The Correlation of Cyclin A with Drug Resistance in Adult Acute Non Lymphoblastic Leukemia

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Abstract

Acute Non Lymphoblastic Leukemia is one of the most common malignant tumors of Haematology. With the recent progress in chemotherapy and supportive therapy, the remission and survival rate have been markedly improved.

In this study, Cyclin A2 and multidrug resistance expression was measured by Flow Cytometry and RT-PCR in 52 de novo AML patients with acute myeloid leukemia. Their expression was correlated with other prognostic criteria, response to treatment and to overall survival.

The rate of CR and PR was significantly higher in the group of positive expression of Cyclin A2, compared to that with negative expression. However a statistically significant difference was only reached by PCR (p=0.02). By Flow Cytometry, the overall Survival (OS) in the group with positive Cyclin A2 expression is significantly higher than that in the group of negative Cyclin A2 expression, p=0.03.

Regarding MDR1, it was expressed in 39% of our patients and the level of expression was slightly higher by RT-PCR. The rate of CR and PR in the group of negative MDR expression was significantly higher as compared to the group of positive MDR expression, by both Flow Cytometry and RT-PCR (p=0.005, 0.004, respectively). The OS in the group with negative MDR1 expression was significantly higher than that in the group of positive MDR1 expression, p=0.04.

There was a significant inverse relationship between Cyclin A2 and MDR expression in our AML cases by RT-PCR technique (p=0.005), while it showed no significance by Flow cytometry (p=0.12).

There was no agreement (Kappa=0.25) between Flow cytometry and RT-PCR in detection of Cyclin A2. On the contrary, there was an agreement between Flow cytometry and RT-PCR in detection of MDR.

In conclusion, the low expression of Cyclin A2 and high expression of MDR1 are indicators for unfavorable prognosis for individuals with AML. The detection of Cyclin A2 level would predict drug resistance. However, it is one of many other factors.

Key Words: Acute non-lymphoblastic leukemia – Cyclin A2 – Multiple drug resistance (MDR).

Introduction

ABNORMAL cell cycle regulation can lead to uncontrolled cell growth and cancer. Cyclins are positive regulators of the cell cycle and their expression is associated with uncontrolled cell growth and cancer [1].

The A-type family of cyclins consists of two members: cyclin A1 and cyclin A2. Cyclin A2 (formerly known as cyclin A) is a member of the G2 cyclins that are involved in the control of the G2/M cell cycle transition and mitosis, as well as S-phase progression [2]. Cyclin A (A2) was considered previously as a marker of proliferation [3]. Cyclin A2 is particularly interesting as it can activate two different cyclin-dependant kinases (Cdk2 and CDC2) which correspond to the different stages of the cell cycle [4]. It participates in the synthesis and replication of DNA and propels cells into mitosis phase [5].

The synthesis of cyclin A is mainly controlled at the transcriptional level, involving E2F and other transcriptional factors. Removal of cyclin A is carried out by ubiquitin-mediated proteolysis [4]. Cyclins A2 may be associated with chemosensitivity of leukemic blast cells [6].

Considerable interest has focused on resistance of leukemic cells that is due to increased efflux of a drug. A classical multi drug resistance (MDR) occurs due to a membrane glycoprotein, termed P170 or P-glycoprotein (P-gp). P-gp consists of two homologous domains containing ATP-binding sites. It is encoded by MDR1 gene. It acts as a
drug efflux pump in the plasma membrane. After binding of the drug on the intracellular side of P170, the drug is exported from the cell via a channel formed between the two domains. In AML, a reduced intracellular concentration of cytotoxic drugs attributable to the action of P-gp in AML blasts may therefore be related to resistant disease and failure of AML therapy [7].

The mechanism by which P-gp is up-regulated is still a matter of debate [7]. Altered activity of certain transcription factors have been implicated in MDR1 gene regulation [8]. Another possibility is the hypomethylation of the MDR1 promoter [9]. In addition, it has been assumed that the extent of P-gp expression may be influenced by structural variation of the MDR1 gene [10,11].

P-gp is not the only MDR-related protein; several proteins of the ATP binding cassette family are involved in the intracellular transport of a variety of molecules. Besides P-gp, the other two major members are multidrug associated protein (MRP) and the lung resistance protein (LRP) (Kris-ahan et al., 1997).

