RESEARCH ARTICLE

EVALUATION OF CD9 & CD56 ANTIGENS EXPRESSION IN ADULT ACUTE MYELOID LEUKAEMIA.

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Abstract

Background: -Acute myeloid leukaemia (AML) is a malignant clonal disorder resulting from the neoplastic proliferation of a clone of myeloid cells. Flow cytometry is used for confirming diagnosis, identifying prognostic differences, staging of AML and detecting an aberrant immunophenotype that can be used for monitoring of complete remission (CR) achievement.

Objectives: -To evaluate the expression of aberrant CD9 and CD56 in newly diagnosed adult AML patients and their association with clinical and haematological parameters and with CR achievement.

Methods: -Thirty adult patients (>15 years) who were newly diagnosed de novo AML were selected from the Baghdad Teaching Hospital and Al-Imam-ein-Kadhimein medical city from July 2015 to March 2016. All patients were grouped according to FAB classification and evaluated individually and the diagnosis was based on the morphology, cytochemistry or flow cytometry. Aberrant antigens CD9 and CD56 expressions were investigated by four-colours flow cytometry at the time of diagnosis. The patients were evaluated at day 28 from the start of chemotherapy to assess complete remission achievement. Verbal consent was taken from the patients.

Results: -The aberrant expression of CD56, CD9 were observed in 23.3% and 33.3% of AML patients respectively. CD56 was expressed more with monocytic differentiation and CD9 was expressed more with M2 cases. CD56 expression was significantly associated with high total WBC count, high peripheral blood and bone marrow blast cells and the extramedullary manifestations. There was significant association between CD56 and CD9 expression regardless of their intensity of the markers with non-responsiveness to induction therapy.

Conclusion: -Aberrant CD9 and CD56 antigens were associated with adverse clinical and hematological parameters at presentation as well as with low cure rate.

Introduction: -

Acute myeloid leukaemia (AML) is a hematopoietic stem cell disorder, characterized by a block in differentiation of haematopoiesis which results in the growth of a clonal population of neoplastic cells or blasts. This malignant alteration in hematopoietic stem cells leads to a loss of normal hematopoietic function. The peak incidence rate...
occurs in the first year of life and then decreases steadily up to the age of 4 years and remains relatively constant in childhood and early adulthood; AML is thus a disease of adults. (1)

AML represents approximately 90% of all acute leukemias in adults but accounts for only 13% of leukaemia cases in children younger than 10 years. (2)

Recurring chromosomal aberrations and gene mutations are frequently found in AML and contribute greatly to the pathogenesis of the disease. (2) One of these is the t (8; 21) (q22; q22) translocation which produces RUNX1-RUNX1T1 fusion product that blocks hematopoietic differentiation and enhances self-renewal of hematopoietic stem cells.

The inv (16) (p13q22) or t (16; 16) (p13; q22) produces the leukaemogenicCBFβ-MYH11 fusion gene which blocks differentiation of hematopoietic stem cells by inhibiting the function of Runt-related transcription factor 1 (RUNX1). APL cells usually have t(15;17)(q22;q11-21) producing PML-RARA fusion products which also behave as a transcriptional repressor.

Accurate diagnosis and classification in AML are essential for treatment decisions and assessment of prognosis. Initial assessment requires a careful history, physical examination, complete blood count (CBC) with peripheral blood (PB) smear review, bone marrow (BM) examination, Flow cytometry (FC), cytogenetic and selected molecular genetic analysis. (3)

Classification of AML: -
Acute myeloid leukaemia can be classified in many ways: (4, 5)
1. By morphology and cytochemistry supplemented by immunophenotyping, as proposed by the FAB group.
2. The morphologic, immunologic, and cytogenetic (MIC) classification, which was the first to recognize the usefulness of cytogenetic for identifying subgroups of acute leukaemia.
3. The WHO Classification which takes into account morphologic and immunologic features plus well-studied, common non-random chromosomal abnormalities.
4. By immunophenotyping alone, as proposed by the European Group for the immunological classification of leukaemias (EGIL).

