Impact of Ascorbic Acid Adjuvant Therapy on CD117 (C-Kit Gene) Expression and Apoptosis in Adult Acute Myeloid Leukemia: Egyptian Single Centre Analysis

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Abstract: Background: It has been established that growth of leukemic progenitor cells from patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) can be profoundly modulated by L Ascorbic Acid (AA). CD117 (c-kit gene) is a receptor tyrosine kinase and its expression is important in the pathogenesis of AML. In this study we evaluated the effect of AA adjuvant therapy on apoptosis and CD117 (c-kit gene) expression in AML patients. **Design and Methods**: 47 newly diagnosed AML had been divided into 2 groups according to the use of AA adjuvant treatment versus use of conventional 7+3 chemotherapy alone. **Results**: AA had a significant negative impact on CD117 expression (p value 0.03 and 0.02) for % and MFI respectively while apoptosis wasn't affected significantly (p value 0.37 and 0.83) for % and MFI respectively. **Conclusion:** AA adjuvant therapy can decrease expression of the adverse prognostic marker CD117 (c-kit gene) which may be another possible mechanism in inducing apoptosis.

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1. Introduction

Acute myeloid leukemia (AML) is a heterogenous group of malignant diseases arising as a result of progressive genetic damage occurring in hematopoitic progenitor cells**[1]**. Inspite of the vast advances in the therapeutic approach to AML, chemotherapy based on total cell killing is still the mainstay of treatment with fatal infections and bleeding as possible treatment related morbidities **[2]** . Complete remission (CR) is achieved in 60-70% of adults after initial treatment but without consolidation with hematopoitic stem cell transplantation (HSCT) there are few long-term survivors **[3].**

The primary cause of treatment failures in AML, whether failure of induction of remission or early relapse is frequently attributed to defective apoptotic pathways [1]. Perturbation of tyrosine kinases (TK) signaling has been implicated in the pathogenesis of neoplasia including leukemias, FLT□ mutations as a vivid example. c-kit another member of type III receptor TK has gained focus in the process of hemopoietic cell development and leukomogenesis . High c-kit expression has been identified in 33-45% of AML with inversion of chromosome 16 and 12.8-46.8% of AML M□ with t (8;21) **[4].**

The proto-oncongene c-kit encodes a transmembrane receptor (kit or CD117 or stem cell factor), CD 117 is the c-kit gene product **[5]**. Reliability of Flow cytometric in assessing c-kit expression is established by comparing it to gene expression arrays and it was found to be accurate and reliable **[6]**.

L-ascorbic acid (LAA) at 10nM-1mM induces apoptosis in neuroblastoma, melanoma and human myelogenous leukemia cell lines within 24 h by oxidative stress. A long series of studies have solidly demonstrated that the growth of leukemic progenitor cells from patients with acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS) can be profoundly modulated by LAA[7].Also ascorbic acid increases the apoptotic effect of arsenic trioxide on AML cells in vitro and can be safely combined in vivo in treatment of AML **[8]**.

In this study we evaluated the impact of CD 117 (c-kit gene) expression in AML patients at presentation and ascorbic acid adjuvant therapy on apoptosis of myeloid blasts ,we also assessed their effects on the outcome of those patients .

2. Design and Methods:

Patients:

This follow-up study was carried out over 48 month period on 47 newly diagnosed adult AML patients (22 males and 25 females with their ages ranged between 16-78 y).

Patients with secondary AML (MDS, MPD) or those not eligible for conventional chemotherapy according to ECOG performance were excluded.

The study was approved by local ethical committee and informed consent was obtained from all patients before enrollment.

Diagnostic criteria for acute myeloid leukemia patients:

Patients enrolled in this study had been diagnosed –after detailed history and physical examination – using standard morphological assessment (PB and B.M.examination) with cytochemical staining for myeloperoxidase (MPO) and flow cytometric immunophenotyping using CD34, CD33, CD13, CD14, CD15, HLA-DR, MPO, CD10, CD19, CD20,CD2, CD3, CD5 and CD7 using Epics XL Flow cytometer (coulter, Haileah, Florida, USA).

