Original Article

Evaluation of Natural killer activated receptor NKG2D, Inhibitory receptor KIR2DL1 and soluble ligand MICA in Iraqi patients of Acute myeloid leukemia.

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Abstract

Background: AML is a leukemic disease that related to the rapidly myeloid proliferation progenitor cells are transformed and leading to stop proliferation and self-renewing. AML have many abilities to evading immune system one of them down expression of NK cells activating receptors NKG2D which is elaborate in in cooperation innate and adaptive immunities, and occupations as a "principal switch" in influential the stimulation of natural killer (NK) cells. NKG2D can binds to a various ligand molecule, which are only expressed at small concentration in usual cells but can be increase by a cellular tension response. And the realizing soluble NKG2D ligands MICA which is express by tumor cells and by binding with NKG2D lead to activated NK cells.

Patients and methods: This study consists of 30 new diagnosed patients with AML and 24 out of 30 patients follow up at 14 days after treatment, 20 healthy looking persons match age and sex as control group. NKG2D detection by Flowcytometry and soluble MICA detection by ELISA.

Results: The NK cells activation receptor down expression in patients of AML as well as increase releasing of soluble ligand MICA.
Conclusion: The NKG2D in patients with AML down expression which may due to increase soluble ligand MICA which lead to mask NKG2D receptors. There was relationship between down expression of NKG2D besides releasing soluble ligand MICA.

Keywords: AML, Natural killer cells, NKG2D, KIR2DL1, sMICA

INTRODUCTION:

Acute myeloid leukemia (AML) is a diverse illness categorized by a wide range the genetic modifications and molecular abnormalities that impact clinical consequences and afford prospective therapeutic goals. The 2017 European Leukemia Net (ELN) risk stratification recommendations, which combine cytogenetic abnormalities and genetic alterations, have been widely utilized to predict AML patients' prognosis (Döhner et al., 2017). The yearly incidence of AML is 4 per 100,000. Although it can occur at any age, the median age is 70 years old .Secondary AML, or AML that developed as a result of chemotherapy or radiation treatment or as a transition from myelodysplastic syndrome (MDS) or myeloproliferative neoplasia in the past represent one-third of AML (Johansson & Harrison, 2010). AML, like most hematologic malignancies, is more frequent in men than in women, with the greatest prevalence in men aged 60 to 90 years (Juliusson et al., 2020). Natural Killer cells are "large granular lymphocytes ascending from the lymphoid source which are deemed the 3rd largest residents of lymphocytes following T and B cells include 10-15% of all peripheral blood lymphocytes "intricate in protection against firm virus-infected and malignant cells(Taha, 2022). They also lyse target cells by "antibody-dependent cellular cytotoxicity (ADCC)", a serious manner of action of numerous therapeutic antibodies used to delicacy cancer(Carlsten & Järås, 2019). Without previous sensitization, the NK cells remain innate lymphoid cells that subsidize to the immune system's initial line defense counter to infections and malignant cells (Hallner et al., 2019; Spits et al., 2013). Natural killer group 2 member D (NKG2D) receptor is expressed in NK cells as well as numerous T cells, including NKT cells, CD8+ T cells, and T cells, and γδT. NKG2D normally serves solely as a costimulatory receptor in T cells and does not directly cause cytotoxicity. NKG2D ligand (NKG2DL), which is abundant in cancerous cells, may trigger NK cells by connecting the NKG2D receptor, and activated NK cells can subsequently destroy tumor cells. Costimulatory signals can be delivered when paired with the NKG2D receptor on T cells. However, there are several ways that block the function of the NKG2D receptorNKG2DL to allow tumor cells to elude immune detection(Duan et al., 2019; Peng et al., 2013). NKG2D ligands (MICA/MICB and ULBPs1–3) missing or expressed at

awfully low concentration on the cell superficial of myeloid blasts, as shown by many research groups (Pende et al., 2005). The ligands expression is dependent on the subtype of the cells, with low or absent expression in AML blast cells and a extra concentration in monoblastic cells, indicating that ligands of NKG2D is attained thru the last phases of maturation(Diermayr et al., 2008). The ligands shedding of in solvable form from the cell shallow by metalloprotease cleavage or into exosomes is an essential technique evolved by AML cells to elude immune identification. Two investigations showed that ligands are often found in the sera of patients of AML , resulting in NKG2D down expression and the impaired cytotoxicity of NKs (Hilpert et al., 2012). Consequently, AML patients thru decreased concentration of soluble MICA, MICB, and ULBP1 had a greater chance of achieving complete remission CR and surviving additional than a year (Hilpert et al., 2012).

