“A study on the expression pattern of key apoptotic regulators in Acute Myeloid Leukemia patients from the North-East Indian regions”

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Abstract: Cancer is the result of succession of genetic changes during which a normal cell is transformed into a malignant one. Evasion of cell death is one of the essential changes in a cell that causes this malignant transformation. Acute Myeloid Leukemia is a cancer of the hematopoietic cells which occurs due to mutational inactivation of some important genes or due to genome rearrangement. It is a malignant disease characterized by an accumulation of immature myeloid blast cells in the bone marrow and most often in the peripheral blood. Apoptosis in cancer occurs due to disrupted balance of pro-apoptotic and anti-apoptotic proteins, reduced caspase function and impaired death receptor signaling. In this study, we studied the role of apoptosis and activation of apoptosis signaling molecules in of AML. 20 blood samples were collected from which RNA was isolated and then cDNA was synthesized. Three apoptotic genes i.e. Bcl-2, Apaf-1 and P53 were used to analyze the samples in AML along with a reference gene (β-actin) for comparative purpose. The results showed a low expression pattern for all the three genes in the studied samples. Bcl-2 is an anti-apoptotic factor which helps in blocking apoptosis action, but in our samples it clearly shows down regulation. Apaf-1 is down regulated and it may disrupt the formation of apoptosomes. P53 helps in blocking the other apoptotic regulators like Bcl-2 but again, in our samples it shows low expression while B-actin has been used as the normal reference gene for comparative studies since it is a house-keeping gene. Usually, for apoptosis to occur we need high expression of the genes Apaf-1 and P53 but the unavailability of these genes could project towards error in apoptosis.

Keywords – Acute Myeloid Leukemia, apoptosis, cancer, expression, gene.

I. Introduction
Cancer is a form of disorder which is characterized by an excessively unnatural and uncontrolled proliferation of cells and has the potential to spread across different parts of the body. It is fundamentally a disease of tissue growth regulation [1]. In order for a normal cell to transform into a cancer cell, the genes that regulate cell growth and differentiation must be altered. Cancer harms the body by dividing the altered cells at a rapid speed to form lumps or masses of tissue called tumors except in the case of leukemia, where cancer restricts the function of normal blood flow by an abnormal cell division in the blood stream [2]. Various physical, chemical and biological components along with different genetic factors are mainly responsible for the chaotic, unconstrained cell proliferation that has the built-in ability to invade or destroy other healthy cells of the body. Some of the carcinogenic components merge with the DNA of the normal cells that cause prohibition of DNA synthesis or gene mutation and develop into tumors.

1.1 Acute Myeloid Leukemia (AML):
Acute myeloid leukemia (AML) is the cancer of the myeloid line of blood cells, characterized by the rapid growth and differentiation of abnormal/immature WBCs in the the bone marrow and peripheral blood cells and interfere with the production of normal blood flow [3]. AML is the most common acute leukemia affecting adults and although AML is a relatively rare disease, its incidence is expected to increase as the population ages. The hematopoietic stem cells produce two types of cell lineages during hematopoiesis such as the myeloid lineages and the lymphoid lineages in the blood cells. The myeloid progenitor constructs megakaryocytes, erythrocytes, mast cells and myeloblasts. A normal myeloblast gives rise to basophils, neutrophils, eosinophils and monocytes and these monocytes in return produce macrophages. These myeloblastic cells are therefore called white blood cells (WBC).
1.2 Role of apoptosis in cancer i.e. AML:

Apoptosis is basically a gene-directed program where the cell numbers can be regulated by factors that influence cell survival as well as those that control proliferation and differentiation. Apoptosis refers to cell death, like any other metabolic or developmental program, that can be disrupted by mutation. In fact, defects in apoptotic pathways are now thought to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy.

