

Interleukin-27 and interleukin-35 in de novo acute myeloid leukemia: expression and significance as biological markers

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Background and objectives: IL27 and IL35 are regulatory T cells (T-regs) related cytokines; they were accused in eukemogenesis of acute myeloid leukemia (AML). This study aimed to assess the expression of these cytokines in de novo AML and investigate their role as biomarkers.

Subjects and methods: Seventy newly diagnosed patients with primary AML and 30 matched healthy volunteers were recruited. AML diagnosis was confirmed with flowcytometric and immunophenotypic analyses, while ELISA was used to assess serum levels of IL27 and IL35 in patients and controls. Receiver operating characteristic curve analysis was used to estimate IL27 and IL35 optimum cutoff values for predicting AML.

Results: Serum levels of both cytokines were significantly higher in AML patients than controls ($P<0.001$), with no effect of gender or French-American-British subtypes. Significant correlations of IL27 and IL35 with poor prognostic factors and with each other were detected in patients only. IL27 optimum cutoff for predicting AML was >43 , AUC (0.926) with a sensitivity 74% and specificity 96.6% ($P<0.001$), while for IL35 >27.8 , AUC (0.972) with 88% and 98% sensitivity and specificity, respectively ($P<0.001$).

Conclusion: Conclusively, this study proved that IL27 and IL35 could identify AML patients from healthy subjects, and their overexpression denotes poor prognosis. Based on the simplicity and wide availability of their detection technique we recommend the inclusion of IL27 and IL35 in the diagnostic/prognostic workup of AML; however, further longitudinal research is needed to prove their exact prognostic value.

Keywords: acute myeloid leukemia (AML), regulatory T cells, flowcytometry, IL27, IL35

Introduction

AML is a hematopoietic neoplasm derived from early progenitor myeloid cells; it is characterized by clonal expansion of myeloblasts, which are immature myeloid progenitors that accumulate in the bone marrow (BM). AML can arise in patients with antecedent hematological disorder and named secondary AML, or as primary disorder in normal individuals (de novo AML) or as a consequence of prior therapy (therapy-related AML). Even with the use chemotherapy, 70% of patients 65 years old, or more, may die because of their disease or its complications, within 1 year of diagnosis.¹⁻⁴ Immune system impairment and immunotolerance were reported in patients with AML. T cells are the most active part of the immune system, nevertheless they were found to be functionally and numerically defective in AML.⁵

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Tregs suppress the function and multiplication of T helper (Th) cells. AML patients show paradox high level of Tregs in their BM and peripheral blood compared with healthy subjects.⁵ Tumorigenesis of AML has not been fully clarified, tumor immune escape mechanism controlled by CD4+ & CD25+ Tregs was proposed as the hall mark of AML pathogenesis.⁶ Circulating Tregs in AML patients mediate vigorous suppression, through contact dependent and independent mechanisms. Furthermore, increased expression of T-reg might enhance tumor development or growth and affects the natural history of the disease. Better response to induction regimens was found in patients with lower Tregs at diagnosis. Based on these findings, depletion of Tregs was suggested as a future therapy that could offer great hope for AML patients.^{7,8}

The family IL12 are heterodimeric cytokines characterized by three alpha subunits (p19, p28, p35) and two beta subunits (EBI3 and p40), that led to six different pairings. There is concluding evidence that the pairing occurs in vivo for IL12 (p35/p40), IL23 (p19/p40) and IL-27 (p28/EBI3).⁹ Structure similarity within the IL12 family leads to very different biological properties. In general, IL27 and IL35 function mainly as anti-inflammatory or immunoregulatory cytokines.¹⁰ IL27 has antitumor effects in different cancer models, therefore it has been proposed as a potential agent in cancer immunotherapy studies.¹¹ IL27 also displays pleiotropic functions, which comprise both immune-enhancing and immune-regulatory effects.¹² Several studies found correlation between increased serum levels of IL27 and disease progression or cancer growth in different types of tumors.^{13–16}

IL35 is specifically expressed by resting and activated CD4+ Foxp3+ Tregs and is considered to be a crucial anti-inflammatory cytokine, which could suppress Th1, Th2 and Th17 cell-responses in a context-dependent manner.¹⁷ IL35 induces the conversion of naïve T cells into regulatory T cells and downregulates Th17 cell development and differentiation as well suppression of autoimmune inflammation.¹⁸ Tao et al found that IL35 exerted a pro-proliferative and an anti-apoptotic effect on AML blasts. These findings pointed to the role of IL35 in the mechanism of immune escape in AML.¹⁹

This study aimed to assess serum levels of IL27 and IL35 in patients with primary AML, and to investigate their role as biological markers.

Subjects and methods

Study settings and subjects

AML patients were recruited among those attending the Hematology/Oncology Clinics at Sohag University Hospital,

during the period from January 2016 to June 2018. They were included after fulfilling the following inclusion criteria:

1. Newly diagnosed (ND) AML patients.
2. De novo AML.
3. Willingness for participation in the study.

Another 30 healthy volunteers were collected as controls they were age and sex matched with the patients, and were among medical students, medical and paramedical professionals.

Study tools

Medical history, clinical examination and laboratory investigations

Both patients and controls were subjected to thorough history and physical examination, next blood samples were withdrawn for complete blood count (CBC) using Cell-Dyn 3700, automated cell counter (Abbott diagnostic, Dallas, USA).

The diagnosis of AML in the study patients was according to the WHO recommended criteria,²⁰ accordingly patients were subjected to further investigations as:

1. Leishman-stained peripheral blood smears.
2. BM aspirates or occasionally trephine biopsy.
3. Cytochemical staining of BM specimens using myeloperoxidase (MPO), Esterase and periodic acid schiff.
4. Immunophenotyping (IPT): monoclonal antibodies (MoAb) used for staining of blast cells were labeled with fluorescein Isothiocyanate, phycoerythrin. Markers looked for to prove or disprove diagnosis of AML including: myeloid markers: CD13, CD33, MPO. Monocytic markers: CD14. B cell markers: CD19, CD20, CD22 and CD79a. T cell markers: CD3, CD5, CD2 and CD7. Common progenitor marker: HLA-DR, CD34. All Moab were purchased from Becton Dickinson Bioscience, San Jose, CA, USA. FACS acquisition and analysis were performed with FACS Cell Quest software (BD Biosciences).

The diagnosis of AML subtype was based on the standard morphological and IPT criteria.²¹

Karyotyping and assessment of other molecular prognostic markers were not available for all patients in our center so they were not included in the current research.

Measurement of serum levels of IL27 and IL35 in the study subjects

Serum was obtained from plain blood sample tubes that were kept at -80°C by centrifugation. Serum levels of IL-27 and IL-35 were acquired by ELISA, for both patients and controls, using the commercially available kits (Elabscience Systems, Houston, Texas, USA, catalogue numbers E-EL-H2338 and E-EL-H2443, respectively). Absorbance was recorded at 495 nm, IL-27 assay range was 31.25–2000 pg/mL, and IL-35 assay range was 15.63–1000 pg/mL.

Data analysis

Data were analyzed using IBM SPSS Statistics for Windows version 24 and Medcalc version 18.1. Quantitative data were expressed as means \pm standard deviations, while qualitative data as numbers and percentages. Shapiro–Wilk test was used to test for normality of quantitative data, on the other hand Mann–Whitney U test, Wilcoxon signed-ranks test and Spearman's correlation were used for not normally distributed data. Independent Samples *t*-test and Pearson's correlation were used for assessing differences and associations among normally distributed data and Chi-square (χ^2) test for categorical variables. Receiver operating characteristic (ROC) curve was constructed to detect IL27 and IL35 optimum cutoff points in predicting AML, and the area under the ROC curve with 95% CI was calculated, sensitivity and specificity were also calculated. A 5% level was chosen as a level of significance in all statistical tests used in the study.

Ethical considerations

The study was consistent with the World Medical Association declaration of Helsinki, and the study design was approved by the Research Ethical Committee of Faculty of Medicine, Sohag University. The study design protocol and significance was explained to the study participants. All the study participants signed an informed consent and confidentiality was addressed.

Results

Characteristics of the study participants

A total of 100 subjects were enrolled in the study, 70 ND patients with de novo AML and 30 age and sex matched healthy controls. Table 1 shows a comparison between patients and controls regarding demographic, clinical and CBC data. There were no significant differences between

patients and controls as regard age and gender distribution ($P=0.552$ and 0.513 , respectively), which ensured perfect matching. The median age of the study patients was 36 years and the range was 18–80 years. Females comprised 52.9% of the patient group with a female to male ratio 1:1. There were significant differences between AML patients and controls ($P<0.001$) in CBC parameters (WBCs, hemoglobin and platelets).

Figure 1 illustrates the distribution of the French-American-British (FAB) subtypes of AML patients which was, 3 patients with M0 (4.2%), 7 with M1 (10.1%), 22 with M2 (31.4%), 20 with M3 (28.6%), 10 with M4 (14.3) and 8 with M5 (11.4%).

Serum levels of IL27 and IL35 in AML patients compared with the controls

Table 2 shows a significantly higher levels ($P<0.001$) of IL27 and IL35 in AML patients compared with the control subjects, with median values of 77 and 51 pg/mL compared to 37 and 22.2 pg/mL, respectively.

On the other hand, there were no significant differences in levels of IL27 and IL35 between males and females, whether in patients or the controls, as in Table 3. Figure 2 illustrates no significant differences in mean values of IL27 and IL35 among different FAB subtypes of the study patients ($F=0.93$ and 0.25 ; $P=0.47$ and 0.94 , respectively).

Associations between serum levels of IL27 and IL35 and other variables in the study subjects

Table 4 shows the correlations between serum levels of IL27 and IL3 in AML patients ($n=70$), it revealed a significant positive correlation between IL27 with, WBCs count, peripheral blasts percent and MPO positivity ($r=0.269$, 0.328 and 0.237 ; $P=0.024$, 0.030 and 0.048 , respectively). However, IL35 levels showed a significant negative correlation with platelets count ($r=-0.267$, $P=0.025$) and significant positive correlations with age, CD45, HLA-DR, CD34 ($r=0.294$, 0.273 , 0.240 and 0.259 ; $P=0.038$, 0.022 , 0.045 and 0.030 , respectively).

Table 5 shows no significant correlations between cytokine levels and other quantitative variables of the control group.

Figure 3 and Table 5 reveal a significant positive correlation between IL27 and IL35 levels with each other ($r=0.253$ and $P=0.042$) for the patient group only.

Table 1 Comparison between AML patients and the control group as regarding age, gender, clinical and laboratory criteria

Parameter Mean \pm S.D. Median (Range)	Cases group (N=70)	Control group (N=30)	P-value
Age (years)	39.74 \pm 16.44 36 (18–80)	41 \pm 15.19 45 (29–80)	0.552
Gender			
Female N (%)	37 (52.9%)	43.3 (52%)	0.513**
Male N (%)	33 (47.1%)	56.7 (48%)	
WBCs ($\times 1000/\text{mm}^3$)	40.4 \pm 40.06 27 (1–168)	6.32 \pm 1.63 5.9 (4–10)	P<0. 001*
Hemoglobin (g/dL)	7.41 \pm 1.65 8 (3–11)	13.52 \pm 1.41 13.4 (12–16)	P<0. 001*
Platelets ($\times 1000/\text{mm}^3$)	57.3 \pm 34.47 58 (6–139)	255.2 \pm 57.17 242.5 (177–350)	P<0. 001*
Peripheral blast (%)	69.5 \pm 21.68 70 (33–97)	–	NA
Bone marrow blast (%)	81.42 \pm 12.29 80 (45–98)	–	NA
Splenomegaly			
No	59 (84.6%)	–	NA
Yes	11 (15.4%)	–	
Lymphadenopathy			
No	62 (88.8%)	–	NA
Yes	8 (11.2%)	–	

Notes: P-value was calculated by Mann–Whitney test. *P-value was calculated by Independent Samples t-test. **P-value was calculated by Chi-square test. P-value <0.05 is statistically significant.

Abbreviations: NA, not applicable; AML, acute myeloid leukemia.

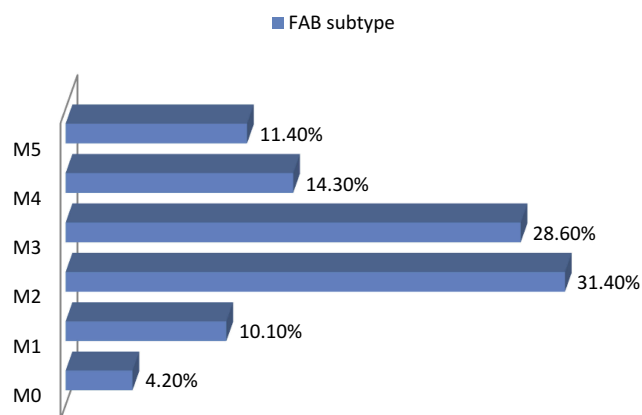


Figure 1 Distribution of the French-American-British (FAB) subtypes in the study patients.

Predictive values of IL27 and IL35 in de novo AML

For detection of optimum IL27 and IL35 cutoff level that is needed for predicting AML a ROC curve analysis was done that demonstrated that IL27 optimum cutoff is >43, AUC

0.926 with 74% of 96.6% sensitivity and specificity 100% P-value <0.001. IL35 optimum cutoff is >27.8 AUC 0.972 with 88% of 98% sensitivity and specificity 100%, as shown in Figure 4. Table 6 shows the results of the logistic regression analysis of predictors in de novo AML, P-value <0.001.

Discussion

AML is a type of blood cancer that mostly has poor consequences. Over the past 10 years the image of AML was albeit changed, this was in part due to advances in understanding AML tumorigenesis and detection of new molecular markers.^{22–24} Nevertheless there is a lack in the literature regarding the exact pathogenetic mechanism of AML development and pathogenesis. Recently, the T-reg related cytokines, IL27 and IL35 were implicated in pathogenesis of AML either directly or indirectly. However, their role in AML is similar to a buzzle where some researchers proven their antileukemic effects whereas others reported their leukemogenic potential.^{25–28} Therefore, the role of cytokines IL27 and IL35 in AML

Table 2 Comparison of serum levels of IL27 (pg/mL) and IL35 (pg/mL) among AML patients and the control group

Parameter	Case group	Control group	P-value
IL27			
Mean±SD	80.72±28.68	38.9±11.69	<0.001*
Median (range)	77 (32–158)	37 (21–68)	
IL35			
Mean±SD	53.64±16.07	22.37±7.54	<0.001*
Median (range)	51 (28–88)	22.2 (10.5–36.8)	

Notes: P-value was calculated by Wilcoxon signed-Ranks Test. *Statistically significant.

Abbreviation: AML, acute myeloid leukemia.

is a fertile research area, particularly after the success of immunotherapy in other hematological malignancies.^{29,30}

This study aimed to assess the expression of IL27 and IL35 in de novo AML and explore their role as biological markers of the disease, to do so 70 ND AML patients and 30 matched controls were included in the study. Both patients and controls were subjected to a panel of clinical and laboratory investigations. Analysis of the obtained results ensured perfect matching between patients and

controls. Strikingly, this study showed a younger age female predominant AML, compared to most research in AML.^{31,32} The explanation for these conflicting results that willingness for participation was a prerequisite before enrollment in the current study.

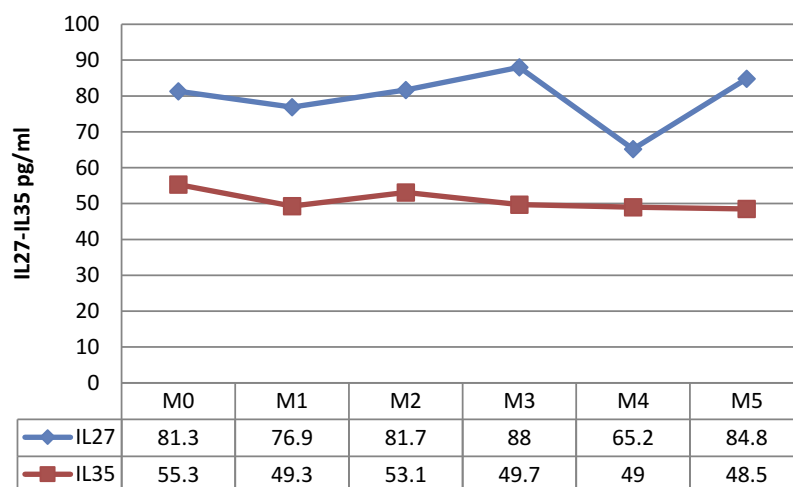
To investigate the expression of our target cytokines in AML patients, we compared serum levels of IL27 and IL35 in ND patients with AML with those in the controls. Notably, the serum levels of cytokines in patients were in the reference range supplied by the kit. Results of the current study showed significantly higher levels of IL27 and IL35 in ND AML patients compared with the control group. However, there were no significant effect of gender or FAB subtypes on cytokine values. Absence of gender effect was further confirmed when we compare interleukin values in males and females of the control group and find similar results. Our findings were in agreement with Zorzoli et al, Tao et al and Wu et al who estimated IL27 in AML pediatric patients and IL35 in adult patients, respectively, they demonstrated a significantly higher serum concentration of IL27 and IL35 in patients compared to the controls.^{19,27,33} These findings demonstrated

Table 3 Comparison of IL27 (pg/mL) and IL35 (pg/mL) serum levels among males and females of AML patients and the control group

Parameter	Cases group (N=70)	Mean difference	P-value	Control group (N=30)	Mean difference	P-value
IL27	Male 83.36±28.27 Female 78.89±28.24	4.47	0.511	Male 39.59±13.65 Female 30.00±8.97	1.588	0.0716
IL35	Male 50.76±14.83 Female 50.70±15.62	0.05	0.988	Male 22.59±7.98 Female 23.57±7.92	-0.0988	0.740

Note: Values were calculated with Independent Sample t-test.

Abbreviation: AML, acute myeloid leukemia.

**Figure 2** Comparison of IL27 and IL35 among different FAB subtypes of the study patients.

Note: One way analysis of variance was used (F=0.93 and 0.25; P=0.47 and 0.94, respectively).

Abbreviation: FAB, French-American-British.

Table 4 Correlations of IL 27 (pg/mL) and IL35 (pg/mL) serum levels with age, laboratory and immunophenotypic parameters of AML patients (n=70)

Parameter	IL27		IL35	
	R	P-value	R	P-value
Age (years) [^]	0.101	0.407	0.294	0.038*
WBCS ($\times 1000/\text{mm}^3$) [^]	0.269	0.024*	0.261	0.049
HB (g/dL) [^]	0.109	0.370	0.160	0.166
Platelets ($\times 1000/\text{mm}^3$)	0.137	0.258	-0.267	0.025*
Peripheral blast cells % [^]	0.328	0.030*	0.081	0.503
BM blast%	0.226	0.061	0.026	0.761
CD14	0.074	0.550	-0.040	0.744
CD45	0.177	0.143	0.273	0.022*
CD13	0.059	0.630	0.043	0.724
MPO	0.237	0.048*	0.030	0.801
CD33	0.050	0.679	0.030	0.807
HLA-DR	0.060	0.621	0.240	0.045*
CD34	0.013	0.912	0.259	0.030*

Notes: r=spearman correlation coefficient. *Statistically significant. [^]Pearson correlation coefficient.

Abbreviations: MPO, myeloperoxidase; CD, cluster of differentiation; HLA-DR, human leucocyte antigen D-related.

Table 5 Correlations between IL27 (pg/mL) and IL35 (pg/mL) with age and laboratory parameters of the control group (n=30)

Parameter	IL27		IL35	
	R	P-value	r	P-value
Age (years) [^]	0.017	0.939	0.052	0.783
WBCS ($\times 1000/\text{mm}^3$) [^]	0.182	0.336	0.071	0.771
HB (g/dl) [^]	0.030	0.875	0.012	0.950
Platelets ($\times 1000/\text{mm}^3$)	0.050	0.794	0.424	0.151
IL35	0.119	0.531

Notes: r=spearman correlation coefficient. [^]Pearson correlation coefficient.

the value of IL27 and IL35 as diagnostic markers in ND patients with AML.

To explore the value of IL27 and IL35 as prognostic markers in AML, we analyzed their correlation with other prognostic factors in the study patients. To validate our results we studied the correlation between IL27 and IL35 levels with age and CBC parameters of the controls. Interestingly, our results showed no significant correlation of cytokine levels with any parameter in the control group. On the contrary, we noted significant positive correlations of IL27 and IL35 with poor prognostic factors in AML as WBCs count, MPO and peripheral blasts %, and age, platelets count and CD34, respectively.

In the current study IL27 level seems to be higher in M3 subgroup, despite the usually low WBC/blasts number

at onset in this FAB group. This could be explained by the different source of origin of the cytokine other than blast cells, furthermore differences in cytokines were not significantly different among FAB subtypes.

The above-mentioned results suggested the value of IL27 and IL35 as prognostic markers in AML and accused them in development and disease progression of AML. In addition, they reinforced the work of Shenghu et al, who found that high levels of IL35 increased the suppressive function of T-reg cells in peripheral blood and BM in patients with AML, this was suggested to play a role in immunotolerance in AML.³⁴ Also the work of Tao et al, who concluded that increased IL35 levels facilitated the immune escape of AML blasts, adult AML blasts, enhanced blast proliferation and inhibited blast apoptosis, as such they suggested IL35 to be a future therapeutic target in AML.¹⁹ On the contrary, our findings contradicted with Nasreldin et al who found no significant association between IL35 and platelets count,⁷ this difference could be explained by the difference in type of AML patients between the two studies, wherein Nasreldin et al's study included ND patients with AML and those in complete remission.

Next, we go further and analyzed the correlation of IL27 and IL35 with each other in both patients and controls. Our results revealed, for the first time, a significant positive correlation of IL27 with IL35 serum levels in AML patients which was obviously absent in the control group. The most logical explanation for these results relies on the findings of Ersvaer et al, who revealed that in ND patients with AML, frequencies of T-regs are higher compared to the controls, and remain high during treatment. They believed that high levels of T-regs at AML diagnosis could denote poor prognosis.³⁵ IL27 and IL35 were found to be T-regs related cytokines that belonged to the same family and performed albeit similar biological functions.^{9,10} Accordingly, upregulation of T-regs in AML will subsequently result in increased expression of both IL27 and IL35.

IL35 was found to be produced by B-cells (B-regs) as well as T-regs.^{36,37} This means that serum levels of IL35 could be affected by up- or downregulation of both T-regs and B-regs. This could explain the modest increase of IL35 levels noted in AML patients.

In order to check for the effectiveness of both IL27 and IL35 as diagnostic markers in ND AML, we follow other researchers and use ROC curve analysis.^{38,39} Interestingly, our results reported a new finding that both IL27 and IL35

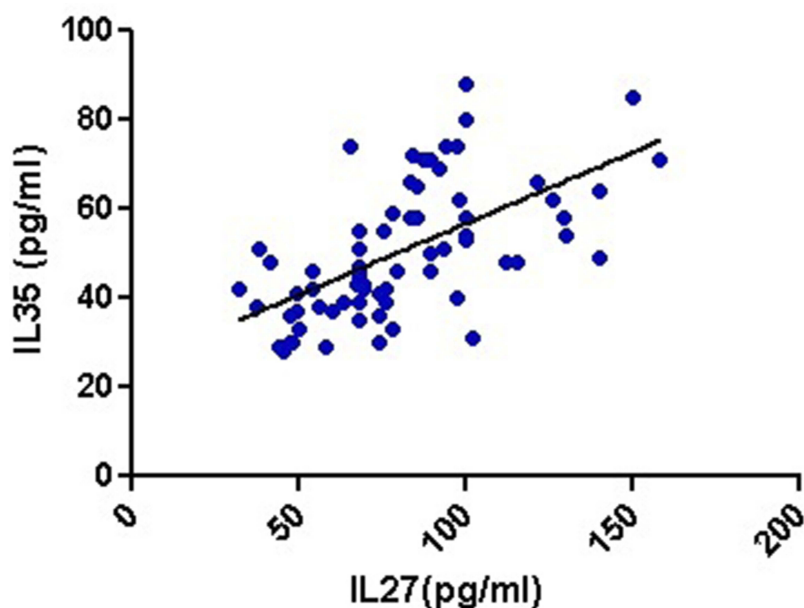


Figure 3 Correlation of IL27 serum levels (pg/mL) with IL35 (pg/mL) in the study patients ($r=0.253$ and $P=0.042^*$).

