

ORIGINAL ARTICLE

Molecular responses during chemotherapy in acute myeloid leukemias in predicting poor-response to standard chemotherapy

Abdullah MAHA *PhD*, Soon-Keng CHEONG* *FRCPE, FRCPA*, Chooi-Fun LEONG* *FRCPE, FRCPA*, and Heng-Fong SEOW *PhD*

*Department of Pathology, Faculty of Medicine and Health Sciences, University Putra Malaysia, and *Faculty of Medicine, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia*

Abstract

Signal transduction pathways are constitutively expressed in leukaemic cells resulting in aberrant survival of the cells. It is postulated that in cells of chemo-sensitive patients, chemotherapy induces apoptotic signals leading to cell death while survival signals are maintained in cells of chemo-resistant patients. There is very little information currently, on the expression of these mediators in patients immediately after chemotherapy initiation. We examined the expression pattern of proinflammatory cytokines, signaling molecules of the PI3K and MAPK pathways molecules and death receptor, DR5 on paired samples at diagnosis and during chemotherapy in acute myeloid leukaemia patients treated with cytosine arabinoside and daunorubicin. The results were correlated with remission status one month after chemotherapy. We found that in chemo-sensitive patients, chemotherapy significantly increased the percentage of cases expressing TNF- α ($p=0.025$, $n=9$) and IL-6 ($p=0.002$, $n=11$) compared to chemo-resistant cases. We also observed an increased percentage of chemo-sensitive cases expressing DR5 and phosphorylated p38, and Jnk. Thus, expression of TNF- α , IL-6, DR5, phospho-p38 and phospho-Jnk may regulate cell death in chemo-sensitive cases. In contrast, a significantly higher percentage of chemo-resistant cases expressed phospho-Bad ($p=0.027$, $n=9$). IL-1 β and IL-18 were also found to be higher in chemo-resistant cases at diagnosis and during chemotherapy. Thus, expression of various cellular molecules in leukaemic blasts during chemotherapy may be useful in predicting treatment outcome. These cellular molecules may also be potential targets for alternative therapy.

Keywords: Interleukin, signal transduction, apoptosis, treatment response

INTRODUCTION

Signal transduction pathways (STP) including phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/Raf/MEK/ERK (also known as Ras-MAPK, mitogen activated protein kinase) regulate normal cell proliferation, survival and cell death. Phosphorylated Akt leads to phosphorylation and inhibition of pro-apoptotic molecules FKHR (transcription factor Forkhead) and Bad resulting in protection against apoptosis.¹ STPs may be activated by proinflammatory cytokines such as TNF- α , interleukin (IL)-1 β , IL-6 and IL-18.² Proinflammatory cytokines and environment stresses also stimulate other members of the MAPK family, p38 and Jnk.³

Aberrant activation of STPs plays a role in tumorigenesis. Constitutive expression of PI3K/Akt mediators was frequently observed in cancers including acute myeloid leukaemias (AML) contributing to proliferation and survival of cells.⁴ Ras/Raf/MEK/ERK was activated in over 50% of AMLs.⁵ The role of p38 and Jnk in growth regulation of acute leukemia blasts is less established⁶ but recent studies has implicated p38 as an important regulator of cancer progression.⁷ Proinflammatory cytokines were observed to promote growth, attenuate apoptosis and facilitate invasion and metastasis of tumour cells.^{2,8}

The conventional method of leukaemia treatment is chemotherapy. Chemotherapeutic drugs induce leukaemic cell death by altering

signaling pathways and activating apoptotic molecules. Arabinosylcytosine (Ara-C) was observed to induce apoptosis in HL-60 cell lines through the activation of p38.⁹ Adriamycin was shown to activate JNK in a T cell leukemia cell line.¹⁰ Leukemia cell lines (TF-1 and K562) primed for apoptosis were also revealed to stimulate Jnk and p38 phosphorylation.¹¹

