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Flavopiridol induces *BCL-2* expression and represses oncogenic transcription factors in leukemic blasts from adults with refractory acute myeloid leukemia

Dwella M. Nelson, Ph.D.¹, Biju Joseph, Ph.D.¹, Joelle Hillion, Ph.D.¹, Jodi Segal, M.D.³, Judith E. Karp, M.D.^{2,3}, and Linda M. S. Resar, M.D.*^{1,3,4}

¹Division of Hematology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

²Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

³Division of General Internal Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

⁴Division of Hematologic Malignancy; The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

⁵Department of Oncology; The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

⁶Department of Pediatrics; The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract

Flavopiridol is a cyclin-dependent kinase inhibitor that induces cell cycle arrest, apoptosis, and clinical responses in selected patients with acute myeloid leukemia (AML). A better understanding of the molecular pathways targeted by flavopiridol is needed to design optimal combinatorial therapy. Here, we report that *in vivo* administration of flavopiridol induced expression of the *BCL-2* anti-apoptotic gene in leukemic blasts from adult patients with refractory AML. Moreover, flavopiridol repressed the expression of genes encoding oncogenic transcription factors (*HMGAI*, *STAT3*, *E2F1*) and the major subunit of RNA Polymerase II. Our results provide mechanistic insight into the cellular pathways targeted by flavopiridol and suggest that blocking anti-apoptotic pathways could enhance cytotoxicity and improve outcomes in patients treated with flavopiridol.

Keywords

Flavopiridol; acute myeloid leukemia; *BCL-2*; *HMGAI*; *STAT3*; *E2F1*

BACKGROUND

Acute myeloid leukemia (AML) in adults remains a formidable clinical challenge that demands further investigation to identify more rational therapy [1-5]. This year, almost

*Corresponding author: The Johns Hopkins School of Medicine Ross Building, Room 1015 720 Rutland Ave., Baltimore, MD 21205, USA Telephone: 410-614-0712 Fax: 410-955-0185 lresar@jhmi.edu .

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13,000 new cases will be diagnosed, and AML will claim over 9,000 lives in the U.S. alone [5]. AML is also a frequent, fatal complication of myelodysplastic syndrome (MDS), a clonal myeloid malignancy that affects over 40,000 individuals in this country [5]. In newly diagnosed AML without poor risk features, only 70% of adult patients under the age of 60 years will achieve complete remission (CR) after induction therapy and more than half of these CR patients will ultimately relapse and die from their disease [5]. Certain subgroups of AML patients have an even worse prognosis with cure rates less than 15%, including older patients (over 60 years), individuals with underlying MDS or other hematologic disorders, or those with AML linked to prior chemotherapy, environmental, or occupational exposures [5]. These abysmal epidemiologic data underscore the dire need for further research to investigate novel therapeutic targets and optimize therapy [1-5].

Flavopiridol is a small, semi synthetic, cytotoxic flavone derived from two Indian plants used for herbal therapy (*Amoora rohiuka* or *Dysoxylum binectariferum*) [6-10]. Previous studies show that flavopiridol induces apoptotic death in diverse hematologic malignancies [11-14]. Flavopiridol is thought to function through at least three distinct mechanisms [13-18]. First, it is a potent cyclin dependent kinase (cdk) inhibitor that blocks cell cycle progression [13,14]. In the setting of pan-cdk inhibition, E2F is released and drives apoptosis [12,13]. Second, by blocking cdk 7 and 9 function, flavopiridol prevents phosphorylation and activation of the RNA Polymerase II (Pol II) protein [9,12]. This is thought to result in the global down-regulation of gene expression, including genes that promote leukemic cell survival and proliferation [13]. Previous studies also show repression of *Cyclin D1* or *CCND1* [13-14], *MCL-1* [16] and *BCL-2* [16,18] at the RNA and protein level. Third, studies show that flavopiridol disrupts STAT3 function by blocking its binding to DNA in tumor cells [15]. Because STAT3 is constitutively active in many subtypes of leukemia and serves as a central regulator in multiple pro-survival and oncogenic signaling pathways [19-21], flavopiridol could block the tumor promoting pathways induced by STAT3. Given the pleiotropic effects of flavopiridol, it is likely that it functions through additional, unknown molecular pathways.

