

Myeloid-derived suppressor cells level and *MUC1* expression in de novo acute myeloid leukemia

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Abstract

Background: Acute myeloid leukemia (AML) is the most common acute leukemia occurring in adults. It is an aggressive myeloid neoplasm with maturation arrest of myelopoiesis, leading to an accumulation of myeloblasts in the bone marrow and peripheral blood. Objective: To evaluate alterations in myeloid-derived suppressor cells level and *MUC1* gene expression in patients with de novo acute myeloid leukemia concerning disease characteristics and response to induction chemotherapy. Patients and methods: The study was performed on 50 AML patients and 50 healthy controls. Detection of myeloid-derived suppressor cells (MDSCs) in peripheral blood was performed by mononuclear separation and flow cytometry. *MUC1* gene expression was performed by RNA extraction, reverse transcription, and real-time PCR at Hematology Department Medical Research Institute, Alexandria University. Results: We have demonstrated that AML patients had both increased presence of MDSCs in peripheral blood as well as *MUC1* overexpression in comparison to normal controls. MDSCs showed a significant correlation regarding response to induction chemotherapy on day 28. While MDSCs and not *MUC1* are associated with inferior response to induction chemotherapy on day 28. Conclusion: The current data suggested that AML patients exhibit an increased presence of MDSCs as well as *MUC1* gene overexpression in comparison with normal controls. While MDSCs showed a significant correlation regarding response to induction chemotherapy on day 28, MDSCs and not *MUC1* are associated with inferior response to induction chemotherapy on the same day.

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Keywords:

acute myeloid leukemia, induction response, *MUC1* gene, myeloid-derived suppressor cells

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal hematopoietic stem cell malignancy in which immature hematopoietic cells proliferate and accumulate in bone marrow, peripheral blood, and other tissues. Most cases of AML have no apparent cause. Some patients may have the emergence of abnormal myeloid clones in the bone marrow, termed clonal hematopoiesis, years before diagnosis [1]. Myeloid-derived suppressor cells (MDSCs) are a critical component of the tumor microenvironment that modulates interactions between immune effector cells and malignant cells. These MDSCs are newly identified, heterogeneous population of immature myeloid cells that are characterized by the ability to suppress both innate and adaptive immune responses [2]. MDSCs play a critical role in promoting immune tolerance and disease growth. The role of MDSCs in AML has not been well described. The role of MDSCs in solid tumors has been extensively characterized as protumorigenic [2, 3, 4]. In intensive clinical studies, circulating and/or infiltrating MDSCs at the tumor site were associated with poor prognosis in patients with solid tumors [5]. Removing MDSCs might contribute to restoring immune surveillance. Meanwhile, conflicting roles have been reported in hematological malignancies [6–10]. Mucin1 (*MUC1*) is a cell membrane glycoprotein, and it is normally expressed at low levels on the apical surfaces of

epithelial cells. *MUC1* is translated as a single polypeptide that undergoes autocleavage into two subunits, which in turn form a stable noncovalent heterodimer at the cell surface. The *MUC1* N-terminal subunit (*MUC1-N*) contains glycosylated tandem repeats, which are characteristic features of the mucin family [11, 12]. *MUC1-N* forms a complex with the transmembrane *MUC1* C-terminal subunit (*MUC1-C*). *MUC1-C* interacts with receptor tyrosine kinases such as FMS-like tyrosine kinase 3 (*FLT3*) at the cell membrane [13]. In these ways, *MUC1-C* has been shown to mediate critical aspects of oncogenesis, including cell proliferation, autonomous self-renewal, tissue invasion, and resistance to apoptosis and cytotoxic injury [14–19]. It has been recently identified that *MUC1* may play an important role in modulating the immunosuppressive milieu of the tumor microenvironment [20].