PATIENTS AND METHODS:

Thirty patients diagnosed with AML were divided into 19 male and 11 female, and follow-up at day 14 of treatment and at day 30 of treatment were collected from Baghdad Medical City, National Center of Hematology from February 2021 to November 2021. Twenty healthy-looking people matched by age and sex were considered as control subjects. In this study, the inclusion criteria were that the patients were newly diagnosed with any type of acute myeloid leukemia except AML-M3 with pre-treatment Bone marrow aspirate sample available for immunophenotyping study with multicolor FC, of any sex, age 18 - 65 years. The treatment 7+3 drug regimen.

Exclusion criteria were age under 18 years, AML -M3 patients, another treatment protocol.
The patients peripheral blood (PB) and bone marrow aspirate (BMA) samples were received as fresh as possible and for each patient the following analysis were performed in diagnostic settings: complete blood count, blood film and morphology of BMA smear with SBB staining if morphology of blast cells was not showing clear myeloid differentiation, BMA multicolor FC testing for Using multicolor Flowcytometry to estimate the countenance of" CD7, CD11c, CD13, CD14, CD19, CD33, CD34, CD45, CD64, CD117 and MPO" in mature patients with morphologically, cytogenetic analysis and genetic mutation (NPM-1 and FLT-3), newly diagnosed AML.

Automated complete blood count (CBC): Was performed using standard procedures be autohemtolyser 5 differential (mindary, China). In present study 8-collor Flow cytometry BD FACSCANTO II instrument was used to detect Immunophenotyping NK cells and activator receptor (NKG2D) receptors and inhibitory receptor KIR2DL1 in patients with AML and control subjects.

The Surface receptors used for detection NK cells is (CD45 conjugated Violate 500 (V500) , CD3 conjugated Violate 450 (V450) , CD56 conjugated allophycocyanin (APC) ,CD16a conjugated (PerCP - Cyanines 5.5), The NK cells immunophentype is (CD45+,CD3-,CD56+,CD16+) The activator receptor is NKG2D conjugated- Alexa flour 400 , inhibitory receptor KIR2DL1 conjugated PE. All antibodies were products either Elabscience and Santa cruz biotechnology.

Sample processing for BD FACSCANTO II flow cytometer: Using the following techniques with the principle of Stain–Lyse–Wash.

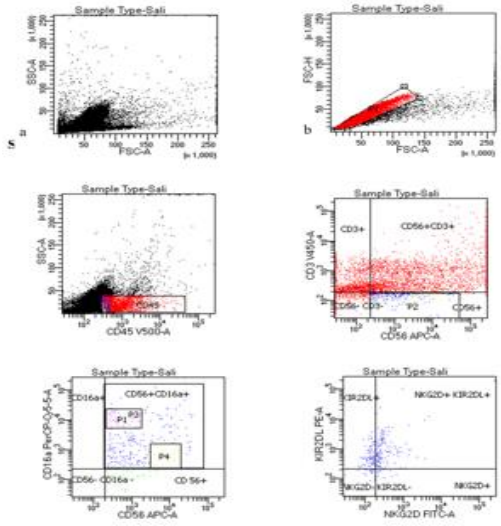


Figure 1: Flow cytometry analysis of AML patients at zero day .a) dot plot to of unstained cells **b)** dot plot to choose singlets cells **c)** dot plot to identify CD45+ **d)** dot plot to identify CD3- CD56+ population **e)** dot plot to identify CD56+CD 16+ (NK cells) as well as detection **of $CD56^{bright}$ $CD16^{dim}$ (p4) and $CD56^{dim}$ $CD16^{bright}$ (p3) f)** dot plot to identify NK cells that express KIR2dL1 + ,NKG2D+ , KIR2dL1 + NKG2D+ and KIR2dL1 - ,NKG2D-