Previous *in vitro* studies demonstrated that flavopiridol causes apoptotic cell death in leukemic blasts from patients with poor-risk AML or acute lymphoblastic leukemia (ALL) [11]. A subsequent clinical trial with flavopiridol for 3 days, followed by ara-C and mitoxantrone, resulted in a response rate of 31% in adults with relapsed or refractory AML or ALL [22]. Correlative studies of leukemic bone marrow blasts obtained before and after flavopiridol showed decreases in the protein levels of at least one putative flavopiridol target, including phosphorylated RNA Pol II, phosphorylated STAT3, CCND1, BCL-2, or MCL-1 in some cases [22]. These observations were translated into a Phase II clinical trial and CRs were achieved in 75% (12/15) of newly diagnosed, secondary AML patients [23]. Notably, 10 (67%) of these secondary AML patients had MDS and CRs were achieved in 90% (9/10) of these high-risk patients [23].

The *high mobility group A1* (*HMGA1*) gene is a member of the *HMGA* gene family [24-44] and encodes a potent oncogenic transcription factor that is highly overexpressed in diverse, high-grade malignancies, including ALL [29,33], AML [20,29,41], and Burkitt's lymphoma [20,24-25]. *HMGA1* induces a transformed phenotype in cultured, hematopoietic cells [20,24-25] and causes aggressive leukemia in transgenic mice [33-34]. Conversely, inhibiting *HMGA1* expression blocks transformation phenotypes in diverse cancer cells, including those from hematopoietic malignancies and solid tumors [20,24,27,35,38,40]. In addition, recent gene expression profile analyses indicate that *HMGA1* is a key transcription factor enriched in human embryonic stem cells [36], hematopoietic stem cells [41,44-45], and leukemic stem cells [46]. In hematopoietic malignancies, *HMGA1* induces expression of *STAT3* [20,]. More recent preliminary data suggests that *HMGA1* up-regulates *E2F1*

expression (Resar, unpublished data). HMGA1 also enhances global gene expression by interfering with histone H1-mediated repression of transcription [40]. Because flavopiridol affects these HMGA1 pathways by down-regulating transcription through inhibition of Pol II phosphorylation [12], promoting apoptosis through E2F1 [12-13], and blocking STAT3 activity [15], we hypothesized that flavopiridol will be cytotoxic in tumors dependent upon *HMGA1* overexpression.

Here, we investigate expression of pro-oncogenic transcription factors and anti-apoptotic pathways in primary, AML blasts from adults with refractory or high-risk AML before and after *in vivo* flavopiridol administration. We found that flavopiridol induces expression of the gene encoding the anti-apoptotic protein, BCL-2, while it represses expression of the genes encoding the oncogenic transcription factors, HMGA1, STAT3, and E2F1. In addition, expression of the gene encoding the major subunit of RNA Polymerase II (*POLR2A*) was repressed. Our findings provide mechanistic insight into cellular pathways targeted by flavopiridol and potential combinatorial strategies to optimize therapy in AML.

MATERIALS AND METHODS

Patient selection, treatment schema and responses

Adult patients with relapsed or refractory AML received flavopiridol as part of an NCI-sponsored clinical trial (NCI 00470197) in accordance with the Johns Hopkins Medical Institutional Review Boards and guidelines. Flavopiridol was administered daily for three consecutive days by a bolus-infusion schedule developed by Byrd *et al.* and based on pharmacologic data [47]. Flavopiridol doses were escalated from a total flavopiridol dose of 50 mg/m² (level 1) up to 100 mg/m² (level 6) after treating 2-10 patients at each dose level (Table I). On day 6, patients received a 72 hour continuous infusion of ara-C (667 mg/m²/24 hours from days 6-9). Mitoxantrone (40 mg/m²) was given as a single intravenous bolus over 60-120 minutes on day 9 [23].

Patient response was designated as CR if the bone marrow aspirate performed after peripheral blood counts recovered showed no evidence for leukemia by morphologic examination and flow cytometry, and the absolute neutrophil count was $\geq 1000/\text{mm}^3$, the platelet count was $\geq 100,000/\text{mm}^3$, and there were no blasts in the peripheral blood by morphologic examination [48]. Clearance of cytogenetic abnormalities was not required for CR. No response (NR) was defined as persistent leukemia in the marrow and/or peripheral blood and no significant decrease in the % blasts from pretreatment levels. Partial remission (PR) was defined as the presence of trilineage hematopoiesis in the marrow with normalization of peripheral counts, but with persistent abnormal blasts (5-25%). In this study group, there were 5 CRs, 2 PRs, and 8 NRs. One patient had complete tumor clearance on day 14, but died of infection while neutropenic on day 14, and is therefore designated not evaluable (NE). Results of the full clinical trial showed similar response rates and are reported elsewhere [41].

