Interleukin-3 Receptor Alpha Chain as a Unique Marker for Leukemic Stem Cells in Acute Myeloid Leukemia

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Abstract: Recent studies suggest that the population of malignant cells found in human acute myeloid leukemia (AML) arises from a rare population of leukemic stem cells (LSCs). In the present study, we investigated the presence of interleukin-3 receptor alpha chain in the bone marrows of 30 newly diagnosed AML cases versus 20 normal bone marrow donors as a control group; both on fresh bone marrow samples and on post culture ones. Flow cytometric study showed that the interleukin-3 receptor alpha chain was strongly coexpressed with CD34 in 91.7% of primary AML specimens and in 96% of post culture ones. Conversely, normal bone marrow derived stem cells showed virtually no detectable expression of the interleukin-3 receptor alpha chain antigen. Collectively, these data indicate that the interleukin-3 receptor alpha chain represents a unique marker for primitive leukemic stem cells. Given the strong expression of this receptor on leukemic stem cells, we propose that targeting of the interleukin-3 receptor alpha chain may be a promising strategy for detection of minimal residual disease, as well as for the preferential ablation of AML cells.

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1. Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous clonal disorder characterized by the accumulation of acquired genetic alterations in the hematopoietic progenitor cells. These alterations disturb normal mechanisms of cell growth, proliferation and differentiation resulting in the accumulation of Leukemic cells in the bone marrow, ultimately replacing most of the normal hematopoietic cells and their functions, resulting in signs and symptoms of the disease [(1)].

Acute leukemias account for approximately 2% of all cancers in the United States, but have a disproportionately large effect on cancer survival [(2)]. In Egypt, the incidence of acute leukemia is higher; representing 7% of newly diagnosed cancer cases, with AML accounting for 41.5% of all leukemias and 4.6% of all new cancer cases [(3)].

Cancer stem cells (CSCs) are a small population of tumor cells capable of self-renewal, giving rise to all the heterogeneous components of a tumor. These cells generally represent fewer than 5% of all cells in a tumor and are believed to be tumorigenic (tumor-forming), in contrast to the bulk of cancer cells, which are thought to be non-tumorigenic. CSCs have stem cell properties such as self-renewal and the ability to differentiate into multiple cell types [(4)]. These rare cells with a self-renewal potential and the capacity to form a tumor and maintain its growth were isolated in hematological cancers such as leukemia, multiple myeloma and a few solid tumors such as breast cancer and brain tumors. Much of the knowledge of CSC biology has come from experiments in normal and malignant hematopoiesis that led to the identification of the hematopoietic stem cell (HSC) and its malignant counterpart, the leukemia stem cell (LSC).

Interestingly, the $CD34^{+}CD38^{-}$ cell surface phenotype of LSCs is shared by immature hematopoietic precursors including HSCs, which raises the possibility that LSCs arise from HSCs. This theory provides an attractive model for leukemogenesis since the long lifespan of the HSC allows for multiple genetic hits to occur. Additionally, based on their physiologic capacity for self-renewal, HSCs would require fewer genetic hits to become LSCs than other hematopoietic cells, which must aberrantly acquire self-renewal capacity. AML can be thus viewed as newly formed, abnormal hemopoeitic tissue initiated by few leukemic stem cells (LSCs) that undergo an aberrant and poorly regulated process analogous to that of normal hemopoietic cells [(5)].

Identifying the LSCs for each type of leukemia is a current challenge and a critical step in understanding their respective biology [(6)] and in providing a powerful diagnostic, prognostic, and therapeutic tool [(5)].

Interleukin-3 receptor alpha chain (CD123) represents a unique marker for primitive leukemic

stem cells. Interleukin-3 is a growth factor whose main biologic activity is exerted at the level of the progenitor compartment, where this cytokine stimulates the survival and proliferation of multipotent cells. Particularly, IL-3 stimulates the development of multilineage colonies from normal bone marrow, but displays also effects at the level of the compartment of hemopoietic precursors, restricted to the granulocytic and monocytic lineages [7]. IL-3 promotes development of hematopoietic cells through activation of the IL-3 receptor (IL-3R) complex consisting of alpha and beta subunits. The β subunit plays a major role in signal transduction; being responsible for transmitting various intracellular signals such as activation of the Ras pathway, which is involved in the suppression of apoptosis [8].