Laboratory investigation of acute myeloid leukaemia:

CBC and peripheral blood Smear: -
The leukocyte count is elevated in more than one half of patients but is >100,000 cells/mm3 in <20%. Blasts usually are identified on peripheral smear; Auer rods are considered pathognomonic of AML (which are reddish rod-like filaments of aggregated primary granules). A leukemic leukemia (no blasts in the peripheral smear) is rare. (6)

BM Morphology: -
A bone marrow aspirate is part of the routine diagnostic work-up of a patient with suspected AML. A marrow trephine biopsy is optional, but it should be performed in patients with a dry tap. For a diagnosis of AML, a marrow blood blast count of 20% or more is required, except for AML with t (15; 17), t(8; 21), inv (16) or t (16; 16). Myeloblasts, monoblasts, and megakaryoblasts are included in the blast count. In AML with monocytic or myelomonocytic differentiation, monoblasts and promonocytes, but not abnormal monocytes, are counted as blast equivalents. Erythroblasts are not counted as blasts except in the rare instance of pure erythroid leukaemia. (7)

Cytochemistry: -
Cytochemistry encompasses the techniques used to identify diagnostically useful enzymes or other substances in the cytoplasm of haemopoietic cells

A: Myeloperoxidase: Myeloperoxidase is a lysosomal enzyme present in granulocytic and monocytic cells. The basis of the stain is breakdown of hydrogen peroxide by the enzyme MPO.

B: Sudan Black B (SBB) stain: which is a lipophilic dye that binds irreversibly to an undefined granule component in granulocytes, eosinophils and some monocytes. The intensity of a positive reaction with SBB in general parallels MPO activity; however, SBB is preferable as it is slightly more sensitive in the detection of myeloblasts. (8)
C: Specific esterases: Specific Esterases are a group of enzymes capable of hydrolyzing halogenated naphthol esters. The most commonly used substrate is naphthol AS-D chloroacetate. Chloroacetate esterase (CAE) is most frequently used for the identification of neutrophilic series; it is negative for monocytes, megakaryocytes, erythroblasts and lymphocytes. (8)

D: Nonspecific esterases: are a group of enzymes capable of hydrolyzing various aliphatic and aromatic short chain esters. The substrates used to detect NSEs activity include α-naphthyl butyrate, α-naphthyl acetate, naphthol AS-D acetate and naphthol AS acetate. They are most frequently used because they do not stain for granulocytes. Beside monocytes and histiocytes, α-naphthyl acetate esterase is also positive in megakaryocytes and platelets. (9)

E: Periodic Acid–Schiff (PAS) Reaction: Periodic acid specifically oxidizes 1–2-glycol groups to produce stable aldehydes which give a red reaction product when exposed to Schiff’s reagent (leucobasic fuchsin). A positive PAS stain in erythroblasts is a common finding in erythroleukemia where it has a coarsely granular pattern in cells of early stage and a finely granular pattern in cells of later stage. (9)

Immunophenotyping: -
Leukaemic cells express characteristic nuclear, cytoplasmic and cell surface antigens, this is referred to as the immunophenotype of the cell. Characterization of the immunophenotype is referred to as immunophenotyping and is achieved by means of labeled antibodies that recognize specific epitopes of cellular antigens by:

A: immunocytochemistry methods.

B: multiparameter immunophenotypic flow cytometry.
Flow cytometry is the measurement of numerous cell properties as the cells move in single flow in a fluid column and interrupt a beam of laser light. The method allows the quantitative and qualitative analysis of several properties of cell populations from body fluids. In an individual patient, the role of flow cytometric (FC) immunophenotyping may be: confirming a diagnosis, identifying prognostic differences within a diagnostic category, staging a disease, and detecting an aberrant immunophenotype that can be used for monitoring minimal residual disease, i.e. expression of an antigen inappropriate to a lineage. (10)

Leukaemic myeloblasts express a variety of leucocyte differentiation antigens, which reflect commitment to the myeloid lineage as well as a level of maturation. In the EGIL classification, AMLs are defined immunologically by the expression of 2 or more of the following myeloid markers: MPO, CD13, CD33, CDw65, and CD117. (11) as shown in (Table 1).

Table 1: Expression of cell-surface and cytoplasmic markers for the diagnosis of acute myeloid leukemia. (7)

<table>
<thead>
<tr>
<th>Myeloid stage</th>
<th>CD markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor stage</td>
<td>CD34, CD38, CD117, CD133, HLA-DR</td>
</tr>
<tr>
<td>Granulocytic markers</td>
<td>CD13, CD15, CD16, CD33, CD65, cytoplasmic myeloperoxidase (cMPO).</td>
</tr>
<tr>
<td>Monocytic markers</td>
<td>Nonspecific esterase (NSE), CD11c, CD14, CD64, Lysozyme, CD4, CD11b, CD36, NG2 homologue.</td>
</tr>
<tr>
<td>Megakaryocytic markers</td>
<td>CD41 (glycoprotein IIb/IIIa), CD61 (glycoprotein IIIa), CD42 (glycoprotein 1b).</td>
</tr>
<tr>
<td>Erythroid marker</td>
<td>CD235a (glycophorin A)</td>
</tr>
</tbody>
</table>