Apoptosis occurs principally via two separate yet interlinked signaling mechanisms: the extrinsic pathway, activated by proapoptotic receptor signals at the cellular surface, and the intrinsic pathway, activated by mitochondrial signals from within the cell. Proapoptotic ligands such as FasL (or CD95L) triggers Fas receptor (or CD95) while Apo2 ligand/TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) binds to DR4 and DR5 receptors. The main function of TNF- α is to stimulate proinflammatory gene expression through TNFR1-mediated activation of the transcription factor NF- κ B. However, this ligand can stimulate apoptosis under special circumstances through its death domain-containing receptor TNFR1 when NF- κ B activation is blocked.¹² Chemotherapy induced TNF- α transcripts in AML blasts were observed by Devemy and others.¹³ In human leukaemic cell lines, DR5 was upregulated by chemotherapeutic drugs such as etoposide, cytosine arabinoside (Ara-C) or doxorubicin resulting in cell death¹⁴.

The study by Devemy and others¹³ also showed an increase in IL-6 transcripts during remission induction therapy of AML patients who were accompanied by a fall in blood count and bone marrow cellularity. When cells die *in vivo*, they also trigger an inflammatory response. While often stated apoptotic cell death does not provoke inflammation, in abnormal induction of apoptosis, intracellular molecules released from the late-phase dying cells may stimulate other cells to produce proinflammatory cytokines including IL-1, TNF- α and IL-6.¹⁵

Stress stimuli however, may not always induce cell death in leukaemic cells. Ionizing radiation¹⁶ on Friend erythroleukemia cells and daunorubicin¹⁷ on human acute myeloid leukemia cell lines were also found to stimulate survival mediators such as Akt of the PI3K pathway. These prevented cell death and resulted in drug resistance. High levels of IL-1 β bone marrow samples taken on day 5 of induction were found associated with treatment failure.¹³

Resistance to chemotherapeutic drugs remains a major problem in the successful treatment of

AML. The cure rate for patients younger than 60 years is 20-75% and less than 10% among the elderly patients.¹⁸ The mechanism of resistance in acute leukaemia is still a current subject of interest.

Our aim of the study was to determine if chemotherapy altered the expression pattern of initiators and mediators of STPs in AML samples, and whether the alteration if it exists correlated with treatment outcome. We determined the expressions of proinflammatory cytokines, mediators of MAPK and PI3K signaling pathways and DR5 in blasts of chemo-sensitive versus chemo-resistant AML patients treated with standard induction therapy.

MATERIALS AND METHODS

Leukemia samples

Peripheral blood or bone marrow samples were collected from newly diagnosed acute myeloid leukemia patients admitted into haematological wards in Hospital Universiti Kebangsaan Malaysia between January 2000 and December 2002. All procedures were performed in accordance with the principles of the Declaration of Helsinki (1964). Samples were collected with informed patient consent. The study was approved by the institutional review board. No patient was on growth factor therapy.

Inclusion criteria: Consecutive patients diagnosed to have acute myeloid leukaemia by morphology (French-American-British Classification), cytochemistry and immunochemistry by flow cytometry.

Exclusion criteria: Leukaemia patients whose febrile state was established to be infective in origin. Leukaemic patients who were on antibiotics.

Blood samples during induction therapy

A corresponding peripheral blood sample was obtained from patients undergoing induction therapy. The sample was collected 24 hours after chemotherapy was initiated. Sampling was done between 1 and 4 days. All patients in this study underwent a standard induction therapy which consisted of 100 mg/m² cytosine arabinoside for 5 days and 60 mg/m² daunorubicin for 2 days.

Treatment outcome

Remission status was determined from bone marrow aspirations one month after induction therapy. Criteria for complete remission included targeted values for absolute neutrophil

count (>1000/microL) and platelet count (>100,000/microL), absence of extramedullary disease and less than 5% blasts present in bone marrow. Patients who could not achieve complete remission status by one month was defined as chemotherapy resistant (5 patients), whilst patients who could do so was defined as chemotherapy sensitive (7 patients).

Cell isolation

Mononuclear cell isolation was performed by gradient density centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Cells were used immediately or cryopreserved in RPMI 1640 (Gibco, BRL, USA) containing 20% heat inactivated fetal calf serum (Gibco, BRL, USA) and 10% dimethylsulfoxide (DMSO) (Amresco, USA).