Although only a few AML blasts are actually clonogenic as they hardly show any DNA synthesis, they remain in the proliferative stage and eventually assemble due to decreased cell death as much as increased proliferation. In the transformed phenotype stage, they gain the ability to overcome cell-death promoting stimuli, such as cytotoxic chemotherapy, the clinical consequences of which are refractory disease, relapse and death from leukemia [4]. Also, resistance towards chemotherapy frequently encompasses drugs with distinct mechanisms of action (such as multiple drug resistance or MDR) and may arise in many ways. Recently it was observed that alterations in return to apoptosis-inducing stimuli, by disruption of the apoptotic pathway or by a modified DNA repair, may result in resistance to chemotherapy and treatment failure.

1.3 Significance of biomarkers in Apoptosis:

A biomarker is a characteristic objectively measured and evaluated to indicate normal or pathogenic biological processes or pharmacologic response. Its potential to enhance translational science progress and accelerate drug development is becoming recognised. Nowhere is this more pertinent than in the complex arena of anticancer drug development, where the rate of compound attrition is high and success rates in the clinic are low [5]. Biomarkers may facilitate rational decision-making during drug discovery and in pre-clinical drug evaluation [6]. In addition, pharmacodynamic biomarkers allow real-time monitoring of drug efficacy and identify early signs of toxicity during clinical drug evaluation, while stratification biomarkers should facilitate selection of patients most likely to respond [7]. Ideal biomarkers should therefore aspire to the specificity for the biological process/target, accurately quantifiable in clinical samples with sufficient dynamic range to detect change upon drug treatment, provide a rapid, reliable and robust measurement, must be validated to internationally recognised standards, should exhibit little overlap in levels between untreated patients and treated patients, have baseline levels not subject to wide variations between patients, have levels that correlate closely with the proximal or distal effects of therapy and be measurable in a readily obtainable clinical sample.

1.4 Significance of the apoptotic genes used in our study:

- β-actin: It is a highly conserved cytoskeletal protein involved in cell structure formation, motility, and cohesion. They are also used as loading controls in protein assays. β-actin is known as a “housekeeping” protein, i.e. it is expressed constantly, and at high levels, in all the cell types used in protein research. It is a useful loading control in Western blot analysis, RT-PCR etc. In our study this has been used as the reference gene.
- P53: The function of P53 is related to its transcriptional activity. P53 activates or suppresses transcription of specific genes. Transcriptional suppression is achieved via several mechanisms, including the recognition of specific nucleotide sequences in particular genes. To exert most of its effects, P53 acts as a specific transcription factor and induces transcription of various genes. The set of genes controlled by P53 depends on many factors, including the P53 level, cell type, cell state, etc. The P53-dependent mechanisms continuously monitor the cell state and prevent proliferation of abnormal cells. This activity provides a reliable barrier to an accumulation of mutations and induction of carcinogenesis [8].
- Bcl-2: This protein determines the commitment of cells to apoptosis, essential for development, tissue homeostasis and immunity. Bcl-2 helps in the physiological control of apoptosis, the pathological consequences of its disregulation and the promising search for novel cancer therapies that target the Bcl-2 family. Too little apoptosis can promote cancer and autoimmune diseases; too much apoptosis can augment ischaemic conditions and drive neurodegeneration [9]. It was first discovered in follicular b-cell lymphoma as a gene linked to the immunoglobulin heavy chain breakpoints of t(14;18) translocation.
- Apaf-1: This gene encodes a cytoplasmic protein that initiates apoptosis. It contains several copies of the WD-40 domain, a caspase recruitment domain (CARD), and an ATPase domain (NB-ARC). Upon binding cytochrome c and dATP, this protein forms an oligomeric apoptosome that binds and cleaves caspase 9 preproprotein, releasing its mature, activated form. Activated caspase 9 stimulates the subsequent caspase cascade that commits the cell to apoptosis.

1.5 Aim and Objectives:

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Aim: To study the expression pattern of key apoptotic regulators in AML patients samples from North-East Indian Regions.

