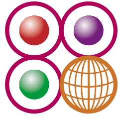

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ORIGINAL ARTICLE

Investigating the expression pattern of the angiopoietin-Tie system in ALL and its correlation with baseline characteristics

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Background
Acute lymphoblastic leukemia (ALL) is the most common type of leukemia in children. Several environmental and genetic factors are known to be involved in its development and progression. The angiopoietin-Tie system is one of the most critical factors in angiogenesis, and its possible role in solid tumors and leukemia has been previously investigated. In this study, we examined the expression of these genes in ALL patients (early pre-B-ALL and pre-B-ALL) and compared them with normal samples.

Methods
Bone marrow samples were collected from 40 patients (aged 0–19 yr) newly diagnosed with early pre-B-ALL or pre-B-ALL using molecular and flow cytometric tests and from 15 control individuals. For molecular tests, RNA extraction and cDNA synthesis were performed, and *Ang1, Ang2, Ang4, Tie1*, and *Tie2* gene expression was examined by real-time polymerase chain reaction.

Results
Ang2, Tie1, and *Tie2* gene expression were significantly increased in patients with ALL, whereas *Ang1* gene expression was decreased. The *Ang4* gene did not show significant expression changes between the two groups.

Conclusion
Changes in the expression of the Ang-Tie system indicate a possible role of angiogenesis in ALL prognosis. Moreover, such changes can be considered as potential diagnostic biomarkers or therapeutic targets.

Key Words Angiopoietin, Leukemia, Tie receptor, Acute lymphoblastic leukemia

INTRODUCTION

Leukemia is caused by the unrestrained proliferation of hematopoietic stem cells and was reported to account for 3.8% of cancer deaths as of 2020 [1]. Acute lymphoblastic leukemia (ALL) is a clonal malignancy of the lymphoid line of blood cells. It is the most common childhood cancer and the most common cause of cancer death in individuals younger than 20 years in the United States [2]. Based on the involved cell line, the World Health Organization divides ALL into B-ALL and T-ALL, each of which can be detected by immunophenotyping. B-ALL is the most prevalent type among both children (88%) and adults (75%) [3]. Despite numerous studies on the etiology of ALL, its causative agents have not been precisely identified. However, heredity, infections, and environmental factors such as radiation exposure play an essential role in ALL pathogenesis [2, 3]. Numerous studies have shown angiogenesis in leukemia by reporting increased levels of various angiogenic factors in patients and have suggested that anti-angiogenic drugs can act as a treatment agent for leukemia [4, 5]. The balance between angiogenic and anti-angiogenic factors secreted by vascular endothelial cells regulates angiogenesis, thus de-

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termining leukemia progression. Different types of angiogenic factors and their role in angiogenesis have been previously described; these factors include VEGF, FGF, angiopoietin, TGF, PIGF, HGF, HIF, and TNF [4, 6, 7].

The angiopoietin family (Ang1, Ang2, and Ang4) and its receptors (Tie1 and Tie2) are some of the most significant and well-known angiogenic factors [8]. Ang1 and Ang2 play antithetical roles in the process of angiogenesis, and *Ang4*, the human ortholog of *Ang3*, has a similar role to Ang1 [9, 10]. Ang1, one of the most noteworthy members of the Ang-Tie system, is a Tie2 agonist. By binding to and phosphorylating Tie2, this protein induces survival, proliferation, and migration signals in endothelial cells and, subsequently, maturation. It is also essential in inflammation [9, 11-13]. Ang2 acts as a natural Ang1-Tie2 antagonist. In the presence of VEGF, Ang2 causes the separation of pericytes from arteries. It also increases vascular permeability and prepares endothelial substrates for angiogenesis [10]. However, Ang2 can act as a relative Ang1 agonist with anti-apoptotic effects in cases of decreased Ang1 levels or high concentrations of Ang2 *in vitro* [14]. Ang4, much like Ang1, phosphorylates and activates Tie2 and its downstream pathways such as the PI3K signaling pathway. Furthermore, it causes endothelial cell survival, migration, and angiogenesis and increases blood vessel permeability. Ang4 also dilates lymph vessels and exhibits increased levels under hypoxic conditions *in vitro* [15]. However, little research has been done on this member of the angiopoietin family.

