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ORIGINAL ARTICLE

Soluble angiopoietin-2/sTie2 receptor ratio is an independent prognostic marker in adult acute myeloid leukemia

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Abstract

Aim Angiogenesis is the formation of new blood vessels from preexisting one. The angiopoietins act a central role in these process. The aim of the present study is to the assess the impact of the circulating levels of angiopoietin-1 (Angi-1), Angiopoietin-2 (Angi-2), soluble Tie2 receptor (sTie2), and calculated Angi-2/sTie2 ratio on overall survival in 71 acute myeloid leukemia patients and 19 normal controls.

Materials and methods Ang-2, and calculated angi-2/sTie values were significantly higher in AML patients as compared to healthy volunteer (P= 0.002, 0.015 respectively) on the other hand Angi-1 was not significantly different in patients and control.

Results In univariate Cox proportional hazards model Angi-2, sTie2, angi-2/sTie2 ratio were predictive of poor survival. In multivariate analysis the calculated angi-2/sTie2 ratio is independent prognostic factor, with relative risk of 3.939, with 95% confidence interval 0.002–0.001.

Conclusion The calculated angiopoietin-2/sTie2 ratio represent an independent prognostic factor in AML and its value should be considered when considering anti angiogenic therapy.

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Introduction

Acute myeloid leukaemia (AML) is an aggressive disease with high mortality rate and disease free survival is rare [1]. It has been clarified that interaction between hematopoietic cells and endothelial cells is important for proliferation and differentiation of both cell lineage [2].

Several studies are concerning critical role of angiogenesis in development and growth of solid tumors and hematological malignancies [3]. Moreover, angiogenic mediators produced by AML cells act through external or internal autocrine loops, thereby directly promoting cell survival, proliferation and disease progression [4]. It has been established that vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b FGF) are major regulators of tumor associated angiogenesis in AML, cellular VEGF represents an adverse prognostic factor [5].

Recently many studies focused on angiopioetins (Angi) that is a novel family of angiogenic mediator which have been shown to be important regulators of angiogenesis and vascular stability [6]. Angi-1 and its antagonist Angi-2 act via the receptor tyrosine kinase sTie 2, which is expressed in endothelial cells (ECs) of the vasculature and in subset of hematopoietic stem cells [7]. Binding of Angi-1 causes phosphorylation of sTie2 and ensures the integrity of the vasculature by promoting interactions between ECs and periendothelial support cells [8]. In contrast, the non-signal transducing ligand Angi-2 disrupts sTie2 activation, resulting in vessel destabilization and facilitating the angiogenic response to mitogeneic factors [9]. In tumor, the balance between both angiopiotin is shifted toward Angi-2, rendering the vasculature susceptible for mitogenic growth factors

such as VEGF or bFGF and resulting in extensive neovascularization [10].

Over expression and prognostic significance of cellular angiopiotin in AML bone marrow and isolated peripheral AML blast is not clarified Loges 2005 [11] showed patients with high cellular Angi-2 had extended overall survival compared to patients with low Angi-2 expression.

The aim of this study is to assess the pretreatment levels of plasma Angi-1, Angi-2 and sTie2, and the calculated ratio of Angi2/sTie2 receptor in a cohort of 71 AML patients and to study the impact of their levels on the AML patients overall survival

Material

This study was carried out on 71 AML newly diagnosed patients. The patients characteristics are shown in table 1. The patients were followed up to 24 months or until death (table 1). All patients were treated with approved protocols and were followed regularly in oncology department in Mansoura Cancer Institute. The AML patients were treated by 3+7 protocol (Daunorubicin 45 mg/m2 iv. days 1–3; Cytarabine 100 mg/m2/day continous infusion for 7 days). Post remission high dose cytarabine. For patients who did not response to induction therapy; salvage therapy (HAM protocol) was applied (Cytarabine 3 gm/m2 bid iv 3hours infusion days 1–3; Mitoxantrone 10 mg/m2 iv days 3–5. the patients achieving complete hematological remission (Bone marrow blast cells <5% in Bone marrow) underwent consolidation therapy containing high dose cytrabine containing regimen (2 g/m2/2hours/day × 4 days) + Daunorubicin 45 mg/m2/day × 3 days. The M3 patients received all-trans retinoic acid in addition to chemotherapy. Nineteen normal healthy subjects were taken as a normal control group.