**Aim of the work:**

In the present study, we investigated the expression of Cyclin A2 and MDR in de novo AML patients. We analyzed the correlation between Cyclin A2 and MDR and their value in clinical outcome, prognosis and survival of the patient.

**Patients and Methods**

**Patients:**

The present study was carried out in the clinical pathology department of the National Cancer Institute, Cairo University. Patients were selected from the outpatient clinic of medical oncology department. It included 34 males (64%) and 18 females (36%) with an age range of 18 to 65 years, a mean of $38.7 \pm 14.7$ and median of 37.5 years.

Ten healthy age and sex matched subjects were taken as control for Cyclin A2.

After an informed consent, Bone marrow (BM) and peripheral blood (PB) samples were obtained from 52 newly diagnosed acute myeloid leukemia patients. Seventeen patients (33%) were classified as AML M0-M1, twenty two (43%) AML M2, nine patients (17%) AML M3, four (7%) AML M4.

According to response to treatment, patients were divided into 2 groups, complete remission group and refractory and partial remission group. Complete remission in AML has been defined using the following criteria developed by an International Working Group as follow:

- Normal values for absolute neutrophil count (>1000/microL) and platelet count (>100,000/microL), and independence from red cell transfusion.
- A bone marrow biopsy which reveals no clusters or collections of blast cells. Extramedullary leukemia (e.g. central nervous system or soft tissue involvement) must be absent.
- A bone marrow aspiration reveals normal maturation of all cellular components (i.e. erythrocytic, granulocytic, and megakaryocytic series).
- There is no requirement for bone marrow cellularity.
- Less than 5 percent blast cells are present in the bone marrow, and none can have a leukemic phenotype (e.g. Auer rods).
- The persistence of dysplasia is worrisome as an indicator of residual AML but has not been validated as a criterion for remission status.
- The absence of a previously detected clonal cytogenetic abnormality (i.e. complete cytogenetic remission, CRc) confirms the morphologic diagnosis of CR but is not currently a required criterion.

However, in a report from the CALGB, conversion from an abnormal to a normal karyotype at the time of first CR was an important prognostic indicator, supporting the use of CRc as a criterion for CR in AML [12,13,14].

Some patients may fulfill all of the above criteria for CR but may not recover peripheral blood counts to the required level. These are denoted as CRi, or CR with insufficient hematological recovery (platelets or neutrophils). CRp describes a subset of patients with CRi, wherein patients fulfill all criteria for CR except that platelet counts are <100,000/microL [12,14].

Patients who fail to achieve CR or CRi may experience a partial remission (PR), defined as a ≥50 percent decrease in bone marrow blasts with normalization of peripheral blood counts, or some other measure of hematologic improvement. A PR in AML is generally expected to be of short duration, and, in most circumstances, is unlikely to serve as a surrogate reasonably likely to predict for clinical benefit [4] in our study two case were in partial remission and we add them to complete remission group for statistical reason.
Methods:

Sampling:

Peripheral blood samples were collected on EDTA (5ml) for complete hemogram, immunophenotyping by Flow Cytometric analysis (Partec III) of the studied parameters. Three ml of PB were collected on Heparin for PCR.

Detection of Cyclin A2 and MDR-1 mRNA by Reverse transcription polymerase chain reaction (RT-PCR):

RNA extraction:

Cell from peripheral blood were separated by Ficol-Hypaque density gradient centrifugation, washed twice in PBS. Cells were dissolved in 0.2 ml PBS. RNA was extracted by homogenization (Polytron; Kinematica, Lucerne, Switzerland) in TRIzol reagent (Gibco BRL) at maximum speed for 90-120s. The homogenate was incubated for 2 min at room temperature. A 1:5 volume of Chloroform was added and the tube was vortexed and subjected to centrifugation at 12000g for 5 min. The aqueous phase was isolated and one half of the volume of Isopropanol was added to precipitate the RNA. The purified total RNA was finally eluted in 10 ml Diethylbicarbonate-treated H2O and the quantity was characterized using UV Spectrophotometer.

cDNA synthesis:

Reverse Transcriptase (RT) of the isolated total RNA was done in a 20ml reaction volume containing 1ml of superscript-II RT enzyme (Gibco-BRL, Gaithersburg, MD, USA), 2.5ml 10X RT-buffer (250 mM Tris-HCl pH8.3, 50 mm KCl, 1.5 mm Mgcl2, 0.1% Triton X-100, 0.2 mm deoxynucleotides each and 2 units Taq-polymerase). One microliter of the cDNA product was amplified by PCR using specific primers. Then add 0.5 µmol/l in 50 µl of mixture consisting of 10 mmol/l Tris-HCl (pH8.3), 50 mmol/KCl, 1.5 mmol/dNTPs. (dATP, dTTP, dGTP, dCTP). The oligonucleotides used in the PCR amplification were as follows: sense strand 5’ - TCCATGTCAGTGCTGAGAGGC- 3’ and antisense strand 5’- GAAGGTCCATGACAAGGC-3’. Based on the sequence information surrounding the intron-exon junctions of each gene, the primers were designed to span an intron; therefore the PCR product specifically detects mRNA. PCR comprised 30 cycles for cyclin A2 and 30 cycles for GAPDH, with denaturing at 94ºC for 1 min and extension at 72ºC for 30 sec. The level of expression of Cyclin A2 mRNA was quantitated as the ratio of band intensity of Cyclin A2 versus GAPDH. Control samples were subjected to the same amplification procedures and analysis.

MDR1:

Amplification of MDR1 was carried out by polymerase chain reaction using 2 µl of cDNA (corresponding to 5 ng of total RNA) in a volume of 50 µl containing 10 mm Tris-HCl, pH 9.0, 50 mm KCl, 1.5 mm Mgcl2, 0.1% Triton X-100. 0.2 ml deoxynucleotides each and 2 units Taq-polymerase (Promega), β-microglobulin was used as a control for employing similar amounts of RNA in the experiment. The following primers were used (Microsynth, Balgach, Switzerland): MDR1 (product length 157 base pairs): 5’ CCC ATC ATT GCA ATA GCA GG 3’ (sense) and 5’ GTT CAA ACT TCT GCT CCT GA 3’ (antisense); B2-mg (product length 120 base pairs): 5’ ACC CCC ACT GAA AAA GAT GA 3’ (sense) and 5’ ATC TTC AAA CCT CCA TGA TG 3’ (antisense). Thirty cycles were performed, with a denaturation temperature of 94ºC for 25s, an annealing temperature of 57ºC for 30s and an extension temperature of 73 ºC for 1 min. This is followed by complete extension cycle at 72ºC for 10 min. The reaction products were separated on a 10% polyacrylamide gel (BioRad, Hercules, CA, USA) and stained with ethidium bromide. Intensity of the DNA bands was determined by densitometry (Vilber-Lourmat, Marne La Vallee, France). mRNA levels were quantified as ratio MDR1/B2-mg.

Detection of Cyclin A2 and MDR-1 protein expression by flow Cytometry:

A. Cyclin A2:

Transfer 50 µl of the diluted anticoagulated blood to be analyzed to each of 2 tubes, labeled Cyclin A2 and control. Add 100 µl of intra-stain reagent A (fixation) to each tube, vortex gently. Incubate at room temperature for 15 minutes. Add 2ml PBS and mix well. Centrifuge at 1200 rpm for 5 minutes, and then aspirates the supernatant, leaving approximately 50 µl of fluid. Add 100 µl of Intra-stain reagent B (Permeabilization) (FixativeA and permeabilizingB, DAKO Cytomation, Lot no. 000 43662) to the test tubes, add 10 µl of PE-conjugated monoclonal mouse anti-human Cyclin A (SC-239PE, Lot#C0303), to test tube labeled cyclin A. Vortex...
gently to ensure that the cells are in suspension. To control tube add 10 µl unconjugated monoclonal antibodies as negative control. Incubate in the dark at room temperature for 15 minutes. Wash twice by PBS. Resuspend pellet in a sheath fluid for flow cytometric analysis.

The relative Florescent Intensity (RFI) ratio was calculated by dividing RFI of patient by the RFI of the isotypic control. A patient is considered positive if the RFI of Cyclin A2 is >0.2.