The primary receptor of angiopoietins is Tie2, which upon binding with ligands, is activated by autophosphorylation and activates its downstream pathways [16, 17]. A soluble type of the Tie2 protein regulates angiopoietin activity by binding to one angiopoietin type (most commonly, Ang1) to increase the activity of the other angiopoietins [18]. Tie1 is another angiopoietin receptor. No ligand is known for this receptor, and its function is induced through the formation of heterodimers with Tie2. Tie1 binds to Tie2 at the cell surface and prevents angiopoietins from binding to Tie2 [17]. The Ang-Tie system plays a key role in the angiogenesis, proliferation, and maturation of vascular endothelial cells and is a useful therapeutic target in solid tumors and hematologic malignancies [19]. However, few studies have been performed to determine expression level changes and the function of the angiopoietin family in ALL [20, 21].

In this study, we aimed to quantify the expression levels of the genes in the Ang-Tie system (*Ang1, Ang2, Ang4, Tie1*, and *Tie2*) in newly diagnosed ALL patients compared with those in normal samples.

MATERIALS AND METHODS

Study population

Forty bone marrow (BM) samples from newly diagnosed ALL patients (aged 0–19 yr) and 15 normal BM samples (from individuals aged 0–19 yr) were obtained from the bone marrow transplantation section of Taleghani Hospital in Tehran from March to October 2020. Pre-B-ALL and early pre-B-ALL cases, which are the most common types of ALL, were included in this study. Normal BM specimens were selected from individuals who were referred to our hospital because of high peripheral blood (PB) WBC count, but their BM specimen examination and flow cytometric tests did not confirm any malignancy or other diseases influencing the BM. The patients signed a consent form, and questionnaires were filled out for demographic data collection. The study protocol was approved by the ethical committee of Tabriz University of Medical Sciences (code: IR.TBZMED.REC.1397.1040).

Quantitative real-time polymerase chain reaction

The nucleotide sequences of designed primers for the target and normalizer genes are shown in Table 1. Total RNA was extracted and purified using the Qiagen RNeasy Mini Kit (Cat. No: ID: 74104; Qiagen, Germany) according to the manufacturer's protocol. Quality and concentration assessments of the RNA samples were carried out using a

Table 1. The forward and reverse primer sequences and polymerase chain reaction product lengths used in this study.

Gene	Direction	Sequence	Length	Product
Ang1	F	GCCAGAACCCAAAAAGGTGT	20	188
	R	GCCTCTGACTGGTAATGGCA	20	
Ang2	F	ACTGGGAAGGGAATGAGGCTTAC	23	167
	R	TTTGTCGTTGTCTCCATCCTTTGTG	25	
Ang4	F	ATTACAAACAGGGCTTCGGAGA	22	174
	R	ATAGCTGGTTCTCACTGCCC	20	
Tie 1	F	GGTTCTTGCGGACAGTGGGTTC	22	140
	R	GCTGGCGGCTCTGCTTGG	18	
Tie 2	F	ACCCTTAGTGACATTCTTCCTCCTC	25	155
	R	TGCTGGTCTTCATTCTTGCCTTG	23	
ABL	F	ACACTTCTAAGCATAACTAAAGGTGAAAAGC	31	117
	R	GATGTAGTTGCTTGGGACCCA	21	

Abbreviations: F, forward primer; R, reverse primer.