Flow cytometry

Prior to staining, cells were quick-thawed in a 37°C water bath and washed in phosphate buffered saline (PBS). The wash step consisted of diluting cells with approximately 2-3 mls of PBS followed by centrifugation at 2,000 rpm for 5 min to obtain a cell pellet. Viability and cell count was determined using the trypan blue exclusion test. Only samples with viability of 70% and above were used for further analysis.

Cell permeabilization for intracellular staining

Cells were fixed in PBS containing 2% formaldehyde at 4°C for half an hour. Cell permeabilization was carried out with an equal volume of 0.4% of Triton-X (in PBS) for 5 min at room temperature (RT). After washing, cells were resuspended with fresh PBS to obtain a cell concentration of 5×10^6 cells/ml.

Antibodies

A three-step indirect staining procedure was performed. The primary antibody used were rabbit anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473), anti-phospho-FKHR (Ser256) (Forkhead), anti-phospho-Bad (Ser112), anti-phospho-p38 (Thr180/Tyr182) and anti-phospho-Jnk (Thr183/Tyr185) from Cell Signaling Technologies (USA). Antibodies chosen were those that were directed specifically against phosphorylated peptides of respective human proteins. Antibodies were diluted 1:10 or 1:20 times and 4 µl was taken to stain each sample. The secondary antibody consisted of biotin-conjugated swine anti-rabbit, anti-mouse and anti-goat (Dako Corporation, USA) antibodies.

Phycoerythrin (PE) conjugated streptavidin (Transduction Lab, Becton Dickinson, USA) was directed against the secondary antibody. Half a million cells were used for each test. Step-wise incubation with the appropriate volume of antibody was carried out for 20-30 min in the dark at RT. After incubations, cells were washed and resuspended in 100 µl of PBS. Cells were re-suspended into 400 µl of PBS and analyzed on the FACSCalibur (Becton Dickinson, USA) at final wash. Negative controls were similarly stained but omitted for the primary antibody. Samples with > 20% fluorescence staining were regarded as positive cases.

RNA isolation

RNA was isolated according to the manufacturer's instructions (Tri-Reagent, Life Technology, USA). Briefly, 1 ml of Tri-Reagent was added to the cell pellet containing 2 million cells. To this mixture 0.2 ml of chloroform was added. After vigorous shaking the tube was left to stand in room temperature for 2-15 min followed by a spin at 12,000 g for 15 min at 4°C. The upper aqueous phase containing RNA was transferred into a new tube and mixed with 0.5 ml isopropanol. The RNA was precipitated and centrifuged at 12,000 g for another 8 min at 4°C. Isopropanol was discarded and replaced with 75% cold ethanol. After a second wash with cold ethanol and a second spin, ethanol was discarded and the RNA pellet was left to dry briefly. RNA pellet was dissolved in an appropriate amount of diethylpyrocarbonate (DEPC)-treated ultra-pure water containing RNase inhibitor (0.1 U/µl) and stored at -80°C.

The purity and quantity of RNA were determined on a spectrophotometer. The ratio of wavelength reading at 260:280 nm was between 1.8-2.0. The RNA concentration was estimated by measuring absorbance at 260 nm.

Reverse transcription (RT)

Two µg RNA, 0.5 µg of oligo(dT)₁₅ primer (0.5 µg/ml) and DEPC-treated water to make a total volume of 9.0 µl was heated at 70°C for 5 min. After heating, tube was cooled on ice for another 5 min. A cocktail of the following reagents were made: 4.0 µl 5x reaction mix, 1.0 µl dNTP mix (10 mM) (Fermentas, USA), 0.5 µl RNase inhibitor (40U/ml) (Promega, USA) and DEPC-water to make up total volume of 9.0 µl. Cocktail was added to tube and tube was incubated at 37°C for 10 min. Two µl of M-MLV RT (200 U/ml) (Promega, USA) was added and

TABLE 1: Primer sequences and expected amplicon sizes of the cDNA used in this study