RNA isolation and cDNA synthesis

Peripheral blood blasts were obtained on day 1 prior to flavopiridol therapy and within 2 hours after the end of the first infusion [22]. Patients were eligible if the pretreatment circulating, peripheral blood blasts prior to and following the initial flavopiridol infusion were $>400/\text{mm}^3$ to ensure adequate RNA. All studies were conducted following blast enrichment by Ficoll-Paque density gradient separation (Pharmacia, Piscataway, New Jersey) with the blast percentage following enrichment estimated to be $\geq 70\%$ based on prior experience [11]. Total RNA from the blasts was then isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA) and quantified on a Nanodrop ND-1000 spectrophotometer

(NanoDrop Technologies, Wilmington, DE). RNA (500 ng) was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) in 100 μ L reaction containing 10 μ L 10X reverse transcription buffer, 4.0 μ L 25X dNTP and 5 μ L murine moloney reverse transcriptase as previously described [33,35]. A negative control reaction without reverse transcriptase amplification was included in all experiments. Although we initially planned to assess proteins by Western analysis in addition to gene expression, the limited cell number precluded protein analysis. The gene expression results from the peripheral blood blasts were compared to gene expression in CD34+ hematopoietic stem cells (HSCs); mRNA levels from the HSCs were arbitrarily assigned a value of 1.0. HSC RNA was obtained from a commercial source (ALLCELLS, CA, USA).

Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) of cDNA was performed as previously described [33] with the following modifications. For most genes, we used Taqman PCR reagents (Applied Biosystems, NJ, USA) and primers (*E2F1*, *BCL-2*, *VEGF-A*, *RNA polymerase II 2A* or *POLR2A*, *CCND1*, or *MCL-1*) (Applied Biosystems, CA, USA). The gene of interest was compared to a housekeeping gene to control for sample loading and reaction conditions were optimized for maximal efficiency. Human *phosphoprotein (PO)* was used as the control for *HMGA1*, *E2F1*, and *MCL-1* as previously described [33,35]; β -*actin* was used as the control for *BCL-2*, *VEGF-A*, and *POLR2A*. Because the Taqman primers do not distinguish *STAT3* from *STAT3 β* , we assessed *STAT3* using SYBR green master mix (Applied Biosystems, UK) as we previously described [36] with β 2 *microglobulin* as the control gene for sample loading. Reactions were performed in triplicate and repeated at least once if there were discordant results. Results indicate the mean from all results \pm the standard deviations. The qRT-PCR results were analyzed using the software provided by the manufacturer (Applied Biosystems, CA, USA) using the $\delta\delta$ CT method (according to the manufacturer's instructions).

Statistical analysis

GraphPad Prism version 5.0 for windows (GraphPad software, CA, USA) was used for statistical analysis and graph preparation. The Wilcoxon signed-rank test was used to compare the expression of each gene before and after flavopiridol therapy.

RESULTS

Patient characteristics, cytotoxicity following flavopiridol and clinical responses

Leukemic blasts were isolated from peripheral blood before and after flavopiridol in 36 patients enrolled in the Phase I part of the NCI 00470197 protocol [41]. Sufficient RNA was available for further analysis from 16 patients. The remaining 20 samples had either degraded RNA or blast counts that were too low to obtain adequate quantities of RNA. (Clinical characteristics of the 16 patients are summarized in Table I). Flavopiridol resulted in a 50% or greater decrease in the peripheral blood blast counts in 9/16 (56%) of cases after the first infusion and in 15/16 (94%) of cases after all 3 doses, indicating significant cytotoxicity from flavopiridol alone. Of the 16 patients studied in this cohort, there were 5 CRs (31%), 2 PRs (12.5%), and 8 NRs (50%); and; one patient (6%) died during aplasia and was therefore not evaluable (NE). The clinical responses of all 55 patients treated with this protocol are reported separately [42] and were similar to the results from this cohort of 16 (CRs in 40% of all cases with an overall and disease-free survival of \geq 60% at over 2 years in patients achieving a CR).

