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Original Article

The Relationship between the *Connexin 32* and *Connexin 43* Genes and the Pretreatment Stage and Short-term Follow-up of Patients with Acute Myeloid Leukemia

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Abstract

Background: Connexins (Cxs) are gap junction proteins involved in the communication between acute myeloid leukemia (AML) and stromal cells. They consist of intercellular channels termed "connexions", which can cause uncontrolled cell proliferation if dysregulated. This study aimed to evaluate the expression levels of the *Cx32* and *Cx43* genes and their correlations with other prognostic markers in patients with AML.

Methods: This cross sectional study was performed on peripheral blood samples from 60 newly diagnosed patients with AML and 40 healthy control subjects at Kasr Alainy School of Medicine, Cairo University, from June 2016 to December 2017. The quantitative real-time polymerase chain reaction (qRT-PCR) test was used to examine the relative expression level of *Cx43* and *Cx32* genes in the patients and the control subjects. The Chi square test or the Fisher exact test was employed to examine the relationship between qualitative variables, while the independent *t* test or the Mann–Whitney test was employed for quantitative data. All the tests were two-tailed, and a P value of less than 0.05 was considered significant.

Results: Among the patients with AML, 65% had a high *Cx32* expression level, whereas 63.3% had a low *Cx43* expression level. There was a statistically significant difference in the fold change values of *Cx32* and *Cx43* expression between the patient group and the control group (P=0.009 vs P=0.013, respectively). There was a remarkable association between both Cxs and CD34 and HLA-DR cells.

Conclusion: Cx expression in samples may add to the diagnostic workup of AML. Although we found a negative correlation between *Cx43* expression and the peripheral blood blast percentage, the response after the first induction of chemotherapy showed no significant relationship with *Cx43* and *Cx32*.

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Keywords ● Leukemia, Myeloid, Acute ● *Connexin 43* ● Gap junctions ● Gene expression

What's Known

- Gap junctions are involved in the communication between acute myeloid leukemia and stromal cells during disease development.
- The expression and significance of connexins in acute myeloid leukemia are currently unclear.

What's New

- Acute myeloid leukemia samples showed high expression levels of *connexin 32* and low expression levels of *connexin 43*.
- Response (remission, relapse, and death) after the first induction of chemotherapy showed no significant relationship with *connexin 32* or *connexin 43*.
- Both connexins were highly related to CD34 expression.

Introduction

Acute myeloid leukemia (AML) is the most common type of hematological malignancies, and it is characterized by key

properties such as blocked differentiation, enhanced self-renewal, and increased proliferation. Leukemia is an aggressive malignancy in which both disease development and chemosensitivity tend to be regulated by adjacent stromal cells (i.e., the cells that form the hematopoietic stem cell niche). Gap junction (GJ) proteins can play a role in the communication between AML and stromal cells during disease progression.[1]

GJ proteins constitute one of the several structures involved in intercellular communication.[2] Each of the two communicating cells contributes to these GJ proteins by their own connexin hemichannels (CxHcs), also termed "connexons", and each CxHc consists of six transmembrane proteins, called "connexins (Cxs)".[3] Each Cx shows tissue- or cell-specific expression, with most organs and many cell types expressing more than one Cx.[4] Since Cxs can oligomerize with other Cxs, a variety of CxHcs can be developed. GJ proteins have structural heterogeneity due to the large number of potential Cx combinations in each CxHc, which may also be the basis for functional heterogeneity.[5, 6] At least 21 different human Cxs have been identified, and they have been regrouped into five subfamilies.[5, 7] It has been posited that the dysregulation of Cx expression is related to uncontrolled proliferation and carcinogenesis.[2, 5, 7]

Cx32 is expressed by vascular endothelial cells and participates in endothelial GJ intercellular communication.[1, 2] *Cx43* is named according to the molecular weights in kDa of the proteins predicted from its complementary DNA (cDNA). Although GJ proteins are usually not present in circulating blood cells, *Cx43* is found to be expressed on leukocytes during inflammatory reactions in blood vessels in humans, and it has been shown to mediate coupling between bone marrow stromal cells (BMSCs) and CD34-positive blood cell precursors.[8] CD34 is expressed on leukemic stem cells and plays an important role in disease relapse and prognosis by minimal residual disease.[9] Many modulators of survival and death are exchanged through GJs. The disruption of normal Cx functions has an essential role in many solid tumors, but the expression and significance of Cxs in AML are currently unclear. Previous studies on Cxs have mainly focused on leukemic cell lines rather than patient samples.[10, 11] Clarifying this issue is of major clinical interest, because it might be linked to patients' prognosis and could help guide the optimal choice of therapy.