MDR-1 (P-glycoprotein):
Mix 100 µl of the heparinized blood with 10 µl anti MDR-1 (Monoclonal mouse antihuman MDR-1 (SC-1313, Lot#C1104, FITC). An irrelevant monoclonal antibody of the same iso-type and protein concentration should be used as a negative control. Incubate at room temperature in the dark for 30 min. Wash twice with PBS; aspirate the supernatant, leaving approximately 100 µl fluid. Add sheath liquid and analyze by Flow Cytometry. Results of p-gp expressed by the relative Fluorescence Intensity (RFI). The RFI ratio was calculated by dividing RFI of patient by the RFI of the isotypic control. A patient is considered positive if the MFI of Pgp is ≥1.1 [29].

Statistical methods:
Data was analyzed using SPSSwin statistical package version 12. Numerical data were expressed as mean±standard deviation (SD), median and range. Qualitative data were expressed as frequency and percentage. Chi-square test (or Fisher’s exact test) was used to examine the relation between qualitative variables. Regarding numerical variables, comparison between two groups was done using Mann-Whitney test while comparison between more than two groups was done using Kruskal-Wallis Test. Correlation between numerical variables was done using Spearman's rho method. Survival analysis was done using Kaplan-Meier method. Comparison between two survival curves was done using Log-rank test. p-value <0.05 was considered significant.

Results
This study was carried out on fifty two de novo AML patients. Patients were studied at diagnosis and during the course of treatment. It included 34 males (64%) and 18 females (36%), with an age range of 18 to 65 years, a mean of 38.7±14.7 and median 37.5 years. Patients' clinical and laboratory data are summarized in Table (1).

In 52 AML patients, 2 cases (4%) showed hepatomegaly, 1 case (2%) with splenomegaly, 8 cases (15%) with hepatosplenomegaly and 1 case (2%) with splenomegaly and lymph node enlargement. Forty cases (77%) had no clinically evident hepatosplenomegaly.

The median total leucocytic count was 74x10⁹/L (range, 4.3-470x10⁹/L). The median haemoglobin concentration was 7.1g/L (range, 3.1-11.2g/L) and median platelet count was 34.5x10⁹/L (range, 5-134x10⁹/L).

Thirty-nine patients (75%) presented with hypercellular BM and thirteen (25%) presented with normo- and hypocellular BM. The median marrow blast count was 74% (range, 83%-91%).

Cyclin A2 expression by flow cytometry and RT-PCR:
By flow cytometry, according to control, patient is considered positive if cyclin A2 level of expression ≥0.2, Fig. (4). Cyclin A2 was expressed in 37 (72%) patients as compared to 15 (28%) showed no expression of Cyclin A2, Fig. (1). By RT-PCR, Cyclin A2 mRNA was positive in 29 (57%) while it was negative in 23 (43%) patients, Table (2).

In our patients group, By flow cytometry, Cyclin A2 showed the lowest expression in AML (M3) and (M4) (mean=0.64±1.13 and 0.62±0.58, respectively) as compared with other groups. The same result was obtained by RT-PCR where Cyclin A2 showed the lowest expression in AML (M3) (mean= 94.33±68.27) as compared with other groups although it did not reach the significant level, data not shown.

Relationship between Cyclin A2 expression and clinical and laboratory data:
We analyzed the association between Cyclin A2 expression and age, sex, Haemoglobin, WBCs, BM cellularity and marrow blast count and platelets. There was no significant association detected between groups.

Response to treatment and cyclin A2:
Thirty patients (59%) showed CR and PR while twenty one (41%) were refractory or resistant to treatment, one case is missing.

By RT-PCR, the rate of CR and PR was significantly higher in the group of positive expression of Cyclin A2 compared to that of negative expression (p= 0.02).

By flow cytometry, the rate of Cyclin A2 expression was higher in the group of CR and PR
23/30 (77%) patients as compared to the resistant group 13/21 (62%), but the difference was not statistically significant ($p=0.255$) Table (3).

**Survival:**

In our patients, the Overall Survival (OS) in the group with positive Cyclin A2 expression (37/52) was significantly higher than that in the group of negative Cyclin A2 expression (15/52), $p=0.03$. This significant relationship was detected only by Flow Cytometry. However, by RT-PCR technique, it did not reach a statistically significant level, $p=0.48$, Table (4), Fig. (1).

In our patients, the Disease Free Survival (DFS) in the group with positive Cyclin A2 expression (23/30) was higher compared to the group of negative Cyclin A2 expression (7/30), however the difference was statistically non-significant either by RT-PCR or by FCM ($p=0.14, 0.34$, respectively), Table (5).