Clinically, high CD123 expression in AML is associated with higher blast counts at diagnosis and a lower complete remission rate that results in reduced survival [9]. The increased expression of CD123 on LSCs compared with HSCs presents an opportunity for selectively targeting AML-LSCs with a therapeutic antibody. Besides the possibility that IL-3 is required for LSC functions, an antibody to CD123 could stimulate host immune-mediated mechanisms for cell killing [10]. Thus IL-3R may be an appropriate target for cytotoxic drugs designed to selectively kill AML cells while sparing their normal hematopoietic-cell counterparts [11].

2. Subjects and Methods

Subjects: This study was carried out in the Clinical Pathology Department of the National Cancer Institute (NCI), Cairo University, where cases were randomly selected from the outpatient clinic of the Medical Oncology Department. Thirty cases of adult de novo acute non lymphatic leukemia (ANLL), fifteen males (50%) and fifteen females (50%), with male to female ratio 1:1 were incorporated in the study. Their ages ranged from 18 to 66 years with a mean of 48 years. In addition, twenty normal bone marrow age and sex matched donors; as a control group; were included.

Methods:

I- Cases were subjected to thorough history taking, full clinical examination, particularly for hepatomegaly, splenomegaly and lymphadenopathy. Complete blood picture and bone marrow aspiration as well as cytochemical stains such as Myeloperoxidase (MPO) or Sudan Black Stain (SBB), Esterases, Acid Phosphatase and PAS when indicated. Immunophenotyping was performed by Flow cytometer: to confirm the diagnosis of AML with a wide panel of myeloid markers (MPO, CD13, CD33, CD117, CD64 CD14 and CD15), lymphoid markers (CD10, CD19, CD22, CD79a, CD20, Cyto IgM, Kappa and Lambda for B lymphoid series, and CD3, CD2, CD4, CD8, CD7 and CD5 for T lymphoid series) and the stem cell marker CD34 as well as CD56 and HLADR on routine basis. Cytogenetic examination was also done.

Response to induction therapy was assessed between days 15 and 28 after induction therapy, and follow up of cases was done for a period of at least 20 months to calculate their disease free survival and overall survival. Complete response was defined in accordance with standard criteria by **Cheson *et al.*** [12].

Fresh bone marrow samples were analysed at the time of diagnosis, as well as post culture samples for CD34, CD38 and CD123 as well as proper isotype control. Viable, antibody-labeled cells were identified according to their forward- and side-scattering, electronically gated and analyzed on a Flow cytometer DAKO Cytomation. The Monoclonal Ab used for the immunophenotypic detection of CD123 was RPE conjugated MoAb against the surface IL-3 receptor α chain (mouse IgG CDw123), that for the detection of CD34 was FITCI conjugated MoAb against the surface CD 34 (mouse IgG) and that for CD38 was CY5 conjugated MoAb against the surface CD 38 (mouse IgG). The antibodies were purchased from RD System Products. The technique used for the detection of the three surface markers was the direct staining technique.

Bone Marrow Sample Culture

Fresh bone marrow samples were cultured for 3 days in 37°C/CO_2 incubator. The BM samples were added to a tissue culture graduated flasks containing 10 ml of the culture media, which was prepared via dividing 100 ml of fetal calf serum and 7ml tetracycline, ampicillin and penicillin as antibiotics and 15 ml L glutamine to 4 flasks each containing 100 ml of RPMI medium (provided by GIBCO, lot number 21875) and 5ml of Alpha Minimum Essential Medium α MEM (provided by GIBCO, lot number 22561). After the 3 days, the cultured samples were analyzed in the same manner like the fresh samples.

Statistical Methods:

Data was analyzed using SPSS win statistical package version 17 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non parametric t-test). Comparison of repeated measures

was done using Friedman test followed by Wilcoxon signed-ranks test. Spearman-rho method was used to test correlation between numerical variables. The Receiver Operating Characteristic (ROC) curve was used for prediction of cut off values. A p-value < 0.05 was considered significant.