Flavopiridol up-regulates BCL-2 in leukemic blasts

To elucidate molecular pathways disrupted by flavopiridol in AML, we investigated gene expression of in primary leukemic blasts obtained from patients with refractory or poor-risk AML before and after therapy with flavopiridol (Figure 1A). Previous studies have shown that expression of the anti-apoptotic gene, *BCL-2*, at the mRNA [18] and protein [16,22] level decrease in cultured HL60 cells and leukemic blasts after flavopiridol therapy. Strikingly, we found that the gene encoding *BCL-2* was induced in the leukemic blasts from all patients (16/16) following flavopiridol (Figure 1A). Before therapy, the mean mRNA level of *BCL-2* in the peripheral blood leukemic blasts and HSCs were similar (3.58 ± 3.32 for AML blasts versus 1.0 ± 0.87 for HSCs). After flavopiridol treatment, *BCL-2* expression increased significantly in the blasts to 19.38 ± 18.40 ($p=0.0005$; Table II).

Flavopiridol results in the down-regulation of genes encoding oncogenic transcription factors (HMGA1, STAT3, E2F1) and the major subunit of RNA Pol II (POLR2A)

We investigated expression of oncogenic and cell cycle regulatory genes before and after therapy with flavopiridol. The *HMGA1*, *STAT3*, and *E2F1* mRNA levels were similar in the leukemic blasts before therapy to those observed in the CD34+ HSCs. Strikingly, there was a statistically significant decrease in gene expression for *HMGA1*, *STAT3*, *E2F1*, and *POLR2A* in most patients (Figure 1B; Table II). Flavopiridol resulted in the down-regulation of *HMGA1* expression in all cases (16/16 or 100%). The mean *HMGA1* mRNA decreased from 2.48 before flavopiridol to 1.07 after flavopiridol ($p=0.0005$). In addition, *STAT3* and *E2F1* mRNA decreased in most patients studied (13/16 or 81% for *STAT3* and 15/16 or 94% for *E2F1*) (Table II). The mean *STAT3* mRNA decreased from 3.73 to 2.68 ($p=0.041$). The mean *E2F1* mRNA levels also fell from 0.55 before therapy to 0.11 after therapy ($p=0.009$). *POLR2A* expression in pre-treatment blasts was also similar to expression in HSCs and decreased from 1.36 to 0.76 ($p=0.034$). These results indicate that flavopiridol significantly repressed expression of three oncogenic transcription factors (*HMGA1*, *E2F1*, and *STAT3*) involved in leukemogenesis. In addition, the gene encoding a major subunit of RNA Pol II (*POLR2A*) was also down-regulated in leukemic blasts by flavopiridol. For all genes studied, the mean changes in expression after flavopiridol were similar in the patients achieving CRs or NRs (Table II). The other genes investigated in this study did not change significantly after flavopiridol, including *VEGF-A*, *CCND1*, and *MCL-1* (Figure 2; Table II).

DISCUSSION

To define the molecular pathways targeted by flavopiridol in AML, we investigated gene expression from primary human leukemic blasts before and after therapy with flavopiridol. Notably, *BCL-2* expression was induced, which contrasts from previous studies demonstrating down-regulation of *BCL-2* at the mRNA and protein level following exposure to flavopiridol [16,18,22]. Our results suggest that flavopiridol induces a *BCL-2*-mediated anti-apoptotic response, potentially as a protective mechanism in the leukemic blasts during cell cycle arrest driven by cdk inhibition.

We also found that flavopiridol significantly represses expression of *HMGA1*, *STAT3*, and *E2F1*, three oncogenes previously shown to be involved in hematopoietic malignancies [20-21,24-25,29,40-41]. These findings are important because recent studies also identified *HMGA1* as a key transcription factor enriched in human embryonic stem cells [36], leukemic stem cells [46], and poorly differentiated solid tumors [40], suggesting that *HMGA1* could drive a primitive, stem-like phenotype in AML. Like normal embryonic stem cells, leukemic stem cells appear to have long-term self-renewal and other stem-like properties that could confer drug resistance. Of note, the *HMGA1* expression levels in the AML blasts before therapy were similar to levels in HSCs. *STAT3* also appears to play a

pivotal role in murine HSC self-renewal and could regulate similar pathways in leukemic cells [20,49]. In addition, *E2F1* is a master regulator of cell cycle progression and a recent study found that E2F1 promotes a poorly differentiated state in AML by repressing the *microRNA-223* [50]. Taken together, our results suggest that targeting these pathways with drugs like flavopiridol could be efficacious in leukemia and other malignancies with stem cell-like molecular signatures.