In the present study, we aimed to evaluate the expression levels of the *Cx32* and *Cx43* genes and determine their correlations with the severity of AML and different parameters, especially CD34, in the blood of patients with AML. We also sought to find relationships between the expression levels of the *Cx32* and *Cx43* genes and the short-term outcome after the induction of chemotherapy.

Patients and Methods

This cross-sectional study was conducted on peripheral blood samples from 60 newly diagnosed patients with AML and 40 healthy control subjects. The samples were collected from the Medical Oncology Department, Hematology Department, Internal Medicine Hospital, and French Hospital of Kasr Alainy, School of Medicine, Cairo University (Egypt), from June 2016 to December 2017, after taking written consent from the participants and obtaining approval from the Research Ethics Board of Kasr Alainy School of Medicine (ethics code I-361014).

The diagnosis of AML was established by consultants of hematological pathology, who work at the hospitals of Kasr Alainy School of Medicine. It was based on a full clinical assessment of the morphology, cytochemistry, and immunophenotype of leukemic blast cells. The classification of AML cases was based on the French–American–British (FAB) criteria.[12]

The quantitative real time polymerase chain reaction (qRT-PCR) test via the SYBR green technique was carried out to examine the relative expression level of *Cx43* and *Cx32* genes in the patients with AML and the control subjects, with the *β-actin* gene being used as an endogenous reference. RNA extraction from whole blood samples was performed using the QIAamp RNA Blood Mini Kit (QIAGEN, Germany, Catalog No. 52304). The conversion of the extracted RNA to cDNA was done by using the High Capacity cDNA Reverse Transcription Kit (QIAGEN, Germany, Catalog. No. 205313) according to the manufacturer's instructions. The qRT-PCR test was performed on a Thermal Cycler (Applied Biosystems, 7500, USA) using QuantiTect SYBR Green Master Mix (QIAGEN, Catalog No. 204141).[13] The reaction mixture (25 µL) contained 12.5 µL of SYBR Green qPCR Mix (2X), 2 µL of cDNA, and 1 µL of each primer pair. The relative mRNA expression level of the *Cx43 and Cx32* genes in each sample was calculated by using the ΔΔ cycle time (Ct) method, where the target PCR Ct value is normalized by subtracting the *β-actin* Ct value from the target PCR Ct and relative to the calibrator. The formula is applied as follows:

target amount=$2^{-\Delta\Delta CT}$

where $^{\Delta\Delta Ct}$ is the Ct value (cycle threshold), defined as the number of cycles required for the fluorescent signal to cross the threshold.[5] The expression levels of the *Cx43* and *Cx32* genes were expressed as a fold difference relative to the calibrator. Therefore, a $2^{-\Delta\Delta CT}$ value of greater than one was considered a high expression level compared with the control, and a value of less than one was considered a low expression level.

Statistical Analysis

Data were analyzed with IBM SPSS Advanced Statistics Software, version 22 (SPSS Inc., Chicago, IL). Numerical data with parametric distributions were expressed as mean±SD, while those with nonparametric distributions were expressed as the median and the range. Qualitative data were expressed as frequencies and percentages. The Chi square test or the Fisher exact test was utilized for the between-group comparisons of qualitative variables. The independent *t* test was employed for the between-group comparisons of quantitative data with parametric distributions, while the Mann–Whitney test was drawn upon for the between-group comparisons of quantitative data with nonparametric distributions. Additionally, the Kruskal–Wallis test was used to compare quantitative data with nonparametric distributions between more than two groups; and if the results were significant, *post hoc* analysis via the Dunn-Bonferroni procedure was carried out. Univariate and multivariate logistic regression analyses were applied to assess the factors associated with the patient group. The confidence interval was set to 95%, and the margin of error accepted was set to 5%. A P value of less than 0.05 was considered significant.

