

Original Article

## Differential Expression Patterns of Leukaemia Associated Genes in Leukaemia Cell Lines Compared to Healthy Controls

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### ABSTRACT

**Introduction:** The phenotype and genotype of cancer cells portray hallmarks of cancer which may have clinical value. Cancer cell lines are ideal models to study and confirm these characteristics. We previously established two subtracted cDNA libraries with differentially expressed genes from an acute myeloid leukaemia patient with poor prognosis (PP) and good prognosis (GP). **Objective:** To compare gene expression of the leukaemia associated genes with selected biological characteristics in leukaemia cell lines and normal controls. **Methodology:** Expression of 28 PP genes associated with early fetal/embryonic development, HOX-related genes, hematopoiesis and aerobic glycolysis/hypoxia genes and 36 GP genes involved in oxidative phosphorylation, protein synthesis, chromatin remodelling and cell motility were examined in B-lymphoid (BV173, Reh and RS4;11) and myeloid (HL-60, K562) leukaemia cell lines after 72h in culture as well as peripheral blood mononuclear cells from healthy controls (N=5) using semi-quantitative polymerase chain reaction (PCR) method. Cell cycle profiles were analysed on flow cytometry while MTT cytotoxicity assay was used to determine drug resistance to epirubicin. **Results:** Genes expressed significantly higher in B-lymphoid leukaemia cell lines compared to healthy controls were mostly of the GP library i.e. oxidative phosphorylation (3/10), protein synthesis (4/11), chromatin remodelling (3/3) and actin cytoskeleton genes (1/5). Only two genes with significant difference were from the PP library. Cancer associated genes, *HSPA9* and *PSPH* (GP library) and *BCAP31* (PP library) were significantly higher in the B-lymphoid leukemia cell lines. No significant difference was observed between myeloid cell lines and healthy controls. This may also be due heterogeneity of cell lines studied. PBMC from healthy controls were not in cell cycle. G2/M profiles and growth curves showed B-lymphoid cells just reaching plateau after 72 hour culture while myeloid cells were declining. IC50 values from cytotoxicity assay revealed myeloid cell lines had an average 13-fold higher drug resistance to epirubicin compared to B-lymphoid cell lines. Only *CCL1*, was expressed at least two-fold higher in myeloid compared to B-lymphoid cell lines. In contrast, *MTRNR2*, *EEF1A1*, *PTMA*, *HLA-DR*, *C6orf115*, *PBX3*, *ENPP4*, *SELL*, and *IL3Ra* were expressed more than 2-fold higher in B-lymphoid compared to myeloid cell lines studied here. **Conclusion:** Thus, B-lymphoid leukaemia cell lines here exhibited active, proliferating characteristics closer to GP genes. Higher expression of several genes in B-lymphoid compared to myeloid leukaemia cell lines may be useful markers to study biological differences including drug resistance between lineages.

**Keywords:** Leukaemia cell lines, Gene profiling, Protein synthesis associated genes, Chromatin remodelling genes

### INTRODUCTION

Heterogeneity in clinical characteristics of cancer cells such as response to treatment may be the result of transformation or may be natural characteristics possessed by the unique cell. Thus, cancer cell phenotype or genotype provide biological markers that may be useful for management of the disease. Hallmarks of cancer include self-sufficiency

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in growth signals, insensitivity to antigrowth signals, ability to evade apoptosis, acquisition of limitless replicative potential, sustained angiogenesis and subsequent tissue invasion and metastasis (1). In addition, other characteristics that have emerged of equal importance are reprogramming of energy metabolism, evading immune destruction as well as creating the tumor microenvironment by manipulating surrounding normal cells to aid in its persistence (2). These phenotype translates into genetic diversity where large scale examination identifies useful biomarkers.

Cancer cell lines provide a continuous source of cancer cells for comparative study with normal cells. Screening of potential cancer associated genes in cancer cell lines provide preliminary support for their relevance. They are used as models to perform mechanistic studies to understand gene function.

Acute myeloid leukaemia (AML) is the malignant proliferation of cells of the hematopoietic system involving precursors of the myelo-monocytic lineage. Patient response to treatment is heterogeneous and survival remains poor especially among the older age group (3). In some patients no prognostic markers are available to predict outcome to conventional therapy.

We isolated two cDNA libraries containing genes highly expressed in an AML with good prognosis (GP) and poor prognosis (PP). Gene ontology analysis identified distinct gene functions which may be relevant to leukaemia development. Heterogeneity in the clinical and biological characters of cancer cell lines provide a means to examine these genes. PP and GP genes were screened on leukaemic cell lines as well as PBMC from healthy controls. The biological characteristics such as chemosensitivity to epirubicin, cycling cell profiles of these cell lines were also determined and compared with gene expression.

## METHODS AND MATERIALS

### Cell lines and peripheral blood from healthy controls

The leukaemia cell lines Reh, RS4;11, HL-60 and K562 were purchased from the American Type Culture Collection (ATCC, Virginia, USA) while BV173 (DSMZ-German Collection, Braunschweig, Germany). Cell line characteristics are shown in Table 1. Healthy controls (N=5) were individuals not diagnosed with cancer and currently with no other acute or chronic disease. This study was approved by the Institutional Medical Ethics Committee. Informed consent were obtained before blood taken. All procedures are in compliance with the Declaration of Helsinki, 2013 which details ethical principles for medical research involving human subjects.