Participants and methods

Participants

This study was conducted on 100 subjects that were classified into 50 de novo adult AML patients where promyelocytic leukemia cases were excluded from the present study. All patients were recruited from the Hematology Department, Medical Research Institute, Alexandria

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University. A total of 50 healthy controls with similar age and sex are identified as a control group. This study was performed in the period from September 2018 to October 2019. All patients enrolled in the present study received induction chemotherapy (which includes standard-dose cytarabine 100–200 mg/m² continuous infusion × 7 days with idarubicin 12 mg/m² or daunorubicin 60–90 mg/m² × 3 days) [21]. All patients in our present study were subjected to a full medical checkup, such as clinical examination, complete blood count (CBC) with blood film examination, bone marrow examination, and the World Health Organization (WHO) classification. Bone marrow examination was performed at diagnosis and on day 28 post-induction chemotherapy was performed to evaluate response to induction chemotherapy. This study was approved by the local ethics committee at the Medical Research Institute, Alexandria University, Egypt. Informed consent from the patients was obtained before sample collection and after a brief explanation of research objectives.

Response assessment

Response assessment was performed on day 28. Where morphologic complete response (CR) was defined as an absolute neutrophil count >1,000/mL, with platelets ≥100,000/mL, bone marrow <5% blasts in an aspirate with spicules, no blasts with Auer rods, and no residual evidence of extramedullary disease were found. Partial response (PR) decreases of at least 50% in the percentage of blasts to 5%–25% in the bone marrow aspirate and the normalization of blood counts. No response is defined as the failure to attain CR or PR after induction chemotherapy [21].

Detection of MDSCs by flow cytometry

The sample is a fresh EDTA anticoagulated peripheral blood sample, and peripheral blood mononuclear cells were isolated using the Ficoll mononuclear separation principle [22]. After two washes, labeled antihuman monoclonal antibodies (mAbs) were used for stainings, such as HLA-DR FITC (Immunostep, Spain), CD11b FITC (Immunostep, Spain), and CD 33 PE (Immunostep, Spain) monoclonal antibodies. The three tubes were incubated in the dark for 40 min and washed twice by adding 1–2 mL of phosphate-buffered saline (PBS) to each tube.

Samples were acquired and the frequency of the MDSCs subsets was analyzed by flow cytometry Sysmex Partec CUBE 8 using Cyflow software. Sequential gating was performed; first, AML blasts are gated out based on previously clinically defined phenotype (HLA-DR positive blasts were gated out) [23]. Second, gating of HLA-DR negative/low population of cells and then sequential gating of CD 11b and CD 33 double-positive cells [23]. The gating strategy is shown in figure 1.

MUC1 gene expression by real-time PCR

RNA extraction was performed via Qiagen Blood QIAamp genomic RNA extraction kit and under sterile conditions using a UV laminar flow cabinet according to manufacturer instructions. The quality and quantity of RNA samples were analyzed and controlled at the end of

Table I. Primers and product size: Quantitect primer assay – Qiagen

Target gene	MUC1
Catalog number	QT00015379
Lot number	269867127
Forward	5'-CTCACCAGCCCAACAGG-3'
Reverse	5'-TGCCGCCGAAAGAACTAC-3'
Product size	312
Housekeeping gene	GAPDH
Forward	5'-GTCAACGGATTGGTCCGTATT-3'
Reverse	5'-AGTCTCTGGGTGGCAGTGAT-3'

extraction by NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse transcription (RT) was performed using QuantiTect Reverse Transcription Kit (Qiagen). The quality and quantity of DNA samples were analyzed and controlled at the end of RT by NanoDrop 2000 spectrophotometer. SYBR Green real-time PCR preparation was performed under sterile conditions using a UV laminar flow and the reaction was performed using Rotor-Gene Q cyclor from Qiagen. Primers and product size (Quantitect primer assay – Qiagen) details are supplied in table I. Ct values were evaluated for cases and controls, $\Delta\Delta C_t$ values (ΔC_t case - ΔC_t control), and the relative gene expression was calculated using $2^{-\Delta\Delta C_t}$ [24].

Results

With regard to the age of AML patients, it had a mean of 45.38 ± 14.35 years. The hemoglobin value of the studied group had a mean of 7.87 ± 2.05 g/dL, while the WBC count mean value was $64.34 \pm 94.19 \times 10^9/\mu\text{L}$, where 60% of the patients had leucocytosis, while 20% had leucopenia. Also, 90% of AML patients had thrombocytopenia. The blast percentage in peripheral blood was $40.10 \pm 33.25\%$. All cases were diagnosed and subtyped according to WHO classification including morphology, immunophenotyping, and cytogenetic studies as shown in table II. Also, FAB classification was performed and showed the following frequencies: M1 24%, M2 26%, M4 24% (6% of which were M4eso), and M5 24%. Regarding the response to induction chemotherapy, 44% of AML cases were responsive to chemotherapy, 32% achieved CR1, and 12% achieved PR, while 12% were nonresponsive to induction chemotherapy and 42% died very early before evaluation of treatment response. The results are shown in figure 2.