Notes: r =Pearson correlation coefficient. *Statistically significant. Figure was generated with GraphPad Prism V.5.

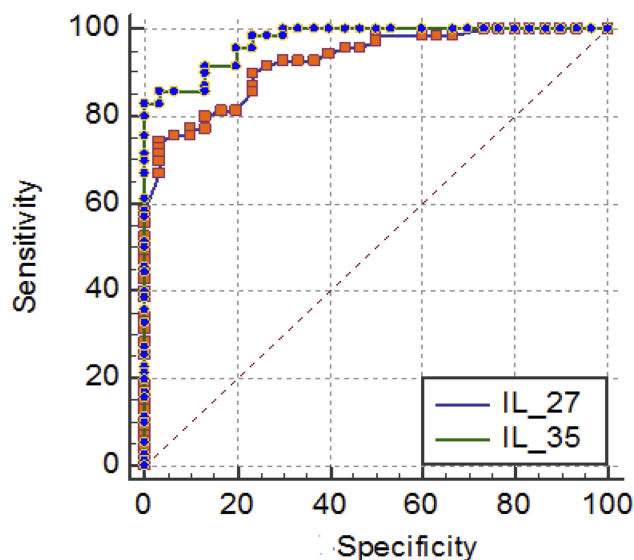


Figure 4 Receiver operating characteristic (ROC) curve of IL27 and IL35 for optimum cutoff point in predicting AML.

have a low false negative rate (specificity), which mean that their use as diagnostic tool in AML could help rule in the disease.

This study proved the value of IL27 and IL35 as biomarkers in de novo AML, and generated a cutoff value for IL27 and IL35 as predictor in AML, from ROC curve, it was 43 (pg/mL) and 27.8 (pg/mL), respectively with high statistical significance.

Table 6 Logistic regression analysis of predictors in de novo acute myeloid leukemia

Characteristics	OR (CI _{95%})	P-value
Age (years)	0.993 (0.96–1.025)	0.668
Gender	0.875 (0.461–1.661)	0.683
IL27 (pg/mL)	1.134 (1.074–1.195)	0.000*
IL35 (pg/mL)	1.396 (1.185–1.607)	0.002*
Bone marrow blast (%)	0.872 (0.815–0.933)	0.000*
WBC ($\times 1000/\text{mm}^3$)	1.136 (1.046–1.233)	0.041*
Platelets ($\times 1000/\text{mm}^3$)	0.97 (0.96–0.98)	<0.001*

Note: *Statistically significant.

Conclusion

From this study, we proved, for the first time, that serum levels of IL27 and IL35 might be served as highly sensitive and specific diagnostic/prognostic markers in patients with de novo AML. Also, we suggested that IL27 and IL35 over-expression in ND AML would be associated with poor prognosis. In addition, we reported strong association between the two cytokines in AML, and we generated cutoff values for IL27 and IL35 as predictors for AML. As such we claim IL27 and IL35 in AML development and pathogenesis.

On the light of these results, we recommended assay of IL27 and IL35 in ND AML, as the method for their measurement is relatively cheap, simple and widely available. Furthermore, based on our finding of the intimate association between IL27 and IL35, we assumed

measuring one cytokine only to save time and reduce cost. However, further wide scale longitudinal studies are mandatory to link certain cytokine values to disease progression, response to treatment and survival analyses.

Based on the results of this study it was indirectly suggested that cytokine levels can predict relapse probability but unfortunately we focused only in de novo cases to elicit diagnostic/prognostic value of cytokines. Accordingly, future research is needed to investigate cytokine levels in relapsed patients.

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Disclosure

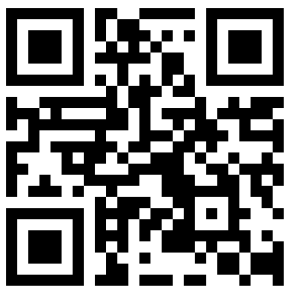
The authors report no conflicts of interest in this work.

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