**MDR1 by flow cytometry and RT-PCR technique:**

Twenty out of fifty two (39%) patients were positive for MDR (P-glycoprotein expression) while 24/52 (47%) were positive by RT-PCR, Fig. (3).

In correlation to the FAB subgroups, the level of MDR expression was the highest in AML (M3) group (mean=2.72±5.55) as compared to other FAB groups, although it does not reach the statistical significant level, data not shown.

**MDR in relation to response:**

The rate of CR and PR in the group with negative MDR expression was significantly higher than the group with positive MDR expression. This result was detected by both techniques, flow cytometry and RT-PCR ($p=0.005, 0.004$, respectively), Table (3).

**Survival:**

The OS in the group with negative MDR1 expression (28/52) was significantly higher than that in the group of positive MDR1 expression (24/52), $p=0.04$. This relationship is detected only by RT-PCR however, by Flow cytometry technique it was non significant, $p=0.95$, Table (4), Fig. (2).

In case of MDR1, the DFS in the group with negative MDR1 expression (23/52) is significantly higher than that in the group of positive MDR1 expression (7/52), $p=0.13$. This relationship is detected only by Flow cytometry however, by RT-PCR technique, it was non significant, $p=0.46$, Table (5).

### Correlation between cyclin A2 and MDR expression:

There was a significant inverse relationship between Cyclin A2 and MDR expression in our AML cases by RT-PCR technique ($p=0.005$) while it showed no statistically significance correlation by Flow cytometry, $p=0.12$.

### Comparison between flow cytometry and RT-PCR techniques:

There was no agreement (Kappa=0.25) between Flow cytometry and RT-PCR in detection of Cyclin A2. There were 6 cases, that were negative by Flow cytometry, showed positive expression by PCR. On the other hand, there were 13 cases that were positive for Cyclin A2 by Flow cytometry and found to be negative by RT-PCR, Kappa=0.23, data not shown.

On the contrary, there was an agreement between Flow cytometry and RT-PCR in detection of MDR. There are 5 cases that were positive for MDR by Flow cytometry showed negative expression by RT-PCR. On the other hand, there were 9 cases, that were negative by Flow cytometry, showed positive expression by PCR, Kappa=0.45, data not shown.

### Table (1): Clinical and pathologic characteristics of 52 AML patients.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std deviation</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>38.7</td>
<td>14.7</td>
<td>37.50</td>
<td>18.00-65.00</td>
</tr>
<tr>
<td>TLC (X10^9/L)</td>
<td>73.01</td>
<td>76.05</td>
<td>74.0</td>
<td>4.3-470.0</td>
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<tr>
<td>BM Blasts (%)</td>
<td>69.17</td>
<td>20.84</td>
<td>74.00</td>
<td>0.83-91.00</td>
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<tr>
<td>Hb (g/dL)</td>
<td>7.15</td>
<td>1.87</td>
<td>7.10</td>
<td>3.10-11.20</td>
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<tr>
<td>Plt (X10^9/L)</td>
<td>46.27</td>
<td>35.58</td>
<td>34.5</td>
<td>5.0-134.0</td>
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<table>
<thead>
<tr>
<th></th>
<th>Number</th>
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<tbody>
<tr>
<td>Sex</td>
<td>34 male</td>
<td>(64%)</td>
</tr>
<tr>
<td></td>
<td>18 female</td>
<td>(36%)</td>
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</table>

<table>
<thead>
<tr>
<th>Clinical data:</th>
<th>Number</th>
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<tbody>
<tr>
<td>No HSM</td>
<td>40</td>
<td>(77%)</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>2</td>
<td>(2%)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>1</td>
<td>(2%)</td>
</tr>
<tr>
<td>HSM</td>
<td>8</td>
<td>(15%)</td>
</tr>
<tr>
<td>Splen+LN</td>
<td>1</td>
<td>(2%)</td>
</tr>
<tr>
<td>Response</td>
<td>CR and PR: 30</td>
<td>(56%)</td>
</tr>
<tr>
<td></td>
<td>Refractory: 21</td>
<td>(39%)</td>
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<table>
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<tr>
<th>FAB subtypes:</th>
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<th>Percent</th>
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<tbody>
<tr>
<td>M0-M1</td>
<td>17</td>
<td>(33%)</td>
</tr>
<tr>
<td>M2</td>
<td>22</td>
<td>(43%)</td>
</tr>
<tr>
<td>M3</td>
<td>9</td>
<td>(17%)</td>
</tr>
<tr>
<td>M4</td>
<td>4</td>
<td>(7%)</td>
</tr>
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</table>
The Correlation of Cyclin A with Drug Resistance

Table (2): Expression of cyclin A2 and MDR1 by flow cytometry and RT-PCR.