Aberrant immunophenotyping expression: -
In the majority of cases of AML, leukemia cells have immunophenotypet that distinguish them from myeloid progenitor cells found in normal marrow. Comparison of antigen expression on AML cells with that in normal bone marrow reveals: (a) expression of non-myeloid antigens; (b) asynchronous expression of myeloid-associated antigens; (c) over expression of myeloid associated antigens; and (d) absence of expression of myeloid-associated antigens. (12)

CD9: -
A transmembrane-4 super family that are characterized as having four transmembrane domains with cytoplasmic N and C termini and the conserved cysteine motif, which act as “molecular facilitators” and linkers for
transmembrane proteins forming tetraspanin web. (13)

CD56: -
CD56 antigen, a 200–220-kDa cell surface glycoprotein, belongs to the immunoglobulin supergene family and has been identified as an isoform of the neural cell adhesion molecule (NCAM). This antigen mediates cell-to-cell interactions and is possibly involved in cell-mediated cytotoxicity. (14)

Aims of the study: -
1. To detect the frequency of aberrant CD56 and CD9 expression in de novo newly diagnosed cases of adult AML.
2. To evaluate the correlation of CD56 and CD9 expression with certain clinical and hematological parameters and their initial response to the induction therapy.

Patients, Materials and Methods: -
This prospective cross-sectional study was conducted on thirty adults newly diagnosed de novo AML patients from July 2015 to March 2016.

The patients were admitted to the Hematology Department of Baghdad Teaching Hospital of the Medical City and Al-ImameinKadhimein medical city. Diagnosis was based on morphology and cytochemistry of the PB and/or BM samples by an expert hematopathologist in the Teaching Laboratories of the Medical City in Baghdad and Al-ImameinKadhimein medical city. FC was done by four-color (PartecCyflow®, Germany).

Flow cytometric Immunophenotyping: -
After AML cases have been documented in the Teaching Laboratories, the samples were transferred in cool box (6 hours being the maximum time since obtaining the sample) to be investigated for the aberrant expression of surface marker antigens CD56 and CD2 by using four-color (PartecCyflow® Cube 6, Germany).

Gating of the cells of interest was done depending on FSC/SSC gate. Device software based on Windows™ FC software (CyView™), and the optics of the instrument employing 6 optical parameters: FSC and SSC work in combination with 4 fluorescence channels (FL1-FL4). (15)

Assay Procedure: -
Includes 3 steps: -
1. Antibody labeling: 100μl of whole blood was mixed with 10μl conjugated antibodies in a test tube, and after thoroughly mixing the mixture was incubated for 15 minutes in the dark at room temperature.
2. Leucocyte fixation: 100μl of reagent A was mixed thoroughly with the mixture obtained in step 1 and was incubated for 10 minutes in the dark at room temperature.
3. Erythrocyte lysis: 2.5 ml of reagent B was added to mixture in step 2 and was shaken gently and incubated for 20 minutes in the dark.

Determination of the aberrant marker. Identification of blast cells was performed using FSC versus SSC parameters. Basically, antigen expression is considered to be positive when the percentage of positive blast cells is equal or greater than 20%. Similarly, aberrant phenotypes are defined when at least 20% of the blast cells expressed that particular phenotype. (16)(Figure 1, 2, 3).

Statistical Analysis: -
Statistical Package for Social Science (SPSS) version 17 was used to present, describe and analyze data included in the present study. Numeric variables were presented as mean and standard deviation. Nominal variables were expressed as frequency (number) and percentage out of total. Pearson's chi-square and Fisher exact tests were used to evaluate nominal variable frequency difference between groups. Independent sample student t-test was used to compare the mean of numeric variables between groups. The level of (≤ 0.05) was considered significant for interpretation of P values.
Figure 1: Aberrant expression of CD56 by FC
Figure 2: Aberrant co-expression of CD56 and CD9 by FC
Results:
Age Groups:
The mean age of AML patients included in this study was 41.33± 16.6 SD, with a median of 38 years (range of 16-75 years). Half of the cases (50%) being in the age group 21-40 years (Figure 4).