3. Results:

This study included thirty cases of newly diagnosed adult acute non lymphatic leukemia (ANLL) presenting to the Medical Oncology Department of the National Cancer Institute (NCI), Cairo University. The median age of cases in our study was 48 years with a mean of 44.6 ± 15 years (range 18 - 66 years). Sex distribution was 15 males (50%) and 15 females (50%). Hepatomegaly was encountered in 96.7% of cases, splenomegaly in 86.7% of cases and lymphadenopathy in 100% of cases. In addition, twenty normal bone marrow age and sex matched donors; as a control group; were included.

The presenting total leucocytic count in patients ranged from 1.4 to 66.8 with a mean of 40.3 ± 18.3 and a median of 47.7 $x10^9$ /L. The control group showed a mean of 7.5 ± 1.3 $x10^9$ /L. The difference between patients and controls was found to be statistically significant (P = 0.001). The platelet count in patients ranged from 8.0 to 87.00 with a mean of 43.1 ± 22.88, and a median of 43.5 $X10^9$ /L, while the control group showed a mean of 275.1 ± 73.27$x10^9$ /L. The difference between patients and controls was found to be statistically significant (P=0.001). Hemoglobin in patients ranged from 5.8 to 9.1, with a mean of 6.5 ± 1.8, and a median of 6.8 gm/dl, while the control group showed a mean of 12.8 ± 0.7 gm/dl. The difference between patients and controls was found to be statistically highly significant (P < 0.001). Blasts in peripheral blood were detected in the 24 out of 30 patients (80 %). The mean percentage of blasts in peripheral blood was 18.1% ±18.1; the median was 16% and the range was 0-62 %. On bone marrow examination, twenty three patients out of 30 (76.7%) had ≥ 50% leukemic cells encroaching on their normal hematopoietic cells while seven ones (23.3%) had <50% leukemic cells. The mean percentage of blasts in marrow was 66.6 %± 27.4, the median was 74% and the range was 23-99%.

The myeloid lineage markers including CD13, CD33 and MPO were detected on thirty patients (100%), myeloid with monocytic markers including CD14, CD64, CD4 were detected on six out of 30 (20%) of patients and myeloid markers with aberrant expression of lymphoid markers were detected on one out of 30 patients (3.3%). Conventional cytogenetic study was done to the 30 patients. Fourteen cases out of 30 (46.6%) were of normal karyotype. One out of 30 cases (3.3%) was positive to t (15:17), four cases out of 30 (13.3%) were positive to inv (16) and nine out of 30 cases (30%) were positive for t (8:21). While two cases out of 30 (6.7%) showed different cytogenetic abnormalities which were -20, +21, del11q23 and -14. Cytochemistry was done for all cases. According to FAB classification the cases included 6 cases (20%) M1; 16 cases (53.3%) M2; 1 case (3.3%) M3; 4 cases (13.3%) M4 (1 M4 and 3 M4 with abnormal esoinophils ; 2 cases (6.6%) M5 (1 M5a and 1 M5b) and one case M7 (3.3%).

Patients were followed up for a period of at least 20 months. Seven out of thirty (23.3%), died from leukemia and chemotherapy. Twenty three (76.7%) were alive by the end of the study period. Six out of thirty (20%) had complete remission (CR), while seventeen (56.7%) had CR followed by relapse (R). Tables (1 and 2) show the range, the median, the mean and the SD of expression of CD123, CD34, CD38 and CD34/CD38 coexpression in AML patients versus the control group (pre and postculture respectively). Tables (3 and 4) show the range, the median, the mean and the SD of mean fluorescent intensity of CD123, CD34, CD38 and CD34/CD38 coexpression in AML patients versus the control group (pre and postculture respectively). Figure (1) shows AML cases with positive coexpression of CD34 and CD123.