Thermo Scientific NanoDrop One (Thermo Fisher Scientific Inc., Waltham, MA, USA). The Thermo Scientific SuperScript IV Reverse Transcriptase kit was used for cDNA synthesis. We chose the *ABL* gene as a normalizer gene, and relative expression of the *Ang1, Ang2, Ang4, Tie1*, and *Tie2* genes were measured in BM samples of ALL patients and in normal samples. A total reaction volume of 15 μL was used for quantitative real-time polymerase chain reaction (qPCR). We used 7.5 μL of RealQ Plus Master Mix Green, without Rox (AMPLICON, Odense, Denmark) in each reaction, which was performed in the ABI StepOne Plus system in duplicates. As the first step of the qPCR, initial activation was performed at 95°C for 10 min. The thermal cycling program was carried out for 40 cycles with the following: denaturation (95°C for 10 s), annealing (58‒64°C depending on the gene for 20 s), and extension (72°C for 30 s). After cycling, the final extension step (72°C for 10 min) was performed.

Statistical analysis

Real-time PCR efficiencies and the cycle threshold (CT) were used in the ^^CT formula [Δct (treated sample)‒Δct (untreated sample)] to calculate the relative expression levels of the *Ang1, Ang2, Ang4, Tie1*, and *Tie2* genes in ALL patients and normal samples. Data normalization of gene expression levels was performed using the *ABL* gene as a housekeeping gene. All statistical analyses were performed using Prism software version 8 (GraphPad, San Diego, CA, USA) and the SPSS statistical software package (version 18.0). The Kolmogorov‒Smirnov test and the Mann-Whitney U test were used to check data normalization and to measure the differential expression levels of targeted genes in each sample type, respectively. The association between relative gene expression and demographic data was evaluated by Pearson's chi-square and Kruskal-Wallis tests. The diagnostic power of the ANG-TIE system genes was evaluated using receiver operating characteristic (ROC) curve analysis. Statistical significance was set at $P<0.05$.

Table 2. General baseline characteristics and clinical data of patients and controls.

Variable	Value	
	Patients	Controls
Age (yr, mean±SD)	9.3±5.4	10.6±6.5
First decade (N)	23	8
Second decade (N)	17	7
Sex (male/female)	1.2	1.5
Male (N)	22	9
Female (N)	18	6
WBCs in PB (×10^3/μL, mean±SD)	81.4±40.2	11.2±5.7
<50 (N)	15	15
>50 (N)	25	None
BM blasts (%, mean±SD)	79±17.7	<5%
25‒50% (N)	4	None
51‒75% (N)	7	None
76‒100% (N)	29	None
WHO classification (pre-B-ALL/early pre-B-ALL)	1.5	None
Early pre-B-ALL (N)	16	None
Pre-B-ALL (N)	24	None

Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; PB, peripheral blood; SD, standard deviation; WBC, white blood cell; WHO, World Health Organization.

RESULTS

Clinical and demographic data of patients

In this study, 40 samples from newly diagnosed ALL patients and 15 samples from control individuals were collected. Demographic characteristics and data obtained from laboratory diagnoses of patients were collected using diagnostic tests or questionnaires filled out by the patients. Demographic and laboratory information of patients are shown in Table 2.

Expression levels of the Ang-Tie system genes in ALL patients

Statistical analyses were performed using the *ABL* gene as a housekeeping gene and 2^(‒ΔΔ$_{ct}$) to compare angiopoietin gene expression levels in ALL patients with control samples. *Ang1* showed a significant decrease in expression levels in patients with ALL (fold change=0.3, P=0.0165) (Fig. 1).

Conversely, *Ang2* (fold change=3.1, $P\leq0.0001$), *Tie1* (fold change=2.6, P=0.0014), and *Tie2* (fold change=2.8, P=0.0001) were significantly upregulated in ALL samples compared to control samples (Fig. 1). However, *Ang4* did not show a significant difference in expression levels between the two sample groups (P=0.09, Fig. 1).

