ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Flow Cytometric Detection of Angiopoietin Receptor Tie-2 in Acute Myeloid Leukemia

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Manuscript Info

Manuscript History:

Received: 18 October 2015
Final Accepted: 16 November 2015
Published Online: December 2015

Key words:

AML, Tie-2, flow cytometry, angiogenesis.

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Abstract

Background: Angiogenesis is required for the growth of solid tumors and hematologic neoplasia such as Acute Myeloid Leukemia (AML). Several endothelium-specific receptor tyrosine kinases (RTKs) such as TIE RTKs, Tie-1 and Tie-2, as well as, the vascular endothelial growth factor receptor kinases (VEGFRs) are known to play key roles in several important physiological and pathological angiogenesis.

Objective and Methods: This study aims to investigate the expression of tyrosine kinase Tie-2 receptor in Acute Myeloid Leukemia by flow cytometry in 60 adult patients with newly diagnosed Acute Myeloid Leukemia (AML) and in 20 normal controls and correlate the angiogenic factor expression with other hematological variables and immunophenotyping.

Results: According to the results of Tie-2 expression, AML patients were classified into 2 groups: high expression in 20 patients (33.3%), whereas 40 patients (66.7%) were designated as low expression. It was found that there was a highly significant difference between both groups as regarding Tie-2 expression ($P<0.001$). Moreover, there was a highly significant difference between the two patient groups as regarding age, WBC and Hb level ($P<0.001$) and a significant difference as regarding platelets` count and bone marrow blast cell count ($P<0.05$), while no significant difference as regarding gender. Tie-2 was highly expressed in M2 than M3 in low expression group. While, in high expression group, it was highly expressed in M5 than M1. A highly significant correlation was found between Tie-2 expression with WBC, bone marrow blast cell count and CD34 ($P<0.001$), a significant positive correlation with HLA-DR ($P<0.05$) and a highly significant negative correlation with both Hb levels and platelets` count. There was no correlation with CD13, CD14, CD33 and CD7.

Conclusion: We concluded that elevated Tie-2 is a distinctive feature in Acute Myeloid Leukemia associated neo-angiogenesis and could be used as a prognostic factor to predict AML outcome.

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INTRODUCTION

Acute Myeloid Leukemia is a clonal malignant disease of haematopoietic tissue resulting from somatic mutation in a pluripotent stem cell or a slightly more differentiated progenitor cell. It is characterized by proliferation of abnormal leukemic cells, principally in the bone marrow, and impaired production of normal blood cells **Redaelli et al., 2003**. Numerous studies have been attempted to define the prognostic factors that can predict the outcome of the disease. These factors involve both patient and disease related variables. The Age of the patient, performance status, some

morphological variation, FAB type and immunophenotypical variations can be considered as important prognostic factors in AML **Buchner et al., 1996.**

Angiogenesis plays a key role in the pathogenesis and progression of haematological malignancies. It is thought to control the growth and metastatic potential of most tumours, both solid and haematological **Michael and Jakop 2014.**

In AML, Endothelial cells from pre-existing venules within the bone marrow are activated, proliferate, migrate and form new blood vessels which in turn support tumor proliferation **Dias et al., 2000.**

VEGF is the most well characterized proangiogenic factor. It has different isoforms all of which are secreted as dimeric glycoproteins. VEGFR-1 and VEGFR-2 are predominantly expressed on vascular endothelial cells, and the activation of VEGFR-2 appears to be both necessary and sufficient to mediate VEGF-dependent angiogenesis and the induction of vascular permeability **Ferrara et al., 2003**.

VEGF isoforms (VEGF-1, -2, -3 and 4) share the same receptor in there action. This receptor is known as Tyrosine receptor Tie-2 **Lee et al., 2007.**

Blocking VEGF activity has been shown to be particularly effective at sensitising the vasculature and improving the delivery of cytotoxic drugs to tumour and endothelial cells. **Michael and Jakop 2014.**

The Tie2 receptor is a transmembrane tyrosine kinase highly enriched in the endothelium. It is considered as an important regulator of vascular barrier function in health and in disease. The principal ligands of Tie2, Angiopoietins 1 and 2, exert opposite effects on this receptor as Angiopoietins -2 antagonizes Angiopoietins -1 binding to the receptor **Milam and Parikh 2015.**

To demonstrate the value of Tie-2 expression in AML patients and to correlate this expression with other prognostic factors, we investigated 60 newly diagnosed AML patients for Tie-2 expression using Flow Cytometry.

PATIENTS AND METHODS:

Case selection

The current study was conducted on 60 patients newly diagnosed as suffering from de novo Acute Myeloid Leukemia (AML) and 20 healthy individuals as a control group. The patients were selected from the Hematology Unit of Internal Medicine Department in Zagazig University Hospitals between July 2011 and June 2014.

Diagnosis of AML patients was based on WHO classification of myeloid neoplasms. (**Vardiman et al.,2009).** All cases were subjected to the following: Full history taking, clinical examination laying stress on the presence and extent of leukemic involvement including: pallor, purpuric eruptions, hepatomegaly, splenomegaly, lymphadenopathy and CNS involvement**.**

Sample collection:

Peripheral blood (PB) and bone marrow (BM) samples were obtained from patients in accordance with local Institutional Ethical protocols and were collected from each patient under complete aseptic conditions using sterile EDTA vacutainer for performing CBC, Leishman stained PB film, and bone marrow smears. One ml of BM aspirate or two ml of PB were collected on EDTA vacutainer and were used for immunophenotyping of AML cases. Flow cytometric detection of Tie-2 receptor was done using FACScan flow cytometry (Becton Dickinson, LA, USA).

Methods:

- **Complete blood picture:** using Sysmex Xs-500i (Roche) hematology analyzer.
- **Bone marrow aspiration and examination of leishman stained smears.**
- **Cytochemical staining:** using Myeloperoxidase, Esterases and Periodic Acid Schiff.
- **Immunophenotypic analysis:**

In the present study, the monoclonal antibodies (MoAb) used for staining of blast cells were combined single, dual and triple color. Monoclonal antibodies labeled with Fluorescein Isothicyanate (FITC), Phycoerythrin (PE) and Peridinin-Chlorophyll-Protein-Complex (PerCP) were used for each sample to be diagnosed as AML including: Myeloid markers: CD13, CD33, MPO. Monocytic markers: CD14, CD64. B cell markers: CD19, CD20, CD22 and CD79a. T cell markers: CD3 and CD7. Common progenitor marker: CD34, HLA-DR and TDT. All Moab purchased from Dako (Denmark). FACS acquisition and analysis were performed with FACS CellQuest software (BD Biosciences).

Staining method:

One hundred µl of whole blood or cell suspension containing up to 1 x 10^6 leucocytic cells completed to 1 ml by ammonium chloride lysing solution (NH4cl 8 gm, EDTA 1 gm and KH2po4 0.1 gm) in a tube at room temperature for 10 – 20 minutes. Then centrifuged for 5 min at 400g, the supernatant was discarded and the pellet

was washed with phosphate buffer solution (PBS) two times to obtain white pellet which was resuspended in 1 ml buffer. After lysing and washing techniques, each tube was stained with a cocktail of antibodies. Isotypic control was used to define the negatively stained cells. Gating was done on the blast cell population based on forward and side scatter properties. The percentage of blast cells positive for the relevant studied marker was determined as a percentage from the gated blast cells population. The negative isotopic control was set at 5%. Cells were considered positive for a certain marker when ≥20% of cells expressed it, except for CD34, TDT and intracellular MPO where their expression by 10% of cells was sufficient to confer positivity.

Detection of Tie-2 expression using flow cytometry:

One hundred µl of whole blood or cell suspension containing up to 1 x 10^6 leucocytic cells were first treated with FcR blocking reagent (Miltenyi Biotec, Auburn, CA) to block unwanted binding of antibodies to the cells and then incubated with PE-conjugated mouse anti-human Tie2 (83715, R&D Systems), antibodies for 30 min in the dark at room temperature, and results were expressed in the form of percent.

Statistical analysis:

Data were collected, revised, verified then edited on personal computer and then analyzed using SPSS version 10.

Qualitative variables were given in percentages and number of cases. Quantitative variables were expressed as mean, range and standard deviation. Cut off value was calculated according to this equation: (cut off= mean +2SD). P≤ 0.05 was considered as significant, P≤ 0.001 was considered as highly significant while P> 0.05 was considered not significant.

RESULTS:

Sixty patients of de novo AML were enrolled in this study. They were selected for the study on the basis of standard clinical, hematological and immunophenotypical criteria for diagnosis of AML. Their ages ranged from 18 to 69 years, with a mean age of 43.3 ± 13.4 years. They were 44 (73.3%) males and 16 (26.7%) females.

Twenty apparently healthy adults (age and sex matched) were included in the study as a control group. Their ages ranged from (22-62) years with mean±SD of (38.3±11.9), they were 12 males (60%) and 8 females (40%)**.**

Expression of Tie-2: Normal control group showed that Tie-2 expression was 10±2.5 with range of 7-14%. In AML patients, the level of Tie-2 ranged from 5% to 30% with a mean of 11.8±9.5%. A cut off value of 15% was defined for Tie-2 expression using the mean±SD of control group. According to this cut off value, the sixty studied AML patients were classified into 2 groups: the expressions of Tie-2 were positive in 20 patients (33.3%), their levels were equal or above this cut off , so it was designated as "high expression" group. Whereas 40 patients (66.7%) were below this level and were designated as "low expression" (Table 2). There was a highly significant difference between the two groups as regarding Tie-2 expression (P<0.001).

There was a highly significant difference between the two patients groups as regarding age (P<0.001), while there was no statistically significant difference between the two groups as regarding gender. Moreover, a highly significant difference was found between the two patient groups as regarding WBC and Hb levels (P<0.001) and a significant difference as regarding platelets` count and bone marrow blast cell count (P<0.05) (Table 3).

In low expression group, Tie-2 was highly expressed in M2 than M3 while, in high expression group, it was highly expressed in M5 than M1 (Table 4).

There was a highly significant positive correlation between Tie-2 expression with WBC and bone marrow blast cell count (*P*<0.001), while a highly significant negative correlation was found between Tie-2 expression with both Hb and platelets` count (*P*<0.001) (Table 5) (Fig. 1).There was a highly significant positive correlation between Tie-2 expression with CD34 (*P*<0.001) and a significant positive correlation with HLA-DR (*P*<0.05). However, there was no significant correlation was found between Tie-2 expression with CD_{13}, CD_{14}, CD_{33} and CD_7 *(P> 0.05)*. (Table 6) (Fig.1).

Table (1): Hematological data of patient group.

Hematological parameters	**AML group (no= 60)**
***WBC (×10^3/µl)**	16.9 ± 11.05 (12.1-33)
***Hb (g/dl)**	7.98 ± 1.3 (5.1-10.7)
***Platelets (x 10^3/µl)**	37.4 ± 17.2 (18-80)
***Peripheral Blood (Blast %)**	57.8 ± 15.7 (28-84)
BM (blast) (%)	80.1 ± 9.7 (58-95)
FAB	
M1	6(10%)
M2	18(30%)
M3	12(20%)
M4	10(16.7%)
M5	14(23.3%)

**Data presented as mean±SD (range)*

Table (2): Comparison between groups with low Tie-2 and high Tie-2 positivity

	low Tie-2 (n=40)	**High Tie-2 (n=20)**	***T***	***P***
Mean ± SD	5.75 ± 3.37	24.1 ± 4.2	12.98	< 0.0001*
Range	1-12	19-30		

**Highly significant*

Table (3): Comparison between expressions of Tie-2 in both groups as regards to patients' data

	low Tie-2 (n=40)	**High Tie-2 (n=20)**	T	*P*
Age	36.6 ± 10	57. ± 17.5	5.7	< 0.001*
Gender M/F	28/12	16/4	$X^2 = 0.02$	0.88
WBC (x10^{3}ųl)	11.7 ± 9.1	27.05 ± 2	4.6	< 0.001*
Hb (g/dl)	8.5 ± 1.1	6.89 ± 1.05	3.8	< 0.001*
Platelets(x10^{3}ųl)	43.3 ± 17.9	25.5 ± 5.9	3.03	<0.05**
Blast cell percentage	51.9 ± 15	68.6 ± 9.2	3.38	<0.05**

**Highly significant* ***Significant*

Table (4): Relation between FAB and Tie-2 expression in both groups

	low Tie-2 (n=40)		**High Tie-2 (n=20)**	
	No	%	No	%
M1	0	0	6	30
M2	16	40	2	10
M3	8	30	0	0
M4	6	15	4	20
M5	6	15	8	40

Table (5): Correlation between hematological parameters and Tie-2

	r	***P***
Hb	-0.56	< 0.001*
WBC	0.6	< 0.001*
Platelets	-0.56	< 0.001*
Blast cell count (BM)	0.112	< 0.001*

*Highly significant

Table (6): Correlation between Tie-2 and immunophenotyping

	r	P
CD13	-0.136	> 0.05
CD14	0.23	> 0.05
CD33	0.15	> 0.05
CD34	0.44	< 0.001*
CD7	0.153	> 0.05
HLA-DR	0.41	< 0.05*

**Highly significant*

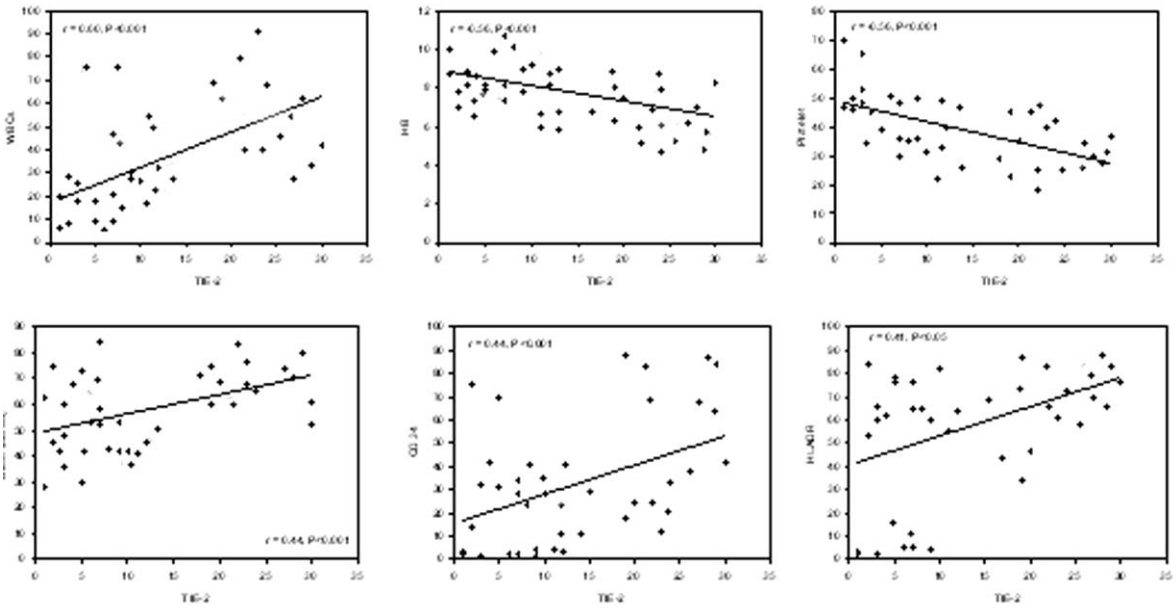

Figure 1: Correlation between Tie-2 expression with some haematological parameters and immunophenotyping.

DISCUSSION:

Acute Myeloid Leukemia (AML) is a clonal malignant disease which finds its origin in the transformation of uncommitted stem cells **Lichtman and liesveld 2001.** It is an aggressive disorder that results from a block in the differentiation of hematopoietic progenitor cells along with uncontrolled proliferation. It has also the lowest survival rate of all leukemias **Redaelli et al., 2003.**

Assessment of the prognostic factors in AML is very important and discovery of therapeutic targets is now increasing. Interaction between hematopoietic cells and endothelial cells is important for proliferation and differentiation of both cell lineages **Gregory et al., 2009**.

Aref et al., 2009 reported that several studies were done as concerning the critical role of angiogenesis in the development and growth of solid tumours and haematological malignancies.

Bone marrow neoangiogenesis in AML is a complex process involving the interplay of different angiogenic growth factors. The vascular endothelial growth factors (VEGF) and angiopoietin family are believed to represent the most specific inducers of angiogenesis **Sonja et al., 2005.**

Increased numbers of endothelial cells (ECs) have been detected on histologic sections of bone marrow biopsies of AML patients compared with those with reactive disease **Riccioni et al., 2007**.

Nowicki et al., 2006 have attempted to elucidate the impact of a single angiogenic factor on the pathogenesis or prognosis of haematologic malignancies. They found that the absence of VEGF-C in blast cells predicts long-lasting remission in all leukaemic children.

Angiopoietin family includes four members: Ang-1, -2, -3, and -4. However, they share only a single receptor the Tie-2 **Lee et al., 2007**.

Tie-2 is a cell membrane receptor involved in transmembrane receptor protein tyrosine kinase signaling pathway **Saber et al., 2011**. It has an important function in the regulation of angiogenesis, vessel remodeling, cell survival, cell migration, and cell-to-matrix and cell-to-cell adhesion. It is expressed on the ECs, early haematopoietic stem cells (HSCs), and the so-called leukaemic cells. Its expression was described to have a prognostic impact in AML **Liu et al., 2009**.

High levels of Tie-2 are found in placenta, lung, spleen and heart tissues. It is also expressed by endothelial cells, hematopoietic stem cells, monocytes and certain tumor cells **Martin et al., 2009**.

Leukemic bone marrow is likely to demonstrate a level of hypoxia which causes an increased expression of Tie-2 on leukemic blasts. Hypoxia exerts inhibitory effects on the activity of the angiopoietin-1/Tie-2 receptor pathway through reduction of angiopoietin-1 and upregulation of angiopoietin-2 and -3. The variation in angiopoietin levels and the degree of hypoxia can explain different levels of Tie-2 positivity on leukemic blasts **Riccioni et al., 2007**.

It has become increasingly clear that angiogenic factors produced by leukemic blasts may act in an autocrine or intracrine fashion, thereby stimulating cell proliferation and survival through a mechanism independent from angiogenesis **Schliemann et al., 2006.**

Riccioni et al., 2007 showed that leukemic cells release Ang-1 and VEGF. They reported also that incubation of AML blasts with agents able to inhibit binding of angiopoietins to Tie-2 results in moderate but significant increase of apoptosis. This observation suggested that the Tie-2/Ang-1 autocrine pathway can play a relevant role in the survival of Tie-2 positive AML patients.

Schliemann et al., 2006 suggested also that the switch in angiopoietin balance may play an important role in the angiogenic process occurring during leukemic transformation. The modulation of the autocrine angiopoietin/Tie-2 axis may be a promising approach to improve outcome in AML patients.

To demonstrate the value of Tie-2 expression in AML patients and to correlate this expression with other prognostic factors, we investigated 60 newly diagnosed AML patients and 20 normal controls for the expression of Tie-2 using flow cytometry.

In the current study, we found that all control group expressed Tie-2 receptor with a range of (7%-14%). The 60 studied AML patients expressed Tie-2 with a range of (5%-30%). They were classified into 2 groups according to the level of surface expression of Tie-2; low expression group (40/60) (66.7%) (Low Tie-2% expression) displayed < 15% and high expression group (20/60) (33.3%) (High Tie-2% expression) displayed ≥ 15%. There was a highly significant difference between the two groups as regarding Tie-2 expression ($P<0.001$).

Riccioni et al., 2007 observed Tie-2 expression in only 56% of AML studied samples. It was expressed in normal level in 39/111(35%) and at high level in 23/111 (21%) of patients.

In our study, there was a highly significant difference between Tie-2 high expression group and Tie-2 low expression group as regarding age ($P<0.001$). **Schliemann et al., 2006** found also that the scores of Tie-2 expression were slightly higher in AML patients under 60 years old than in AML patients over 60 years old, In contrary, **Aref et al., 2009** found that Tie-2 expression was not related to the age of the studied patients.

In this study, Tie-2 expression was not related to sex. This finding was similar to a study done by **Aref et al., 2009**. However, **Lee et al., 2007** demonstrated that Tie-2 level was higher in males than in females.

At diagnosis, WBC represents one of the important prognostic factors for AMLs. In the present study, there was a highly significant difference between high expression group and low expression group as regarding WBC ($P<0.001$). **Riccioni et al., 2007** found that Tie-2 positive patients had increased WBC count compared with Tie-2 negative patients, while not all Tie-2 positive AMLs displayed a high WBC count.

In the present study, there was a significant difference between high expression group and low expression group as regarding platelets` count ($P<0.05$). As far asa we are aware, this finding was not reported by any other investigator.

In our study, low expression group showed that Tie-2 was highly expressed in M2 than M3, while in high expression group it was highly expressed in M5 than M1. **Riccioni et al**., **2007** found a preferential occurrence of M4 and M5 leukemias among Tie-2 positive AMLs. While **Lee et al., 2007** found that Tie-2 seemed to be expressed in AMLs in most of French-American-British (FAB) subtypes.

In the present study, there was a highly significant positive correlation between Tie-2 expression with BM blast cell count ($P<0.001$). **Lee et al., 2007** found that marrow level of Tie-2 did not correlate with the absolute

number of immature cells in the PB. **Schliemann et al., 2006, also,** found that Tie-2 scores did not correlate with the percentage of BM blasts.

In our study, there was a highly significant positive correlation between Tie-2 expression with CD34 ($P<0.001$) and a significant positive correlation with HLA-DR ($P<0.05$), while there was no significant correlation between Tie-2 expression with CD_{13}, CD_{14}, CD_{33} and CD_7 ($P> 0.05$). In contrast, **Murdoch et al., 2007** found that Tie-2 positive AML blasts were CD14 positive and also **Riccioni et al., 2007** found that a high percentage of Tie-2 positive AMLs were CD14 positive, whereas only a minority of Tie-2 negative were CD14 positive.

Riccioni et al., 2007 found that Tie-2 positive AML displayed the tendency to be less positive than Tie-2 negative AML for CD34 antigen. **Watari et al., 2002** found that CD34 did not correlate with the frequency of Tie-2 expression.

Koenecke et al., 2010 stated that the difference in the studied sample size and the methods of detection of Tie-2 may be the cause of the difference in opinions regarding in the effect of Tie-2 on outcome of AML patients.

In conclusion, our study reveals that elevated Tie-2 is a distinctive feature in Acute Myeloid Leukemia associated neo-angiogenesis and could be used as a prognostic factor to predict AML outcome.

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