Primer	Sequence	Band Size (bp)	Ref
Growth factors /Cytokines:			
IL-1 β	F: ACTACAGCAAGGGCTTCAGG R: CATATCCTGTCCCTGGAGGT	290	Zhou and Tedder, 1995 ¹⁹
IL-6	F: ACGAATTCACAAACAAATTCGGTACA R: CATCTAGATTCTTTGCCTTTTTCTGC	335	Cochet and others, 1998 ²⁰
IL-18	F: GATTACTTTGGCAAGCTTGAA R: GTCTTCGTTTTGAACAGTGAA	480	Wong and others, 1999 ²¹
TNF- α	F: GCGAATTCCTCCTGGCCAATGGCGTGG R: CTAAGCTTGGGTTCCGACCCTAAGCCCC	507	Cochet and others, 1998 ²⁰
TNF receptors:			
DR5	F: GGGAGCCGCTCATGAGGAAGTTGG R: GGCAAGTCTCTCTCCAGCGTCTC	181	Kim and others, 2000 ²²
Housekeeping Gene:			
18S	F: GTAACCCGTTGAACCCATT R: CCATCCAATCGGTAGTAGCG	188	

incubated for another 2 hours at 37°C. At the end of incubation, tube was again heated at 70°C for 5 min followed by chilling on ice for 5 min. First strand cDNA were stored at -20°C.

Polymerase chain reaction

Two μ l of first strand cDNA was used for PCR amplification. The sequences of the primers used for amplification of IL-1 β , IL-6, IL-18, TNF- α , DR5 and 18S RNA and the expected size of the amplicons are as listed in Table 1. A cocktail of the following was made and added to the tube: 2.0 μ l 10x buffer, 1.0 μ l (0.5 μ M) each of forward and reverse primers, 1.0 μ l MgCl₂ (25mM), 0.4 μ l dNTP mix (10mM), made up to a total of 16 μ l with DEPC treated water. Lastly, 1U of Taq DNA polymerase in 2.0 μ l of water was added. Tubes were then subjected to a hotstart which consisted of heating to 94°C for 5 min. The thermocycling conditions were denaturation at 94°C for 1 min, annealing at 55°C for amplification of 18S and 56°C for the cytokines and DR5 (Table 2) and extension at 72°C for another 1 min. Thirty-five cycles were performed for amplification of cytokine and DR5 cDNA and 25 cycles for 18S cDNA (Table 2). At the end, a second extension at 72°C for carried out for 7 min after which product was left at 4°C.

Multiplex PCR was carried out with as many sets of primers as possible. Whenever a multiplex was performed, the amount of each primer was reduced to 0.5 μ l and volume of MgCl₂ increased to 1.5-2.0 μ l. Table 2 shows the combination of primers, annealing temperature and number of cycles and volume of MgCl₂ used in PCR. PCR was carried out on a gradient thermocycler (Biometra, Germany).

At the end of PCR, 10 μ l of the reaction was loaded onto a 1.5 % agarose gel in TAE buffer containing ethidium bromide. PCR products were electrophoresed on the gel together with the Generuler 100 bp DNA ladder (Fermentas, USA) in an empty well. Bands were visualized on an imaging system utilizing the GeneSnap software program from SynGene, a division of Synoptics Ltd (Cambridge, UK).

Statistical analysis

Non-parametric Mann-Whitney *U* test was used to compare between two groups. Correlation of two variables was analysed using the Spearman non-parametric correlation test, indicated by *R* (ρ) coefficient for continuous variables and Fisher's Exact Test, indicated by ϕ (Φ) to determine association between dichotomous variables (+/-).