Results

The present study evaluated 60 patients with newly diagnosed AML, comprising 39 men (57.4%) and 21 women (42.6%). Their age ranged between 14 and 82 years with a mean value of 49.68±16.1 years. Additionally, 40 age and sex-matched control subjects, consisting of 21 men (52.5%) and 19 women (47.5%), were included. Their age ranged between 29 and 55 years with a mean value of 43.47±7.26 years (P=0.211 for sex and P=0.024 for age).

The multivariate logistic regression analysis showed that age had no effect on the difference between the patients and the controls regarding *Cx32* and *Cx43*. The results also showed a significant association between *Cx32* and the patient group (OR: 1.699, 95% CI: 1.053 to 2.741; P=0.030) and between *Cx43* and the patient group (OR: 0.327, 95% CI: 0.102 to 0.648; P=0.036).

Clinical Details of the Patients with Acute Myeloid Leukemia

The FAB classification of the 60 newly diagnosed patients with AML revealed that one case (1.7%) was FAB-M1, 23 (38.3%) were FAB-M2, 10 (16.7%) were FAB-M3, 23 (38.3%) were FAB-M4, and three (5%) were FAB-M5.

The immunophenotyping of AML blasts revealed the expression of CD34 in 89.9%patients (53/59), myeloperoxidase (MPO) in 91.6% (44/48), CD13 in 88.3% (53/60), CD33 in 86.6% (52/60), CD14 in 51.1% (23/45), CD11b in 62.9% (17/27), CD11c in 80.9% (17/21), and HLA-DR in 79.6%(47/59).

Cytogenetic analysis was performed for 28 patients in the AML group, and the results demonstrated t(8;21) in nine patients, t(15;17) in six, and inversion (16) in eight.

FMS-like tyrosine kinase 3/internal tandem duplication (FLT3/ITD) was performed on 16 samples of the patients with AML. It was detected in six samples (37.5%), while 10 samples (62.5%) showed normal (wild type) FLT3.

The patients with AML were categorized according to cytogenetic and molecular analyses into a favorable group (four patients), consisting of those with t(8;21) or t(15;17) but without FLT3/ITD mutation; an intermediate-risk group (six patients), composed of those with normal karyotyping but without FLT3/ITD mutation; and an unfavorable-risk group (six patients), comprised of those with FLT3/ITD mutation.

Connexin 32 Expression

Among the patients with AML, 21/60 (35%) had a low *Cx32* expression level, while 65% (39/60) showed a high *Cx32* expression level. The median *Cx32* expression level was 18 and ranged from 0.02 to 144. There was a statistically significant difference in the values of *Cx32* expression between the patients and the controls (P=0.009).

There was no significant relationship between *Cx32* expression and organomegaly (P=0.245), sex (P=0.525), the different risk groups (P=0.580), or the response after the first induction of chemotherapy (P=0.600) (table 1).

The *Cx32* gene expression showed a statistically significant difference between the patients with AML allocated to the different FAB categories (P=0.033). Notably, a statistically significantly lower *Cx32* expression level was encountered in the AML FAB-M3 patients (median [range])=0.48 [0.06 to 11]) than the AML FAB-M1

Table 1: Comparisons of the different categories of patients with acute myeloid leukemia as regards *connexin 32* expression

Patients/Clinical Details		n (%) (n=60)	Median (Range)	P value
Sex	Male	39 (65%)	30 (0.04–144)	0.525•
	Female	21 (35%)	14 (0.02–112)	
Organomegaly	Present	21 (35%)	11 (0.03–112)	0.245•
	Absent	39 (65%)	25 (0.02–144)	
FAB Subtype	M1 & M2	24 (40%)	22.5 (0.78–41.75)	0.033*
	M3	10 (16.70%)	0.48 (0.06–11)	
	M4 & M5	26 (43.30%)	26.5 (0.70–50)	
CD34	Negative	6 (10.20%)	0.16 (0.06–0.70)	0.045•
	Positive	53 (89.20%)	20 (0.76–39)	
Risk Groups	Favorable	4 (25%)	29.5 (14–41.75)	0.580*
	Intermediate	6 (37.50%)	0.73 (0.4–39)	
	Unfavorable	6 (37.50%)	15.5 (0.7–51)	
Response after First Induction	Remission	18 (41.90%)	15.5 (0.05–108)	0.600*
	Relapse	17 (39.50%)	20 (0.04–73)	
	Death	8 (18.6%)	8.77 (0.02–68)	