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
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<tbody>
<tr>
<td>Cyclin A2:</td>
<td></td>
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</tr>
<tr>
<td>Flow cytometry</td>
<td>38 (72%)</td>
<td>15 (28%)</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>29 (57%)</td>
<td>23 (43%)</td>
</tr>
<tr>
<td>MDR1:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>20 (39%)</td>
<td>32 (61%)</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>24 (47%)</td>
<td>28 (53%)</td>
</tr>
</tbody>
</table>

Table (3): Expression of cyclin A2 and MDR1 in relation to response.

| Flow cytometry: | CR and PR | Resistant | p-value |
| Cyclin A2 | 23/30 (77%) | 13/21 (62%) | 0.25 |
| MDR1 | 7/30 (23%) | 13/21 (62%) | 0.005* |
| RT-PCR: | Cyclin A2 | 21/30 (70%) | 8/21 (38%) | 0.02* |
| MDR1 | 9/30 (30%) | 15/21 (71%) | 0.004* |

*: Significant.

Table (4): Overall survival (OS) in relation to cyclin A2 and MDR.

<table>
<thead>
<tr>
<th></th>
<th>Cumulative survival (ms)</th>
<th>Median± SE (m)</th>
<th>Confidence interval</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole group</td>
<td>0.36</td>
<td>4±0.99</td>
<td>2.05-5.95</td>
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</tr>
<tr>
<td>Flow cytometry:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin -ve (15p)</td>
<td>0.14</td>
<td>1</td>
<td>0.00-0.00</td>
<td>0.03*</td>
</tr>
<tr>
<td>Cyclin +ve (37p)</td>
<td>0.44</td>
<td>6±1.24</td>
<td>3.58-8.42</td>
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<tr>
<td>MDR -ve (32p)</td>
<td>0.34</td>
<td>5±0.8</td>
<td>3.43-6.57</td>
<td>0.95</td>
</tr>
<tr>
<td>MDR +ve (20p)</td>
<td>0.41</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR:</td>
<td></td>
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</tr>
<tr>
<td>Cyclin -ve (22p)</td>
<td>0.31</td>
<td>3±1.1</td>
<td>0.85-5.15</td>
<td>0.48</td>
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<tr>
<td>Cyclin +ve (30p)</td>
<td>0.39</td>
<td>5±1.02</td>
<td>2.99-7.01</td>
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<tr>
<td>MDR -ve (28p)</td>
<td>0.42</td>
<td>5±1.26</td>
<td>2.53-7.47</td>
<td>0.04*</td>
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<tr>
<td>MDR +ve (24p)</td>
<td>0.29</td>
<td>2±1.14</td>
<td>0.00-4.24</td>
<td></td>
</tr>
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</table>

SE = Standard error. *: Significant.

Table (5): Disease free survival (DFS) in relation to cyclin A2 and MDR.

<table>
<thead>
<tr>
<th></th>
<th>Cumulative survival (ms)</th>
<th>Median± SE (m)</th>
<th>Confidence interval</th>
<th>p value</th>
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<tbody>
<tr>
<td>Whole group</td>
<td>0.43</td>
<td>5±1.1</td>
<td></td>
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<tr>
<td>Flow cytometry:</td>
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<tr>
<td>Cyclin -ve</td>
<td>0.14</td>
<td>5±1.3</td>
<td>2.43-7.57</td>
<td>0.14</td>
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<tr>
<td>Cyclin +ve</td>
<td>0.52</td>
<td>7±4.04</td>
<td>0.00-14.92</td>
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<tr>
<td>MDR -ve</td>
<td>0.56</td>
<td>5±0.79</td>
<td>3.45-6.55</td>
<td>0.34</td>
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<tr>
<td>MDR +ve</td>
<td>0.38</td>
<td>0.00±0.00</td>
<td>0.00-0.00</td>
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<td>RT-PCR:</td>
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<tr>
<td>Cyclin -ve</td>
<td>0.56</td>
<td>18±0.00</td>
<td>0.00-0.00</td>
<td>0.13</td>
</tr>
<tr>
<td>Cyclin +ve</td>
<td>0.38</td>
<td>5±0.74</td>
<td>3.55-6.45</td>
<td></td>
</tr>
<tr>
<td>MDR -ve</td>
<td>0.42</td>
<td>5±1.53</td>
<td>2.01-7.99</td>
<td>0.46</td>
</tr>
<tr>
<td>MDR +ve</td>
<td>0.44</td>
<td>6±1.49</td>
<td>3.08-8.92</td>
<td></td>
</tr>
</tbody>
</table>