TABLE 2: Thermocycling conditions for multiplex PCR

Primers	T _{ann} (°C)	No. of cycles	Time *(sec)	MgCl ₂ 25mM (µl)
Housekeeping gene: 18S	55	25	30	1.0
Multiplex 1: IL-1β and IL-18	56	35	60	1.5
Multiplex 2: TNF-α, IL-6 and DR5	56	35	95	2.0

Note: T_{ann} = annealing temperature * Annealing time

RESULTS*Patients and biological characteristics*

Paired blasts samples from 12 AML patients were collected for analysis. Seven were regarded

chemo-sensitive while five were chemo-resistant cases. Table 3 lists the biological characteristics including age, sex and FAB classification of each patient at diagnosis. Expression of

TABLE 3: Biological characteristics and immunophenotype of AML patients

	Ref	Age	Sex	FAB	Site	CD34	CD13/CD 33	CD11c
Chemo-sensitive								
1	168	5	F	M2	BM	70	98	ND
	Day 2				PB	65	98	ND
2	103	49	M	M4	PB	60	99	25
	Day 1				PB	9	99	ND
3	110	45	F	M4	BM	81	98	35
	Day 4				PB	82	98	ND
4	135	32	F	M4	BM	72	99	55
	Day 1				PB	75	98	ND
5	164	47	M	M4	PB	99	0	63
	Day 1				PB	99	0	ND
6	262	9	F	M5a	BM	90	46	ND
	Day 1				PB	70	ND	ND
7	263	18	M	M4	Aph	0	87	18
	Day 1				PB	0	96	ND
Chemo-resistant								
8	30	51	M	M4	PB	56	93	30
	Day 1				PB	23	98	
9	196	45	F	M2	Aph	99	25	77
	Day 4				PB	99	25	ND
10	220	25	M	M4Eo	BM	98	53	ND
	Day 2				PB	90	90	ND
11	13	36	M	M4	PB	10	99	99
	Day 2				PB	0	95	ND
12	39	21	M	M1	BM	78	98	11
	Day 3				PB	97	97	ND

BM=bone marrow PB=peripheral blood Aph=apheresis ND=not done

an early marker of haemopoiesis, CD34 and myeloid lineage markers, CD13/CD33 and peroxidase reaction on leukaemic cells were also shown in Table 3. The majority of samples (83%) expressed CD34. Myeloid lineage was identified by the expression of CD13 or CD33 and peroxidase reaction on leukameic cells in all samples.

Differential expression/phosphorylation of proinflammatory cytokines, proapoptotic molecule, DR5 and mediators of signaling pathways

Expression of pro-inflammatory cytokines, IL-1 β , IL-18, IL-6, TNF- α , and proapoptotic molecule, DR5 transcripts were determined by RT-PCR. Qualitative positive or negative results were based on the presence or absence, respectively, of an amplified PCR band of the expected band size. Fig. 1 shows the percentage of chemo-sensitive and chemo-resistant cases positive for these markers before and during induction therapy.

Before induction therapy a statistically higher percentage of chemo-resistant cases expressed IL-1 β and IL-18 transcripts ($p=0.047$ and $p=0.047$, respectively) compared to chemo-sensitive cases. Following induction therapy however, a significantly higher percentage of chemo-sensitive cases expressed IL-6 ($p=0.002$) and TNF- α ($p=0.025$) transcripts compared to chemo-resistant cases. There was also an increased percentage of chemo-sensitive cases expressing DR5 (57%) compared to chemo-resistant cases (25%).

Phosphorylation status of signaling pathway mediators, Akt (Thr308), Akt (Ser473), FKHR, Bad, p38 and Jnk were determined on

flowcytometry. The percentages of chemo-responsive and chemo-resistant cases expressing these markers before and during induction therapy are presented in Figs. 2 and 3.

None of the mediators were differently expressed in samples before induction therapy. Following induction therapy however, a significant higher percentage of chemo-resistant cases were phosphorylated for Bad ($p=0.027$) compared to chemo-sensitive cases. A lower percentage these cases were also phosphorylated for p38 and Jnk (33% vs 100% and 25% vs 50% respectively) compared to chemo-sensitive cases.

Correlation in expression of proinflammatory cytokines, proapoptotic molecule, DR5 and mediators of signaling pathways expressed/phosphorylated.

Tables 4 and 5 show molecules significantly correlated in expression in AML samples before and during induction therapy, respectively.