•Mann–Whitney test; *Kruskal–Wallis test; P<0.05 is considered significant. FAB: French–American–British

Table 2: Comparisons of patients with acute myeloid leukemia showing low and high *connexin 32* expression levels as regards different clinical and hematological data

Variables		High *Cx32* (n=39)	Low *Cx32* (n=21)	P value
Sex	Female	14 (35.90%)	7 (33.30%)	0.843*
	Male	25 (64.10%)	14 (66.70%)	
Organomegaly	No	23 (59%)	16 (76.20%)	0.182*
	Yes	16 (41%)	5 (23.80%)	
FAB Classification	M1 & M2	17 (43.60%)	7 (33.30%)	0.191*
	M3	4 (10.30%)	6 (28.60%)	
	M4 & M5	18 (46.20%)	8 (38.10%)	
CD34	Negative	1 (2.60%)	5 (23.80%)	0.010
	Positive	37 (97.40%)	16 (76.20%)	
Risk Group	Unfavorable	4 (40%)	2 (33.30%)	0.099*
	Favorable	4 (40%)	0 (0.0%)	
	Intermediate	2 (20%)	4 (66.70%)	
Disease Course	Lost	12 (30.80%)	5 (23.8%)	0.632*
	Remission	13 (33.30%)	5 (23.8%)	
	Relapse	10 (25.60%)	7 (33.3%)	
	Died	4 (10.30%)	4 (19.1%)	
Peroxidase	Weak	18 (46.20%)	2 (9.50%)	0.004*
	Positive	21 (53.80%)	19 (90.50%)	

*Chi square test; P<0.05 is considered significant. FAB: French–American–British; MPO: Myeloperoxidase

and M2 patients (median [range]=22.5 [0.78 to 41.75]) and the AML FAB-M4 and M5 patients (median [range]=26.5 [0.7 to 50]) (table 1).

As regards the flow cytometry results, a statistically significantly lower *Cx32* expression level was observed in patients with AML, but without HLA-DR (P=0.018).

Furthermore, patients with a *CD34*-positive expression showed a statistically significantly higher *Cx32* expression level than those with a *CD34*-negative expression (P=0.045) (table 1).

Both high and low expression levels of the Cxs were significantly higher on *CD34*-positive cells than *CD34*-negative cells (P=0.010) (table 2).

No significant relationships were found between *Cx32* and CD33 (P=0.363), CD11c (P=0.303), CD11b (P=0.581), CD14 (P=0.420), CD13 (P=0.0.363), and MPO (P=0.668).

Comparisons of the high and low expression levels of *Cx32* with respect to age, the hemoglobin level, the platelet count, the total leukocyte count, the peripheral blood blast percentage, and the bone marrow blast percentage showed no significant relationship between them (P=0.35, P=0.203, P=0.101, P=0.595, P=0.723, and P=0.911), respectively.

No statistically significant value was found in the expression level of *Cx32* concerning the

cytogenetic analysis results: t(8;21) (P=0.758), t(15;17) (P=0.317), and FLT3-ITD (P=0.871).

No significant correlation was found between *Cx32* expression and the patients' age (r=-0.070, P=0.595), the hemoglobin level (r=-0.064, P=0.627), the platelet count (r=0.212, P=0.104), the peripheral blood blast percentage (r=-0.224, P=0.086), and the bone marrow blast percentage (r=-0.130, P=0.320).

Connexin 43 Expression

Regarding the expression level of *Cx43*, 63.3% of the patients with AML showed a low expression level. The median fold change of *Cx43* expression from the controls ($2^{-\Delta\Delta CT}$) was 0.6 and ranged from 0.001 to 22. There was a statistically significant difference in the fold change values of *Cx43* expression between the patient group and the control group (P=0.013).

There was a statistically significant value in *Cx43* expression in the patients with AML regarding the presence of organomegaly (P=0.030).

Comparisons between the *Cx43* fold change gene expression in FAB-M1 and M2 (median [range]=0.75 [0.07 to 9.9]), FAB-M3 (median [range]=0.105 [0.001 to 0.7]), and AML with monocytic differentiation (M4 and M5) (median [range]=0.7 [0.01 to 22]) showed a statistically significant difference (P=0.006).