MDSCs showed a significantly higher level in peripheral blood of AML patients with a mean value of $8.02 \pm 8.11\%$ among the gated HLA-DR negative cells, to normal controls with a mean of $0.20 \pm 0.42\%$. MDSCs showed significant correlation as regard WBCs count and blast percentage in peripheral blood as well as bone marrow (Tab. III).

MDSCs showed a significant correlation regarding response to induction chemotherapy on day 28 ($p = 0.002$). As regards patients achieving CR, MDSCs showed a lower level with a mean value of $2.44 \pm 2.58\%$ compared with those achieving a PR (mean = $8.67 \pm 8.50\%$). Also, patients achieving CR had

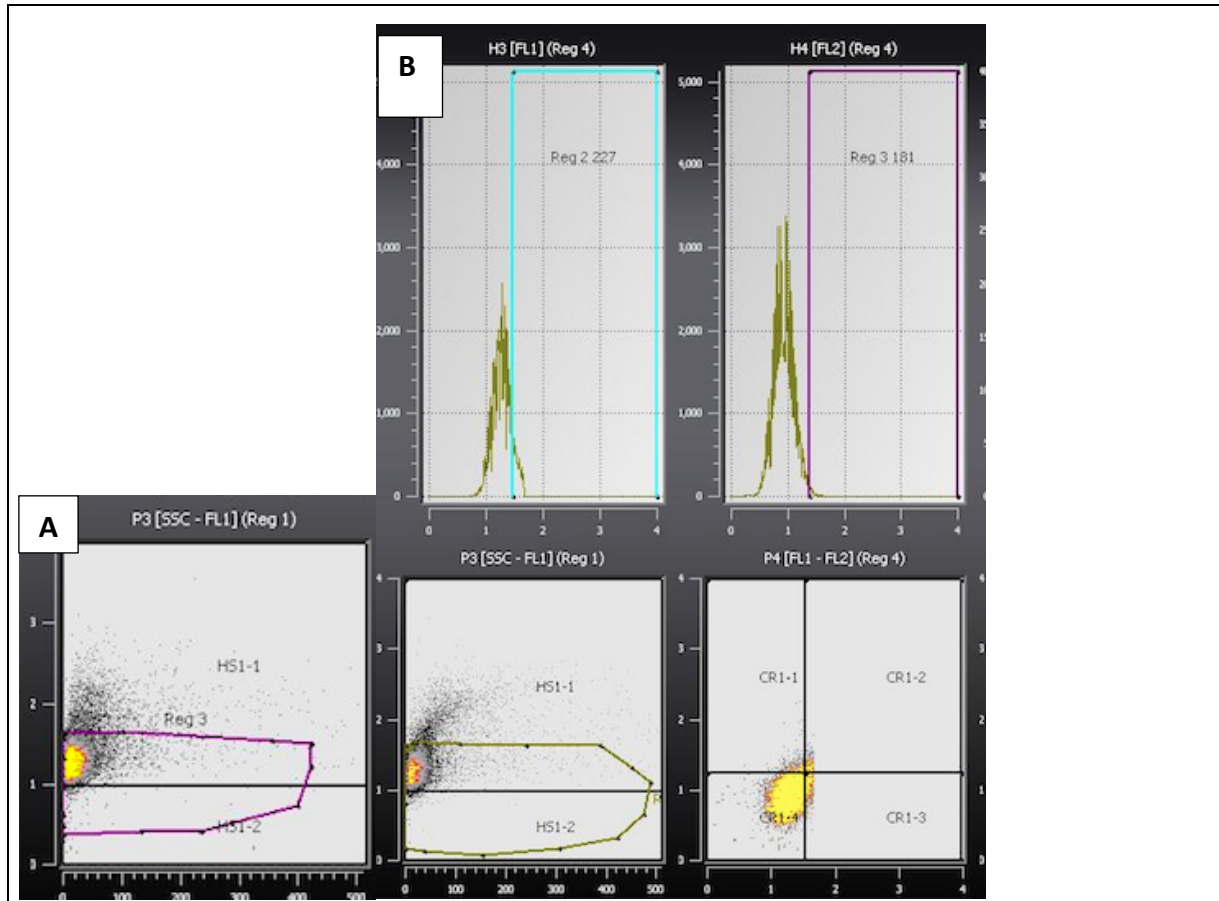


Fig. 1. Gating of HLA-DR negative/low population of cells (A). Sequential gating of CD 11b and CD 33 double-positive cells (B)