**Table 1:** Characteristics of leukaemia cell lines obtained from providers datasheet

Cell line	Origin	Cell lineage
BV173	Established from the peripheral blood of a 45-year-old man with chronic myeloid leukemia (CML) in blast crisis. Philadelphia chromosome (Ph1)-positive acute leukemia	Precursor B-cell line CD3 -, CD10 +, CD13 +, CD19 +, CD37 -, CD80 -, CD138 -, HLA-DR +
Reh	Pediatric patient with pro B-lineage ALL*; positive for TEL-AML1	Precursor B-cell line CD3 (17%) CD4 (15%), CD10 (55%)
RS4;11	Established from the bone marrow of a 32-year old female patient with acute lymphoblastic leukemia.	TdT+++ , CD24+, CD9+, HLA-DR+, CD10 neg
HL-60	Peripheral blood leukocytes were obtained by leukopheresis from a 36-year-old Caucasian female with acute promyelocytic leukemia.	Myelocytic cell line
K562	Pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises	Erythrocytic

\* Tsuganezawa et al. 1988(4)

### Cell culture

Leukaemic cells (BV173, HL-60, K562, Reh and RS 4;11) were cultured as recommended in Roswell Park Memorial Institute (RPMI) media 1640 containing L-glutamine and 25 mM HEPES buffer with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 0.1% gentamycin, 0.5% fungizone (all from Gibco, Invitrogen, USA), and 1.5 g/L sodium bicarbonate. Cultures were maintained at 37 °C in 95% humidified CO<sub>2</sub> incubator (Galaxy S, RS Biotech, UK).

Cell viability was determined by Trypan Blue Exclusion test method using Trypan blue dye (Sigma, USA) according to standard method.

### Generation of growth curve

Cells with a starting density of 0.5 x 10<sup>6</sup> cells/ml were prepared and seeded into a 24-well plate. Cell number and viability were monitored at 24, 48 and 72 h after culture. Cells were mixed with an equal volume of diluted trypan blue and cell number counted manually on a haemocytometer according to standard method.

### Cell cycle analysis

Experimental design was the same as that to determine growth curve. After 24h, 48h and 72h in culture, cells were harvested and fixed in 1 ml of 70% ethanol (Sigma, USA) and kept overnight in -20 °C freezer. Cells were pelleted by centrifugation at 3000 rpm for 5 minutes. The supernatant was discarded and the cells were re-suspended in 1 ml of 1X PBS/0.1% BSA, then followed by centrifugation at 2000 rpm for 5 minutes. The supernatant was again discarded and the cells re-suspended in 450 µl of 1X PBS/0.1% BSA, 25 µl of 10 mg/ml RNase and 50 µl of 1mg/ml propidium iodide (PI). The mixture was incubated in the dark room for 30 minutes prior to analysis by FACS Calibur cytometer (BD Biosciences, CA). Data acquisition was conducted using CellQuest Pro software (Becton Dickinson, USA). For each sample, 10,000 gated events were recorded. Cell cycle data analysis was performed using the ModFit LT software.

### Cell cytotoxicity assay

Cell cytotoxicity to epirubicin was conducted with the MTS assay to determine IC<sub>50</sub> value of each cell lines. Cell Titer 96® Aqueous One Solution (Promega, USA) was used in this assay and performed in a 96-well plate format. Epirubicin was diluted 1:1 with RPMI 1640 (without FBS) starting with 1000 ng/ml as initial concentration and continued with 2-fold dilutions until 10 points were generated. Leukaemic cells were serum-starved overnight to synchronize the cells into G<sub>0</sub> phase before drug treatment. The cells were seeded at density of 1000 cells/ml in 100 µl complete medium of RPMI 1640 with the different dilutions of epirubicin. The treated cells were then maintained at 37 °C in 95% humidified incubator for 72 hours.

Twenty microlitres of MTS reagent was added to the cultures three hours prior to ending at the designated time point. The plate was incubated for three hours at 37 °C in 95% humidified air. The absorbance readings for formazan product were obtained by using Dynex MRX II microplate reader with 490 nm detector. The percentage of cell viability was calculated based on untreated controls.

### Peripheral blood mononuclear cell (PBMC) isolation and RNA extraction

PBMC were isolated from anticoagulated blood samples using density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare, Sweden) according to manufacturer's protocol.

RNA was isolated from 1x10<sup>6</sup> PBMC or cell line using guanidinium thiocyanate-phenol-chloroform extraction method according to the manufacturer's instructions (Tri-Reagent; Molecular Research, USA). RNA was dissolved in an appropriate amount of DEPC treated ultra-pure water containing RNase inhibitor (0.16 U/µl) prior to being stored at -80 °C. Extracted RNA was then subjected to DNase treatment to remove DNA contamination. A total of 20.5 µl RNA was resuspended in 2.5µl of 1X DNase I reaction buffer, 1 µl of 2000 U/mL DNase I (New England Biolabs, UK) and 1 µl of 40 U/mL recombinant RNasin ribonuclease inhibitor (Promega Corporation, WI, USA). The mixture was mixed thoroughly and incubated at 37 °C for 10 minutes. 0.5 µl of 0.25 M EDTA (to a final concentration of 5 mM) was added into mixture and heat inactivated at 75 °C for 10 minutes. Purity and quantity of RNA was determined using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The 260:280 nm ratio ranged from 1.8-2.0. RNA quality was confirmed through gel electrophoresis to show intense 18S and 28S bands and non-degradation of RNA.

### Reverse transcription (RT)

In a sterile RNase-free microcentrifuge tube, 4 µg of total RNA was converted to cDNA with oligo(dT) 15 primer (Promega Corporation, WI, USA) and M-MLV RT enzyme in the presence of recombinant RNasin® ribonuclease inhibitor (Promega Corporation, WI, USA) and dNTP mix (Fermentas, Lithuania, MBI). Products were stored at -20°C unless used immediately.

### cDNA libraries

In an earlier study, two subtracted cDNA libraries containing differentially expressed genes were obtained from an AML patient with poor prognosis (disease free survival, DFS, <12 months) and a good prognosis patient (DFS >12 months). These libraries were cloned and transformed into bacteria. Subsequently, colony PCR and DNA sequencing were performed from colonies randomly selected from agar plates and identified 39 and 37 known genes, respectively, in the GP and PP library (Supplemental Table S1).

### Primer Design

Primers were designed from the DNA sequences identified in each clone with primer design software available online. PCR conditions were optimized for each primer pair using cDNA from the Reh cell line. All PCR products were sent for DNA sequencing to confirm the gene amplified. Only 36 and 28 PCR conditions were successfully optimized from the GP and PP library, respectively. Contig sequences (unidentified genes) were not included in the analysis. Primer sequences and amplicon size for the genes and reference gene are provided in Table 2 and Table 3.