Correlation of Ang-Tie system expression levels across clinical and demographic data

We performed a correlation analysis between the ex-

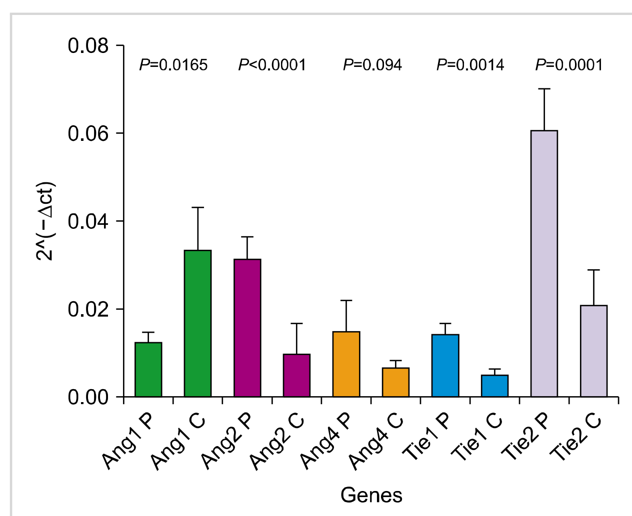

Fig. 1. Relative expression of Ang-Tie system genes (*Ang1, Ang2, Ang4, Tie1*, and *Tie2*) in patient samples and control samples. Abbreviations: C, control samples; P, patient samples.

pression levels of Ang-Tie system genes and ALL subtypes, patient age, blast count, sex, and PB WBC count. Table 3 shows that Ang-Tie system gene expression levels did not show a significant statistical associations across either patient demographic or clinical data.

ROC curve analysis

ROC curves were plotted to evaluate the diagnostic roles of *Ang1, Ang2, Ang4, Tie1*, and *Tie2* in ALL. Except for *Ang4*, the Ang-Tie system genes appeared to be appropriate diagnostic markers for ALL because their specificity, sensitivity, and the area under curve (AUC) values were higher than 70. In addition, the AUC values were statistically significant ($P<0.05$). Table 4 illustrates detailed information on the ROC curve analysis, and Fig. 2 depicts the diagnostic power of the genes.

DISCUSSION

ALL is caused by excessive proliferation and lack of differentiation in lymphoid blast cells, which eventually overwhelm the BM and PB. It is the most common cause of cancer-related deaths in individuals younger than 20 years

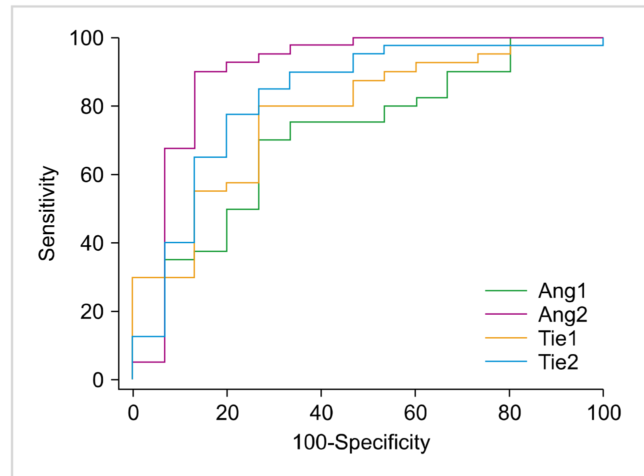

Fig. 2. Receiver operating characteristic curves of *Ang1, Ang2, Tie1*, and *Tie2* genes for predicting their respective diagnostic potential in acute lymphoblastic leukemia.

Table 3. *Ang1, Ang2, Agn4, Tie1*, and *Tie2* expression levels (means of ΔCt±standard deviation) according to the demographic and clinical data of the patients.