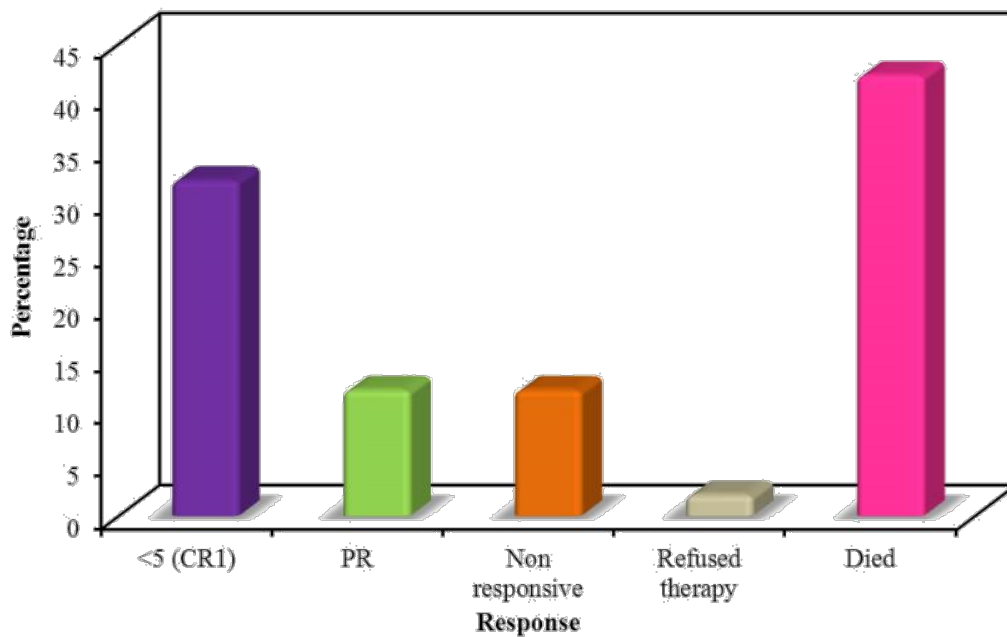


Fig. 2. Distribution of the studied AML patients according to response to induction chemotherapy on day 28 ($n = 50$). Accordingly, they were divided into those achieving complete response (CR1), partial response (PR), nonresponsive patients, and those who died early before the evaluation of treatment response as well as those who refused chemotherapy