In the correlation tests, two forms of data were tested. Firstly, percentage of positive blasts expressed for each molecule was used in the correlation test for results obtained using the flowcytometry method (A, Table 4 and Table 5). To correlate RT-PCR and flowcytometry results, the percentage positive blasts of flowcytometry results were first converted to dichotomous options of positive (+) or negative (-) to equate with PCR results (B, Table 4 and Table 5). An arbitrary cutoff value of 20% was used for each marker. A sample with more than 20% positive cells was indicative of a positive case. Positivity of 20% or less is indicative of a negative sample.

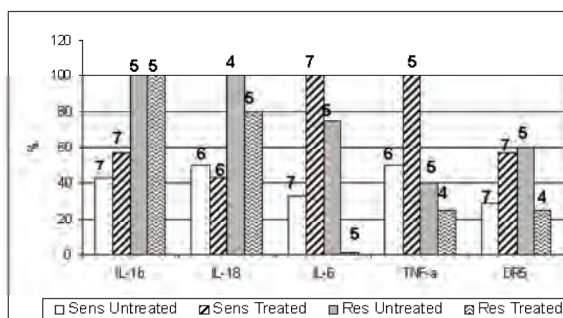


Fig 1: Percentage of AML cases expressing IL-1 β , IL-18, IL-6, TNF- α and DR5 in chemo-sensitive cases before (□) and during (▨) induction therapy and chemo-resistant cases before (■) and during (▩) induction therapy. All genes were not analysed for each sample because of insufficient patient material. The number on top of the bar represents the number of cases.

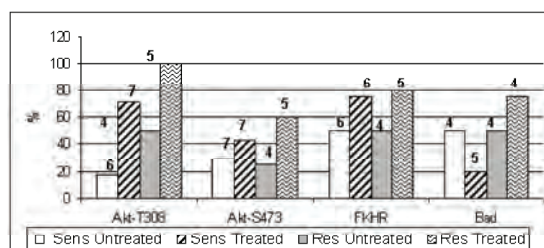


Fig 2: Percentage of AML cases with phosphorylated Akt, FKHR and Bad in chemo-responsive cases before (□) and during (▨) induction therapy and chemo-resistant cases before (■) and during (▩) induction therapy. All genes were not analysed for each sample because of insufficient patient material. The number on top of the bar represents the number of cases.

DISCUSSION

We found that induction therapy altered the expression of cytokines and signaling molecules in AML blast cells resulting in significant differences in expression of some markers when compared between chemo-sensitive and chemo-resistant cases. The increased percentage of chemo-sensitive cases expressing IL-6 transcripts during induction therapy is consistent with Devemy and others¹³ who reported that increase of IL-6 transcripts during remission induction therapy of AML patients was accompanied by a fall in blood count and bone marrow cellularity. IL-6 has the ability to induce differentiation in many cell types including B-cells and myeloid cells.^{23,24} Mature cells are more susceptible to the cytotoxic effect of drugs which may explain the higher percentage of positive cases in chemo-sensitive samples. IL-6 however, is

also known to promote lymphoproliferation and has a role in tumour pathogenesis.² We found a higher percentage of chemo-resistant patients expressing IL-6 before induction therapy.

The significantly increased percentage of cases expressing TNF- α in chemo-sensitive cases is consistent with the study by Devemy and others,¹³ who observed increased TNF- α transcripts in AML cells during chemotherapy. TNF- α like the other proinflammatory cytokines induces an inflammatory response in human monocytes. In NF- κ B-inhibited monocytes however, TNF stimulated apoptosis.²⁵ Thus in this instance, TNF- α played a role as a death receptor ligand, functioning in an autocrine or paracrine manner to induce cell death.²⁶

In this study, a strong association between TNF- α expression and phosphorylation of p38 were observed in the AML cases ($\phi=1.00$,