CD117(c-kit gene) at presentation and after induction therapy.

Some aspects of the immune system CD4/CD8 ratio and CD57% (NK cells) were evaluated at presentation and after induction therapy.

Cytogenetic study with standard karyotyping and FISH detection for t (8; 21), t (15;17) and inv16 were performed .

Ancillary tests: as biochemical analysis for liver and renal functions, LDH and uric A. levels, CSF examination, radiological assessment with pelvi-abdominal U/S and or CT scan,echocardiography were all underwent.

Quantification of apoptosis at single cell level:

Using Tunnel reaction at presentation and after induction therapy: In situ cell death detection kit,fluorescein-Roche molecular biochemicals, Germany).

Quantification of Annexin V positive /CD117 postive blasts using tri-color flow cytometry

Utilizing Argon laser flow cytometry based concepts of hydrodynamic focusing and monolayer flow, direct immunoflurescence immunostaining tri-color flow cytometric analysis was performed.

B.M was diluted with phosphate buffered saline (PBS) and a volume containing an equivelant of 106 cells was incubated with 10µ of each of anti-CD34/PC 5 (pyocyanin 5) labeled and anti-CD117 PE (phycoerytherin) labeled reagents (Becton Dickinson reagents ,USA) for 20 min at 4 C° in the dark.Then incubation with Annexin V/FITC (fluorescin isothiocyanate) labeled reagent was performed in presence of a cold calcium buffer for 10 min at 4 C° in the dark. Erythrocyte lysis was performed by adding 100µL of 0.82% ammonium chloride lysis reagent of PH 7.2±0.2 and incubation for another 5min at room temperature in the dark. Cells were washed with the cold calcium buffer 3 times and then suspended in 500µL PBS .10µL of propdium iodide reagent were added 5 min before flow cytometric analysis.

Preparation of isotype matched negative controls:

Isotype matched negative controls were used for each patient or control sample.

Flow Cytometric Analysis:

Quantification of annexin V and CD117 expression by CD34 postive blasts was performed on Epics XL flow cytometer (Coulter electronics, Hialeah, FL, USA) utilizing tri-color flow cytometry protocols constructed using SYSTEM II software.

Gating of lymphocytes was initially performed using forward scatter (FS) and side scatter (SS) to exclude cellular debris and to identify mononuclear cell population. Further gating of blasts according to their surface expression of CD34 was performed using flurescence /side scatter histograms. CD34 +ve blasts were evaluated according to their expression of annexin V and CD117 surface markers using dual color histograms where percent of cells expressing each marker as well as the intensity of expression was quantified.Positive analysis was set at 2% and each isotype matched control was run prior to its corresponding sample.

Quantification of annexin V and CD117 positive blasts:

The percent of annexin V and CD117 positive blasts, the intensity of each marker and the percent of blast cells co-expressing both markers were quantified.

Statistical analysis:

IBM SPSS statistics (V.19.0, IBM Corp., USA, 2010) was used for data analysis. Date was expressed as Median and Percentiles for quantitative non parametric measures.

The following tests were done:

1-Comparison between two independent groups for non-parametric data using Wilcoxon Rank Sum test.

2-Ranked Sperman correlation test to study the possible association between each two variables among each group for non-parameteric data.

The probability of error at0.05 was considered significant, while at 0.01 and 0.001 are highly significant.

3. Results:

47 adult patients with newly diagnosed AML were enrolled in this study.

25patients were female and 22 patients were males. Their ages ranged between 16-78 y with a mean of 43.9

The patients were classified into 3 risk groups according to the conventional cytogenetic and FISH analysis: favorable, intermediate and unfavorable.