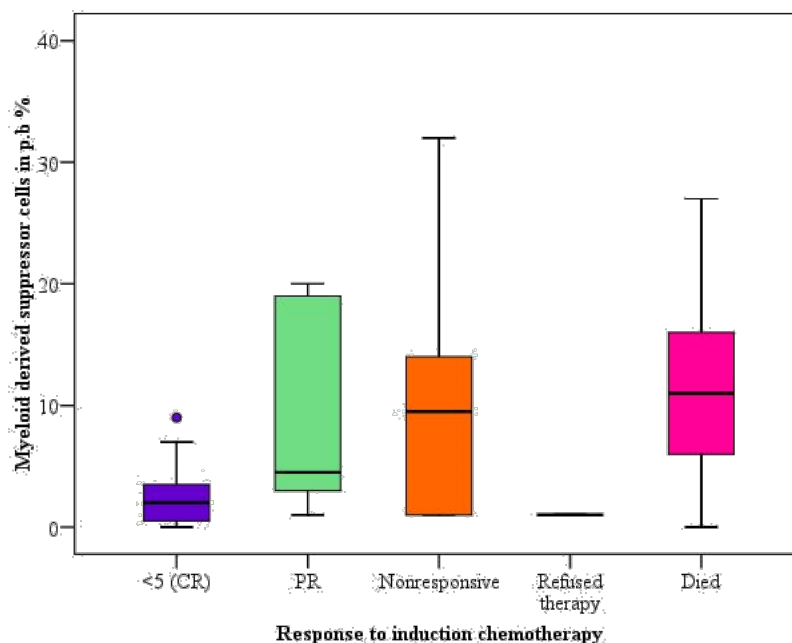


Fig. 3. The relation between response to induction chemotherapy and myeloid-derived suppressor cells in peripheral blood % in cases group (n = 50). They were classified into those achieving complete response (CR), partial response (PR), nonresponsive, and those who died before the evaluation of treatment response as well as those who refused treatment

Table II. Distribution of the studied AML cases according to immunophenotyping by flow cytometry (n = 50)

	Negative		Positive	
	No.	%	No.	%
CD13	4	8.0	46	92.0
CD33	2	4.0	48	96.0
CD7	42	84.0	8	16.0
CD19	46	92.0	4	8.0
CD11C	25	50.0	25	50.0
CD64	26	52.0	24	48.0
CD14	31	62.0	19	38.0
CD4	43	86.0	7	14.0
CD34	13	26.0	36	72.0
CD11B	30	60.0	20	40.0
GLYCOPHORIN and CD71	49	98.0	1	2.0

significantly lower MDSCs level compared to nonresponsive patients (mean = 11.17 ± 11.65%) ($p = 0.037$) as well as patients who died (mean = 11.52 ± 7.79%). As shown in figure 3. However, there was no significant correlation between MDSCs and specific morphologic subtypes as well as blasts phenotype.

A receiver operating characteristic (ROC) curve for the level of MDSCs in peripheral blood was performed at diagnosis to distinguish

Table III. Comparison between the two studied groups according to myeloid-derived suppressor cells in peripheral blood

Myeloid-derived suppressor cells in peripheral blood %	Cases (n = 50)	Control (n = 50)	p
Min.-Max.	0.0–32.0	0.0–1.0	<0.001*
Mean ± SD	8.02 ± 8.11	0.20 ± 0.42	
Median (IQR)	4.50 (1.0–13.0)	0.0 (0.0–0.0)	

Mann–Whitney U test; p – value for comparing between the studied groups; * – statistically significant at $p \leq 0.05$

AML patients achieving CR from nonresponsive patients (NR) after induction chemotherapy. A cutoff value of >10% MDSCs in peripheral blood (sensitivity of 66.67% and specificity of 62.5%) between patients achieving CR and nonresponsive patients.

As regards *MUC1* gene expression using $2^{-\Delta\Delta Ct}$, it showed a significantly higher level in AML patients with a median value of 8.80 (3.57–18.82) than normal controls with a median of 0.75 (0.39–1.41).

A ROC curve was performed as regards *MUC1* gene expression to distinguish AML cases from normal controls showing a cutoff value of >5.38 having a sensitivity of 66% and a specificity of 90% (Fig. 4). Where, AML cases showed positive *MUC1* gene expression in 88%, while only 12% showed *MUC1* gene under expression. As regards the correlation between *MUC1* gene expression and the patients' age, CBC parameters, and CRP, there was no significance ($p > 0.05$). Also, there was no significant correlation between *MUC1* gene expression level at different laboratories, and clinical data as

well as therapeutic response on day 28 post-induction chemotherapy (Tab. IV). Also, there was no significant correlation between MDSCs and *MUC1* gene expression.