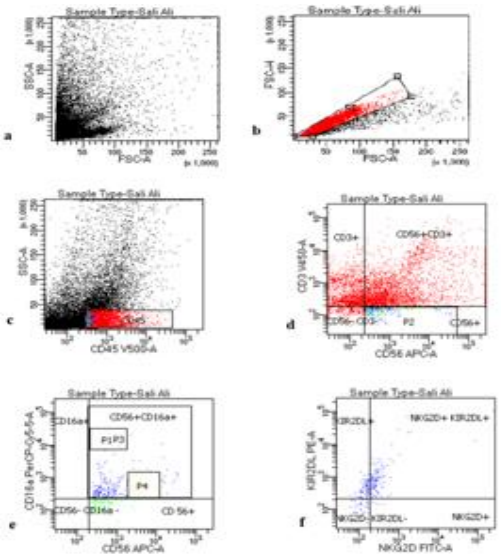
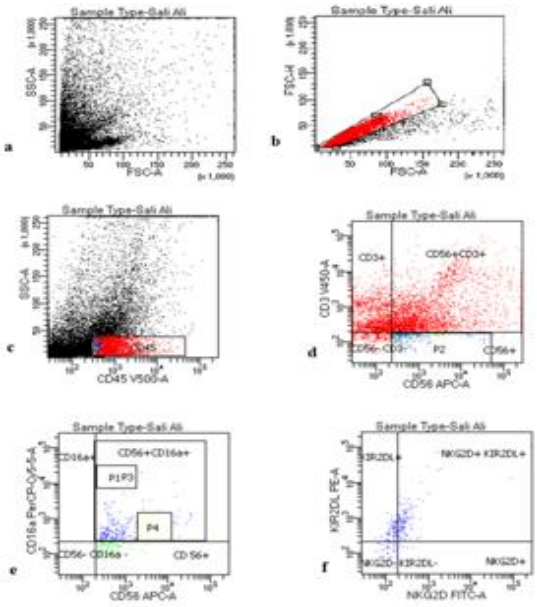


Figure 3: Flow cytometry analysis of AML patients after 14 days treatment .a) dot plot to of unstain cells **b)** dot plot to choose singlets cells **c)** dot plot to identify CD45+ **d)** dot plot to identify CD3- CD56+ population **e)** dot plot to identify CD56+CD 16+ (NK cells) as well as detection **of $CD56^{bright}$ $CD16^{dim}$ (p4) and $CD56^{dim}$ $CD16^{bright}$ (p1) f)** dot plot to identify NK cells that express KIR2dL1 + ,NKG2D+ , KIR2dL1 + NKG2D+ and KIR2dL1 - ,NKG2D-



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Detection of soluble ligand MICA:

Detection of soluble ligand MICA using sandwich ELISA technique (SUNLONG/ CHINA) by using standard curve to detection concentration of sMICA in serum.

Statistical analysis:

Statistical analysis was performed using "Statistical Package for the Social Sciences (SPSS)" version 23.0.0. Diagnostic statistics were approved out using a t-test with a p-value of <0.05 was considered significant, and <0.01was considered highly significant. Descriptive statistics were obtainable in tables, and trend-line mainly in the form of a range, mean and standard deviation. Person correlation (r) to improve the correlation between studied parameters.

RESULTS:

The patients enrolled in this study 54 patients diagnosed with AML divided in to, 30 patients were assessed at day zero of therapy and 24 out of 30 patients were followed up at day 14 after therapy, 19 males and 11 females with male: female ratio 1.72:1, table (1). Six patients out of 30 were deceased. 20 age and gender matched healthy looking subjects considered as a control group.

Mean patients with AML age was (40.2±15.2) years and range from 18 - 65 years, while the mean for the healthy-looking subjects was 41.1±13.3 years and range from (24 – 64) years, table (2).

Table 1: Distribution gender of patients with AML:

Sex group	Patients (n=30)		Control subjects (n=20)	
	No.	%	No.	%
Males	19	63	13	65
Females	11	27	7	25
Male: Female	1.72:1		1.86:1	

Table 2: Distribution of patients with AML age:

Age group (years)	Patients (n=30)		Control subjects (n=20)	
	No.	%	No.	%
16-25	8	26.6	2	10
26-35	4	13.3	6	30
36-45	7	23.4	6	30
46-55	5	16.7	2	10
56-65	6	20	4	20
Range	18-65		24-64	
Mean ± SD	40.2 ± 15.2		41.1 ± 13.3	

Hematological abnormalities in AML patients' group

In this study, there were two or more abnormalities including anemia, thrombocytopenia (<100 x 10^9 /l), leucopenia (<4.0x10^9/l) or leukocytosis (>70x10^9/l), neutropenia and presence of blast cells with newly diagnosed patients with AML. All patients had blasts (>20 %) at diagnosis, and after 14 days treatment 7 (29.2%) out of 24 patients had blasts.

Presence of NK cells expression activation receptor NKG2D in patients with AML.

According to data presented in table (3), there was a statistically significant variation in proportion of cells in the **CD56+ CD16+ (natural killer cells)** in patients with AML at diagnosis and at day 14 of treatment (mean±SD) (2.82±2.96, 1.308±1.928) respectively, with (P-value=0.04). While there was no statistically significant difference in patients with AML at diagnosis and at day14 after treatment, and control subject P-value (0.134, 0.56), respectively.

There was no statistically significant difference in proportion of NK cells, which express activator receptors NKG2D (**CD56+ CD16+ KIR2DL1- NKG2D+)** in patients with AML at diagnosis and AML at day 14 after treatment with mean±SD (2.3 ±2.44; 2.5 ± 2.32), respectively (P-value= 0.772). While there were statistically significant variation between patients of AML at diagnosis mean±SD (2.3 ± 2.44), AML at day 14 after treatment (2.5 ± 2.32) and control subjects mean±SD (9.93 - 8.49)(P-value= 0.001, 0.001). There were no statistically significant variation between expression inhibitory receptor **CD56+ CD16+ KIR2DL1+ NKG2D-)** among studied groups p-value (0.6, 0.23, 0.455), respectively.

Table 3: Comparison of presence of total NK cell and NKG2D, KIR2DL1 expression on NK cells surface in study groups.

CD expression (%)	Range (Mean ± SD)					
	Patients		Control subjects (n=20)	p-value compare to		
	At diagnosis (n=30)	At day 14 (n=24)		at diagnosis and at 14 days after treatment	At diagnosis and control subjects.	At 14 days after treatment and control subjects.
CD45+CD3-CD56+CD16+	0.1 – 12.5 (2.82±2.96)	0.3 – 9.6 (1.308±1.928)	0.3 – 5.6 (1.66±2.03)	**0.04**	**0.134**	**0.56**
CD56+CD16+KIR2DL1-NKG2D+	0 - 10.4 (2.3 ± 2.44)	0 - 8.3 (2.5 ± 2.32)	0.7 - 29.4 9.93 - 8.49)	**0.772**	**<0.001**	**0.001**
CD56+CD16+KIR2DL1+NKG2D-	0 – 52.0 (15.22 ± 6.02)	0.3 - 46.3 (17.3 ± 14.1)	2.9 - 35.1 (20.02 ± 8.93)	**0.60**	**0.23**	**0.455**
significant p value <0.05 highly significant p- value <0.01						

Estimation of sera concentration of sMICA in studied groups

According of data represented in table (4), there are no statistically differences between AML at diagnosis and AML at 14 days after treatment in the serum level of sMICA, P-value= (0.128).

There were highly statistically differences between patients of AML at diagnosis and control subjects in serum level of sMICA with P-value = (<0.001).