Table 1 Patients chracteristics

Parameter	AML Patients
No	71
Median age /years (range) Sex	34 (16–55)
Male	40
Female	31
FAB subtypes	
M0	2
M1	12
M2	19
M3	3
M4	18
M5	10
M6	4
M7	3
Karyotypes	
Favorable; t(8:21),t(15-17), inv(16)	21
Intermediate; normal,+8,+22, others	41
Poor complex,–5,–7	9
Peripheral WBCs × 10^3/cmm	16.0 (9.5–89.2)
Peripheral blast cells %	20 (12.0–70.0)
Bone marrow Blast cells%; median(range)	55% (12–88%)
Follow up	24 months

Methods

Six milliliters of peripheral blood were collected in a sterile tube with EDTA from each AML patient at presentation and before start of induction chemotherapy. Plasma was separated by centrifugation at 1500 Xg for 10 minutes in a refrigerated centrifuge. The plasma was stored at –70ºC and was thawed before assay**.** Enzyme-linked immunosorbent assay (ELISA) were performed using commercially available kits from R&D systems (Minneapolis, MN,USA) according to the manufacturer's instruction.

Briefly, patient samples were collected using the anticoagulant ethylene diaminetetra- acetic (EDTA) and stored at 80°C. Plasma samples were added to separate microplates, each containing a specific antibody for Angi-1 Angi-2 or sTie2. Mixtures were incubated at room temperature for 2 h. Plates were washed four times to remove unbound antigen. Enzyme linked polyclonal antibodies specific for each angiogenic factor were then added and the mixture incubated for 2 h, followed by another washing step. Subsequently, the substrate solution was added to the wells. Color development was stopped, and the intensity of color was measured and compared with a standard curve. Optical density of each well was determined at 570 nm.

Statistics

All calculations were performed using the software package SPSS version 10. Differences in angiogenic factors level between AML and control groups, were analyzed using Mann-Whitney rank sum test for independent groups. In addition correlations between continuous variables were assessed by the spearman rank correlation coefficient (rs).

Survival curves were estimated using the Kaplain-Meier method. Overall survival was the primary outcome studies and was calculated from the date of first diagnosis to death from any cause. Univariate and multivariate Cox regression analysis were performed to evaluate the predictive effect of each angiogenic factor. Optimal cut off points were depend on 50% percentile of each angiogenic factor.

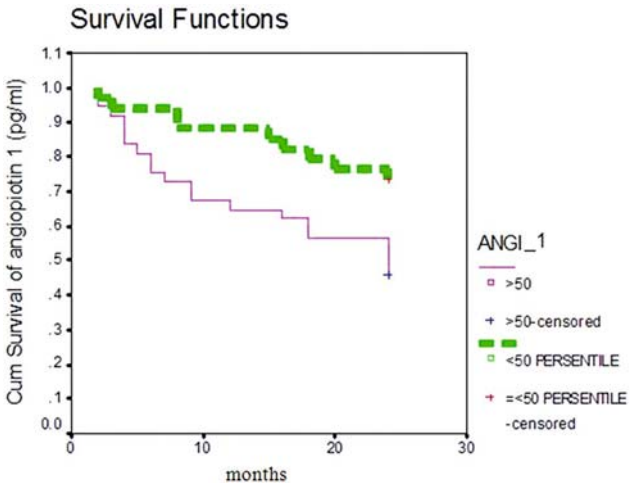

Fig. 1 Kaplan-Meier of overall survival analysis of the AML patients according to Ang1 levels. AML patients with high Angi1 levels >260 = 260 displayed significantly poor survival rates than those with low ≤ = 260 (P = 0.018)