Objective:
1.) Synthesis of cDNA from total RNA (using first strand cDNA synthetic kit) isolated from 20 blood/bone marrow samples of AML patients.
2.) Expression study of key apoptotic genes (such as P53, Bcl-2, Apaf-1) using cDNA samples.
   a.) Standardization of RT-PCR for the studied genes.
   b.) Comparative analysis of relative expression pattern of the studied genes with respect to a reference gene or internal control gene.

II. Materials And Methodology

Non duplicate, fresh blood samples from 20 AML patients enrolled in the Department of Haematology, Gauhati Medical College and Hospital (GMCH) were collected with written informed consent with reference to their clinical history. RNA was isolated from fresh blood samples using QIAamp RNA Blood Mini Kit (Qiagen, Germany) and according to the manufacturer’s protocol. Instruments like temperature control centrifuge (Eppendorf, Germany), vortex (Tarsons, Kolkata), incubator (Biocraft, Agrag), multiskan gro microplate spectrophotometer (Thermo Scientific, Finland), 50mL falcon tubes (Tarsons, Kolkata), 1.5mL centrifuge tubes (Eppendorf, Germany), EDTA vials, micropipettes (Eppendorf, Germany), micro tips (Tarsons, Kolkata), beaker (Borosil, India), 4°C refrigerator (Samsung, South Korea), measuring cylinder (Borosil, India), and weighing balance (Merck Millipore, Germany) were used.

For a cDNA synthesis, total RNA was isolated from AML blood samples and reagents like RevertAid Reverse Transcriptase enzyme kit contains Reaction Buffer, Ribolock, Reverse Transcriptase enzyme and dNTP mix (Thermo Fisher Scientific) and DEPC treated water were used. Thermal cycler (Life Technologies, Singapore), water bath (Relitech, India), vortex (Tarsons, Kolkata), temperature control centrifuge (Eppendorf, Germany), micropipettes (Eppendorf, Germany) and micro tips (Tarsons, Kolkata) were used during synthesis of cDNA. For Real-Time PCR reaction the reagents used were cDNA, Power SYBR green master mix solution (Applied Biosystems, USA), gene specific primers and DEPC treated water. StepOne Plus Real-Time PCR machine (Applied Biosystems,USA), minicentrifuge, vortex (Tarsons, Kolkata), MicroAmp Fast Reaction Tubes (8 Tubes/Strip) and MicroAmp Optical 8-Cap Strip (Applied Biosystems,USA), micropipettes (Eppendorf, Germany) and micro tips (Tarsons, Kolkata) were used.

2.1 Primer Sequences for Real-Time PCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Orientation</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5′-ACGTGGGACATCCGCAAAGAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CAAGAAAGGGTAGACGCAGAACA-3′</td>
</tr>
<tr>
<td>P53</td>
<td>Forward</td>
<td>5′-GGCCGACTTCACCCTACTCT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GTGGTTTCAAGGCCAGACGT-3′</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Forward</td>
<td>5′-TCCCTCGCTCGACAAATCTC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-ACGCCCGATTGCTATAGAC-3′</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Forward</td>
<td>5′-TGGAATGCCAGGCTGTCGG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-TGCACCTCCCCCTGGGGAACA-3′</td>
</tr>
</tbody>
</table>

Table 1: List of the primer sequences used in our study

2.2 Real-Time Polymerase Chain Reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 X SYBR Green master mix</td>
<td>5 µl</td>
</tr>
<tr>
<td>Primers (sense and antisense)</td>
<td>0.25 µl (each)</td>
</tr>
<tr>
<td>c-DNA template</td>
<td>500 ng/µl</td>
</tr>
<tr>
<td>DEPC treated water</td>
<td>1 Upto 10 µl</td>
</tr>
</tbody>
</table>

Table 2: List of the amount of components used during the Real-Time PCR reactions

2.3 Temperature control profile of the Real-Time PCR for the genes used:
Table 3: RT-PCR Temperature control profile

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holding stage</td>
<td>95</td>
<td></td>
<td>10 minutes</td>
</tr>
<tr>
<td>Cycling stage</td>
<td>95</td>
<td>60</td>
<td>40 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 minute</td>
</tr>
<tr>
<td>Melt curve stage</td>
<td>95</td>
<td>60</td>
<td>15 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 seconds</td>
</tr>
</tbody>
</table>