The cut off values of CD 34 & CD 123 were determined by the ROC curve to determine the level which gives the best discrimination between the positive and negative taking the control as a negative value. The determined cut off values was ≥ 1.45% for CD34, ≥ 0.768% for CD123 and ≤ 1.4850 for MFI 123. For the freshly examined samples, twenty four out of thirty (80%) cases had CD34% expression above the cut off value ≥1.45%. Twenty two out of thirty (73.3%) cases had CD123% expression above the cut off value ≥0.768%. Twenty two out of twenty four (91.7%) cases had coexpression of CD123% and CD34% above the cut off values ≥0.768% and ≥1.45% respectively. For the post culture samples, twenty five out of twenty seven (92.6%) post culture cases had CD34% expression above the cut off value ≥1.45%. Twenty four out of twenty seven (88.9%) post culture cases had CD123% expression above the cut off value ≥0.768%. Twenty four out of twenty five (96%) cases had coexpression of CD123% and CD34% above the cut off values ≥0.768% and ≥1.45% respectively. Twenty one out of twenty seven (77.8%) post culture cases had MFI 123 expression below the cut off value ≤1.4850.

One out of twenty four (4.17%) cases having CD34 expression above the cut off value ≥1.45% was in CR. However, five out of six (83.33%) cases having CD34 expression below the cut off value

≥1.45% were in CR. The difference was found to be statistically highly significant (P <0.001). None out of twenty two cases (0%), having CD123 expression above the cut off value ≥0.768%, were in CR. However, six out of eight (75%) cases, having CD123 expression below the cut off value ≥0.768%, were in CR. The difference was found to be statistically highly significant (P <0.001). One out of twenty one (4.77%) cases, having MFI 123 expression below the cut off value ≤1.4850, was in CR versus five out of six (83.33%) cases, having MFI expression above the cut off value ≤1.4850, that were in CR. The difference was found to be statistically highly significant (P <0.001).

Table (1): Expression of CD123, CD38, CD34 & CD34/CD38 coexpression in AML cases and controls in fresh samples

		CD123%	CD38%	$CD34^+$%/$CD38^-$%	$CD38^+$%/$CD34^+$% coexpression
AML (30)	Mean ± SD Median Range	10.2 ± 16.3 4.3 0.1- 60.2	24.2±26.7 10.9 2.0- 88.9	8.7±10.1 5.5 0.1- 38.3	23.0±27.0 7 0.0- 87.0
Controls (20)	Mean ± SD Median Range	0.6 ± 0.5 0.5 0.1- 1.6	3.5 ± 1.7 3.3 0.5- 6.0	1.4± 1.3 1.1 0.1- 3.9	1.0± 1.0 1.0 0.0- 5.0
P value		<0.001	<0.001	0.001	0.001

Table (2): Expression of CD123, CD38, CD34 & CD34/CD38 coexpression in AML cases and controls in post culture samples

		CD123%	CD38%	$CD34^+$%/$CD38^-$%	$CD38^+$%/$CD34^+$% coexpression
AML (27)	Mean ± SD Median Range	15.3 ± 14.1 13.6 0.1- 56.5	17.7±13.5 12.8 2.3- 48.0	14.5±13.4 12.0 0.7- 48.0	34.1±29.7 21.6 0.2- 92.3
Controls (20)	Mean ± SD Median Range	1.1 ± 0.9 0.8 0.1- 2.9	2.1±2.7 0.9 0.1- 7.5	1.0±1.0. 0.7 0.0- 3.4	0.4±0.5 0.2 0.0- 0.5
P value		<0.001	<0.001	0.012	<0.001

Table (3): Mean fluorescent intensity of CD123, CD38, CD34 & CD34/CD38 Coexpression in AML cases and controls in fresh samples

		CD123 flow intensity	CD38 flow intensity	$CD34^+$/$CD38^-$ flow intensity	$CD38^+$/$CD34^+$ coexpression flow intensity
AML (30)	Mean ± SD Median Range	1.52 ± 1.26 1.15 0.14 - 5.13	0.692±0.566 3.58 0.167 - 3.18	1.71 ± 1.55 1.15 0.12 - 6.56	3.2 ± 3.5 3.0 0.4 - 12.8
Controls (20)	Mean ± SD Median Range	2.06 ± 1.15 1.78 0.75 – 4.94	0.42 ± 0.16 1.60 0.90 - 5.07	1.91 ± 0.68 1.79 0.49-2.9	2.9 ± 1.2 3.76 0.8 - 4.4
P value		0.072	0.095	0.794	0.259