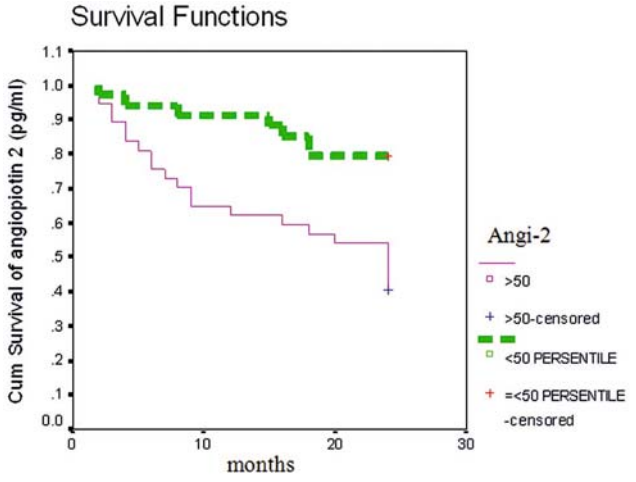

Fig. 2 Kaplan-Meier of overall survival analysis of the AML patients according to Ang1 levels. AML patients with high Angi1 levels >1400 displayed significantly poor survival rates than those with low ≤ = 1400 (P = 0.001)

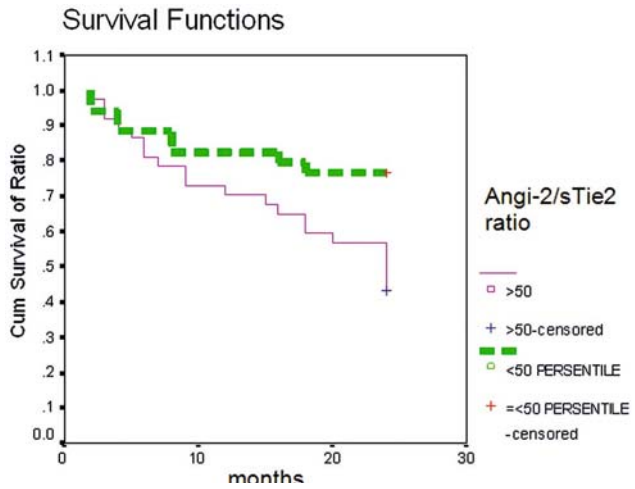

Fig. 3 Kaplan-Meier of overall survival analysis of the AML patients according to Angi-2/sTie2 ratio. AML patients with high Angi-2/sTie2 ratio >279 displayed significantly poor survival rates than those with low ratio ≤ = 279 (P = 0.004).

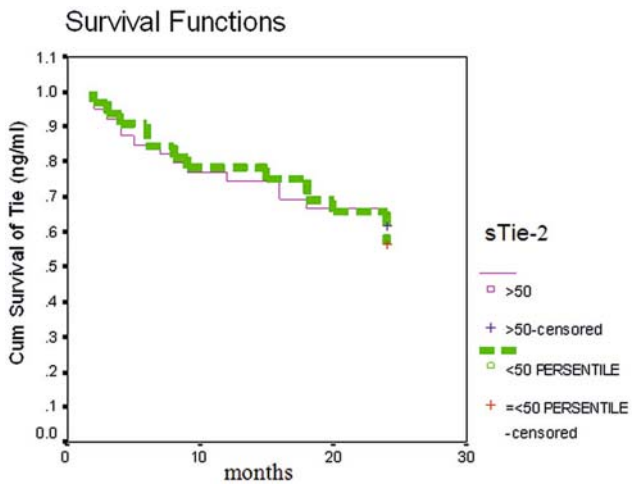

Fig. 4 Kaplan-Meier of overall survival analysis of the AML patients according to sTie receptor levels. AML patients with high sTie receptor>3.9 displayed insignificantly effect on survival rates than those with low levels ≤ = 3.9. (P = 0.652)