**Table 2:** Primer sequences of genes identified in cDNA library from AML with good prognosis (GP). Genes also identified for biological function.

No.	Primer	Sequence	Amplicon (bp)
		Reference gene	(bp)
	<i>B2M</i>	F: 5'- TTCCTGAATTGCTATGTGTCTG-3' R: 5'- TCTCAAACCTCCATGATGCT-3'	259
		Oxidative phosphorylation	
1	<i>COX2</i>	F: 5'- CAGACGCTCAGGAAATAGAAA-3' R: 5'- TAGTCGGTGTACTCGTAGGTT -3'	172
2	<i>COX3</i>	F: 5'- TAGAAGTCCCCTCTAAACA-3' R: 5'- GTTGGCGGATGAAGCAGATAG -3'	288
3	<i>ATP6</i>	F: 5'- CACAACACTAAAGGACGAACC-3' R: 5'- GTGTAAATGAGTGAGGCAGGA -3'	100
4	<i>ATP8</i>	F: 5'- ACAAACACTACCACCTACCTCCC-3' R: 5'- GCAATGAATGAAGCGAACAGA -3'	103
5	<i>MTND4</i>	F: 5'- CACTGCCCAAGAACTATCAA-3' R: 5'- TAAGTGGAGTCCGTAAGAG -3'	100
6	<i>MTND5</i>	F: 5'- GGCCTATCACCACTCTGTT-3' R: 5'- GTTGGTTGATGCCGATTGTA -3'	124
7	<i>ATP5B1</i>	F: 5'- CAGAGGTGTCTGCATTATTGG-3' R: 5'- CACATAGATAGCCTGTACAGAG-3'	139
8	<i>COX7B</i>	F: 5'- CTCCAAGTTCGAAGCATTTCAG-3' R: 5'- CTTGTGTTGCTACATATGTCCA-3'	144
9	<i>C16orf61</i>	F: 5'- GACTTATCTCCACACTTGAC -3' R: 5'- TTCTACGTACTCATTCTTCAGG -3'	152
10	<i>16s RRNA</i>	F: 5'- ACCAGTATTAGAGGCACCG-3' R: 5'- CATAGGGTCTTCTCGTCTTG-3'	209
		Iron binding protein	
11	<i>FTL</i>	F: 5'- CCTCCTACACCTACCTCTC-3' R: 5'- CAGCTGGCTTCTTGATGTC-3'	179
		Ribosome biogenesis/Protein synthesis	
12	<i>NOLA-2</i>	F: 5'- CAAGAAAGCGGTGAAGCAG-3' R: 5'- CACTCATCGTAAGCCTCCT-3'	272
13	<i>RPLP0</i>	F: 5'- TGGCTACTTTGTTTCGCATTAT-3' R: 5'- TCTCCCTGGGCATCACG-3'	136
14	<i>RPL9</i>	F: 5'- CACATCAATGTAGAACTCAGCC-3' R: 5'- CAAGCAACACCTGGTCTC-3'	283

15	<i>RPL21</i>	F: 5'-CATCTTCCAGTAATTCGCCA-3' R: 5'-TTTGAACAGTACCCATTCCCT-3'	169
16	<i>RPL27</i>	F: 5'-TCGTGAAGAACATTGATGATGG-3' R: 5'-GGGATATCCACAGAGTACCT-3'	195
17	<i>RPL36a</i>	F: 5'-TCCGTTTGCCTCGCGTTTC-3' R: 5'-TAGCCACTCTGCTTCTGTGTC-3'	195
18	<i>RPL41</i>	F: 5'-GTGGAGGAAGAAGCGAATG-3' R: 5'-TTTGTTTATGAGCAAGGTGG-3'	248
19	<i>EEF1A1</i>	F: 5'-GTGTGAAACAATAATTGTCCGG-3' R: 5'-GAACCAAGGCATGTTAGCAC-3'	202
20	<i>C14orf166</i>	F: 5'-GTAATGCTTAAGGCAATTCGG-3' R: 5'-GCTTCATTAAGAAGTGCATCTCC-3'	148
21	<i>EIF3M</i>	F: 5'-AAGTAGTTGTGTCAGTCATAGCAC-3' R: 5'-AACTCAGGTATCAGAAAGACTC-3'	136
22	<i>EIF4A1</i>	F: 5'-AACTATATCCACAGAATCGGTC-3' R: 5'-AATGACAAGATGTCCATCCCT-3'	262
Chromatin remodeling			
23	<i>H2AFZ</i>	F: 5'-GCAGAGGTAAGTGAAGTGG-3' R: 5'-TTTCACAGAGATACAGTCCAC-3'	288
24	<i>PTMA</i>	F: 5'-GCCATCTTTGCATTGTTCT-3' R: 5'-GTCCTTGGTGGTGATTTTCG-3'	207
25	<i>OGT</i>	F: 5'-ATTGTCAAGATGAAGTGTCTG-3' R: 5'-TTCTGGTAACCCGTAAGTGG-3'	260
Actin cytoskeleton			
26	<i>MALAT1</i>	F: 5'-TTGGAGGGATGGGAGGAGGG-3' R: 5'-CATTCTAATAGCAGCGGGAT-3'	153
27	<i>RhoA</i>	F: 5'-CATGCTTGCTCATAGTCTTCAG-3' R: 5'-CCAACTCTACCTGCTTTCCA-3'	110
28	<i>MYL6</i>	F: 5'-CCAAGAGTGATGAGATGAATGTG-3' R: 5'-TGATACAACCATTGCTGTCT-3'	254
29	<i>TMSB4X</i>	F: 5'-TCCAAAGAAACGATTGAACAGG-3' R: 5'-TGCCAGCCAGATAGATAGAC-3'	243
30	<i>CALM-2</i>	F: 5'-AGAATCCACAGAAGCAGAG-3' R: 5'-CATTGCCATCCTTATCAAACAC-3'	170
HLA gene			
31	<i>HLA-DRA</i>	F: 5'-CGATACCAATGTACCTCCA-3' R: 5'-GACTGTCTCTGACACTCCTG-3'	166
Cell death regulator			
32	<i>CSTB</i>	F: 5'-CTTCATCAAGGTGCACGTC-3' R: 5'-GATGACTTTGTGCTGCTTCTGG-3'	186
Stress protein			
33	<i>HSPA9</i>	F: 5'-GTGAGCAGCAGATTGTAATCC-3' R: 5'-CAGCTTGTGCACTCATCAG-3'	211
Enzyme			
34	<i>MPO</i>	F: 5'-ATTCAATGTCAGTGTGCTG-3' R: 5'-CGTCTGTTGTTGCACATCC-3'	140
35	<i>PSPH</i>	F: 5'-GTGGCTTTAGGAGTATTGTAGAG-3' R: 5'-AATGAAAGCATCAGCAGGAG-3'	253
Unknown			
36	<i>C6orf115</i>	F: 5'-CCGTCTTCTCTTTGCCTC-3' R: 5'-CACATTCATTGCTGCCTCTC-3'	141