With regard to the flow cytometry results, two markers were significantly related to *Cx43*: HLA-DR (P=0.010) and CD34 (P=0.007). However, no significant relationship was found between *Cx43* expression and CD33 (P=0.415), CD11c (P=0.635), CD11b (P=0.841), CD14 (P=0.340), CD13 (P=0.730), and MPO (P=0.760). In the further evaluation of the difference in the expression of *Cx43*, whether low or high, with the other cell surface markers, a positive relationship was found between both low and high *Cx43* expression levels and HLA-DR (P=0.027).

Patients with a *CD34*-positive expression showed a higher *Cx43* expression level (median [range]=0.70 [0.01 to 22]) than those with a *CD34*-negative expression (median [range]=0.033 [0.001 to 0.65]) with a statistically significant difference between the two groups (P=0.007) (table 3).

A statistically significant difference was observed between patients with low and high expression levels of *Cx43* concerning peripheral blood blasts in that there was a higher blast percentage with the low *Cx43* expression level (P=0.013). No statistically significant difference was observed compared with age (P=0.240), the hemoglobin level (P=0.472), the total leukocyte count (P=0.387), the platelet count (P=0.542), and the bone marrow blast percentage (P=0.154) (table 4).

No significant relationship was found between the expression of *Cx43* and the cytogenetic analysis, molecular analysis, or the different FAB subtypes.

An inverse relationship was found between *Cx43* expression and the peripheral blood blast percentage (r=-0.339, P=0.008), while no correlation was found with the patients' age (r=-0.112, P=0.394), the hemoglobin level (r=-0.126, P=0.336), the platelet count (r=0.150, P=0.252), and the bone marrow blast percentage (r=-0.191, P=0.144).

A positive correlation was found between *Cx43* and *Cx32* expression in the patients with AML (r=0.599, P≤0.001).

Table 3: Comparisons of the different categories of patients with acute myeloid leukemia concerning *connexin 43* expression

Patients/Clinical Details		n (%)	Median (Range)	P value
Sex	Male	39 (65%)	0.50 (0.01–22.00)	0.957•
	Female	21 (35%)	0.70 (0.001–12.80)	
Organomegaly	Present	21 (35%)	1.00 (0.02–22.00)	0.030•
	Absent	39 (65%)	0.36 (0.001–12.80)	
FAB Subtype	M1 & M2	24 (40%)	0.75 (0.07–9.90)	0.006≠
	M3	10 (16.70%)	0.105 (0.001–0.70)	
	M4 & M5	26 (43.30%)	0.7 (0.01–22)	
CD34	Negative	6 (10.20%)	0.033 (0.001–0.65)	0.007•
	Positive	53 (89.20%)	0.70 (0.01–22)	
Risk Groups	Favorable	4 (25%)	0.458 (0.21–1.80)	0.461≠
	Intermediate	6 (37.50%)	0.456 (0.05–7.10)	
	Unfavorable	6 (37.50%)	0.28 (0.001–0.70)	
Response after First Induction	Remission	18 (41.90%)	0.5 (0.05–7.10)	0.795≠
	Relapse	17 (39.50%)	0.6 (0.001–12)	
	Death	8 (18.60%)	0.25 (0.012–10.60)	

•Mann–Whitney test; ≠Kruskal–Wallis test; P<0.05 is considered significant. FAB: French–American–British

Table 4: Comparisons of patients with acute myeloid leukemia showing low and high *connexin 43* expression levels as regards their age, peripheral blood, and bone marrow

		High *Cx43*	Low *Cx43*	P value
Age	Mean±SD	53±14	48±17	0.240•
	Range	25–80	14–82	
Hb	Mean±SD	7.84±2.14	8.24±2.05	0.472•
	Range	4.80–12.70	4.40–12.30	
Plt	Mean±SD	153.59±115.71	135.55±106.38	0.542•
	Range	29–420	10–390	
TLC	Mean±SD	53.78±26.45	66.08±62.8	0.387•
	Range	15.1–110	3.10–257	
PB Blast (%)	Mean±SD	58.50±22.72	71.68±16.89	0.013•
	Range	20–94	29–99	
BM Blast (%)	Mean±SD	76.41±19.81	82.71±13.86	0.154•
	Range	40–100	40–100	

•Independent *t* test; P<0.05 is considered significant. Hb: Hemoglobin; Plt: Platelet count; TLC: Total leukocyte count; PB blast (%): Peripheral blood blast percentage; BM blast (%): Bone marrow blast percentage