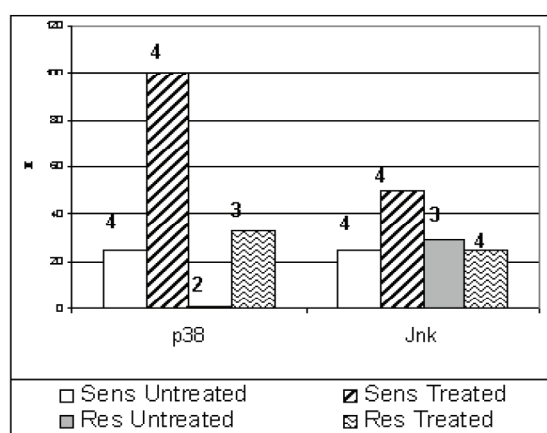


Fig 3: Percentage of AML cases with phosphorylated p38 and Jnk in chemo-sensitive cases before (□) and during (▨) induction therapy and chemo-resistant cases before (■) and during (▩) induction therapy. All genes were not analysed for each sample because of insufficient patient material. The number on top of the bar represents the number of cases.

TABLE 4: Molecules (proinflammatory cytokines, proapoptotic molecule and mediators of STP) significantly correlated in expression in AML samples before induction therapy

(A)

	% positive blasts	
	FKHR*Bad	p38*Jnk
Rho coefficient (R)	0.826	0.943
^a p value	0.011	0.005
N	8	6

a=Spearman's correlation

(B)

	Qualitative (+/-)		
	IL-1 β *IL-18	Akt475*TNF- α	FKHR*Bad
Phi (ϕ) coefficient	0.625	0.655	0.775
^b p value	>0.05	>0.05	>0.05
N	12	10	8

b=Fisher's Exact test

TABLE 5: Molecules (proinflammatory cytokines, proapoptotic molecule and mediators of STP) strongly associated in expression in AML samples during induction therapy

(A)

	% positive blasts	
	Akt 308*FKHR	p38*Bad
Rho coefficient (R)	0.696	-0.812
^a p	0.017	0.050
N	11	6

a=Spearman's correlation

(B)

	Qualitative (+/-)							
	IL-1 β * IL-18	IL-1 β * DR5	IL-1 β * Akt308	IL-1 β * Akt475	IL-6* TNF- α	IL-18* Jnk	Jnk*DR5	TNF- α * p38
Phi (ϕ) coefficient	0.683	-0.671	0.775	0.577	0.791	-0.745	0.745	1.000
^b p value	0.045	>0.05	0.045	>0.05	0.048	>0.05	>0.05	>0.05
N	12	11	12	12	9	8	8	6

b=Fisher's Exact test

n=6) during induction therapy. This association was however not significant. Phosphorylated p38 was also found in a larger percentage of chemo-sensitive cases. This was consistent with another study which reported that p38 mediated TNF-induced apoptosis.²⁷ The author then showed in TNF- α induced apoptosis, p38 regulated phosphorylation of Bad (Ser112).²⁸ Our correlation analysis showed an inverse relationship between phosphorylated p38 and Bad ($R = -0.812$, $p = 0.05$, $n = 6$) supporting these earlier reports. Expression of phospho-Bad in blasts of AML patients was reported to correlate with a negative outcome.²⁹ We found a significantly lower percentage of chemo-sensitive cases phosphorylated for Bad compared to chemo-resistant cases. This suggested downregulation of Bad phosphorylation activated pro-apoptotic pathways favoring cell death as observed in chemo-sensitive cases.

This study also showed the percentage of cases phosphorylated for Jnk was higher in chemo-sensitive cases during induction therapy as was observed by others in leukaemia cell lines primed for apoptosis.³⁰ *In vitro* treatment of leukaemia and lymphoma cell lines with a combination of arsenic trioxide and buthionine sulfoximine was shown to activate Jnk and increase the level of DR5.¹¹ Correlation analysis showed a strong association between activated Jnk and DR5 expression ($\phi = 0.75$, $n = 8$) in AML samples studied here, which however was not significant. DR5 was also expressed more frequently in chemo-sensitive samples during induction therapy. Identification of pro-apoptotic molecules involved in the induction of cell death in cancer is important as targets for alternative therapy. In the search for more selective, tumour-biology driven therapies with apoptotic inducing potential, the focus has been on members of the tumour necrosis factor (TNF) family including Fas-ligand, TNF and TRAIL. TRAIL, the ligand for death receptors DR4 and DR5, stands out as the most promising anti-tumour agent in preclinical models, as it induces apoptosis in a wide range of tumour cells without causing toxicity to normal cells.³¹ Several agonistic humanized or human monoclonal antibodies against DR4 and DR5 have been tested in Phase I and II trials in patients with advanced cancer and have shown positive results.³² Our results showing induction of DR5 leading to cell death supports the use of this therapy in acute myeloid leukaemias.