**Table 3:** Primer sequences of genes identified in cDNA library from AML with poor prognosis (PP). Genes also identified for biological function.

No.	Gene	Sequence	Amplicon (bp)
Aerobic glycolysis/Hypoxia			
1	<i>PGK1</i>	F: 5'-AACAACTGGAGATTGGCAC-3' R: 5'-GGCATTCTCATCAAACCTTGTC-3'	143
2	<i>MAPK8</i>	F: 5'-CAACAACTTAAAGCCAGTCAG-3' R: 5'-TTCAGAAGGATCATAACCAGAC-3'	129
3	<i>PIMI</i>	F: 5'-GATCCTGCTGTATGATATGGT-3' R: 5'-GAGATGCTGACATTCTGAAGAG-3'	111
4	<i>GLUL</i>	F: 5'-ACTTTGGAGTGATAGCAACCT-3' R: 5'-CCTCCTCGATGTACTTCAGAC-3'	125
Iron binding protein			
5	<i>HBD</i>	F: 5'-CTGTTGCTTACACTTTCTTCTG-3' R: 5'-CCACCAGTAATCTGCCCA-3'	161
Glucocorticoid signaling			
6	<i>GLCC11</i>	F: 5'-TCCACTTCCTGCTCATTACC-3' R: 5'-CTACTCCCCTGTCTTTATCAC-3'	194
Ribosome biogenesis/Protein synthesis			
7	<i>MRPS21</i>	F: 5'-GTGAAGGTTTAAATCCAAGGTC-3' R: 5'-AGTGAGGATTCTGTTTAGGG-3'	114
8	<i>RPL19</i>	F: 5'-CAGCCATGAGTATGCTCAG-3' R: 5'-ATTCTCCTCATCCATGTGAC-3'	300
Cell division			
9	<i>ANAPC5</i>	F: 5'-TAAAGCACTTGAAGGAACGA-3' R: 5'-AACCGCTTTCCTATAAACAC-3'	178
Fetal/embryonic			
10	<i>LCOR</i>	F: 5'-TAAAGTAAAGGGACACTTAGTCGG-3' R: 5'-TTTGTTCTTTGTGCTATTGGG-3'	287
11	<i>CNOT1</i>	F: 5'-AGATCACAAGAGTTCTCTTGG-3' R: 5'-GCACAGTGACAAATTCATGG-3'	129
12	<i>ORMDL1</i>	F: 5'-AGTGAATCCAAATACCCGTG-3' R: 5'-CCATAGTCCAGTTGTTCCCA-3'	248
13	<i>CENPC1</i>	F: 5'-CATCACATATTACCCAAGACGA-3' R: 5'-ATTGCTATAAACAGGACTCTCCTC-3'	292
Hematopoiesis and chemokine			
14	<i>SELL</i>	F: 5'-TCTCAATGATTAAGGAGGGTG-3' R: 5'-GGGTCATTACACTTCTCTTGG-3'	144
15	<i>IL3Ra</i>	F: 5'-GAAGGAAGATCCAAACCCAC-3' R: 5'-AATCCAGCAGGTCAGATTCTC-3'	285
16	<i>CCL-1</i>	F: 5'-GAGGGCTTAATATTCAAGCTG-3' R: 5'-CCCACAATGGAAAGAAATCTG-3'	135
<i>HOX</i> -related			
17	<i>HOXA3</i>	F: 5'-ACCAACTAACGCCTAAACCTCG-3' R: 5'-GCAATTCTTCTCCTGACG-3'	133
18	<i>ENPP4</i>	F: 5'-TAATGATCGAATTCAGCCCA-3' R: 5'-CTATGCTTGTAGCCTTTGTG-3'	167
19	<i>PBX3</i>	F: 5'-AATCACAGGTGGATACCCTC-3' R: 5'-TAGGAGAAGTCACAGAAGATGG-3'	149
20	<i>PPIE</i>	F: 5'-GGGACCAGGTCTACTATCCA-3' R: 5'-TGCTGACTCTTCTTGTCTC-3'	168
21	<i>PHF2</i>	F: 5'-GGACTCAGACTACGTTACCC-3' R: 5'-GAGGTGGTGTAGGAGTCAG-3'	281



22	<i>SF3B1</i>	F: 5'-AATGGATAGAGACCTTGACACAG-3' R: 5'-AACTGCCTGAATTACATGAGGA-3'	159
Cell death regulators			
23	<i>PDCD61P</i>	F: 5'-GTTTCATCCAGCAGACTTACC-3' R: 5'-GATCATAATATCTCAGGAGCGT-3'	151
24	<i>SON-b</i>	F: 5'-CATTCCCTTCTCCTTCC-3' R: 5'-TTTGACACTTGGCATT-3'	110
25	<i>DDB2</i>	F: 5'-CCCTTATGAATTGAGGACGA-3' R: 5'-AATGTGGTAACCCATTGCAG-3'	150
26	<i>BCAP31</i>	F: 5'-GGAAACTCGGAAACAAGCTC-3' R: 5'-GCCATAGGACACTAACAACCTC-3'	196
Methyltransferase			
27	<i>TPMT</i>	F: 5'-TGATCGCAAATGCTATGCAG-3' R: 5'-AAGCATCAACCTTCTCAAGAC-3'	184
Calcium transporter			
28	<i>ATP2B1</i>	F: 5'-AACTCAGTTGCTTACAGTGG-3' R: 5'-CACTTAAACCTTCATTGGGAG-3'	198

### Array polymerase chain reaction (PCR)

For each cDNA sample, semi-quantitative PCR was conducted on 65 genes (64 differentially expressed genes and one reference gene,  $\beta$ -2 microglobulin ( $\beta$ 2M)). PCR was carried out in 96-well PCR plates with 20  $\mu$ L reactions containing 1  $\mu$ L of previously synthesized cDNA product, 0.5  $\mu$ M of each primer pair, 0.2 mM dNTP and 1 U of Taq DNA polymerase. Amplification was conducted in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Canada). PCR cycle profile consisted of an initial denaturation at 95°C for 5 min., followed by 30 cycles of denaturation at 95°C, 1 min, annealing at 55°C 1 min and elongation at 72°C for 20 sec and subsequently a final elongation at 95°C for 7 min.

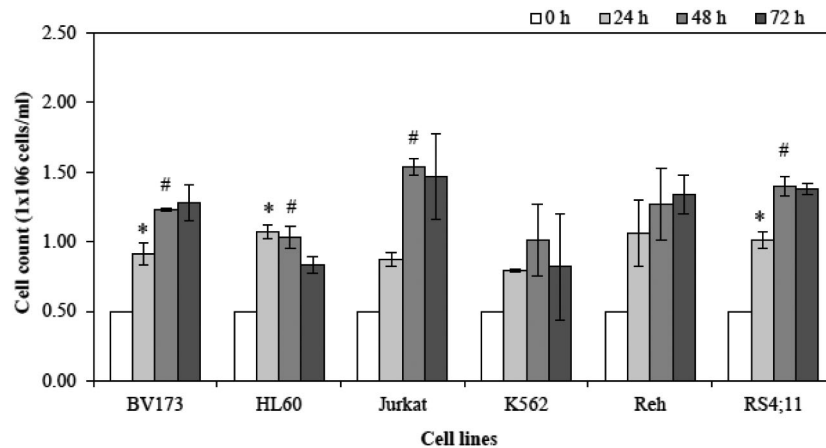
Following PCR, products were visualized by gel electrophoresis on 2% agarose. The amount of PCR product was semi-quantitated using gel imaging software (AlphaEase® FC software, Alpha Innotech, USA). Expression level was determined from the integrated density value (IDV) of the band of interest and reference gene using the spot density tools provided by the image analyzer system. Fold expression was determined by normalizing this value in gene of interest with that of reference gene in the same sample.

### Statistical analysis

Non-parametric Mann-Whitney test was used to compare between two groups while Spearman's correlation test was used to show association between parameters. The IBM SPSS Statistics version 22 software was used for the analysis.  $P < 0.05$  was considered significant.

## RESULTS

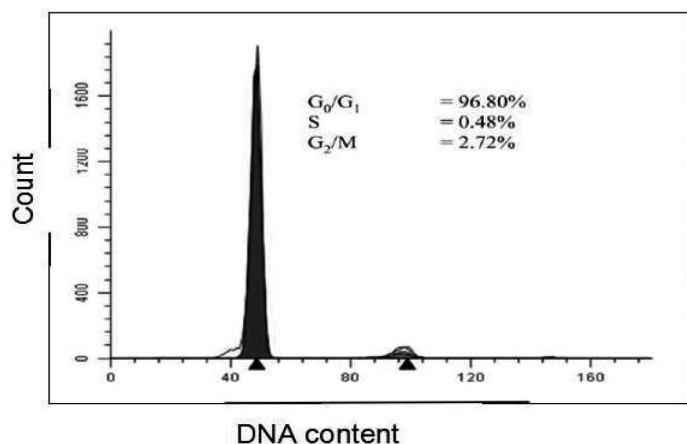
Growth curve and cell cycle analysis of leukaemia cell lines are shown in Figure 1 and Figure 2, respectively. Growth curve showed BV173 and Reh (and Jurkat) still in proliferation while the cell numbers were already declining in other cell lines after 72 h in culture. B-lymphoid leukaemia cell lines achieved cell numbers higher than myeloid cell lines (HL-60 and K562) suggesting shorter doubling time. Myeloblast (16-20  $\mu$ m) are larger in diameter than prolymphocytes (9-18  $\mu$ m) (5).



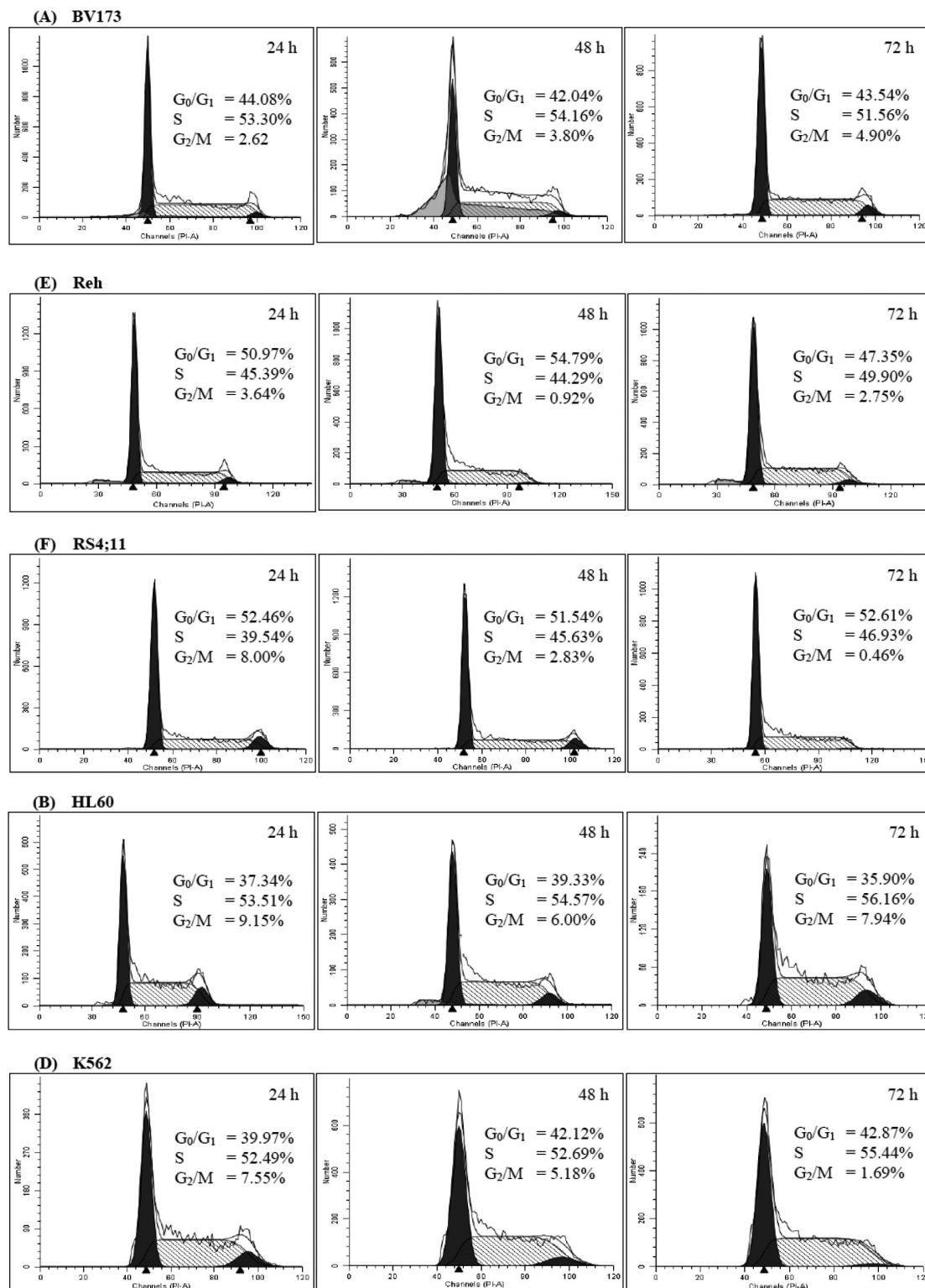
**Figure 1:** Growth curve of leukaemia cell lines monitored for 72 hours in complete media. Results are expressed as mean + SD of two independent experiments. \*Mean significantly different from 0 h. #Mean significantly different from 24 h. (p<0.05).

Cell cycle analysis provide confirmation on growth patterns of the cell lines. The cell cycle phase that correlated best with growth curve in the cell lines was the percentage of G<sub>2</sub>/M, which is where cells are in division (Figure 2). Among B-lymphoid cell lines, the relatively higher percentages of G<sub>2</sub>/M corresponded well with cells still in proliferation at 72h seen for BV173 and Reh. In the RS4;11 cell line which had stopped proliferation at 72h in culture, percentage of G<sub>2</sub>/M was lowest at 72 h. In the myeloid cell lines, reduced number of cells at 72h corresponded to lower G<sub>2</sub>/M percentages for K562. However, for the HL60 cell line, even though number of cells were also reduced by 72h, percentages of G<sub>2</sub>/M cells remained high. A distinct difference between B-lymphoid and myeloid cells was a higher percentage of cells in S-phase in all days including days when cell numbers appeared to be declining in culture. PBMC isolated directly from blood showed these cells were not in proliferation. These different growth characteristics provide an opportunity to observe patterns in expression of the genes studied here.