2.4 RT-PCR analysis:
Expression level of a particular gene was calculated in terms of fold change using the \( C_t \) values. The following formula was used while calculating the fold change:
\[
\Delta C_t \text{ Calibrator} = C_t \text{ Normal control} - C_t \beta\text{-actin}
\]
\[
\Delta C_t \text{ Target} = \text{Average } C_t \text{ Target} - \text{Average } C_t \beta\text{-actin}
\]
\[
\Delta \Delta C_t = \Delta C_t \text{ Target} - \Delta C_t \text{ Calibrator}
\]
So,
mRNA expression level i.e. Fold change = \( 2^{(\Delta \Delta C_t)} \)

III. Experimental Findings

3.1 Documentation of samples:
20 samples from AML patients were collected from Guwahati Medical College and Hospital (GMCH) and the samples were documented with the isolated RNA concentrations. These were then converted to cDNA and the values are as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>( \beta\text{-actin} )</th>
<th>\text{Bcl-2}</th>
<th>\text{Apaf-1}</th>
<th>\text{P53}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total average</td>
<td>16.3765</td>
<td>19.305</td>
<td>23.8705</td>
<td>24.774</td>
</tr>
<tr>
<td>( \Delta C_t \text{ Calibrator} ) (( C_t\text{ Normal control} - C_t \beta\text{-actin} ))</td>
<td>-1.803</td>
<td>-5.753</td>
<td>7.617</td>
<td></td>
</tr>
<tr>
<td>( \Delta C_t \text{ Target} ) (( \text{Average } C_t \text{ Target} - \text{Average } C_t \beta\text{-actin} ))</td>
<td>2.929</td>
<td>7.494</td>
<td>8.3975</td>
<td></td>
</tr>
<tr>
<td>( \Delta \Delta C_t ) (( \Delta C_t \text{ Target} - \Delta C_t \text{ Calibrator} ))</td>
<td>4.732</td>
<td>13.247</td>
<td>0.7805</td>
<td></td>
</tr>
<tr>
<td>Fold Change</td>
<td>0.03763</td>
<td>0.0001</td>
<td>0.5822</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Readings of the \( C_t \) values of the different genes
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Figure 1: Bar diagram representing down-regulation i.e. showing low expression of the three genes in our study.

IV. PRODUCT ANALYSIS OF THE 4 GENES USING RT-PCR

4.1 Real-Time Melt Curve (Derivative Reporter vs. Temperature):

![Fig. 2: β-actin](image2)

![Fig. 3: Bcl-2](image3)

![Fig. 4: Apaf-1](image4)

![Fig. 5: P53](image5)
4.2 Real-Time Amplification Plot (Threshold value, ARn vs. Cycle):

**Fig. 6: β-actin**

**Fig. 7: Bcl-2**

**Fig. 8: Apaf-1**

**Fig. 9: P53**

V. CONCLUSION

In this study, we studied the role of apoptosis and activation of apoptosis signaling molecules in of AML. 20 blood samples were collected from which RNA was isolated and then cDNA was synthesized. Three apoptotic genes i.e. Bcl-2, Apaf-1 and P53 were used to analyse the samples in AML along with a reference gene (β-actin) for comparative purpose. The results showed a low expression pattern for all the three genes in the studied samples. Bcl-2 is an anti-apoptotic factor which helps in blocking apoptosis action, but in our samples it clearly shows down regulation. On the other hand, Apaf-1 is an important factor in forming apoptosome. But if Apaf-1 is down regulated then it may disrupt the formation of apoptosomes which is crucial for apoptosis progression. P53 helps in blocking the other apoptotic regulators like Bcl-2 but again, in our samples it shows low expression while β-actin has been used as the normal reference gene for comparative studies since it is a house-keeping gene. Usually, for apoptosis to occur we need high expression of the genes Apaf-1 and P53 and unavailability of these genes means error in apoptosis. After calculating the average C\textsubscript{t} values of the above and plotting the them, a bar graph was obtained which showed down-regulation for all the four genes as: β-actin (1) > P53 (0.5822) > Bcl-2 (0.03763) > Apaf-1 (0.0001). We studied the product analysis of RT-PCR for all the 20 samples with the respective four genes by generating a series of Real-Time Melt Curve (Derivative Reporter vs. Temperature) and Real-Time Amplification plot (Threshold value, ARn vs. Cycle).