Results

Plasma levels of Angi-1, Angi-2, sTie2 and Ang-2/sTie2ratio in AML patients and healthy controls

Median and range of plasma levels of Angi-1, Angi-2, sTie2 in pre-therapeutic AML patients and healthy volunteer were presented in Table 1. Circulating level of Angi-2 and the calculated Angi-2/sTie2 ratio are significantly higher in AML patient as compared to controls (P=0.002, P= 0.015 respectively). On the other hand the Anig-1 and sTie2 levels were not significantly different in AML patients as compared to controls (Table 2).

Association between plasma levels of Angi-1, Angi-2, sTie2, calculated Angi-2/sTie2 ratio and clinico-pathological features

There is significant correlation between Angi-2, calculated Angi-2/sTie2 ratio and patients age (P < 0.05), but no correlation with sTie2 receptor (P = 0.786). Further more, WBCs is strongly correlated with Ang-2, sTie2 (r = 0.338, P = 0.004,; r = 0.263, P = 0.027) but not with Ang-1 and calculated Ang-2/sTie2 ratio.

Peripheral blood blast cells percentage was positively correlated to Ang-2 and sTie2 receptor (r = 0.365 P = 0.002, r = 0.387 P = 0.001); but was not signifi-

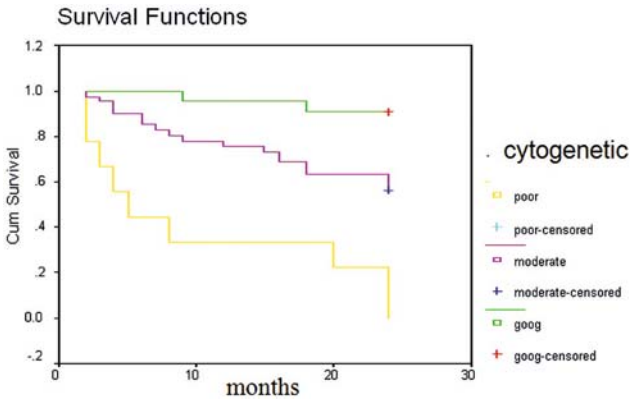

Fig. 5 Kaplan-Meier of overall survival analysis of the AML patients according to cytogenetic grade. AML patients with poor vs intermediate vs high displayed significantly poor survival rates (p = 0.000) .

(p < 0.005). The angiogenic factors Angi-1 (≤ 260 vs > 260), Ang-2(≤ 1400 vs > 1400), Angi-2/sTie ratio(≤ 279 vs > 297) were significantly associated with effect on AML survival but sTie2 show no significant effect. The relative risk (RR) of death of Ang-2 was significant higher when base line > 1400 pg/ml (RR 5.7, 95% confidence interval (CI) 0.061–0.510:, p = 0.001), in addition high significant with ratio. The relative risk (RR) 4.1 95% confidence interval (CI) 0.084–0.653: P = 0.004). In contrast no significant role of sTie2 >3.9 ng/ml (RR 1.24 95% confidence interval 0.481–3.220 : p = 0.652) (Table 4).

Furthermore, we performed multivariate Cox regression analysis incorporating all variables that were of significant effect on univariate analysis. The calculated Angi-2/sTie2 ratio were identified to be as the most prognostic factor with significant independent impact on survival (p = 0.000) (Table 5)

Table 2 Plasma angiopoietins levels in AML patients as compared to control

	Angi-1 (pg/ml)	Angi-2 (pg/ml)	sTie2 receptor ng/ml	Calculated Angi-2/sTie
Patient (n= 71)	260 (0-2250)	1400 (133-4800)	3.9 (1.0-35)	279 (32.4-1233)
Control (n=19)	180 (.0-2195)	392 (185-1520)	3.1 (2-3.9)	139 (78-471)
P value	0.573	0.002**	0.057	0.015*

Table 3 Correlation of angiopoietiens and other prognostic markers

	Angi-1	Angi-2	sTie2 receptor	Angi-2/sTie2 ratio
Age(years)	0.254 0.030*	0.336 0.004*	-0.033 0.786	0.287 0.015*
WBCs (x10^9/L)	0.170 0.155	0.338 0.004*	0.263 0.027*	0.205 0.086
Peripheral blood blast cell %	0.183 0.127	0.365 0.002*	0.387 0.001*	0.169 0.158
Blast in BM %	0.137 0.254	0.305 0.010*	0.317 0.007*	0.187 0.119
LDH	0.513 0.000*	0.362 0.002*	0.262 0.027	0.146 0.225
Cytogenetic	0.258 0.030*	0.426 0.000*	0.170 0.157	0.333 0.005*

cantly correlated with Ang-1, or Ang-2/sTie2 ratio (Table 3). LDH is significantly correlated with Angi-1, Angi-2, sTie2 but not with Angi-2/sTie2 ratio. Angi-2 and Angi-2/sTie2 ratio are positively correlated with cytognetic grades, but not correlated with sTie2 receptor levels (P > 0.1).

Association between plasma levels of Ang-1, Ang-2, sTie2, calculated Angi-2/sTie2 ratio and overall survival

To assess the effect of circulating Ang-1, Ang-2, sTie2 levels and calculated Angi-2/sTie2 ratio on the AML overall survival; we initially do univariate Cox proportional hazard analysis depending on 50% percentile as a cut off value. As previously settled the clinico-pathological variable as cytogenetic (good vs intermediate vs poor), LDH (≤ 450 vs > 450) have a significant effect on the overall survival

Discussion

Continuous efforts were done in order to predict the AML patients outcome. Over the past few years, several attentions was directed to the prognostic impact of angiogenic growth factors in hematological malignancies. The results of the present study demonstrated that calculated Angi-2/sTie-2 ratio is the independent prognostic parameter that could predict AML patients outcome. Also, there is significant elevation in the levels of Angi-2 and the calculated Angi-2/sTie2 ratio in AML patients as compared to normal controls. These findings are partially in agreement with that of Quartarone et al. [12] who reported similar findings in chronic myeloid leukemia and multiple myeloma; and with the finding of Schliemann et al. [13] in AML patients.

The exact origins of Angi-2 and its soluble receptor in blood stream remain to be elucidated, and further research

Table 4 Univariate analysis of overall survival in AML patients

Variables	No	RR (oddis ratio)	95% CI	P-Value
Age (years)				
≤ 25	33	0.894	0.346–2.311	0.817
>25	38	1.12		
Sex				
Male	38	1.11	0.37–1.16	0.818
Female	33	0.88		
WBCs (× 10^3/l)				
≤ 16.2 × 10^3	36	0.104	0.034–0.320	0.000
> 16.2 × 10^3	35	3.62		
Bone marrow infiltrate				
≤ 50	38	0.202	0.073–0.561	0.002
>50	33	4.95		
Karyotype				
Good	21	2.34	1.11–4.17	0.014
Moderate	41	1.00		
Poor	9	0.27	0.02–2.19	0.235
LDH (U/L)				
≤ 450	36	0.143	0.049–0.417	0.0000
>450	35	6.99		
Angio-1 (pg/ml)				
260	34	0.306	0.113–0.831	0.0180
<260	37	3.27		
Angio-2 (pg/ml)				
≤ 1400	34	0.177	0.061–0.510	0.001
>1400	37	5.65		
sTie-receptor (ng/ml)				
3.9	32	1.2	0.481–3.22	0.652
>3.9	39	44.81		
Ratio				
279	34	0.234	0.064–0.6530	0.004
>279	37	4.27		0

Abbreviations: AML, acute myeloid leukemia ; CI confidence intervals ; RR , relative risk ; sTie2 , soluble Tie2 receptor ; WBC ; white blood cells. Our result depended on optimal cutoff points to define poor or good prognosis (Table 4).

Table 5 Multivariate regression analysis of all variants

Model	95% confidence interval for B		T	P
	Lower bound	Upper bound		
Angiopiotin1	0.000	0.000	–0.068	0.946
Angiopiotin2	0.000	0.000	1.829	0.072
Ang-2/sTie ratio	–0.002	–0.001	–3.939	0.000**
WBCs	–0.007	0.002	–1.092	0.279
Peripheral Blast	–0.016	0.004	–1.266	0.210
cell %	–0.010	0.008	–0.299	0.766
Blast in BM %	0.000	0.000	–0.732	0.467
LDH				

**Multivariate regression analysis of angiogenesis markers with other variant clarified that ratio is the most predictor (p = 0.000)

into this issue is required. However, several explanation were postulated suggesting that the blast cells, and other cell types as endothelial cells might be a source [13]. In fact, it is entirely possible that predominant proportion of circulating Angi-2 could be secreted by activated bone marrow endothelial cells, given the increased angiogenic activity in the leukemic bone marrow. However, the strong correlation between the plasma levels of Angi-2, and sTie-2 levels with bone marrow blast cell counts, peripheral blood WBCs counts, and LDH, suggesting that the blast cells are the main source of plasma levels of these angiogenic factors.

The levels of Angi-2 might affect the prognostic impact of AML. As angiopoietin signaling in AML most likely compromise paracine, autocrine and eventually intracine loops. There is growing evidences that AML blasts express the angiopoietin-2 and receptors Tie-2 [14, 15], and established Angiopoietin/Tie-2 loop. The soluble Tie-2 receptor compete with the cellular Tie receptor for interaction with Angi-2 leading to down regulation of the effect of Angi-2 on AML cells. Therefore, theoretically the increased levels

of sTie2 has a good impact on the AML patients outcome which is not the case in our results.

We observed strong correlation between circulating levels of Angi-1, Angi-2, sTie2 and Angi-2/sTie ratio and overall survival in the those AML patients. Patient with high Angi-2 displayed significantly worse overall survival than with low levels. Although multivariate regression analysis revealed that the prognostic impact of Angi-2/sTie2 ratio not Angi-2 was independent prognostic factor from established prognostic markers as cytogenetic, LDH, WBC which in generally accepted as the most important risk factor in AML. Our finding not coordinated with Loges et al. 2005 in which cellular Angi-2 is identified as an independent predictor of a favorable prognosis in AML patients [14]. These difference between soluble Angi-2 and cellular expression of Angi-2 could be explained in part on the basis that the source of circulating Angi-2 not only from leukemic blast but also from other cell types such as endothelial cells [13].

The studies considering the prognostic relevance of circulating Angi-2/sTie ratio in AML, other hematological malignancies or solid tumors is very scarce. however elevated level of Angi-2 have been detected and linked with angiogenesis in malignancies such as angiosarcoma [16], breast cancer [17], multiple myeloma and chronic myeloid leukaemia [12] and recently in acute myeloid leukemia [13].

In this sense, Angi-2 in circulation may represent an attractive therapeutic target when introducing antiangiogenic strategies into treatment of AML. The therapeutic efficacy of blocking Angi-2 has already been demonstrated in solid tumors [18] and a recombinant Fc fusion protein directed against the action of angiopiotins is currently being evaluated in a phase I study in patients with advanced solid tumors.

Conclusion

The result of our study clearly demonstrate the calculated ratio between Angi-2/sTie is an independent prognostic factor and should be part of the decision making and should be considered the use of antiangiogenic therapy.

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