Figure 3:- Aberrant expression of CD9 by FC
Gender:
Acute myeloid leukaemia were observed more in males (17 males “56.7 %”) than in females (13 females “43.3 %”) with an M: F ratio of 1.3:1.

Distribution of AML cases according to the FAB subtypes:
According to FAB subtypes, of the 30 cases studied, 4 were M1, 9 were M2, 7 were M3, 4 were M4, 4 were M5, one was M6 and one was M7. (Figure 5)
Clinical Features:-
Figure 6: shows the common signs and symptoms of AML patients

Figure 6: Patient’s common signs and symptoms.

Aberrant CD56 and CD9 Expression:-
CD56 was expressed in 7/30 patients (23.3%), 3 of those were with moderate intensity, 2 were weak and 2 were strong. For CD9 expression, 10/30 patients (33.3%) had positive expressions with 6 of those were with weak intensity, 2 were moderate and 2 were strong. And 2/30 patients (6.7%) had CD56 and CD9 co expression (Figure 6).

Figure 7: Percentage of aberrant expression of CD56 and CD9 in AML patients.

Correlation between the aberrant CD56 and CD9 expressions with hematological parameters:-
Table 2 shows that the total WBC count, PB and BM blast cells percentage of AML patients with aberrant CD56 expression were significantly higher than those without aberrant CD56 expression (p value < 0.05), while there was nonsignificant correlation between Hb and platelet count with the aberrant CD56 expression.
Table 2:- Correlation between CD56 expression with hematological parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD56 positive (n=7)</th>
<th>CD56 negative (n=23)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC (×10⁹/L)</td>
<td>88.9 18.8</td>
<td>16.5 17.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>7.3 1.8</td>
<td>8.8 2.8</td>
<td>0.199</td>
</tr>
<tr>
<td>Platelet count (×10⁹/L)</td>
<td>82.3 43.1</td>
<td>53.7 35.7</td>
<td>0.088</td>
</tr>
<tr>
<td>PB blast cells %</td>
<td>68.2 54.6</td>
<td>30.6 10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BM blast cells %</td>
<td>74.6 15.5</td>
<td>45.4 21</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 3. showed that there was no significant correlation between the aberrant CD9 expression and any of the hematological parameters.

Table 3:- Correlation between CD9 expression with hematological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD9 positive (n=10)</th>
<th>CD9 negative (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC (×10⁹/L)</td>
<td>25.7 34.3</td>
<td>37.2 36.8</td>
<td>0.42</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>8.8 2.7</td>
<td>8.3 2.6</td>
<td>0.579</td>
</tr>
<tr>
<td>Platelet count (×10⁹/L)</td>
<td>64.4 33.2</td>
<td>58.4 42</td>
<td>0.699</td>
</tr>
<tr>
<td>PB blast cells %</td>
<td>36.1 19.7</td>
<td>41 18.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>BM blast cells %</td>
<td>46.4 24.2</td>
<td>55.2 22.9</td>
<td>0.336</td>
</tr>
</tbody>
</table>

The Distribution of aberrant CD56 and CD9 expression in relation to AML FAB Classification:-

CD56 was expressed in 57.1% of the cases of AML FAB subtypes with monocytic differentiation (M5 cases (2/4, 50%) and M4 (2/4, 50%)); it was also expressed in other FAB subtypes 1/4 for M1, 2/9 for M2. CD56 is not expressed on M3, M6 and M7 subtypes.

CD9 was expressed more in M2 subtype (4/9, 44.4%). It is also expressed in all other AML subtypes except M7 subtypes.

Correlation between the aberrant CD56 and CD9 expression with the extramedullary manifestations:-

The extramedullary manifestations were present in 57.2% of CD56 positive patients with statistically significant correlation (p-value = 0.016).

There is no significant correlation between CD9 aberrant expression and the extramedullary manifestations (p-value = 1).

Correlation between the aberrant CD56 and CD9 expression with CR achievement:-

Complete remission was achieved in 17/30 patients (56.7%) with standard chemotherapy. For patients with aberrant CD56 expression 6 out of 7 did not respond to induction treatment. It appeared that there was a significant correlation between this expression with the non-responsiveness to the induction therapy with p-value = 0.025 (Table 4).