Table (4): Mean fluorescent intensity of CD123, CD38, CD34 & CD34/CD38 Coexpression in AML cases and controls in post culture samples

		CD123 flow intensity	CD38 flow intensity	$CD34^+$/$CD38^-$ flow intensity	$CD38^+$/$CD34^+$ coexpression flow intensity
AML (27)	Mean ± SD Median Range	1.5 ± 1.35 0.90 0.40 – 4.32	0.81±0.72 2.71 0.13 -3.95	1.65 ± 1.1 1.94 0.13-5.44	3.18 ± 2.5 3.98 0.15 -11.6
Controls (20)	Mean ± SD Median Range	1.8 ± 1.4 1.72 0.38-4.78	0.64 ± 0.20 0.605 0.34 – 1.0	1.37 ± 0.67 1.21 0.36- 2.58	3.72 ± 2.54 2.76 1.53 -9.28
P value		0.013	0.827	0.555	0.555

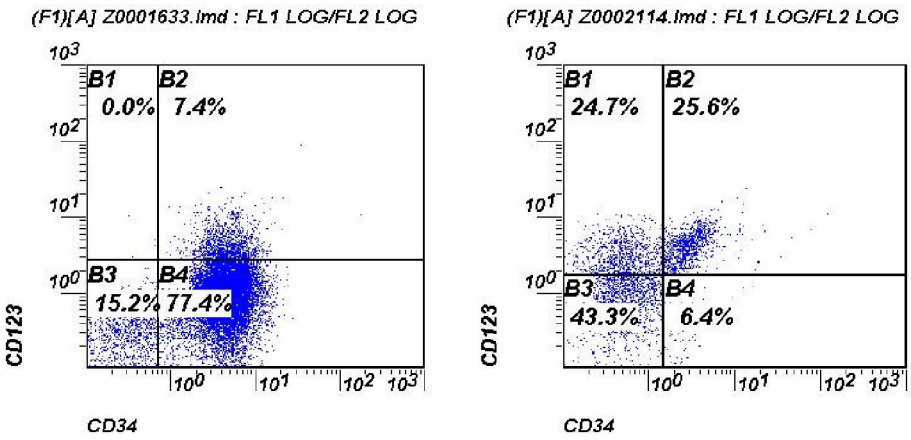

Fig (1): AML cases with positive expression of CD34 and coexpression of CD123 (fresh samples left and post culture right).

4. Discussion

The outcome of adults with AML varies based on a variety of well-defined factors including age of the patient, intensity of post remission therapy (in younger adults), and biologic characteristics of the disease. The karyotype at diagnosis, the presence of transmembrane transporter proteins; which confer multidrug resistance; and mutations or overexpression of specific genes are among the most common factors affecting the disease outcome [13]. In this work, cases were classified according to the FAB classification criteria [14], based on morphological and cytochemical characteristics. The most commonly encountered FAB subtype was M2 (53.3%), followed by M1 (20%), M4 (13.3%), M5 (6.6%) and finally both M3 (3.3%) and M7 (3.3%).

Several recent studies have suggested the presence and importance of stem cells in both the genesis and perpetuation of AML. Phenotypically, cells described as $CD34^+/CD38^-$ or $CD34^+/HLA\text{-}DR^-$ appear to play a central role in the development of leukemic populations. Furthermore, there is evidence suggesting that such cells may be relatively resistant to chemotherapeutic drugs, and consequently contribute to the phenomenon of relapse. The study of cytokine receptor expression by flow cytometry could allow differentiation between normal and tumor cells. Tumor cells show high expression of IL-3Rα chain (CD123) in hematologic malignancies compared to normal precursors [15]. In the study done by Jordan, identification of CD 123 among $CD34^+/CD38^-$ AML stem cells facilitates their discrimination from normal hematopoietic stem cells [16]. The conserved expression of this molecule on AML specimens examined suggests that it is related to a central aspect of AML biology.