Regarding to comparison between patients of AML at14 days after treatment and control subject, there was statistically significant differences in serum level sMICA P-value = (0.001).

Table 4: Serum concentration of sMICA in studied groups.

Parameter/ Serum level	Range (Mean ± SD)					
	Patients		Control subjects	p-value compare		
	At diagnosis	At 14 days after treatment		At diagnosis and at 14 days after treatment.	At diagnosis and control subjects.	at 14 days after treatment and control subjects
sMICA (ng/L)	30.0 - 51.0 (33.68±2.04)	30.0- 35.0 (32.83±1.34)	25.0 - 32.0 (30.3± 1.50)	0.128	<0.001	0.001

In order to expression of activation receptor between patients of AML at diagnosis and at day 14 of treatment, (CD56+ CD16+ KIR2DL1- NKG2D+) there were significant negative correlation with sMICA, (r=-0.464, P=0.011; r=-0.444, P=0.001), respectively. There were no significant correlation among sMICA, The NK cells and inhibitory receptor KIR2DL1 among studied groups.

Table 5: Correlation between NKG2D receptor and sMICA in patient of AML at diagnosis and at day 14 of treatment.

Variables		**sMICA**	
		AML patients at diagnosis	**AML patients at day 14 of treatment**
NK cells	r	-0.08	0.313
	p	0.337	0.068
NKG 2D	r	-0.464	-0.444
	p	0.011	0.001
KIR2 DL1	r	0.021	0.038
	p	0.456	0.43

R: person correlation; p: p-value at 0.05

DISCUSSION:

AML is a malignancy demarcated by the proliferation and differentiation of clonal detention of myeloid progenitor cells. The AML age frequency is 4.3 / 100,000 a year (Shallis et al., 2019). In Iraq, separate studies conducted in Baghdad, Karbala, and Sulaymaniyah revealed that the incidence of AML was , (24.85%, 19.2% and 17%), respectively among the other type of leukemia(Abdulridha, Jawad and Numan, 2021).

AML may affect people of all ages, from infants to the elderly(Abelson et al., 2018; Juliusson et al., 2021). AML is thought to be a malgnancy of the ageing. In the United States, the middle age of AML finding ranged from 62 - 68 years(Song et al., 2018). Similar variations were seen in Europe and Canada(Shysh et al., 2017; Smith et al., 2011).

The AML patients in our study was 19 males and 11 females with (male: female ratio) 1.72:1. This is agree with Juliusson and colleges as with record leukemia, AML is more common hematological malignancy in men than in women (Juliusson et al., 2021).

Leukemia cells in AML may evade NK cell-mediated detection due to NK cell anomalies, immunosuppressant features of myeloblast cells, or relations amongst NK cells and further insusceptible cells that facilitate immune evasion (Lion et al., 2012). The of surface countenance levels of CD 56 and CD 16, as determined by the power of immune-fluorescence, they may be divided into two categories (Xu & Niu, 2020). The classical $CD56^{dim}CD16^{bright}$ NK cell types constitutes about (90 %) of the overall residents in marginal blood and displays potent cytolytic activity by producing cytotoxic granules containing perforin and granzymes. NK cells from AML patients have defective cytolytic activities (Aggarwal et al., 2016). With activating and inhibitory receptors, the function of NK cells is either inhibitory or activating depending on the balance between activating and inhibitory receptors on NK cells. (Lichtenegger et al., 2014). For triggering and repressive receptors, the function of NK cells can be either inhibitory or activating depending on the balance between activating and inhibitory receptors on NK cells (Punt et al., 2019). In this study, there was normal down in the expression of inhibitory receptors KIR2DL1 versus down of the expression of activation receptors NKG2D. Compared with control subjects, human NK cells direct a variety of HLA class I-specific repressive receptors that comprise KIRs and