Among the favorable risk group (16 patients): five patiens had t (8; 21), six patients showed

inversion 16, four patiens with t (15; 17) and 1 patient showed t (16; 16).

In the intermediate risk group (16 patients): all showed normal karyotype.

The unfavorable risk group (15 patients): five patients showed complex karyotype,one patient had inv(3) and one patient with del (5q).

Flow cytometric detection of CD117 (c-kit gene) at presentation was done for all patients. in parallel flow cytometric detection of apoptosis at presentation was done for total blasts and CD117 positive blasts . CD4, CD8, CD57 were assessed to figure out some immune aspects in those patiens.

NB: CD 117 detected as % and MFI. MFI (mean fluorescene index) is a quantitative measurement of c-kit on single cell level.

CD 117 % showed a highly significant correlation with age, OS and CD57 % (with p value 0, 0, and 0.001 respectively) while a non significant correlation with DFS, CD4 and CD 8 (p value of 0.4, 0.949 and 0.087 respectively). Table (1), figure 1(a,b,c).

CD117 MFI had a high significant correlation with age, OS and CD 57 %(p value of 0 for all).Table (1), figure 2 (a,b,c).

CD117 % and MFI showed a non significant correlation with apoptotic blast % and MFI (p value 0.8 and 0.51) respectively.

CD117 % and MFI were not correlated also with CD117 apoptotic blasts % and MFI (p value 0.69 and 0.6) respectively.

The patients were divided into 2 groups upon starting chemotherapy:

Group A (27 patients): received the conventional protocol "seven +three" doxorubicin 25mg/m2/d x3 and cytosine arabinoside 100mg/m2/12 h.x7

Group B (20 patients): received the same conventional protocol + Ascorbic Acid (A.A.) at a dose of 6-8gIV/d x28 d as an adjuvant treatment.

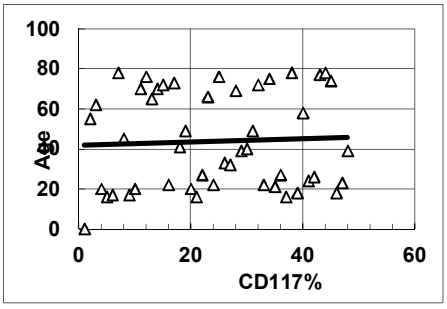
Comparison between both groups ,group B showed a significant increase in CD57 % and significant decrease in the CD117 % and MFI (p value 0.05, 0.03 and 0.02) respectively. apoptotic blasts % and MFI, C117 apoptotic blasts% and MFI were not significantly decreased in group B (p value 0.37 ,0.83 and0.139) respectively, Table (2).

Table 1: showing the correlation between CD117 % ,MFI and age,OS,CD4,CD8 and CD57

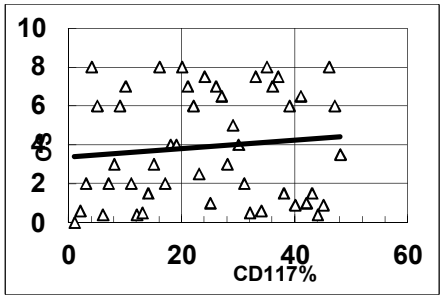
CD117%		Age	OS	CD4	CD8	CD57
	r	0.734	-0.788	0.01	-0.253	-0.482
	p	0	0	0.949	0.087	0.001
	Sig.	HS	HS	NS	NS	HS
CD117 MFI	r	0.657	-0.677	-0.025	-0.008	-0.716
	p	0	0	0.869	0.959	0
	sig	HS	HS	NS	NS	HS

(Table 2) :showing the effect of ascorbic acid(group B)on CD117% CD117MFI,CD57,apoptotic cells % , MFI and CD117 apoptotic cells%.

