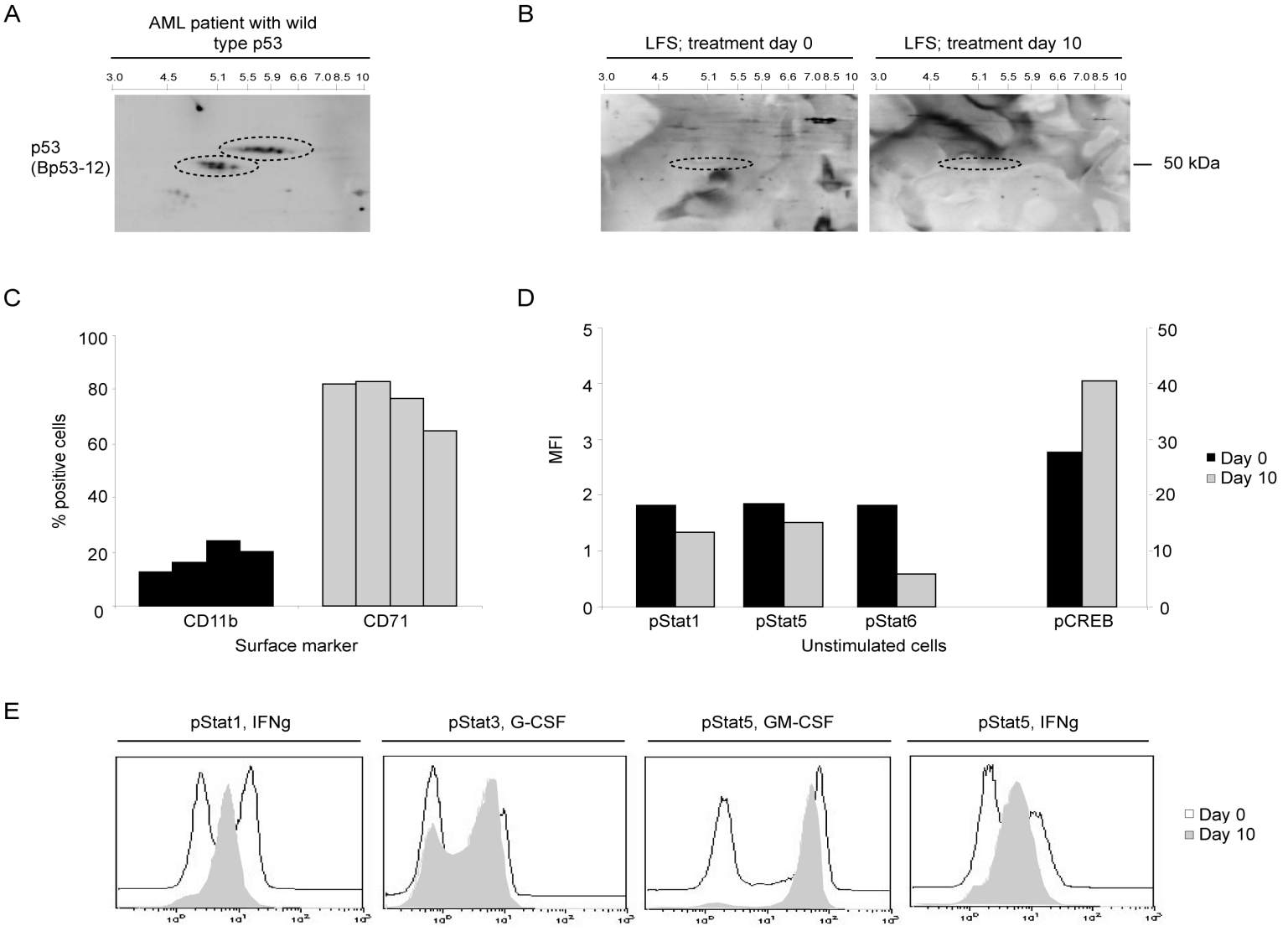

Figure 1. AML cell characteristics before and during treatment

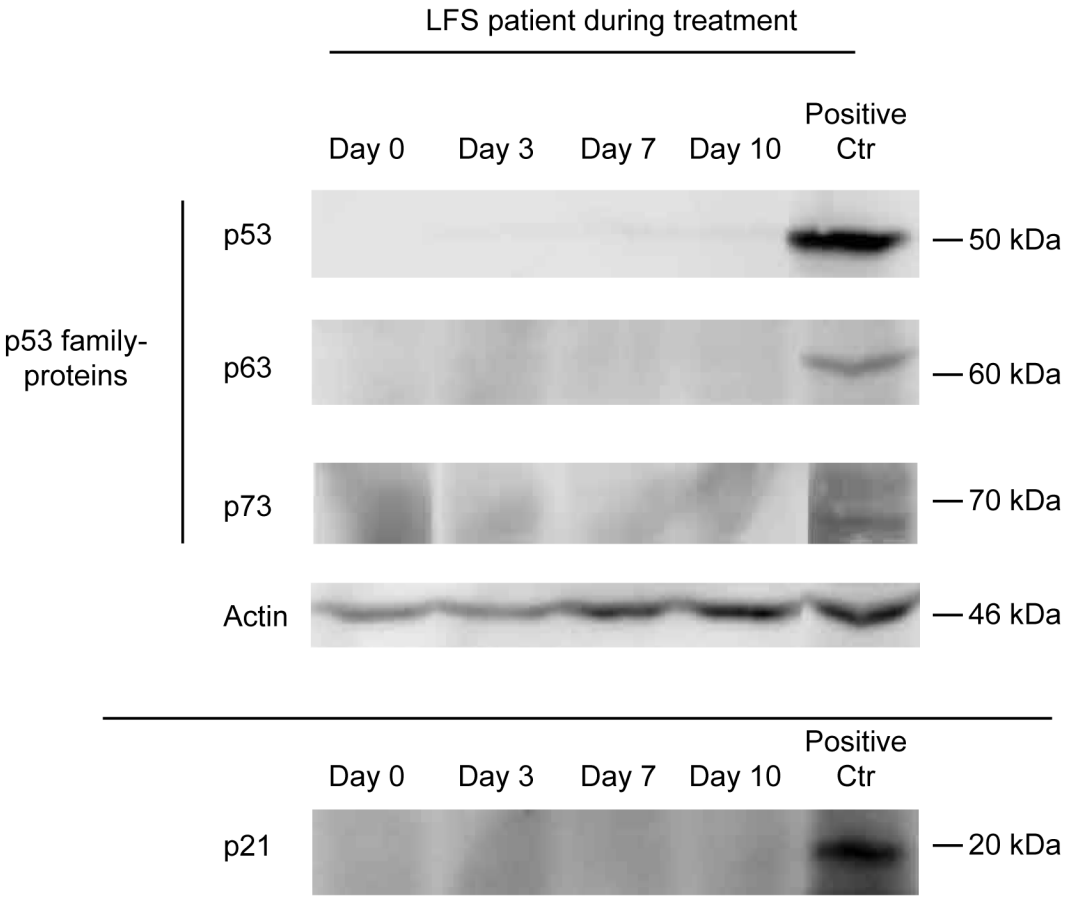

Figure 2. Expression of p53 family members