SE = Standard error.

Fig. (1): The overall survival is significantly different between cyclin A2 positive (>0.2) and negative group (up to 0.2), by flow cytometry, p=0.03.

Fig. (2): The overall survival is significantly different between MDR1 positive and negative group, by RT-PCR, p=0.03.

Fig. (3): Shows positive control (lane 1), negative control (lane 2), PCR marker (lane 7) and results of positive and negative MDR1 patients.
Discussion

With the recent progress in chemotherapy and supportive therapy in Acute Non Lymphoblastic Leukemia patients, the remission and survival rate in AML have been markedly improved. However, drug resistance and relapse are still important factors affecting long survival of these patients. The abnormal regulation of cell cycle is another factor that contribute to the poor prognosis and therapeutic failure, except for primary drug resistance in leukemic cells [5].

In our study, we investigated the relationship between Cyclin A2 and MDR 1, by 2 techniques; Flow Cytometry and RT-PCR, as two important effectors in drug response and survival of the patients.

By Flow Cytometry, Cyclin A2 was expressed in 72% of our AML patients. However, the percentage of expression was lower (59%) by RT-PCR technique.

This finding is not consistent with Paterlini et al. [3] who found a high significant correlation between Cyclin A2 protein and gene expression. On the hand, it has been reported previously that level of Cyclin A2 by RT-PCR is higher than that by Flow Cytometry [15].

This difference in result may be due to difference in monoclonal used and the development of the technology used in their manufacture.

The lowest expression of Cyclin A2, although it does not reach the statistical significant level, was found in AML (M3) as compared with other FAB groups. However, because a small number of samples examined, the significance of this observation needs to be further studied. Our result is not in line with Nakamaki et al. [2] who found that the mean of Cyclin A2 expression was higher in the group of AML (M3) as compared to the other groups. Over expression of Cyclin A2 might prolong the length of the S phase (Ts) and cell cycle time (Tc) in vivo and thus contribute to a better overall survival in AML patients.

Whether Cyclin A2 contributes to the development of certain types of leukemia or associated with these types needs to be clarified.

We could not reach any relationship between Cyclin A2 expression and Clinical and laboratory data of our patients.

Our finding is consistent with Campos et al. [16] and Ma et al. [5]. On the contrary, Yang et al. [1], Nakamaki et al. [2] found an inverse relationship between the expression of Cyclin A2 and the total leucocytic count which represent the tumor cell mass.

We found that the response rate to the initial chemotherapy was higher in patients with high Cyclin A2 expression. It was higher in the sensitive group (CR and PR) as compared to the resistant group.

Our finding is consistent with Ma et al. [5] and Nakamaki et al. [2] who found that Cyclin A2 expression correlates to a high proliferative activity in leukemia cells, better response and prognosis. A higher proliferation rate correlates to a better chemotherapy response [17,18]. So, high Cyclin A2 expression in AML cells can be considered as a favorable prognostic marker associated with a high response rate.

On the contrary, Ekberg et al. [19] found expression of Cyclin A2 did not show any prognostic significance in AML patients. Study of Cyclin A in other malignancies proved that higher expression is associated with poor response [20,21] however, these finding is not consistent with advanced stages of the disease [22].

Expression of Cyclin A2 may predispose leukemic cells to undergo apoptosis through deregulated progression to the S or M phase of the cell cycle [2]. This hypothesis is supported by experimental data as shown by Bortner & Rosenberg [23], Shi et al. [24] and Anderson et al. [25]. Experimental studies have shown that Cyclin A2 efficiently

Fig. (4): Positive Cyclin A2 by flow cytometry with a RFI >0.2
bound to E2F-1, counteract the growth promoting effects of E2F-1 [1]. This result in negative regulation of the S-phase decreasing the ability to bind DNA and transactivate target genes [19]. Differences in results may be due to differences in the monoclonal, techniques used and the intratumoral heterogeneity in S-phase [27].