	Group A (without Ascorbic Acid)	Group B (with Ascorbic acid)	P	Significance
CD117 %	11.5±10	4.9±2.5	0.03	S
CD117 MFI	11±9	3±1.5	002	S
CD57%	10.7±7	17±5	0.05	S
Apoptotic cells %	34±19	25±15	0.37	NS
Apoptotic cells MFI	1.9±1.2	1.8±1	0.83	NS
CD117 apoptotic cells %	25±17	22±18	0.139	NS



(Figure 1(a): Regression analysis showing the correlation between CD 117% and age



(Figure (1b): Regression analysis showing the correlation between CD117 % and OS.

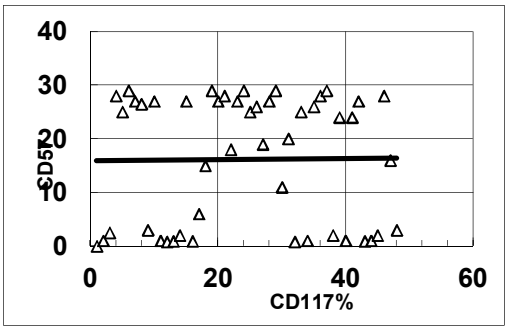


Figure 1(c): Regression analysis showing the correlation between CD117% and CD57

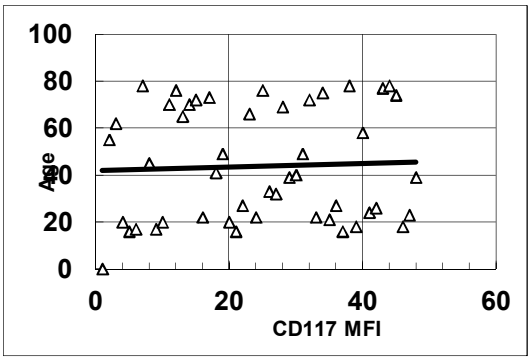


Figure 2(a): regression analysis showing the correlation between CD117 MFI and age .

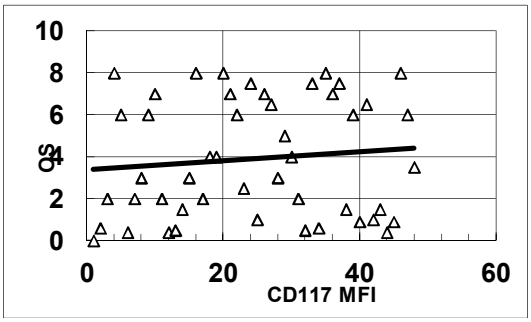


Figure 2(b): Regression analysis showing the correlation between CD117MFI and OS.

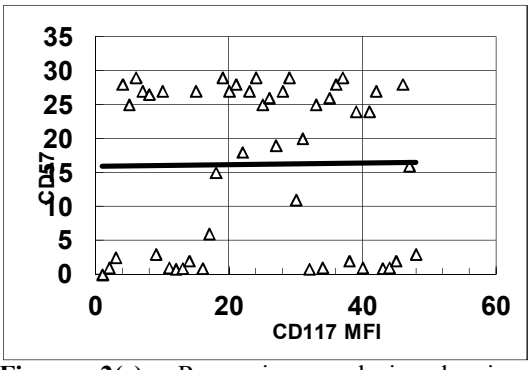


Figure 2(c): Regression analysis showing the correlation between CD117 MFI and CD57

4. Discussion:

The kit protoncogene encodes for a class III transmembrane receptor tyrosine kinase that is composed of an extracellular portion containing 5 immnuglobulin –like domains and intracellular portion consisting of juxtamembrane and 2 protein tyrosine kinase domains (PTK 1 and PTK2) split up by an interkinase domain **[9]**. Activating mutations in kit have been reported in acute myeloid leukemia (AML) and are confined to either extracellular (exon 8 mutations) or the PTK2 domain (D816 mutation)**[10].**

The clinical and prognostic significance of kit mutation in AML preliminary data suggest that kit exon-8 mutation are associated with an increased relapse rate and kit-D816 mutation are correlated to a higher white blood cells (WBCs) in AML pts[4] while kit exon-8 mutations were frequently described in CBFB-MYH 11-positive AML, there is an evidence that kit D816-mutation represent important cooperative mutation in AML 1-ETO positive AML. Kit mutation has been found relatively frequent in AML 1-ETO inspite of an overall low frequency in AML and associated with poor prognosis in those patients **[11]**.