PBMC

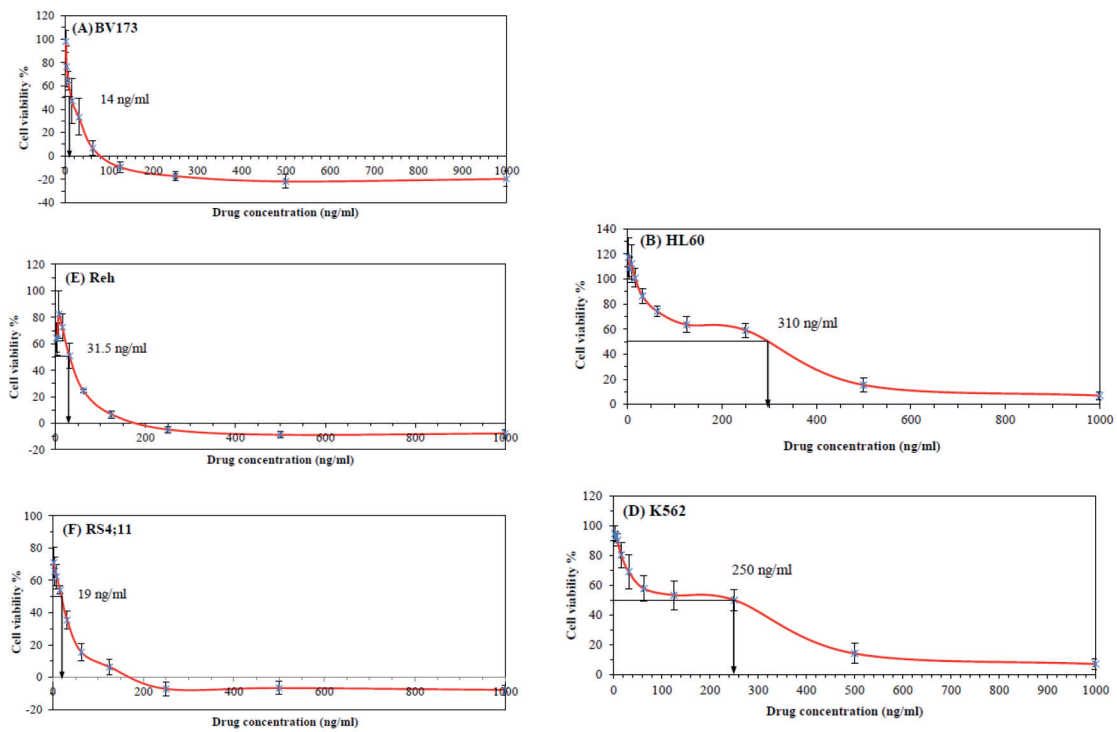






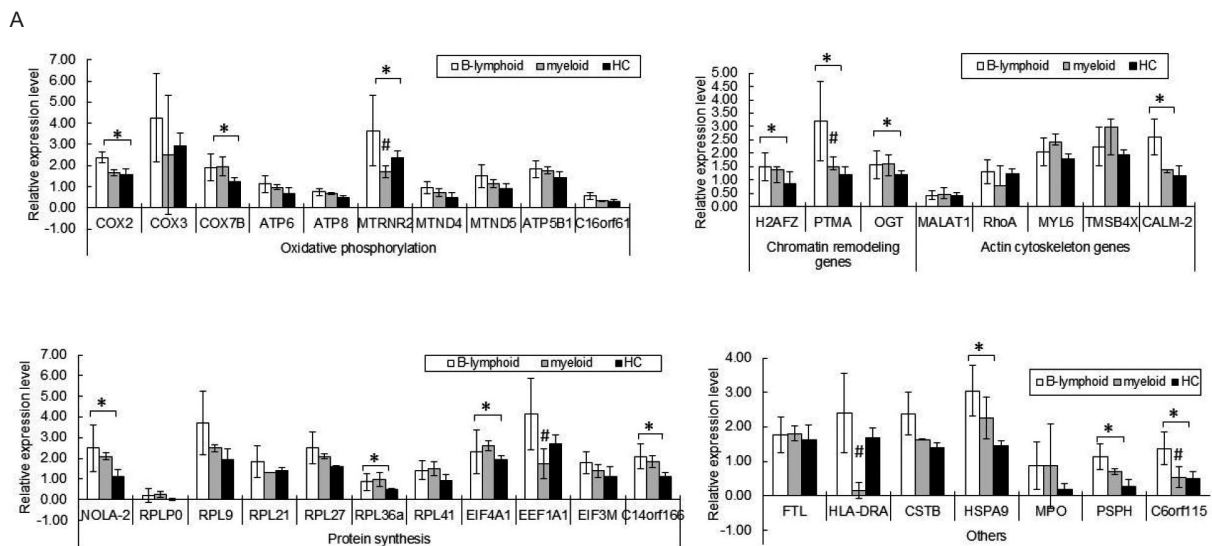
**Figure 2:** Cell cycle profiles of PBMC from healthy control and leukaemia cell lines in culture at 24 h, 48 h and 72 h. Flow cytometry plots were analyzed with ModFit LT software.

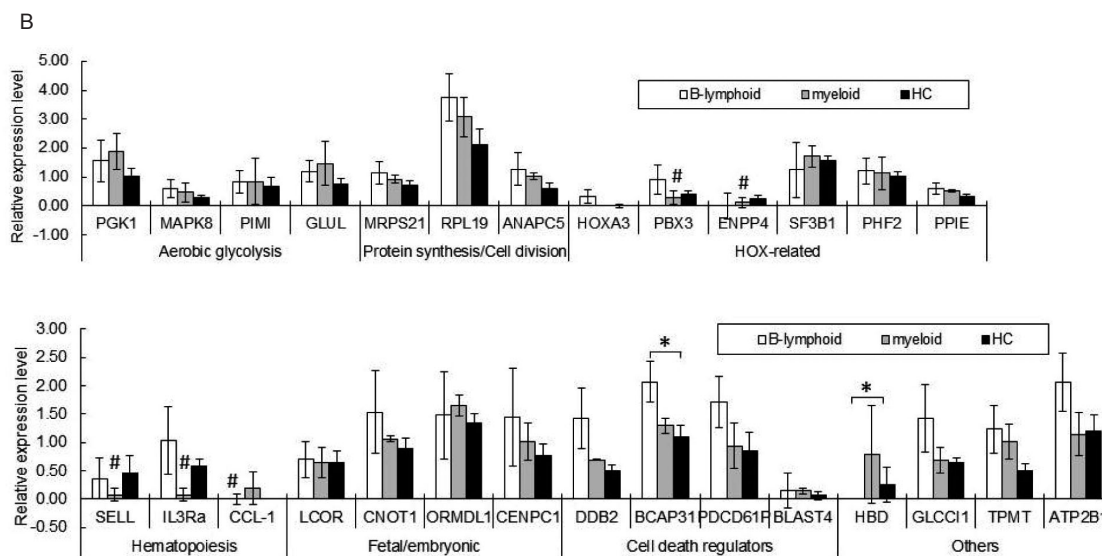
Cell viability of cell lines at increasing epirubicin concentration are shown in Figure 3. From IC<sub>50</sub> values, HL-60 (310 ng/ml) may be most chemoresistant followed by K562 (250 ng/ml), Reh (31.5 ng/ml), RS4;11 (19 ng/ml) and finally BV173 (14 ng/ml).



**Figure 3:** IC50 of leukaemia cell lines treated with chemotherapeutic drug, epirubicin. Cell viability was determined with the MTT assay.