Table 4:- Correlation between aberrant CD56 expression with CR achievement

<table>
<thead>
<tr>
<th>CR achievement</th>
<th>CD56 Positive</th>
<th>CD56 Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
<td>14.3</td>
<td>16</td>
</tr>
<tr>
<td>No</td>
<td>6</td>
<td>85.7</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>100</td>
<td>23</td>
</tr>
<tr>
<td>P-value =0.025</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For patients with aberrant CD9 expression 9 out of 10 did not respond to induction treatment, and it appeared that there was significant correlation between this expression with the non-responsiveness to induction therapy with p-value <0.001 (Table 5).
Table 5: Correlation between aberrant CD9 expression with CR achievement

<table>
<thead>
<tr>
<th>CR achievement</th>
<th>CD9</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>%</td>
<td>Negative</td>
<td>%</td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
<td>10</td>
<td>16</td>
<td>80</td>
</tr>
<tr>
<td>No</td>
<td>9</td>
<td>90</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>100</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
<td></td>
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</table>

Discussion:
This study was conducted on 30 newly diagnosed de Novo acute myeloid leukaemia patients. The mean age of all patients was 41.33 ± 16.6 SD, median of 38 years and ranged between 16-75 years. Those results were comparable to Iraqi studies (17-18).

Pallor and fever are the two most frequent signs, while lymphadenopathy and gingival enlargement are the least frequent. Easy fatigability was the most frequent symptom. Those results were comparable to that published by Alwan AF et al. (19) and Hu R et al. (20) of the 30 cases studied, M2 was the most frequent AML subtype (30%) followed by M3 (23.3%). This result was comparable to studies done by Pouls RK et al. (21) in Erbil, Alwan AF et al. (19).

The current study revealed that CD56 was expressed in 7 cases out of 30 (23.3%) newly diagnosed AML cases, this result was in agreement with Raspadori D et al. (22) E et al. and Dina J et al. (23)

For CD9, it was expressed in 10 cases out of 30 (33.3%) of AML cases. This result is comparable to that obtained by El-Sissy AH et al. (24) who reported CD9 expression in 29.4% of AML cases.

Regarding the haematological parameters; the total WBC count of AML patients with CD56 expression was significantly higher than those without this expression, those findings were comparable with the results obtained by Olteanu H et al. (25). Also the correlations were found between peripheral blood (PB) and bone marrow (BM) blast cells percent of AML patients were significantly higher in patients with aberrant CD56 expression. The cause of the higher total WBC count, PB and BM blast cells percent in AML patients with CD56 expression may be explained by that CD56 expression is associated with an abnormal over expression of the full length p48 RUNX1 isoform in AML cells which block haematopoietic differentiation and enhances self-renewal of haematopoietic stem cells and thus we may predict that the CD56 is a poor prognostic marker.

For the distribution of aberrant expression among FAB subtypes, CD56 expression was expressed in half of the cases of AML FAB subtypes with monocytic differentiation M5 cases (2/4 ; 50%) and M4 (2/4 ; 50%) and those results in agreement with Graf M et al. (26). The increased aberrant CD56 expression in M4 and M5 which are considered as unfavorable AML FAB subtypes (14) confirms that CD56 is associated with a poor prognosis.

About the CD9 expression, it was detected more on M2 subtype (4/9;44.4%). There was a significant correlation between CD56 expression and the extramedullary manifestations and this confirms also that CD56 is a poor prognostic marker as AML patients with increased extramedullary manifestations at presentation generally have a poor outcome. This result was in agreement with Chang H et al. (27)

On the other hand, CD9 expression was no significantly correlated with the extramedullary manifestations.

Regarding the initial response to the induction therapy, CD56 was highly expressed with the non-responsiveness to the induction therapy. This correlation may be explained by observation of more frequent P-glycoprotein (PGP) expression in CD56 positive patients. 106). Those results were consistent with that of Raspadori D et al. (22). Regarding CD9 expression in this study was highly expressed with the non-responsiveness to the induction therapy.
Conclusion:
1. CD56 and CD9 expressions in AML patients were detected in 23.3% and 33.3% respectively.
2. CD56 was expressed more in monocytic AML subtypes.
3. Aberrant CD56 expression was associated with a higher total WBC count, higher PB and BM blast cells, increased extramedullary manifestations at presentation, and a lower response rate to the induction therapy; thus CD56 can be considered as an unfavourable marker.
4. Aberrant CD9 expression was associated with lower response rate to the induction therapy; thus CD9 can be considered as an unfavorable marker.

Recommendations:
1. Studying the correlation of the aberrant expression of CD56 with multidrug related P-glycoprotein (PGP).
2. Studying further aberrant antigens expression in AML as CD10, CD22, and CD4.
3. Further studies for CD9 expression in AML patients.
4. The study should be performed on larger sample size and for longer period of time.

References: