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Clinical value of high expression level of CD71 in acute myeloid leukemia

B. WU‡, N. SHI‡, L. SUN, L. LIU*

Department of Hematology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

**Correspondence: liulinxiang6629@126.com*
‡Contributed equally to this work.

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CD71 (transferrin receptor 1, TfR-1) is a type II membrane glycoprotein and associated closely with tumors. It was recognized as an indication for diagnosing acute erythroid leukemia (AEL). High expression level of CD71 has been identified as a negative prognostic marker for many solid tumors. However, whether CD71 should be identified as an adverse marker in acute myeloid leukemia (AML) remained conflicting. We studied 214 AML patients for analysis of clinical and laboratory data. Taking the CD71 expression level of 60% as a standard, we divided our patients into two groups. We discovered that AML with high expression level of CD71 was prone to linked with severe anemia (P=0.004), thrombocytopenia (P<0.001) and complex karyotype (P=0.024) and had increasing expression level of CD117 (P=0.001). No statistically significant correlations in age, gender, WBC counting, molecular markers between the two groups. And moreover, high expression level of CD71 did not alter the pattern of survival time.

Key words: CD71, acute myeloid leukemia, differentiation, cytogenetics, survival

CD71 is a type II membrane glycoprotein that plays an important role in iron uptake, and its expression is mainly regulated by the iron level in the cell [1]. Most cells have certain expression levels of CD71 in cell membrane; furthermore, CD71 is a well-known marker of cell proliferation [2, 3]. Cell with a higher proliferation rate often expresses higher level of CD71.

To cope with the increasing requirement of iron for DNA synthesis, tumor cells usually express higher levels of CD71 [4]. In normal bone marrow, nearly 80% of CD71 positive cells are erythroid cells. Consequently, CD71 was mainly considered as a typical marker for the diagnosis of AEL [5-8]. High expression level of CD71 has been identified as a negative prognostic marker for many solid tumors [9, 10] in addition for non-Hodgkin's lymphoma [11]. However, there was very little information in relationship between CD71 and acute leukemia. The data available from previous studies on whether CD71 was an adverse marker was conflicting [12-16]. And all these studies concentrated on the positive expression level of CD71 and did not associated with current risk statement assessment. In this study, we reviewed 214 AML patients in order to evaluate the potential clinical significance of high expression level (an expression level of 60%) of CD71 comprehensively.

Patients and methods

Patients. 214 patients diagnosed with AML in the Department of Hematology of the First Affiliated Hospital of Zhengzhou University from July 1, 2014 to October 31, 2015 were enrolled into this study. There were 108 males and 106 females, mean age 45 years.

Diagnostic criteria. The bone marrows were routinely assessed by the hematological diagnostic laboratory for analysis of morphology, immunology, cytogenetics and molecular cytogenetics. The diagnoses were made according to the World Health Organization (WHO) 2008 diagnostic criteria.

Reject criteria. Patients diagnosed with acute promyelocytic leukemia (APL) in the same period.

CD71 analysis. Samples were analyzed by flow cytometry using a Backman EPICS XL instrument. Five data parameters were assessed: forward and side scatter (FSC, SSC), FL-1 (FITC), FL-2 (PE) and FL-3 (PE-Cy5). Taking the CD71 expression level of 60% as a standard, we divided our patients into two groups--CD71-High: the expression level of CD71≥60% and CD71-Low: the expression level of CD71<60%.

Gene detection. The two fusion genes, AML1/ETO and CBFβ/MYH11, were detected by the FISH using the OLYM-

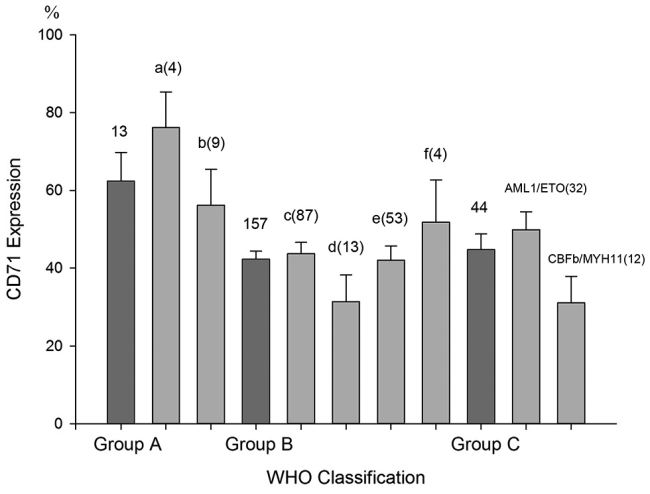

Figure 1. Expression levels of CD71 in different WHO classifications. Group A contained AML with minimal differentiation (a) and AML without differentiation (b). Group B contained AML with maturation (c), Acute myelomonocytic leukemia (d), Acute monoblastic/monocytic leukemia (e), Acute erythriod leukemia (f). Group C contained AML with t(8;21) and AML with inv(16)/t(16;16). The numbers in the graph indicated the cases of patients.

PUS analyzer and the imstar software. The gene mutations of NPM1, FLT3-ITD, CEBPA and C-KIT were detected by the Gene Amp PCR System 9700 and the 3130 genetic analyzer.

Therapy strategy. For the patients whose age were less than 60 years old, those patients were given the therapy of "DA" consisted of daunorubicien 45mg/ m^2 for 3 days and cytarabine 100mg/m^2 for 7 days. Following induction, patients achieving CR1 were consolidated with "HiDAC" contained cytarabine 2g/m^2 for 3 days. Patients assessed as better-risk would be consolidated with 3 circles and intermediated-risk with 4 circles. Autologous HSCT or allogeneic HSCT was implemented in the following situations: (a) patients considered at poor-risk status based on cytogenetics and molecular abnormalities and consolidated after 2-3 therapies of "HiDAC", (b) patients failing to achieve CR1 after "DA" and "HiDAC". The BuCy was used as conditioning regimens for both autologous and allogeneic HSCT.

Table 1. Relationships between CD71 expression and clinical factors

Clinical Factors[a]	CD71-High (N=66)	CD71-Low (N=148)	*P*
Age≥60	14(21.2%)	42(28.4%)	0.271
Male	38(57.6%)	70(47.9%)	0.194
WBC>100×10^9/L	15(22.7%)	22(14.9%)	0.160
Hb<60 g/L	21(31.8%)	22(14.9%)	0.004
PLT<20×10^9/L	30(45.5%)	26(17.6%)	<0.001

a.Normal: WBC 4-10×10^9/L, Hb 120-160 g/L, PLT 100-300×10^9/L

In relapsing patients, rescue therapy mainly included 3-drug fludarabine-based regimens. After that, allogeneic HSCT was the treatment of choice in all patients with a potential HLA-matched donor.

For the patients who aged more than 60 years, they were still intensively treated with curative intent, using either the same regimens as younger patients or their reduced versions.

Statistical analysis. Statistical analyses were done with SPSS 21.0 statistical software. Mann-Whitney U-tests were used for the analyses of CD71 expression in different WHO classifications. The Chi-square tests and Fisher's exact tests were used for the qualitative analyses. The Logistic regression was used in the analyses of relationships between CD71 and CD34, HLA-DR and CD117. Survival curves of patients were prepared by the Kaplan-Meier method, and differences between the survival curves were evaluated using log-rank tests. Binary logistic regression and the Cox model (a method of enter was used) were used for the multivariate analysis of associations between CD71 expression level and OS and DFS. A value $P<0.05$ was considered to be significant.

Results

AMLs with highly CD71 expressed represented adverse biological profiles. The data of age, gender, WBC counting, Hb counting and PLT counting in the time of diagnosis were taken into our analyses. As Table 1 showed, Percentage of severe anemia (31.8% vs 14.9%, $P=0.004$) and thrombocytopenia (45.5% vs 17.6%, $P<0.001$) in CD71-high AMLs (expression level ≥60%) were significant higher than those in CD71-low AMLs (expression level <60%). Especially, nearly half of the patients with CD71 highly expressed had a PLT counting less than 20×10^9/L. There were no significances in age (≥60), gender and WBC counting.

AMLs with higher levels of CD71 expression might be linked with poorly differentiated. We divided the WHO classifications into three groups. Group A contained AML

Table 2. Relationships between CD71 expression and WHO classifications

Morphology	CD71-High (N=66)	CD71-Low (N=148)	*P*
Group A	8(12.1%)	5(3.4%)	0.025
AML with minimal differentiation	3(4.5%)	1(0.7%)	0.088
AML without differentiation	5(7.6%)	4(2.7%)	0.138
Group B	43(65.2%)	114(77.0%)	0.070
AML with maturation	27(40.9%)	60(40.5%)	0.960
Acute myelomonocytic leukemia	2(3.0%)	11(7.4%)	0.353
Acute monoblastic/monocytic leukemia	12(18.2%)	41(27.7%)	0.136
Acute erythriod leukemia	2(3.0%)	2(1.4%)	0.589
Group C	15(31.8%)	29(14.9%)	0.600
AML with t(8;21)	13(22.7%)	19(12.8%)	0.194
AML with inv(16)/t(16;16)	2(3.0%)	10(6.8%)	0.351

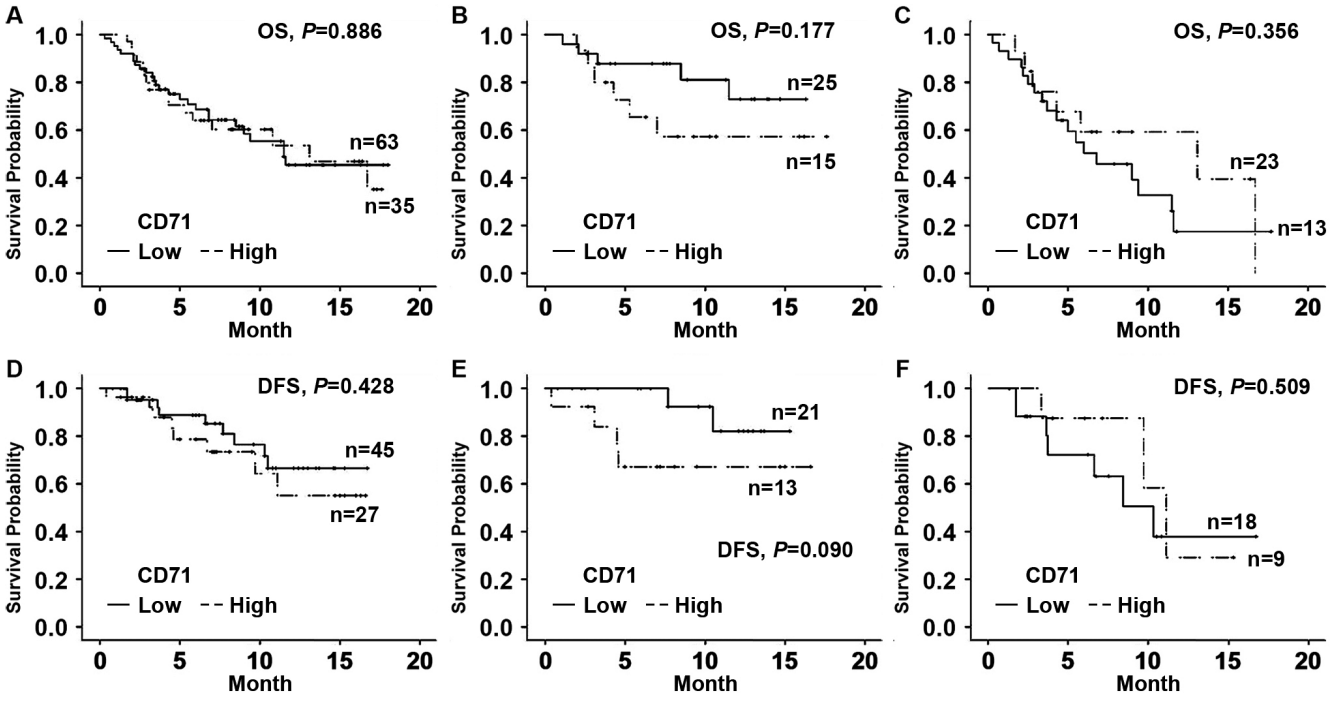

Figure 2. Survival curves of acute myeloid leukemia patients.
(A). OS of the whole samples in CD71-High and CD71-Low. (B). OS of better-risk in CD71-High and CD71-Low. (C). OS of intermediate-risk in CD71-High and CD71-Low. (D). DFS of the whole samples in CD71-High and CD71-Low. (E). DFS of better-risk in CD71-High and CD71-Low. (F). DFS of intermediate-risk in CD71-High and CD71-Low. The risk status assessment was according to the NCCN guideline of AML 2015 based on cytogenetics and molecular markers.

with minimal differentiation and AML without differentiation. Group B contained AML with maturation, acute myelomonocytic leukemia, acute monoblastic/monocytic leukemia, acute erythriod leukemia (no acute megakaryocytic leukemia in our study). Group C contained AML with t(8;21) and AML with inv(16)/t(16;16). In this division, AMLs in Group A were poorly differentiated than those in the remained two groups.

As Figure 1 showed (variables were written as mean± standard deviation, minimum and maximum, median), Group A had the highest level with an expression level of 62.4%±26.2 % (17.2%-96.7%, 64.6%). Group B expressed 44.8%±26.5 % (1.2%-92.8%, 42.9%), which ranked the second. The lowest one was Group C expressing 42.3%±26.7% (0.9%-98.9%, 41.1%). Our results showed that poorly differentiated leukemias often had higher expression levels of CD71 than well differentiated ones (P=0.011, Group A vs Group B; P=0.046, Group A vs Group C).

In Table 2 listed the proportion of each WHO classification in the CD71-High and CD71-Low groups. This analysis demonstrated that the rate of poorly differentiated AML (Group A) in CD71-High group was significant higher than that in the CD71-Low group (P=0.025). The insignificances of AML with minimal differentiation and AML with differentiation were probably caused by the insufficient data. And for the other classification, we did not find any significance.

We then described the correlation of CD71 with the three classical early antigens—CD34, HLA-DR and CD117 to confirm the relationship of CD71 expression and differentiation. Our results (Table 3) demonstrated that CD71 expression had significant positive correlation with CD117 expression (OR=1.024, P=0.001). For CD34 and HLA-DR, the correlations were not significant.

Table 3. Relationships between CD71 expression and CD34, HLA-DR, CD117 expression

Differentiation Antigens	CD71-High[a]	CD71-Low[a]	*P*	OR (95%CI)
CD34	48.6%±34.4%	42.7%±35.1%	0.360	0.994 (0.983-1.006)
HLA-DR	62.0%±34.3%	54.1%±31.8%	0.263	1.007(0.995-1.019)
CD117	65.5%±24.1%	51.6%±29.2%	0.001	1.024(1.009-1.038)

a. variables were written as mean± standard deviation

Table 4. Relation between CD71 expression and cytogenetics[a,b]

Cytogenetics	CD71 -High (n=66)	CD71-Low (n=148)	*P*
Better-Risk	12(18.2%)	24(16.2%)	0.723
inv(16)	3(4.5%)	10(6.8%)	0.758
t(8;21)	9(13.6%)	14(9.5%)	0.362
Intermediate-Risk	41(62.1%)	104(70.3%)	0.239
normal	29(43.9%)	83(56.1%)	0.101
+8 alone	2(3.0%)	7(4.7%)	0.724
t(9;11)	0(0.0%)	1(0.7%)	1.000
other	10(15.2%)	13(8.8%)	0.165
Poor-Risk	10(15.2%)	16(10.8%)	0.348
Complex	11(16.7%)	10(6.8%)	0.024
-5;5q-;-7;7q-	2(3.0%)	9(6.1%)	0.509
inv(3)	0(0.0%)	1(0.7%)	1.000
t(6;9)	0(0.0%)	2(1.4%)	1.000
t(9;22)	1(1.5%)	0(0.0%)	0.308
11q23	1(1.5%)	2(1.4%)	1.000

(a). The data was given in terms of "cases(proportion)".
(b). There was some overlap in this table. For instance, a cytogenetics of "47, xy, +21, del(9)(q23), del(7)(q21)[20]" would be counted in rows of "Complex" and "-5;5q-;-7;7q-" but only tallied once in "poor-risk".

AMLs with highly CD71 expressed were more easily associated with complex aberrant cytogenetics. Cytogenetic abnormalities are the most powerful predictor of outcome and are essential in guiding AML treatment decisions. The NCCN had established a risk status for AML based on validated cytogenetics and molecular abnormalities. We tried to find any possible relationship in CD71 expression and aberrant cytogenetics. As Table 4 Showed, percentage of complex karyotype in group of CD71-high was significantly higher than that in CD71-low (16.7% vs 6.8%, P=0.024). There were no statistic indications in any other cytogenetics.

Some molecular abnormalities have prognostic implications among the cytogenetically normal group of AML. Point mutation or internal tandem duplication in the FLT3 gene means a prognosis similar to that of AML with adverse risk cytogenetics. Isolated mutation in the NPM1 gene and the CEBPA gene confer a favorable prognosis. A C-KIT mutation in CBF-AML drags down the risk status and confers an intermediate prognosis. As Table 5 showed, mutative rates of NPM1, FLT3-ITD, C-KIT and CEBPA in the two groups were not significant neither calculated in normal karyotype nor calculated in the overall sample. In fact, in our study, the proportions of NPM1, FLT3-ITD and CEBPA increased as CD71 expression increased but the differences were not significant. In addition, isolate NPM1 (34.8% vs 21.8%, P=0.232), CEBPA (21.7% vs 18.2%, P=0.757) and FLT3-ITD (8.7% vs 3.6%, P=0.577) mutation was not significant in normal karytpye (variables were given as "CD71 ≥60% vs CD71<60%, P").

High expression level of CD71 seemed not to impact the survival time neither in OS nor in DFS. A total of 98 cases did the therapy and 82 cases got released. Our outcomes showed that the survival curves of OS (P=0.909) and DFS (P=0.649) of the two groups were quite close (Figure 2-A,D). We classified our patients into three groups according to the risk status, we found that high expression level of CD71 dragged down the OS (P=0.177) and DFS (P=0.177) in the groups of better risk (Figure2-B,E) though there was no significance. And the differences were not significant as well in the intermediate risk group (Figure2-C,F). We did not analysis the impact in the poor risk group because there were merely 12 cases did the therapy. In multivariate analysis, factors as age, gender, WBC counting, risk status assessment and CD71 expression were taken in consideration. As Table 6 demonstrated, age and risk status assessment seemed to strongly connect with overall survival time in AML when gender, WBC counting and CD71 expression level seemed not. In the analysis of DFS, the expression level of CD71 also did not have impacts.

Discussion

This thesis aimed to evaluate the probably clinical significance of high expression level of CD71 in AML. We tested CD71 expression in 214 patients by flow cytometry. Taking expression level of 60% as a standard, our result showed that AMLs with high CD71 expressed were easily linking with severe anemia (Hb<60 g/L, P=0.004) and thrombocytopenia (PLT<20×10^9/L, P<0.001). Since CD71 was a well known marker of proliferation, higher expression level of CD71 might mean a higher level of proliferation of leukemic cell. This process might lead to a strong repression to normal hematological generation. Moreover, as PLT counting less than 20×10^9/L meant an increasing rate of fatal hemorrhage, AMLs with

Table 5. Relation between CD71 expression and molecular marker

Molecular	Normal karyotype		*P*	Overall sample		*P*
	CD71-High (n=23)	CD71-Low (n=55)		CD71-High (n=41)	CD71-Low (n=108)	
NPM1	13(56.5%)	21(38.2%)	0.136	14(34.1%)	23(21.3%)	0.105
FLT3-ITD	7(30.4%)	11(20.0%)	0.319	9(22.0%)	14(13.0%)	0.175
C-KIT	0(0.0%)	1(1.8%)	1.000	2(4.9%)	6(5.6%)	1.000
CEBPA	5(21.7%)	10(18.2%)	0.757	6(14.6%)	15(13.9%)	0.907

Table 6. multivariate analysis of CD71 expression in survival time

Factors	P_1(OS)	OR (95% CI)	P_2(DFS)	OR (95% CI)
CD71 expression				
<60%		1.000		1.000
≥60%	0.947	1.022(0.532-1.962)	0.498	0.690(0.236-2.020)
Age				
<60 years		1.000		1.000
≥60 years	0.001	0.274(0.125-0.602)	0.253	0.396(0.081-1.938)
Gender				
Female		1.000		1.000
Male	0.271	0.697(0.366-1.327)	0.891	1.080(0.360-3.233)
WBC counting				
$<100\times10^9$/L		1.000		1.000
$\geq100\times10^9$/L	0.798	0.900(0.404-2.006)	0.312	2.225(0.473-10.472)
Risk status[a]	0.015		0.075	
better		1.000		1.000
intermediate	0.004	2.888(1.402-5.949)	0.032	3.155(1.104-9.015)
poor	0.246	1.818(0.662-4.995)	0.961	0.947(0.107-8.399)

a. The risk status assessment was according to the NCCN guideline of AML 2015 based on cytogenetics and molecular markers.

highly CD71 expressed were exposed to a more dangerous state in the first visit to hospital.

In morphology, our results showed that poorly differentiated AMLs often had higher expression levels of CD71 than well differentiated ones. On the other hand, we also found that the proportion of poorly differentiated AMLs in CD71 highly expressed group was higher than that in the CD71 lowly expressed group. This meant that high expression level of CD71 seemed to be linked with poor differentiation. The result was partially coincident with the previous reporting [17]. The results of FCM demonstrated that CD117 expression was positively correlated with CD71 expression. One thing is for certain, as cellular differentiation is a continuous process, cells in the same stage defined by human can be actually diversely differentiated. In our knowledge, CD117, a receptor tyrosine kinase protein encoded by KIT gene, is mostly expressed on cells that are not mature in morphology such as hematopoietic stem cells [18]. The expression intensity declines as the cells differentiate in the normal bone marrow. Signalling through CD117 plays a role in cell survival, proliferation and differentiation. In this context, our outcomes might show that leukemic cells with CD71 highly expressed were more primitive than the lowly expressed ones.

The cellular labile iron pool (LIP) is a pool of chelatable and redox-active iron, which is transitory and serves as a crossroad of cell iron metabolism [19]. Higher CD71 expression meant an increasing absorption of iron and resulted in the iron overload and the increasing level of LIP. The labile nature of LIP was revealed by its capacity to promote formation of reactive oxygen species (ROS). The ROS was then to impair the DNA [20] and to influence the cell cycle of hematopoietic stem and progenitor cells by MAPK pathway [21]. In erythroid, the ROS was mainly to blockade the differentiation of progenitor cells and to destruct the structure of red blood cells [22]. As a result, AML with highly CD71 expressed showed an enhanced pattern of poorly differentiated and severe anemia.

Proto-oncogene and tumor suppressor gene play a key role in the occurrence and development of tumors. Such translocation, insertion and deletion as chromosome abnormalities are closely linked with gene conversions. One of the activation methods of proto-oncogene is DNA rearrangement such as Burkitt lymphoma or Class II genes AML1/ETO and CBFb/MYH11. Low tumor suppressor gene dosage caused by chromosome deletions or gene mutations also contributes to the tumor generation [23]. Gene transformation result from chromosome abnormality is then lead to the abnormality of survival pathway and eventually result in tumor.

AML is commonly accompanied with chromosome abnormalities. Our results indicated that AMLs with high level of CD71 expression were more easily associated with complex karyotype (P=0.024). Since surface CD71 expression has been proven to be a downstream marker of Notch mediated PDK1 activation [24, 25] and further proposed as a downstream marker of mTOR activity [26], it seems that activity of survival pathway such as PI3K/PDK1/Akt/mTOR is linked with CD71 expression. Kenneth showed that CD71 interacts with the IKK complex and is involved in IKK-NF-κB signaling [27]. Enhanced expression of TfR1 had been showed to contribute to oncogenic signalling by sphingosine kinase 1 [28]. In this context, we supposed that contrasted with uncomplex karyotype, complex karyotype had more frame shift mutations like translocation, insertion and deletion in chromosomes, increasing the incidence rates of the following events: activating or amplifying a proto-oncogene, inactivating a tumor

suppressor gene or reducing its dosage. The comprehensive result was that the abnormality of the signal system controlling cell proliferation and differentiation became more obvious, which then led to the stronger malignant clone ability and earlier differentiation blockade of leukemic cells. Therefore, the expression level of CD71 increased.

Whether CD71 expression should affect survival remained controversial. Our results showed that the survival curves of CD71-high and CD71-low were quite close. And the multivariate analysis as well suggest that high expression level of CD71 did not change the pattern of survival. This meant that the high expression level of CD71 might not influence survival though it linked with poor clinical and laboratory profiles. Our results seemed to support the precious negative studies [12, 14]. Interestingly, we found that highly expressed level of CD71 dragged down the AML with better risk assessment. However, because of the data insufficient and shortly observing time, this difference was also not to be significant. Whether it was a prognostic marker in better risk group remained further studying.

In conclusion, CD71 was a marker for proliferation. AML expressed high level of CD71 might have an adverse clinical and laboratory profile. It was easily associated with severe anemia, thrombocytopenia, poor differentiation and complex cytogenetics. However, high expression level of CD71 might not impact survival time neither in OS nor in DFS.

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