Characteristics	N	*Ang1*	*P*	*Ang2*	*P*	*Ang4*	*P*	*Tie1*	*P*	*Tie2*	*P*
Age			0.790		0.540		0.759		0.425		0.257
First decade	23	6.8±1.5		5.1±1.1		9.2±3.3		6.3±1.1		4.4±1.1	
Second decade	17	6.9±1.4		4.9±1.2		9.0±3.2		6.2±1.1		4.1±1.2	
Sex			0.830		0.236		0.556		0.317		0.688
Male	22	7.2±1.3		4.8±1.2		9.3±3.5		6.5±1.3		4.1±1.2	
Female	18	7.3±1.2		5.0±1.1		9.5±3.6		6.3±1.3		4.2±1.3	
BM blasts			0.431		0.756		0.457		0.345		0.264
25–55%	4	7.3±1.5		5.5±1.2		9.0±3.5		6.6±1.4		4.5±1.3	
56–75%	7	7.2±1.3		5.3±1.2		8.8±3.6		6.3±1.5		4.3±1.5	
76–100%	29	7.2±1.1		5.4±1.1		8.7±3.2		6.4±1.2		4.4±1.2	
PB WBCs			0.322		0.587		0.632		0.435		0.475
<50	15	7.4±1.3		5.4±1.2		9.0±3.3		6.1±1.2		4.2±1.3	
>50	25	7.1±1.2		5.3±1.4		8.9±3.5		6.2±1.6		4.4±1.4	
WHO classification			0.719		0.365		0.235		0.353		0.476
Early pre-B-ALL	16	6.7±1.3		5.2±1.3		8.6±3.4		6.2±1.3		4.2±1.4	
Early Pre-B-ALL	24	6.9±1.4		5.3±1.4		8.5±3.2		6.4±1.4		4.4±1.5	

Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; WBC, white blood cell; WHO, World Health Organization.

Table 4. Receiver operating characteristic curve analysis results.

Genes	Estimate criterion	AUC	J[a]	Sensitivity	Specificity	*P*
Ang1	≤0.012	0.71	0.43	70.0	73.3	0.01
Ang2	>0.007	0.89	0.76	90.0	86.6	<0.0001
Tie1	>0.005	0.77	0.53	80.0	73.3	0.0001
Tie2	>0.015	0.82	0.58	85.0	73.3	<0.0001
Genes combination	>0.045	0.71	0.38	85.0	53.3	0.01

Estimate criterion: optimal cut-off point for gene expression. [a]Youden index.
Abbreviation: AUC, area under the curve.

in the United States [2].

Angiogenesis and its causative factors play critical roles in different types of cancers. In solid tumors, hypoxic conditions follow tumorigenesis, and the balance between pro-angiogenic and anti-angiogenic factors tends to tilt towards pro-angiogenic factors, which cause angiogenesis and tumor spread [4, 22]. Angiogenesis is also responsible for the spread of leukemia, and several studies have been performed to discern the effects of angiogenic factors in various forms of leukemia [4, 5]. The angiopoietin family and their receptors are among the most fundamental angiogenic factors [9, 10]. In the present study, we investigated the expression of genes belonging to this family, as well as of their receptors, in patients with ALL.

Our results indicated that Ang1 was expressed at lower levels in ALL patients than in control samples. The expression level of this gene was not significantly different across demographic or clinical characteristics (Table 3). Ang1 competes with Ang2 for binding to the Tie2 receptor, with Ang1 having a higher affinity. The expression of Ang1 is likely decreased in patients with ALL, leading to Ang2 activation. Examinations of *Ang1* expression levels have shown variable results in different studies. In a study by Schliemann *et al.* [23], the expression of this gene in BM samples from AML (acute myeloid leukemia) patients did not show a statistically significant difference compared to normal samples. Conversely, other studies have shown Ang1 upregulation in AML patients, either in BM or PB samples [24-29]. In a study by Cheng *et al.* [28], increased expression of *Ang1* was reported in patients with myelodysplastic syndrome (MDS). They also demonstrated an association between high *Ang1* expression and short survival in patients with MDS [28]. On the other hand, Atesoglu *et al.* [30] and Karakurt *et al.* [20] demonstrated that the expression of *Ang1* was reduced in AML and ALL patients, similar to the findings of our study. Karakurt *et al.* [20] measured Ang 1 and Ang 2 levels in the BM of 20 patients with ALL. We evaluated the expression of all members of the angiopoietin family (*Ang1, Ang2*, and *Ang4*) and receptors of this family (*Tie1* and *Tie2*) in the BM of 40 ALL patients. Possible causes of inconsistencies in *Ang1* expression levels may include differences in measurement methodology, number of samples, and leukemia type.