Lastly, we found that flavopiridol represses the gene encoding the major subunit (2A) of RNA Pol II, which could lead to a global down-regulation in gene expression. Although this gene is integral to Pol II function and transcription in general, not all genes were repressed, indicating some level of specificity in the effects of flavopiridol.

In summary, our studies demonstrate that flavopiridol induces *BCL-2* expression while it represses expression of the *HMGAI*, *STAT2*, *E2F1*, and *POLR2A* in primary AML blasts from adult patients. This finding suggests that targeting anti-apoptotic pathways in combination with flavopiridol could potentiate flavopiridol's cytotoxic effects and improve patient outcomes, a hypothesis that could be tested in the clinic and laboratory. Because HMGAI is an oncogenic chromatin remodeling protein enriched in stem cells and high-grade, refractory tumors from diverse tissues, flavopiridol could be useful adjunctive therapy in other malignancies with poor prognostic features.

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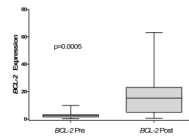


Figure 1. Changes in gene expression following *in vivo* flavopiridol therapy in leukemic blasts from adults with AML

A.) *Bcl-2* mRNA expression was significantly induced following *in vivo* treatment with flavopiridol. Gene expression in leukemic blasts was assessed by qRT-PCR. The relative gene expression is reported as Box and Whisker Plots for n=16 (in triplicate). The minimum and maximum values are shown as the top and bottom values of each whisker, and the median is shown as the center line of each box.

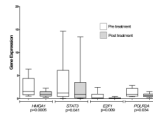


Figure 1B. Changes in gene expression following *in vivo* flavopiridol therapy in leukemic blasts from adults with AML

HMGA1, *STAT3*, *E2F1* and *POLR2A* were significantly down-regulated in leukemic blasts following treatment with flavopiridol. The relative gene expression is reported as Box and Whisker Plots n=16 (in triplicate). The minimum and maximum values are shown as the top and bottom values of each whisker, and the median is shown as the center line of each box.

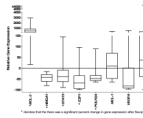


Figure 2. Average percent change in gene expression following therapy with flavopiridol

The relative change in gene expression for all patients was calculated as the difference of the pre-treatment level and post-treatment level divided by the pre-treatment level and multiplied by 100 as follows: $\Delta \text{mRNA level pre-flavopiridol} - \text{mRNA levels post flavopiridol} / \text{pre mRNA level pre-flavopiridol} * 100$. The relative gene expression is reported as Box and Whisker Plots n=16 (in triplicate). The minimum and maximum values are shown as the top and bottom values of each whisker, and the median is shown as the center line of each box.

Table 1

Clinical characteristics of the AML cases

Patient #	Age/Sex	Diagnosis	Dosage Level	Karyotype	% Blasts (Absolute Blast Count)	Response CR/PR/NR/NE
P1	61/M	2° refractory	3	45,XY,-7	50% (1,100)	CR
P2	26/F	2° refractory	3	46,XX,-20q	26% (1,118)	CR
P3	36/M	1° refractory	4	46,XY	12% (966)	CR
P4	20/F	Multi-refractory	5	complex	61% (10,181)	CR
P5	47/F	1° refractory	5	complex	90% (540)	CR
P6	62/M	1° refractory*	2	Trisomy 8	10% (710)	PR
P7	34/M	1° refractory**	2	complex	29% (1,189)	PR
P8	60/F	1° refractory	2	complex	95% (31,873)	NR
P9	63/F	1° refractory	2	46,XX	20% (2,284)	NR
P10	43/M	1° refractory	3	46,XY, t(11;19)	75% (18,120)	NR
P11	55/M	Multi-refractory	4	46,XY	75% (750)	NR
P12	41/F	1° refractory	4	46,XX	41% (2,530)	NR
P13	59/M	1° refractory	5	46,XY	55% (6,914)	NR
P14	53/F	1° refractory	5	complex	41% (18,425)	NR
P15	69/M	Multi-refractory	6	47,XY,+8	32% (403)	NR
P16	23/F	1° refractory	2	Inv 8 t(8;8)	83% (14,757)	NE

Patient characteristics are listed above.

denotes a death during treatment; 20 refers to AML that occurred in the setting of MDS or chemotherapy for a prior malignancy; M-male; F-female; CR-Complete remission; NR-No response; PR-Partial response; NE-Non-evaluable.