Several lines of evidence suggest that the autonomous proliferation of leukemic blasts may be related to autocrine mechanisms of HGF production or to constitutive activation of the signal transduction machinery triggered by HGFR [7]. In this study, IL-3R α was over expressed in AML cases versus controls. Correlation of IL-3Rα over expression with the clinical, hematological and immunophenotypic parameters in our AML cases showed that it was not related to particular sex or age. There were no significant differences in IL-3Rα levels with respect to the initial Hb level, platelet count at presentation and bone marrow cellularity. To the best of our knowledge, no previous studies addressed the correlation of the previous parameters with IL-3Rα expression in AML cases. In the present work, there was no significant difference in IL-3Rα expression levels with respect to the FAB subtypes. This finding was also reported by **Testa *et al.*** [17]. However the increase in IL-3Rα expression was significantly associated with TLC $\geq 50x10^9/L$ (p-value =0.001). Similar results were documented by **Testa *et al.*,** in which IL-3Rα was over expressed at both gene and protein level in about half of the cases of AML patients, and IL-3Rα over expression correlated with high leukocytic count [17]. In this work out of 30 patients, 22 belonged to the group of patients with high IL-3Rα expression while 8 patients belonged to the group of patients with low IL-3Rα expression; the median WBC count at presentation was significantly higher in patients with elevated IL-3Rα levels than in those with low IL-3Rα levels, suggesting a role for IL-3Rα in leucocyte proliferation/survival.

The present work did not reveal any significant differences in IL-3Rα levels with respect to the peripheral blood blasts percentage. Contrary to this finding, **Testa *et al.*,** and **Riccioni *et al.*,** reported that the level of IL-3Rα chain expression is directly correlated with the number of leukemic blasts present in the peripheral blood at diagnosis [17, 18]. In the

present study, IL-3Rα over expression was significantly associated with bone marrow blasts percentage ≥ 50% (p-value =0.002). The same results had been documented by **Testa *et al.***, and **Riccioni *et al.***, in which IL-3Rα was over expressed in about half of the cases of AML patients and was correlated with high bone marrow blasts percentage detected at diagnosis [7, 18]. In their reports, **Testa *et al.,*** discussed two sets of independent observations suggesting that elevated IL-3Rα chain expression may have a role in the proliferation of leukemic cells [7]. The first was that a direct correlation was observed between the level of IL-R3α chain and the number of leukemic blasts observed at diagnosis. The second was that elevated IL-3Rα expression represented a negative prognostic factor and is associated with shorter survival and had explored a possible effect of elevated IL-3Rα expression on Stat5 activation.

In the present study, there was a significant difference in IL-3Rα levels with respect to the early treatment response (CR, No CR and death). None of the cases examined at this work were having CD123 expression above the cut off value ≥0.768%, were in CR. On the other hand 75% of cases, having CD123 expression below the cut off value ≥0.768%, were in CR. The difference was found to be statistically highly significant (P <0.001), similar to **Testa *et al.,*** and **Riccioni *et al.,*** who reported a significant difference in the induction response rate, with a significantly higher rate of complete remissions in the low IL-3Rα group as compared to the high IL-3Rα group [7, 17, 18]. They also reported that the relapse rate was significantly higher with higher IL-3Rα. This finding might suggest a prognostic value of IL-3Rα in AML patients. A significant correlation was found between CD34 expression and IL-3Rα expression levels among AML cases. IL-3Rα expression was significantly higher in CD34+ve compared to CD34-ve leukemic blasts, (p-value <0.001) similar to **Testa *et al.***[7]. 91.7% of preculture cases had coexpression of CD123% and CD34% and 96% of postculture cases had coexpression of CD123% and CD34%.

In conclusion, we have shown that the presence of CD123 on AML cells has several important ramifications. First, expression of this antigen formally demonstrates that LSCs are biologically distinct from their normal stem cell counterparts. Second, because CD123 is not readily found on normal hematopoietic stem cells, it provides a unique marker that can be used to identify malignant tissue. This feature may be useful for detection of minimal residual disease (MRD). Further, the CD123 epitope represents a target to which therapeutic strategies may be directed.

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