Another gene in the angiopoietin family is *Ang2*, which is a natural antagonist of Ang1 and increases vascular permeability and angiogenesis [10]. Our study showed a significantly increased expression of *Ang2* in ALL patients compared to that observed in the control group. Numerous studies have been performed to evaluate the expression of this gene in hematological malignancies, all of which have shown an increase in patient *Ang2* expression levels compared to the control group [20, 23, 30-32]. However, a study by Cheng *et al.* [28] reported decreased expression levels of *Ang2* in MDS patients. Moreover, there was no statistically significant difference in the expression of *Ang2* across demographic and clinical characteristics in the present study. In a study by Aref *et al.* [31], a correlation between increasing the number of blasts and increasing the serum amount of the Ang2 protein in AML patients was shown. Additionally, Schliemann *et al.* [32] have shown a direct correlation between *Ang2* expression levels and the infiltration of leukemic blasts and an inverse association with the survival rate of AML patients. Ang2 also binds to $\alpha5\beta1$ integrin, causing cell proliferation or migration, leading to the spread of leukemia [18].

The least studied member of the Ang-Tie system is Ang4. The precise function of Ang4 in angiogenesis has not yet been defined, due to the operational similarities of Ang4 with Ang1 [15]. However, the expression of *Ang4* in the present study was not significantly different between the control and patient groups. Moreover, there was no statistically significant difference in its expression across patient demographics and clinical data.

In a study by Kivivuori *et al.* [33], no expression of the *Tie1* gene in pediatric or adult ALL patients was reported. In contrast, our study showed a significant increase in *Tie1* and *Tie2* expression levels in newly diagnosed ALL patients. This discrepancy may be due to differences in methodology because we measured gene expression in the present study, whereas Kivivuori *et al.* investigated the presence of protein. Increased expression of the *Tie1* receptor and its role in CLL development were first investigated by Aguayo *et al.* [34] in PB samples of patients with CLL using western blot analysis. Similar to our results, Verstovsek *et al.* demonstrated Tie1 upregulation in AML and CML patients [35, 36] and reported increased levels of Tie1 that were correlated with short survival of CML patients [36]. Increased levels of Tie1 may limit Ang1 function and consequently cause the spread of the tumor. Several studies have shown an increase in *Tie2* gene expression in various hematological malignancies, which corroborates our findings [23, 28, 32]. Previous studies have also considered this increase in *Tie2* gene expression as a marker of poor prognosis, especially in AML [16, 31, 37]. Notably, Schliemann *et al.* [32] reported that high expression of this receptor, along with the Ang2 gene, is directly related to leukemic blast infiltration and that it predicts short survival in AML patients. However, this increase in expression may lead to the production of soluble Tie2 (sTie2), which in turn may inhibit Ang1 activity [18]. However, there was no statistically significant association between *Tie1* and *Tie2* gene expression levels and patient demographics and clinical characteristics.

We performed ROC curve analysis to evaluate Ang-Tie system genes as diagnostic biomarkers in ALL samples. The combination of AUC values, sensitivity, and specificity of the Ang-Tie system genes can be considered as diagnostic biomarkers for patients with ALL. However, since we included only two types of ALL (pre-B and early pre-B-ALL), further studies with a large sample size that includes all types of ALL are needed to improve the sensitivity and specificity criteria of diagnostic markers. We recommend following up on the condition of patients after treatment to measure the effects of these proteins on prognosis, treatment, and survival. Furthermore, we believe it is crucial to investigate

Ang4 expression in order to determine the role of this gene in hematological malignancies, including ALL. We also recommend the evaluation of the effects of angiopoietin and angiopoietin receptor suppressive drugs, such as pexmetinib or nilotinib.

In brief, we observed a significant decrease in *Ang1* gene expression and an increase in *Ang2, Tie1*, and *Tie2* gene expression. With increases in Ang2 and Tie2 levels, a decrease in Ang1 levels, and the possible generation of sTie2, ALL may progress towards exhibiting increased vascular permeability and angiogenesis. It is worth mentioning that since angiopoietins are secreted from both endothelial cells and malignant blasts, the blasts themselves may play a role in the spread of cancer.

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Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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