Most of the studies that have been performed so far agree to these results and show similarities when compared to the analysis performed using the genes Bcl-2, P53 and Apaf-1 to study the key apoptotic regulators in AML patients’ samples which showed down-regulation for the three genes; although few studies show up-regulation of some of these genes in the distinct course of work relating to different forms of leukaemia or myelodysplasia using different techniques which are more or less the same.

In a previous study conducted, it was found that when different transcripts of Apaf-1 were analyzed in the bone marrow samples of 37 patients with AML, tumor suppressor genes are usually down-regulated and proto-oncogenes are up-regulated [10]. Low expression of Apaf-1XL was associated with failure in the therapy of remission induction. To determine if low expression of Apaf-1 was related to lower caspase-9 activation, apoptosis in HL-60 and KG-1 was induced in the cell lines respectively. It was reached out that Apaf-1 between ALL and AML could not find the correlation to its prognostic factors [11]. But, on the other hand it was also reported that the deficiency in Apaf-1 in AML induced resistance to apoptosis [12] and methylation could be responsible for the silencing of Apaf-1 in AML with no relationship studies to prognosis [13].

In the case of Bcl-2, high levels of expression i.e. down-regulation has been reported to have been associated with low complete remission rates and poor survival. All-trans retinoic acid (ATRA) has the ability to increase the sensitivity of AML cell lines [14]. The most critical findings relating to the Bcl-2 gene in AML say that this
gene family is over expressed in AML as Bcl-2 acts as an important regulator for apoptotic cell death and deregulation; they also manipulate their function towards enhancing the antitumor effects in the making of antileukemic therapeutic agents. They are not only necessary for their roles in disease pathogenesis but rather in providing resistance towards chemotherapy as well [15].

Some studies indicate that an increase in the frequency of hypermethylation of Apaf-1 gene in AML leads to its down-regulation and hence, the promoter methylation of Apaf-1 could be considered as a factor contributing to the development of AML. Apaf-1 is deficient in human leukemic blasts and provides resistance to cytochrome-c dependent apoptosis and Apaf-1 protein expression did not correlate with Apaf-1 mRNA levels (some leukemic cells expressed high Apaf-1 mRNA but low Apaf-1 protein) [16]. The factors for P53 activation are widely variable with a common theme being cellular stress [17]. Once activated, P53 enforces cell cycle arrest or apoptosis; however, the factors that influence this process are not well understood. The level to which P53 accumulates may influence whether cells cease to proliferate or undergo cell death. Cells expressing low levels of P53 generally arrest in G1, while those that express high levels undergo apoptosis. Under conditions where cell stress is particularly severe, P53 levels may be sufficiently induced to trigger the expression of apoptotic targets and cell death. The induction of P53 expression in primary fibroblasts is usually associated with cell cycle arrest, whereas the activation of P53 in hematopoietic cells generally results in apoptosis.

In AML cases, the frequency of P53 expression was higher than that in ALL. Bcl-2 protein immunoreactivity has been found in the majority of acute leukemia patients. The marked heterogeneity in the percentage of P53 and Bcl-2 positive cells in individual patients was observed. Comparative analysis of the distinct acute leukemia subtypes according to the percentage of P53 and Bcl-2 positive cells showed no significant differences except for P53 protein positivity in relation between ALL and AML cases [18].

References

Reference for primer sequences:

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