

# Prognostic value of soluble angiopoietin-2 and soluble Tie-2 in Egyptian patients with acute myeloid leukemia

*Akut miyeloid lösemili Mısır'lı hastalarda çözümlü anjiyopoitein -2 ve çözümlü Tie-2'nin prognostik değeri*

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## Abstract

**Objective:** Angiogenesis plays a critical role in the development and growth of solid tumors and hematologic malignancies. The system involving angiopoietin-2 [Ang-2] and its receptor Tie-2 appears to play an important role in tumor angiogenesis and in the biology of hematological and non-hematological malignancies. We evaluated the levels of soluble (s)Ang-2 and sTie-2 in acute myeloid leukemia (AML) patients and investigated the impact of their circulating levels on the overall survival in those patients.

**Materials and Methods:** Ang-2 and Tie-2 were measured in plasma samples from AML patients and controls using enzyme-linked immunosorbent assay (ELISA).

**Results:** The levels of sAng-2 and sTie-2 were significantly higher in AML patients ( $2382.1 \pm 1586.1$  pg/ml and  $6.74 \pm 3.47$  ng/ml, respectively) than in controls ( $649.5 \pm 402.6$  pg/ml and  $2.63 \pm 0.57$  ng/ml, respectively;  $p < 0.01$ ). AML patients with high levels of sAng-2 and sTie-2 ( $\geq 2500$  pg/ml and  $\geq 8$  ng/ml, respectively) had significantly shorter overall survival than those patients with low levels ( $< 2500$  pg/ml and  $< 8$  ng/ml, respectively).

**Conclusion:** The results of our study demonstrated the prognostic significance of circulating sAng-2 and sTie-2 in AML patients. Modulation of the angiopoietin / Tie-2 axis may be a promising approach to improve the outcome in those patients. (*Turk J Hematol* 2010; 27: 282-8)

**Key words:** Ang-2, Tie-2, AML, ELISA

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## Özet

**Amaç:** Anjiyojenez, katı tümörler ve hematolojik hastalıkların gelişimi ve büyümesinde kritik bir rol oynamaktadır. Anjiyopoietin-2 [Ang-2] ve onun reseptörü Tie-2'yi kapsayan sistemin, tümör anjiyojenezinde ve hematolojik ve hematoloji dışı hastalıkların biyolojisinde önemli bir rol oynadığı görülmektedir. AML hastalarında çözümlü sAng-2 ve sTie-2 düzeyleri değerlendirilmiş olup, bu hastalarda dolaşımdaki düzeylerinin toplam sağkalım üzerindeki etkileri araştırılmıştır.

**Yöntem ve Gereçler:** AMLi hastalar ve kontrollerden alınan plazma örneklerinde, Enzim bağlı immünasay (ELISA) ile Ang-2 ve Tie-2 ölçülmüştür.

**Bulgular:** AML hastalarında, sAng-2 ve sTie-2 değerleri [sırasıyla  $2382.1 \pm 1586.1$  pg/ml ve  $6.74 \pm 3.47$  ng/ml], kontrollere [sırasıyla  $649.5 \pm 402.6$  pg/ml ve  $2.63 \pm 0.57$  ng/ml] kıyasla anlamlı derecede daha yüksekti ( $p < 0.01$ ). Toplam sağkalım değeri, yüksek sAng-2 ve sTie-2 [sırasıyla  $\geq 2500$  pg/ml ve  $\geq 8$  ng/ml] değerli AML hastalarında düşük düzeylere [ $<$  sırasıyla  $2500$  pg/ml ve  $< 8$  ng/ml] kıyasla anlamlı derecede daha kısaydı.

**Sonuç:** Çalışmada elde edilen sonuçlarda, AML hastalarının dolaşımdaki Ang-2 ve sTie-2 düzeylerinin prognostik anlamlılığı gösterilmiştir. Anjiyopoietin / Tie2 aksisinin modülasyonu, söz konusu hastalarda sonucu geliştirmeye yönelik ümit verici bir yaklaşım olabilir. (Turk J Hematol 2010; 27: 282-8)

**Anahtar kelimeler:** Ang-2, Tie-2, AML, ELISA

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## Introduction

Angiogenesis, the formation of new blood vessels, plays a critical role in the development and growth of solid tumors and hematologic malignancies [1,2]. Angiogenic activity has been demonstrated to be significantly increased in acute myeloid leukemia (AML) bone marrow as compared to normal BM [3,4]. Padro et al. [5] stated that leukemic blasts release several angiogenic molecules that increase vessel density in neoplastic marrow.

The interplay of BM endothelial cells and growth factors derived from leukemic blasts contributes to the pathogenesis of hematologic malignancies. It has become clear that angiogenic factors produced by leukemic blasts may also act in an autocrine or intracrine fashion, thereby stimulating cell proliferation and survival through a mechanism independent from angiogenesis [6].

Among many angiogenic mediators, the members of the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin (Ang) family have been established as the major regulators of tumor-associated angiogenesis [7]. Cellular VEGF has been found to be upregulated in AML blasts [5,8], and to be negatively correlated with survival [9].

The angiopoietins constitute a novel family of angiogenic mediators, which have been shown to be important regulators of neovascularization, vascular stability and maturation [10]. Ang-1 and its naturally occurring antagonist Ang-2 act via the Tie-2 receptor tyrosine kinase, which is broadly expressed in the endothelium of the adult vasculature and in a subset of hematopoietic stem cells. Although Ang-1 and Ang-2 have very similar protein structure, they elicit opposing responses when binding to endothelial Tie-2 [11]. Binding of Ang-1

causes autophosphorylation of Tie-2 and ensures the integrity of blood vessels by strengthening interaction between endothelial cells and peri-endothelial support cells. In contrast, Ang-2 specifically disrupts Ang-1-mediated receptor activation, resulting in vessel destabilization, thereby facilitating the angiogenic response to mitogenic factors such as VEGF or leading to vessel regression in its absence [12]. With its role in both angiogenesis and vascular maintenance, Tie-2 seems to have a dual function defined by the quantitative balance between Ang-1 and Ang-2 activity. While Ang-1 is constitutively expressed throughout adult tissues, providing a stabilizing signal, normal postnatal Ang-2 expression is only observed at the sites of active vascular remodeling [13]. Thus, angiogenesis is controlled by a dynamic balance between vessel regression and growth that is mediated by the VEGF and the Ang/Tie-2 system.

The role of this complex system has been extensively examined in the neovascularization of a wide variety of tumors, and many reports have documented a correlation between Ang expression and clinical features or prognosis [14]. Loges et al. [15] reported that BM neoangiogenesis plays an important pathogenic and possibly prognostic role in AML. Therefore, this study aimed to evaluate the circulating levels of soluble (s)Ang-2 and sTie-2 in Egyptian patients with AML and to investigate their impact on the overall survival in those patients. We preferred to measure the circulating levels of these parameters because they are easily accessible and applicable for routine use.

## Materials and Methods

The present study was carried out on 60 newly diagnosed AML patients (Table 1) and 30 sex- and

Table 1. Patient characteristics

Parameters	AML patients
No	60
Age/year	15-63
Male	35
Female	25
FAB subtypes	
M0	5
M1	15
M2	10
M4	16
M5a	7
M5b	5
M6	2
WBCs x 10 <sup>3</sup> /cmm	16.0 (9.5-89.5)
Peripheral blood blast cells %	10
Bone marrow blast cells %	50
Follow-up	24 months
Main causes of death	Hemorrhagic complications and infections

age-matched healthy controls in accordance with local institutional ethical protocols. All the patients were admitted to the Hematology/Oncology unit of the Internal Medicine Department of Tanta University Hospital. The diagnosis was made according to the clinical picture and morphological and cytochemical criteria of the French-American-British (FAB) study group and immunophenotypic studies. Patients were treated with a combination of Ara-C 1 g/m<sup>2</sup>/12h (day 1 to day 3) or mitoxantrone 12 mg/m<sup>2</sup> (days 3, 4 & 5) [16].

Patients were considered to be in complete remission (CR) when BM aspirates showed trilineage regeneration with less than 5% blasts by morphological and immunocytochemical analysis in the presence of a normal blood count that persisted for at least one month. All other patients were considered to be non-responsive. Three milliliters of blood were withdrawn from each subject into Vacutainer tubes containing EDTA as anticoagulant. The blood was centrifuged for 15 minutes, and then plasma was collected and stored at -20°C until analysis. Blood samples were obtained from patients at diagnosis before the initiation of induction chemotherapy.

Plasma levels of Ang-2 and Tie-2 were assayed by enzyme-linked immunosorbent assay (ELISA) using

commercially available kits (Quantikine, R&D systems) according to the manufacturer's instructions.

### Statistical analysis

The collected data were organized, tabulated and statistically analyzed using SPSS software statistical computer package version 12. For quantitative variables, the median, range, mean and standard deviation were calculated. The difference between two medians was statistically analyzed using Mann-Whitney test. For comparison between more than two medians, the F value of analysis of variance (ANOVA) was calculated and Scheffe test was performed to compare between two medians if the F value was significant. Pearson's correlation coefficient (r) was calculated to test the association between two variables. Univariate and multivariate Cox regression analysis were performed to evaluate the predictive effects of each angiogenic factor. Values of p<0.05 were considered statistically significant [17].

## Results

### Plasma levels of Ang-2 and Tie-2 in AML patients and controls

Medians, means and ranges of plasma levels of Ang-2 and Tie-2 in AML patients and healthy controls are presented in Table 2. There was significant increase in the circulating plasma levels of both Ang-2 and Tie-2 in AML patients when compared to the control group (p=0.001 for both parameters) (Table 2).

### Correlation between plasma levels of Ang-2 and Tie-2 and clinicopathological features of AML patients

In AML patients, there were significant positive correlations between plasma Ang-2 level and total leukocytic counts (TLC), lactate dehydrogenase (LDH) levels and percentage of blast cells in the BM (r=0.48, 0.54 and 0.48, respectively). On the other hand, there was a significant strong negative correlation between Ang-2 plasma levels and survival in AML patients (r=-0.84) (Table 3). Tie-2 plasma levels were positively correlated with TLC and LDH levels and the percentage of blasts in the BM (r=0.5, 0.52 and 0.52, respectively). Also, a significant negative correlation was found between Tie-2 levels and survival in AML patients (r=-0.8) (Table 3).

### Association between plasma levels of Ang-2, Tie-2 and overall survival

To assess the effect of circulating Ang-2 and sTie-2 levels on the AML overall survival, univariate analysis was done. We had sub-classified the studied patients into risky and non-risky subgroups regarding their age (risk with age  $\leq 20$  or  $\geq 60$  years), TLC (risk  $\geq 50,000$ ) and their response to induction therapy (risk in the absence of CR). LDH level was considered as a risk factor when it was  $>400$  U/L and non-risky when it was  $\leq 400$  U/L.

The angiogenic factors Ang-2 ( $\geq 2500$  vs  $< 2500$ ) and Tie-2 ( $\geq 8.0$  vs  $< 8$ ) were significantly associated with effect on AML survival (Figures 1 and 2, respectively). These cut-off values were statistically calculated using a Receiver Operating Characteristic (ROC) curve. The relative risk (RR) of death of Ang-2 was significant higher when baseline was  $\geq 2500$  pg/ml (RR 6.3, 95% confidence interval (CI) 0.071-0.610,  $p=0.050$ ). In addition, the RR of death of Tie-2 was significant higher when baseline was  $\geq 8$  pg/ml (RR 5.65, 95% CI 0.061-0.710,  $p=0.005$ ) (Table 4).

Furthermore, a multivariate Cox regression analysis incorporating all variables that were found to have a significant effect on univariate analysis was performed (Table 5). Response to treatment was independently and significantly affected by Tie-2 and Ang-2 ( $p<0.001$  and  $p<0.01$ , respectively), while WBC was affected by Ang-2 ( $p<0.01$ ) and LDH was affected by Tie-2 ( $p=0.034$ ).

## Discussion

The system involving Ang-2 and its receptor Tie-2 appears to play an important role not only in tumor angiogenesis, but also in the biology of hematological and non-hematological malignancies. The elevated vessel density in neoplastic BM is the result of the action of several angiogenic molecules released from leukemic blasts. The expanded endothelial microenvironment is able to support leukemic cell survival and growth by secretion of hematopoietic growth factors [18]. Moreover, angiogenic mediators produced by AML cells also act through external and internal autocrine loops, thereby directly promoting cell survival, proliferation and disease progression independently from the mechanisms of angiogenesis [19].

In the present study, the plasma levels of sAng-2 and sTie-2 were evaluated in patients with newly diagnosed AML. Levels of circulating Ang-2 and

Table 2. Plasma Ang-2 and Tie-2 levels in the studied groups

	Control group	AML group	F	P
Number	30	60		
Ang-2 (pg/ml):				
Range	189-1215	345-4210		
Mean $\pm$ SD	649.53 $\pm$ 402.67	2382.10 $\pm$ 1586.12	9.361	0.001*
Median	550	3000		
Tie-2 (ng/ml):				
Range	1.9-3.5	1.4-10.5		
Mean $\pm$ SD	2.63 $\pm$ 0.57	6.74 $\pm$ 3.47	10.592	0.001*
Median	2.6	8.8		

\*Significant

Table 3. Relationship between plasma Ang-2 and Tie-2 levels and measured variables in AML patients

Variables	Ang-2 in AML Group (n=60)		Tie-2 in AML Group (n=60)	
	r	p	r	p
Age in years	0.249	0.185	0.276	0.140
TLC	0.477	0.008*	0.501	0.005*
LDH	0.543	0.002*	0.528	0.003*
Blast cells %	0.483	0.007*	0.520	0.003*
Survival	-0.843	0.001*	-0.806	0.001*

\*Significant

Table 4. Univariate analysis of overall survival in AML patients

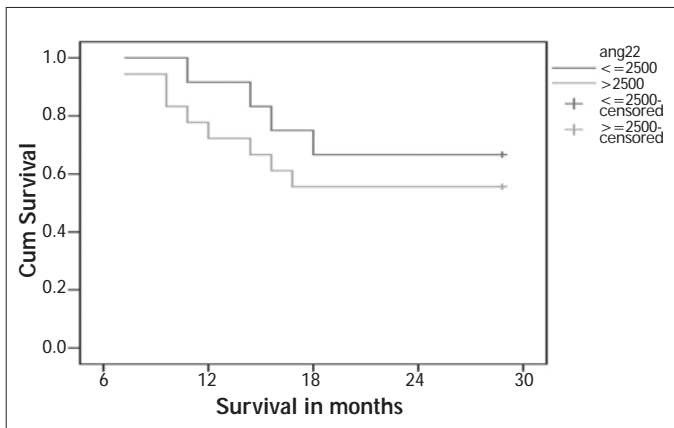
Risk factors	No	RR	95% CI	p-value
Age:				
$\leq 20$ & $\geq 60$	40	1.1	0.346-2.231	0.817
21-59	8	0.9		
TLC:				
$\geq 50,000$	40	0.2	0.033-0.291	$<0.001$
$< 50,000$	8	3.9		
LDH:				
$> 400$	28	1.2	0.051-0.430	0.05*
$\leq 400$	20	5.1		
Response to treatment:				
No remission	36	4.7	0.038-0.420	0.001*
Complete remission	12	0.3		
Tie-2:				
$\geq 8$	28	5.6	0.061-0.710	0.005*
$< 8$	20	6.3		
Ang-2:				
$\geq 2500$	32	6.3	0.0710-0.610	0.05*
$< 2500$	16	1.6		

\*Significant

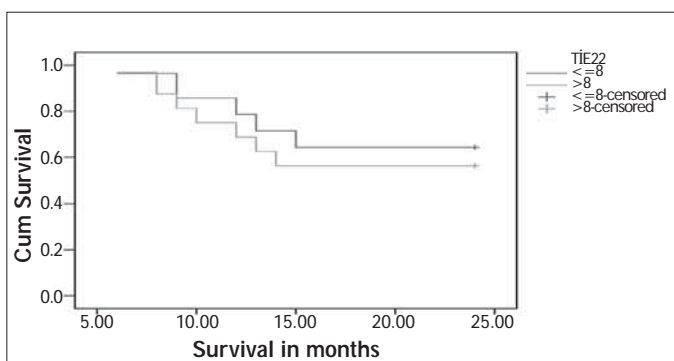
**Table 5. Multivariate analysis for risk factors associated with Tie-2 and Ang-2**

Risk factors	Tie-2		Ang-2	
	Wald	p	Wald	p
TLC	0.656	0.418	6.683	0.010*
LDH	4.471	0.034*	3.285	0.070
Response to treatment	31.137	0.001*	25.138	0.010*

\*Significant



**Figure 1.** Kaplan-Meier curve for overall survival in AML patients in relation to plasma Ang-2 levels. The overall survival is significantly shorter in AML patients with plasma Ang-2  $\geq 2500$  pg/ml than in those with levels  $< 2500$  pg/ml



**Figure 2.** Kaplan-Meier curve for overall survival in AML patients in relation to plasma Tie-2 levels. The overall survival is significantly shorter in AML patients with plasma Tie-2  $\geq 8$  ng/ml than in those with levels  $< 8$  ng/ml

Tie-2 were found to be significantly higher in patients group when compared to the control group. The plasma levels were found to be strongly associated with TLC, LDH levels and the extent of BM infiltrations. The observed differences could be explained in part by the source of circulating Ang-2, which originates not only from leukemic blasts but also from other cell types such as endothelial cells. In fact, it is entirely possible that a predominant proportion of circulating Ang-2 could be secreted by activated BM endothelial cells, given the increased

angiogenic activity in the leukemic BM. Furthermore, it is conceivable that Ang-2 could also be released by the endothelium of the normal vasculature upon dysregulated stimulation by leukemic blasts [19]. Our findings are in line with a recent observation of elevated Ang-2 and Tie-2 levels in patients with chronic myeloid leukemia (CML) and multiple myeloma [14]. It was found that blood levels of sAng-2 and sTie-2 were strongly associated with the extent of BM infiltration, TLC and LDH, providing indirect evidence that the leukemic blasts themselves at least significantly contributed to the plasma levels of the tested angiogenic factors [19].

Furthermore, a strong correlation could be demonstrated between plasma levels of Ang-2 and Tie-2 and the overall survival in AML patients after chemotherapy. The overall survival rate of AML patients was significantly lower in the group having higher Ang-2 and Tie-2 levels ( $\geq 2500$  pg/ml and  $\geq 8$  ng/ml, respectively).

Schliemann et al. [19] found that pre-therapeutic levels of circulating Ang-2 and Tie-2 were significantly higher in AML patients as compared to normal controls, and elevated levels were also observed in patients with CML and multiple myeloma. Furthermore, they could demonstrate a strong correlation between systemic levels of Ang-2 and overall survival in intensively treated AML patients. Patients with high plasma levels displayed significantly worse overall survival than those with low levels. The RR of death was more than four times higher for patients with high Ang-2 levels. The three-year survival rate for AML patients with high levels of Ang-2 was only 14.7% compared to 64.7% for those with low Ang-2 levels. Furthermore, Lee et al. [20] reported that AML patients with lower Ang-2 and Tie-2 levels displayed a survival advantage.

The mechanisms responsible for the difference in prognosis between AML patients with high and low Ang-2 expression remain unclear. The observed differences in survival might result from modulation of BM neovascularization. Angiogenesis is an inevitable step in the development and progression of malignancy. In the absence of vascularization, tumors cannot grow to more than 1-2 mm in diameter, probably because this size is the maximum for allowing oxygen diffusion from vessels. Neoangiogenesis supplies a tumor with oxygen and nutrients, while the newly formed endothelial cells appear to stimulate the growth of adjacent tumor cells by secreting numerous active peptides [21]. It has also been

documented that the tumor cells themselves emit proteins that stimulate neoangiogenesis and this process is correlated with the diffusion of metastases [22]. Elevated levels of circulating Ang-2 have been linked with angiogenesis in malignancies such as angiosarcoma [23], breast cancer [24], and multiple myeloma and CML [14]. In analogy, high levels of Ang-2 in AML may contribute to angiogenesis in the BM in the presence of mitogenic mediators, thereby facilitating leukemic blast proliferation and disease progression. However, Schliemann et al. [13] reported that microvessel counts did not show any association with clinical outcome, and they suggested a potential alternative mechanism independent from angiogenesis, which might have greater impact on prognosis in AML patients intensively treated with chemotherapy.

The findings of our results are not in line with Loges et al. [15], who stated that cellular Ang-2 expression could be identified as an independent predictor of favorable prognosis in AML patients. However, this discrepancy can be explained by the fact that intracellular levels of angiogenic factors may not reflect their blood levels, and circulating Ang-2 most likely originates not only from leukemic blasts but also from other cell types such as endothelial cells. It is possible that a predominant proportion of circulating Ang-2 could be secreted by activated BM endothelial cells given the increased angiogenic activity in the leukemic BM. Ang-2 could also be released by the endothelium of the normal vasculature upon dysregulated stimulation by leukemic blasts [19]. Therefore, different prognostic values may be observed when investigating either cell-associated or circulating Ang-2 separately.

Previous studies have documented that Ang-1 mediates vascular stability while Ang-2 induces vascular instability by overriding Ang-1-mediated Tie-2 activation [25]. Thus, the balance between Ang-1 and Ang-2 determines the grade of endothelial Tie-2 phosphorylation. Ang-1 appears to be the dominant Tie-2 ligand in normal BM. This balance strongly shifts towards Ang-2 during leukemic transformation. Reversal of the normal angiopoietin balance in favor of Ang-2 acting in concert with other angiogenic inducers may be essential for BM angiogenesis in AML [13].

In conclusion, a better understanding of the precise functions of angiopoietin-signaling pathways in AML may open new options of therapeutic interventions, and modulation of the autocrine angiopoietin/

Tie-2 axis may be a promising approach to improve the outcome in AML patients. Furthermore, circulating Ang-2 and Tie-2 may represent attractive therapeutic targets when introducing anti-angiogenic strategies into the treatment of AML.

Finally, measurement of circulating Ang-2 and Tie-2 concentrations at disease presentation may find its way into clinical routine as an additional prognostic tool in the risk-stratified management of AML.

#### Conflict of interest

No author of this paper has a conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included in this manuscript.

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