Table II
Summary of Gene Expression Levels in Patients Pre and Post Flavopiridol Treatment (n=16)

	BCL-2	HMGAI	STAT3	E2F1	POLR2A	MCL-1	VEGFA	CCND1
All (16)								
Mean Pre	3.58	2.48	3.73	0.55	1.36	18.9	1.05	0.88
Range Pre	0.19-9.97	0.81-4.88	0.24-14.64	0.00-1.12	0.54-2.90	3.36-55.75	0.02-0.80	0.12-10.84
Mean Post	19.38	1.08	2.68	0.11	0.76	19.58	0.76	4.22
Range Post	0.54-63.17	0.32-2.28	0.07-13.43	0.00-0.45	0.23-1.54	1.67-39.42	0.00-4.92	0.00-29.24
***p Value % of patients with p≤0.05	p=0.0005 (100%)	p=0.0005 (81.2%)	p=0.041 (68.7%)	p=0.009 (56.3%)	p=0.034 (83.3%)	p=0.518 (93.7%)	p=0.103 (75%)	p=0.104 (37.5%)
CR(5)								
Mean Pre	6.00	2.36	4.31	0.68	1.06	18.49	1.61	0.13
Range Pre	1.35-9.48	0.75-6.42	0.23-11.25	0.05-2.46	0.39-2.90	6.07-27.84	0.51-2.86	0.01-0.33
Mean Post	33.13	0.84	3.26	0.14	0.59	29.87	1.11	5.76
Range Post	4.02-63.17	0.50-1.30	0.07-9.66	0.01-0.31	0.23-1.54	19.69-37.16	0.05-4.83	0.04-25.90
***p Value % of patients with p≤0.05	p=0.0625 (100%)	p=0.0625 (60%)	p=0.0625 (80%)	p=0.0625 (80%)	p=0.0625 (60%)	p=0.62 (80%)	p=0.125 (100%)	p=0.125 (60%)
NR(8)								
Mean Pre	2.79	2.96	2.34	0.52	1.58	12.51	0.45	1.65
Range Pre	0.19-9.97	0.60-5.90	0.07-6.31	0.00-1.84	0.44-2.70	2.67-38.74	0.02-1.94	0.01-10.84
Mean Post	15.89	1.34	1.36	0.073	0.87	10.51	0.73	4.32
Range Post	0.54-54.70	0.43-2.28	0.10-3.59	0.00-0.38	0.43-1.40	1.67-19.04	0.00-4.92	0.00-29.24
***p Value % of patients with p≤0.05	p=0.039 (100%)	p=0.008 (87.5%)	p=0.023 (62.5%)	p=0.078 (37.5%)	p=0.015 (100%)	p=0.039 (100%)	p=0.19 (50%)	p=0.47 (37.5%)
PR(2)								
Mean Pre	2.37	0.68	8.95	0.092	NA	35.03	1.18	NA
Range Pre	1.52-3.21	0.56-0.81	3.25-14.64	0.04-0.14	NA	14.33-55.75	0.41-1.94	NA
Mean Post	7.62	0.33	7.29	0.041	NA	22.95	0.11	NA
Range Post	1.78-13.46	0.32-0.35	1.15-13.43	0.00-0.08	NA	6.48-39.42	0.09-0.13	NA

	BCL-2	HMGAI	STAT3	E2F1	POLR2A	MCL-1	VEGFA	CCND1
***p Value % of patients with $p \leq 0.05$	p=0.50 (100%)	p=0.25 (100%)	p=0.25 (50%)	p=0.25 (50%)	NA	p=0.25 (100%)	p=0.25 (100%)	NA

This table summarizes the gene expression changes (increased or decreased expression) for the 12 AML cases. CR-Complete remission; PR-Partial Response; NR-No response; NE-Non-evaluable.

* (W) p-value; statistical significance assigned by the Wilcoxon signed-rank test.