Average expression levels of B-lymphoid (N=3), myeloid (N=2) leukaemia cell lines and healthy controls N=5 are compared in Figure 4A and 4B, respectively. The majority of genes expressed significantly higher compared to controls were the B-lymphoid leukaemic cell line group. The genes (39%, 14/36) were those examined in the GP library which were involved in oxidative phosphorylation (3/10), protein synthesis (4/11), chromatin remodeling (3/3), actin cytoskeleton (1/5) and others (3/7). In contrast, only 7% (2/28) of genes examined in the PP pool were expressed significantly higher in B-lymphoid cells. Among the PP library, only *BCAP1*, a cell death regulator gene and *HBD* was significantly different between B-lymphoid and controls. No significant difference in gene expression were observed between myeloid cell lines and healthy controls probably due to the low number of samples analysed (Figure 4A and 4B).





**Figure 4:** Median+SD expression levels of differentially expressed genes from A) GP and B) PP in B-lymphoid (N=3), myeloid (N=2) leukaemia cell lines and healthy controls (N=5). \* $p < 0.05$ , #  $> 2$ -fold difference in level of expression between B-lymphoid and myeloid leukaemia cell lines.

Correlation between IC<sub>50</sub> values and gene expression levels in corresponding cell lines did not reveal any significant association. The average IC<sub>50</sub> values in myeloid cell line was 13-fold higher than B-lymphoid cells. A comparison of gene expressions in these two groups of cell lines showed five genes from the GP pool (*MTRNR2*, *PTMA*, *EEF1A1*, *HLA-DR* and *C6orf115*) and four genes from the PP pool (*PBX3*, *ENPP4*, *SELL* and *IL3Ra*) were at least 2-fold higher in expression while *CCL-1* (from PP library) was 2-fold lower in expression in B-lymphoid compared to myeloid cell lines.

## DISCUSSION

Genetic profiling are ideal to identify genes of hallmarks of cancer include self-sufficiency in growth signals, limitless replicative potential and reprogramming of energy metabolism (2) in specific cancers. Screening of isolated markers on cancer cell lines provide preliminary information on its biological characteristics and potential clinical significance.

Several genes co-incidentally isolated from the libraries were useful as 'internal controls' as they demonstrated predicted patterns of expression in the samples analysed here. The myeloperoxidase gene, *MPO*, encodes for a protein associated with the myeloid lineage and used in confirming myeloid differentiation in leukaemias (6). However, it is not expressed in AML with minimal differentiation (7). *MPO* was expressed highest in HL-60, which is of promyelocytic origin. However, lower levels were also observed in B-lineage leukaemia cell lines in particular Reh, which is confirmed by an earlier study which also showed positive expression of *MPO* in Reh (8). Levels in PBMC from healthy controls, in contrast, were expressed at low levels as these were mainly lymphocytes and monocytes. Hemoglobin delta, *HBD* is associated with red blood cells and known to be expressed in K562, which is a cell line which can be induced into the erythrocytic lineage (9). The delta chain constitute HbA-2, which with HbF comprises 3% of adult hemoglobin. Nevertheless, contaminating levels were observed in normal PBMC samples. *HLA-DRA* makes up one of the two proteins in the HLA-DR molecule and is mostly expressed on antigen presenting cells (B cells, monocytes and dendritic cells). As expected, it was expressed in all samples except K562 (erythrocytic) and HL-60 (promyelocytic).

The leukaemic cell lines displayed different patterns in growth curves which is characteristic of cell lines. These cells were harvested 72 h after culture by which time growth curves typically reach a plateau and cells no longer divide. Gene expression analysis showed many genes involved in protein synthesis and chromatin remodelling significantly higher in the B-lymphoid leukaemia cell lines compared to PBMC from healthy controls. While these may reflect the limitless replicative potential of cancer cells, they more likely represents normal characteristics of cells in culture.

PBMC do not proliferate under the same culture conditions as cancer cell lines implying these latter group of cells have attained self-sufficiency in growth signals. Among growth factor genes isolated here, we observed *IL-3Ra*

was expressed highest in BV173 and Reh followed by RS4;11, both were pre-cursor B cell lines. *IL-3Ra* was absent from HL-60 and K562 which is supported by an earlier study (10). IL-3 is a hematopoietic cell growth factor and induced cell growth in control B cells after antigen stimulation (11). We observed *IL-3Ra* expression also in control PBMC but these cells do not grow on their own without external supplementation. Cell lines with self-sufficiency may constitutively express growth factors which act in an autocrine manner to stimulate growth (12).

*In vitro* cytotoxicity assay and IC50 values may be useful in identifying drug resistant cases. Several genes were differentially expressed in the myeloid cell lines which had higher IC50 values compared with B-lymphoid leukaemia cell lines. Mitochondria genes including those involved in oxidative phosphorylation as well as those required for protein synthesis in the mitochondria (e.g. *MTRNR2*) were observed to be mutated in many cancers including haematologic malignancies (reviewed in Carew and Huang, 2002) (13). *L-selectin* (*SELL*) contributes to leukocyte recruitment which together with inflammatory pathways form a permissive microenvironment for metastasis (14). Though these genes were associated with cancers, no report has associated them with drug resistance. On the other hand, several of the genes with lower expression levels in myeloid cell lines were highly expressed in chemo-resistant cancers, which did not support our observation. *PTMA* is overexpressed in various human cancers and associated with drug resistance in hepatocellular carcinoma (15). Overexpression of *eEF1A1* results in chemoresistance in cancer (16). These studies were performed on *in vitro* models. These contradicting results may be because comparison here was made between different cell lineages and relative levels may be more relevant within a lineage. However, higher level of these genes in lymphoid cells was not a defect from normalization with reference gene as these levels were already higher before normalization. Furthermore, both *PTMA* and *eEF1A1* were expressed in both types of cells. Contrasting levels of genes between cells of different lineages nevertheless provide information on the biological characteristics of the cells and require further studies.

## CONCLUSION

B-lymphoid leukaemia cell lines expressed higher levels of genes isolated from the GP sample suggesting high proliferating and active state of the cells (compared to normal controls). Expression of PP genes were no different between the three groups which may be inferred as no stem cell characteristics associated with the cell lines. The results here provide preliminary information for more specific examination in future studies.

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