Discussion

When we stratified the patients with AML in the pretreatment phase according to *Cx32* and *Cx43* mRNA expression, 65% of the patients with AML showed a high expression level of *Cx32*, whereas 63.3% showed a low expression level of *Cx43*. Our finding is concordant with that in a study by Yang and colleagues, who reported that *Cx43*, expressed by BMSCs, induced apoptosis on leukemia cells via caspase three and caspase seven pathways.[14] This can be demonstrated in our results insofar as we found a negative correlation between *Cx43* expression and the peripheral blood blast percentage. Moreover, the results of an investigation by Liu and others showed that the expression levels of *Cx43* and its mRNA in acute leukemia post-chemotherapy BMSCs were significantly higher than the expression levels of *Cx43* and its mRNA in primary acute leukemia BMSCs.[8] Zhang and colleagues found that leukemic Jurkat cells that were co-cultured with *Cx43* overexpressed on BMSCs proliferated at a lower rate and that they were more sensitive to methotrexate than Jurkat cells that were cultured alone or co-cultured with regularly expressed *Cx43*-BMSCs.[15] Our results are contrary to the work of Yi and others, who also used RT-PCR to detect the mRNA expression of *Cx32* and *Cx43* in OCI-AML3 and OCIM2 cell lines and found no difference in the expression of the mRNA of both Cxs in the two cell lines. While there was no difference in the transcription and protein level of Cx32, there was a significantly low expression level of the Cx43 protein.[1] Reikvam and colleagues analyzed the gene expression profile for several Cxs, including *Cx32* and *Cx43*, in patients with AML. Both Cxs showed relatively low mRNA levels, while *Cx45* was the only one to show a relatively high expression level among the patients.[11]

Our results showed high *Cx43* expression levels in FAB subtypes with monocytic differentiation (M4 and M5), when compared with FAB subtypes M1, M2, and M3. This finding is in concordance with the finding in the study by Reikvam and others, who reported that *Cx43* expression was increased in AML cells with monocytic differentiation.[11]

We observed a significantly higher *Cx43* expression level in patients with *CD34*-positive cells than in patients with *CD34*-negative cells, whereas *Cx32*, whether with high or low expression levels, was strongly correlated with CD34 positivity. In contrast, Reikvam and colleagues found no significant correlation between the cell surface expression of both *Cx43* and *Cx32* and CD34.[11]

Both *Cx32* and *Cx43* were highly correlated with the presence of the HLA-DR marker on cells, but they were not significantly correlated with immunophenotypic markers CD11c, CD11b, CD14, CD33, and CD13.

We detected no statistically significant relationship between *Cx32* or *Cx43* expression and the cytogenetic analysis (t[8;21] and t[15;17]), molecular analysis, and the different FAB subtypes. Given that cytogenetics and molecular testing, especially FLT3/ITD mutation, play an important role in the therapy outcome, we categorized our patients according to the results of the aforementioned tests into favorable, intermediate, and unfavorable outcomes. None of these risk groups was significantly related to *Cx32* or *Cx43*.

Studying the response (remission, relapse, and death) after the first induction of chemotherapy, we found no significant relationship with *Cx32* or *Cx43*, suggesting that these Cxs had no role in the short-term follow-up

of AML. A long-term follow-up of patients was not feasible, since most patients are referred to other hospitals specialized in bone marrow transplantation. Another limitation of our study is the unavailability of the full cytogenetic profile, especially for elderly patients, who are not candidates for bone marrow transplantation.

Conclusion

The majority of our patients with AML showed a high expression level of *Cx32* and a low expression level of *Cx43*, which may add to the diagnostic workup of leukemia. However, our short post-therapy follow-up of the patients precluded us from finding a significant effect for *Cx32* or *Cx43* on the treatment outcome, and we could not find a significant relationship with the different risk groups. It would be difficult to regard both Cxs as prognostic markers in this study, and perhaps, long-term follow-ups of patients receiving therapy might find a relationship between these Cxs and the disease condition. Since CD34-positive leukemic stem cells have a negative impact on the long-term outcome of acute leukemia in general, the strong correlation between both Cxs and CD34 in this work might indicate a poor prognosis, which needs to be proven in the future investigations.

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