Discussion

The present work aimed at the study of MDSCs level and *MUC1* gene expression in de novo AML patients. In the present study, MDSCs showed a significantly higher level in AML patients than in normal controls. This was in concordance with Pyzer et al. with a mean value of circulating MDSCs 7.94% (range: 1.70–17.0) and 0.2% (range: 0.02–0.88), respectively [23]. Moreover, similar results were reported by Alex et al. [25], Lv et al. [26], and Sun et al. [27]. Interestingly, MDSCs were elevated in peripheral blood in different hematological malignancies including lymphoma, multiple myeloma, and leukemia [26]. Also, in MDS, there was a higher MDSCs level in PB as well as in MM [28, 29, 30]. Also, CML patients showed increased PMN-MDSCs and M-MDSCs subsets at diagnosis, have been shown to return to normal levels after treatment with the tyrosine kinase inhibitor and M-MDSCs frequency has been proposed as a prognostic factor in CML patients receiving the TKI dasatinib [31]. Besides, CLL and DLBL patients showed the accumulation of myeloid-derived suppressor cells in peripheral blood [32, 33]. In the present series, there was no significant correlation between MDSCs and the age of AML patients ($p = 0.208$) which was consistent with Sun et al. [27]. Also, according to Alex et al. there was no significant correlation as regards patients' age [25]. The present study MDSCs showed a significant correlation with blast percentage in peripheral blood as well as bone marrow blast percentage and WBCs count. Following Pyzer et al. [23] MDSCs are expanded in the presence of AML blasts. According to Alex et al. [25] there was a significant positive correlation of MDSC with WBC counts at diagnosis. On the contrary, Sun et al. [27] stated that there was no significant correlation between the MDSCs level with WBC counts. This difference may be explained by the different ethnic backgrounds and the difference in sample size.

In the present study, there was no significant correlation with different morphologic subtypes according to FAB classification. This was similar to Sun et al., where there was no significant difference of MDSC between AML-M4 and M3, M2 but MDSCs in AML-M5 were significantly lower than that in AML-M2, M3, and M4 [27].

In this study, there was no significant correlation between MDSCs and CD34 expression on blast cells. However, according to Alex et al., there was a significant negative correlation with CD34 expression on blast cells at diagnosis [25]. This difference is related to the fact that CD34 expression was enrolled in this study as a qualitative value but as regards Alex et al. work it was quantitative.

In this study, patients with low MDSCs showed better outcomes as regards the response to induction chemotherapy on day 28. Although in this study, the patient's survival could not be detected, according to Cheong et al. [34] patients with higher MDSCs group had worse outcomes with a significantly shorter overall survival and leukemia-free survival. Besides, Sun et al. [27] observed a significantly lower number of MDSCs in the blood of patients at complete remission. The higher proportion of early deaths in this study was related to

Table IV. Comparison between high and low *MUC1* gene expression in AML cases as regards different clinical and laboratory parameters

	<i>MUC1</i> gene expression		<i>P</i>
	<i>MUC1</i> low ≤ 5.38	<i>MUC1</i> high > 5.38	
Response to induction on day 28	(n = 7)	(n = 21)	^{MC} <i>p</i> = 1.000
Complete response	4 (57.1%)	12 (57.1%)	
Partial response	2 (28.6%)	4 (19.0%)	
Non-responsive	1 (14.3%)	5 (23.8%)	
Blast (%) in peripheral blood	(n = 17)	(n = 33)	0.126
Min.–Max.	3.0–90.0	0.0–98.0	
Mean ± SD	47.94 ± 30.47	36.06 ± 34.34	
Median (IQR)	40.0	26.0	
Bone marrow blast (%)	(n = 17)	(n = 33)	0.367
Min.–Max.	16.0–91.0	20.0–95.0	
Mean ± SD	61.41 ± 23.75	53.36 ± 25.06	
Median (IQR)	65.0	48.0	
CD 19	(n = 17)	(n = 33)	^{FE} <i>p</i> = 0.597
Negative	15 (88.2%)	31 (93.9%)	
Positive	2 (11.8%)	2 (6.1%)	
CD 4	(n = 17)	(n = 33)	^{FE} <i>p</i> = 0.398
Negative	16 (94.1%)	27 (81.8%)	
Positive	1 (5.9%)	6 (18.2%)	
CD 7	(n = 17)	(n = 33)	^{FE} <i>p</i> = 0.699
Negative	15 (88.2%)	27 (81.8%)	
Positive	2 (11.8%)	6 (18.2%)	
CD 34	(n = 17)	(n = 33)	^{FE} <i>p</i> = 1.000
Negative	4 (23.5%)	9 (27.3%)	
Positive	13 (76.5%)	23 (69.7%)	
Strong positive	0 (0.0%)	1 (3.0%)	
FAB classification	(n = 17)	(n = 33)	^{MC} <i>p</i> = 0.616
M1	5 (29.4%)	7 (21.2%)	
M2	3 (17.6%)	10 (30.3%)	
M4	4 (23.5%)	5 (15.2%)	
M4 ESO	2 (11.8%)	1 (3.0%)	
M5	3 (17.6%)	9 (27.3%)	
M6	0 (0.0%)	1 (3.0%)	
Splenomegaly	(n = 17)	(n = 33)	^{FE} <i>p</i> = 0.728
Absent	14 (82.4%)	25 (75.8%)	
Present	3 (17.6%)	8 (24.2%)	
Lymphadenopathy	(n = 17)	(n = 33)	^{FE} <i>p</i> = 0.499
Absent	14 (82.4%)	23 (69.7%)	
Present	3 (17.6%)	10 (30.3%)	