CD94/NKG2Aheterodimers (Marcenaro et al., 2011). Ghasemimehr and collages are enhanced by A 6-fold upsurge in KIR2DL1 gene expression was seen in anew diagnosed patients equated to healthy persons, while a statistically significant reduction in KIR2DL1 receptor countenance was observed in the group of patients following induction treatment. This discrepancy in outcome might be the consequence of a different approach or a smaller sample size in the research (Ghasemimehr et al., 2020) . NKG2D is the utmost essential actuating receptors in the NK cell stock; it identifies the cellular tension ligands MICA/B, and ULBP1-6.NKG2D as well functions as a costimulatory receptor on T cytotoxic cells (Lamb et al., 2021). Further down usual circumstances, If NK cells non significantly activation by cytokines, the two activator receptors must excited concurrently in order to encourage not release granules (Bryceson et al., 2009). Majority of studies have emphasised the down expression of important stim ulation receptors such as "NKG2D, DNAM-1, and the NCRs". However, these downregulations do not appear to associate with the sub-type of AML (Lucas et al., 2007). The incidence of soluble ligands in the tumor surrounded may initiate the loss of NKG2D activating receptors. Boissel and his colleagues showed that" solvable NKG2D ligand (NKG2D-Ls) like MICA, MICB, ULBP1, and ULBP2, which are shed by tumour cells, and tumour exosomes expressing NKG2D-Ls cause a decrease in NK cell surface NKG2D" (Boissel et al., 2006; Fauriat et al., 2007; Hilpert et al., 2012) Myeloblasts of AML, together with Stem cells of AML , might resist NK cell intermediated death through expressing little or no NKG2D Ligand soluble in this environment (Nowbakht et al., 2005; Paczulla et al., 2019). Cytokines such as TGF-β may inhibit the NKG2D receptor (Lazarova & Steinle, 2019).

Artificial initiation of the NKG2D receptors by ligand of NKG2D, and antibody anticancer mixture otherwise NKG2D ligand/cytokine fusion remains best of one strategy to overawed scaping immunity and promote NK cell contact of tumor , besides may also assist a double purpose in T cell activation (Ding et al., 2018).

Current research, coupled with earlier research, reveals that faulty NK cells may not target chemo-resistant tumour cells. Feeble activating NK receptors expression is thus anticipated toward prevent the identification of remnant tumour cells. Analyzing the relationship between the amount of NK cell receptor expression and the incidence of recurrence would be an intriguing measure. Recent research has shown links amongst receptors of NK cell patterns and Over Survivals (OS) and risk of relapse. (Khaznadar et al., 2015). Consistent with the hypothesis that NKG2D ligands realizing is mechanism of tumor evading , higher levels of solvable NKG2D ligands have been found in the sera of patients in tumor , and associations between solvable NKG2D ligands and stage and/or progression tumors have been reported. (Chitadze et al., 2013). The solvable NKG2D ligands may predicament to NKG2 D, induce the down expression of NKG2D on NK cells and CD8+ T cells, and decrease concentration of NKG2D ligands on the superficial of tumours, therefore inhibiting immune recognition by NKG2D then cause eradication(Zwirner et al., 2007). It has been hypothesised that adsorption apheresis may be used to eradicate soluble NKG2D ligands from plasma patients (Döhner et al., 2017). Accumulating data indicates that solvable NKG2D-L, considered biomarker, may restrict blockage by immune checkpoint (Nakamura, 2019; Weil et al., 2017). Some clinically used medicines chemotherapy (e.g., epirubicin) block sMICA flaking by downregulation of disintegrin and metalloproteinase 10 (ADAM10), resulting in decreased solvable MICA in vivo. In circumstances, solvable NKG2D ligands in the sera of cancer patient role has been revealed to be active and able to NKG2D down expression which lead to suppress NKG2D dependent cytotoxicity (Kohga et al., 2009) also low level of NKG2D soluble ligand in complete remission (CR) give chance for survival more than one year(Chitadze & Kabelitz, 2022)

CONCLUSIONS:

The total NK cells decrease in patients with AML and down expression of NKG2D activating receptors versus KIR2DL1 inhibitor receptor which up expression. Soluble MICA elevated in AML patients and lead to masking of NKG2D activator receptor and cause pseudo down expression and leukemic cell evading NK cells activation and killing.

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