We found that, by flow cytometry, Cyclin A2 expression is significantly associated with longer OS. However, there was no statistical significance difference with DFS. We failed to find such relationship by RT-PCR technique.

Our finding is not consistent with Poikonen et al., 2005 who found that high Cyclin A2 is associated short TFS, OS and poor survival in advanced breast cancer. Cyclin A2 may be the most useful marker of proliferation as it is expressed in the late S, G2 and M phases of the cell cycle [20,28]. A major problem in Cytotoxic drug treatment is intrinsic or acquired drug resistance. One potential mechanism of drug resistance is mediated through expression of the P-gp efflux pump, enabling AML blasts to decrease intracellular toxic drug levels and thereby lower the rate of apoptosis [7].

By Flow cytometry, we found 39% of our patients were positive for MDR (P-glycoprotein expression) while by RT-PCR, the percentage was slightly higher (47%) in our AML patients. This can be explained by the different sensitivity of the 2 techniques. Our result is nearly the same as Kamel et al., 2005 who found that MDR1 was expressed in 47% of ANLL patients by Flow Cytometry.

It was found that the level of MDR expression was higher in AML (M3) group, although it does not reach the statistical significant level, as compared to the other AML groups. Our finding is not in line with Paietta et al. [30] and Kamel et al. [29] who found a higher expression of P-gp in M1/M2 compared to other AML groups; M3, M4 and M5.

We could not reach any relationship between MDR1 expression and Clinical and laboratory data of our patients.

In our patients, P-gp expression was associated with lower CR rate and shorter CR duration. Our data suggest that MDR1 expression is highly correlated with the treatment outcome of the patients. This is consistent with Illmer et al. [7], Campos et al. [16], Tothova et al. [31], Meaden et al. [32], Arbelbide et al. [33] and Ma et al. [5] who concluded that patients expressing MDR1 had a lower rate of complete remission, a higher rate of relapse with persistence of post treatment residual disease which produces a short global survival.

The resistance towards chemotherapy may be not only the result of efflux capacity but also of complex interaction with other prognostic markers such as the co-expression of the stem cell marker CD34 [34]. It has been reported that genetic polymorphism of the MDR1 gene may affect the expression and function of P-gp efflux pump in healthy volunteers [7]. Therefore, altered efficacy to drug therapy may be variable P-gp function attributable to the presence of variant MDR1 genotype among AML patients. This polymorphism may also have an impact on therapy outcome [35]. Another factor that contribute to the function of P-glycoprotein function is the coexpression of P-gp with other proteins e.g MRP1 [36,37] and BCRP [38]. Which proved to have a poor prognosis if expressed simultaneously with p-gp rather than P-gp alone.

There was a significant inverse relationship between Cyclin A2 and MDR1. The low expression of Cyclin A2 was associated with multi drug resistance in AML cells i.e. Cyclin A2 may be associated with chemo sensitivity of leukemic blast cells. This was in line with the finding of Beck et al. [6], Ma et al. [5] and Nakamaki et al. [9] who found negative correlation between the gene expression levels of cyclin A and MDR1. Ma et al. found that this was found in patients with drug resistance while there is no such relationship in the sensitive group. So, the lower Cyclin A and lower cellular proliferation activity serves as an additional factor contributing to a lowered vulnerability by chemotherapy to blast cells with enhanced MDR1 gene expression [5].

Ma et al. [5], found that the level of Cyclin A2 mRNA have tendency to decrease after relapse as compared with the primary leukemia. On the other side, Beck et al. also found low level of MDR1 in primary AML patients and it is significantly higher mean RFI in recurrent relapses.

Conclusion:

The present study found that the low expression of Cyclin A2 and high expression of MDR1 are indicators for unfavorable prognosis for individuals with de novo AML. The detection of Cyclin A2 level would predict drug resistance. It plays an important role in leukemogenesis and progression of AML. However, it is one of many other factors.
Further studies are required to understand the precise mechanism by which this Cyclin influence the prognosis of AML patients.

References