We evaluated c-kit gene in our cohort of AML pts as flow cytometric detection of percent and MFI of CD117 (MFI is considered accurate quantitative measurement on single cell level).our observation showed that CD117 % and MFI were strongly correlated with OS though not correlated with DFS. We found also that CD117 % and MFI didn't affect apoptosis (whether apoptotic blasts or CD117 apoptotic blasts).

Kit mutations represents class I mutation which triggers excessive proliferation of aberrant cell clone while in t (8;21) kit +ve AML,AML1-ETO depicts the so-called class II mutations which leads to block in differentiation and a cooperation between both mutations may explain the poor outcome **[11]**.

The low frequency of t (8;21) in our cohort may explain the lack of correlation between CD117 (c-kit gene) and apoptosis .

Our results showed a significant increased expression of CD117 in older AML and those with frequent infectious episodes probably because CD117 was associated with decreased CD 57%.

L-Ascorbic Acid: there are a number of studies demonstrating that L-ascorbic (LAA) one of the major water soluble antioxidant present in cells and plasma can under certain conditions function as a pro-oxidant and increase DNA damage **[12]**.There is also increasing evidence that LAA is selectively toxic to some types of tumor cells functioning as a pro-oxidant rather than antioxidant **[13]**.

Apoptosis plays a role in the response of leukemic patients to chemotherapy and there is an association between therapy induced apoptosis and therapeutic efficiency **[14]**.

Several groups have shown that LAA augments arsenic trioxide As_2O_3 (ATO)-mediated apoptosis **[15]**, it can also overcome resistance to ATO by reducing intracellular GSH levels **[16]**. Other data have proved that LAA alone can modulate malignant cell growth in vitro and can effectively induce apoptosis of the AML cell lines HL-60NB4 and NB4-R1.

LAA resulted in decreased GSH/GSSG ratio accompanied by intracellular H_2O_2 playing a major role in induction of apoptosis. Also LAA induced apoptosis can occur via a decrease in Bcl2/Bax ratio, cytochrome C release from the mitochondria, activation of caspase-9 and caspase-3 and cleavage of PARP**[7]**.

However in our study apoptosis was not affected by LAA when used as an adjuvant treatment to the classic "7+3" chemotherapy of AML yet it had a significant effect on CD117 (c-kit) expression and on the immune status of the AML patients (↑CD57 % expression) who showed a significant less incidence of infectious episodes compared to those who didn't receive LAA.

The contradiction between our results and the previous data can be attributed to dose difference.

Treatment with LAA repressed the constitutive expression of Bcl2 whereas it strongly increased Bax expression in a dose dependent manner **[7]**. Our findings though point to an indirect effect of LAA on apoptosis through a good significant suppression of an adverse prognostic marker CD117 (c-kit) which augment its beneficial role in AML therapy**[17]**.

LAA has been shown to modulate the in vitro growth of leukemic colony –forming cells (L-CFC) from bone marrow cells of patients with AML or MDS. Physiologic concentrations enhance the growth of L-CFC while non physiologic doses (either very low or very high) suppress their growth. This proapoptotic effects have also been demonstrated in various types of malignancies in different species [18,19].

Conclusion:

Our data have shown an indirect effect of AA on apoptosis in AML patients through decreasing the expression of the adverse prognostic marker CD117 (c-kit) gene.further studies to explore its effects and optimum dose on the other bad prognostic markers as Flt3 and WT1 gene are warranted.

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