Correlation studies of markers in AML

blasts during induction therapy showed strong (though not significant) inverse association between pro-inflammatory cytokines IL-1 β and IL-18 with DR5 ($\phi = -0.671$, $n = 11$) and Jnk ($\phi = -0.745$, $n = 8$), respectively, suggesting a reverse role in determining treatment outcome. Devemy and others¹⁸ also observed high levels of IL (interleukin)-1 β in bone marrow samples taken on day 5 of induction and found this to be associated with treatment failure. We were not able to show a significant difference in expression of the cytokines in AML samples with different treatment outcome. Our current approach using RT-PCR needs to be replaced with a more quantitative method such as real-time PCR. Detection and quantitative expression of cytokines could be improved by using flow cytometry. Increasing the number of samples in the analysis may improve the significance of associations.

Various reports have shown that IL- β is able to activate Akt.^{33,34} We also found a strong and significant association in IL-1 β expression with Akt (Thr308) ($\phi = 0.77$, $p = 0.045$, $n = 12$) and positive association with Akt (Ser473) ($\phi = 0.577$, $p > 0.05$, $n = 12$) phosphorylation after induction therapy. Previous report showed constitutive phosphorylation of Akt (Ser473) in the majority of AML samples with high blast counts and it was an adverse prognostic factor for survival.¹ This is in contrast to our finding where the majority of untreated AML samples did not express phosphorylated Akt (Ser473) or Akt (Thr308). However, the percentage of cases phosphorylated for these two molecules increased after induction therapy. Even though the percentages were higher in chemo-resistant cases, the difference was not significant. Our results also showed a significant correlation between phosphorylation of Akt-Thr308 and FKHR ($R = 0.70$, $p = 0.02$, $n = 11$) in these samples. Phosphorylation of FKHR inhibits the pro-apoptotic function of this molecule leading to cell survival. Patients with phospho-FKHR had a significantly shorter overall survival.³⁵

The increased number of chemo-sensitive cases expressing IL-1 β and phosphorylated for Akt (Thr308), Akt (Ser473) and FKHR after induction therapy may also be due to induction of proinflammatory responses observed during cell death.¹⁵

We determined phosphorylation of signaling molecules using flow cytometry as it has many advantages over traditional methods such as Western blotting. In particular, single cell

analysis enables the percentage of cell sample expressing protein to be determined and the amount of protein expression is not averaged over the whole sample as in Western blots.³⁶ However, fluorochromes (and antibodies) selected should have quantitative properties.

In conclusion, we observed that induction therapy of AML patients induced a change in the expression patterns of proinflammatory cytokines, signaling pathway mediators and proapoptotic molecules in leukaemic cells. These molecules may be useful as prognostic markers to monitor treatment outcome and predict response at an early stage in therapy. They may also be useful as novel targets for alternative therapy. The observation of these expression patterns was only evident after induction therapy. The detection of these molecules in untreated and treated samples in this study look promising for early prediction of treatment outcome. Further investigations in a larger scale using more quantitative techniques could validate our results.

ACKNOWLEDGEMENT

This work was supported by IRPA grant, 06-02-04-0083 from The Malaysian Ministry of Science, Technology and Environment. We would like to acknowledge the Heads of the Hematology units of the Paediatric Department, Prof Dr Rahman Jamal, and the Pathology Department, Assoc Prof Datin Dr Hamidah Hussin of Hospital Universiti Kebangsaan Malaysia, for their co-operation and Ms. Sivagengei for technical support. *Author disclosure statement:* No competing financial interest exists.

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