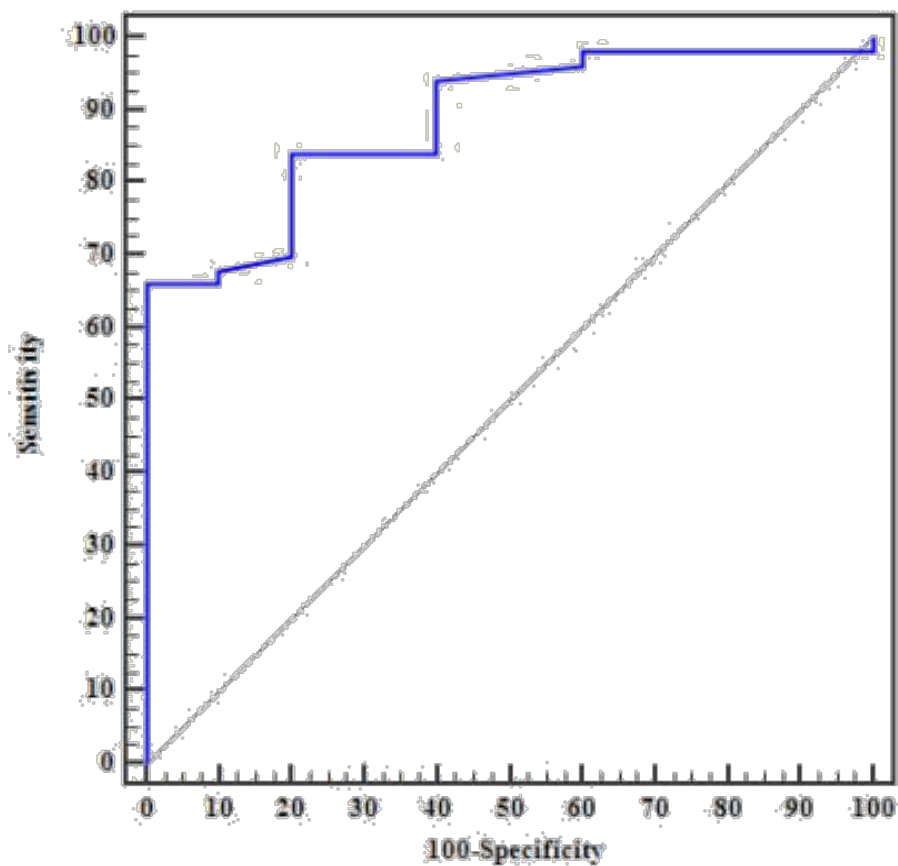


Fig. 4. ROC curve for $MUC1\ 2^{\Delta\Delta CT}$ to distinguish cases from normal controls

infection and febrile neutropenia, which were the major causes of early mortality.

In this study, *MUC1* gene expression showed a significantly higher level in AML patients than in normal controls. This was in accordance with Tagde et al's. work which stated that *MUC1* expression was significantly elevated in AML cells as compared to that in normal bone marrow cells [35]. Moreover, *MUC1* is overexpressed in human cell lines and a majority of primary samples obtained from AML patients at the time of presentation and relapse [36, 37, 38].

Conclusion

We have demonstrated that MDSCs are expanded in AML patients and they appear to impact the clinical course and prognosis of AML cases. Also, the *MUC1* gene is overexpressed in AML patients compared to normal controls. Additionally, using specific MDSC inhibitors or via *MUC1* inhibition, could pave the way for improved responses to immune therapies in AML.

Authors' contributions

SN – manuscript writing. All authors – revision of manuscript and paper design.

Conflicts of interest

There are no conflicts of interest.

Financial support

None.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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