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Methyltransferase-like 3 gene (*METTL3*) expression and prognostic impact in acute myeloid leukemia patients

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Abstract

Background: DNA methylation is involved in pathogenesis of acute myeloid leukemia (AML). N6-methyladenosine (m6A) modification of mRNA, mediated by methyltransferase-like 3 (*METTL3*), is one of the well-identified mRNA modifiers associated with the pathogenesis of AML. High level of *METTL3* mRNA is detected in AML cells, thus can be a potential target therapy for AML. This is a preliminary study that aimed at measuring *METTL3* mRNA expression level in de novo AML patients and correlating it with clinicopathological, laboratory and prognostic markers. *METTL3* expression was analyzed by quantitative reverse transcription polymerase chain reaction in 40 newly diagnosed AML adults and was re-measured in the 2nd month of chemotherapy. Patients were followed up for periods up to 6 months post-induction therapy.

Results: *METTL3* expression was found to be significantly upregulated in AML patients compared to control subjects ($p < 0.001$). *METTL3* gene was significantly expressed among non-responders compared to responders ($p < 0.001$). A cutoff value was assigned for normalized *METTL3* values to categorize AML patients according to response to therapy. Statistically significant association was observed between high pretreatment normalized *METTL3* gene level and failure to attain complete remission at 2nd, 4th and 6th month following therapy ($p = 0.01$, 0.02 and 0.003, respectively). However, insignificant correlation was found between pretreatment normalized *METTL3* gene level and event free survival or clinicopathological prognostic factors.

Conclusion: *METTL3* is overexpressed in AML patients and is associated with adverse prognostic effect and failure to attain hematological remission within 6 months post-induction therapy.

Keywords: AML, Epigenetics, *METTL3*, DNA methylation

Background

Acute myeloid leukemia (AML) is one of the most prevalent hematological malignancies in adults. It occurs due to clonal expansion of undifferentiated myeloid precursors in bone marrow, which leads to defect in normal hematopoiesis [1].

The pathogenesis and clinical outcome of AML are based on different genetic mutations and epigenetic dysregulations [2]. Epigenetic modifiers refer to the regulators of gene expression without an alteration of the DNA coding sequence. While almost 70–90% of the genomic DNA is estimated to be transcribed, only less than 2% of the genomic DNA is translated to proteins [3]. This implicates the leading role of non-coding RNAs (ncRNAs) in the human cell development and survival. The epigenetic dysregulations actively participate in pathogenesis of major hematological malignancies including AML [4]. DNA methylation and histone tail modifications are

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major epigenetic mechanisms that regulate the physiologic process of cell differentiation. However, their functional aberrations lead to silence of critical genes and development of AML [5]. *METTL3,* the m^6A-forming enzyme, is one of the most prevalent regulators of the mRNA nucleotide that occurs regularly in approximately 20% of human cellular mRNAs [6]. It stimulates translation of certain mRNAs including epidermal growth factor receptor (EGFR) and the Hippo pathway effector TAZ in human tumor cells; *METTL3* associates with the reporter mRNA and promotes translation in the cytoplasm. Hence, a depletion of *METTL3* will inhibit translation of the affected genes [7]. M^6A is an internal RNA modification in both coding and non-coding RNAs that is catalyzed by the *METTL3–METTL14* methyltransferase complex [8]. The specific role of these enzymes in leukemia is still largely unknown. However, compared with normal hematopoietic cells, high levels of *METTL3* mRNA and protein are detected in AML cells, which are related to abnormal cell differentiation and development of myeloid hematological malignancies. Furthermore, its depletion induces cell differentiation, apoptosis and delayed progression of leukemia [6, 8]. This effect is primarily mediated by phosphorylated AKT, whose levels are increased as a consequence of *METTL3* depletion [9, 10]. As a result, a new rationale has emerged that targets the writers, erasers and readers of m6A modification, thus representing a potential target therapy for several malignancies, including AML. Inhibition of 2-oxoglutarate (2OG) and iron-dependent oxygenases (e.g., ALKBH5 and FTO), which belong to the 2OG-dependent nucleic acid oxygenase (NAOX) family and suppress m^6A modification demethylation of RNA, have been discussed as promising target therapy of AML [11].

This work represents a preliminary study that measures *METTL3* mRNA expression level by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in newly diagnosed adult AML patients and non-leukemic control subjects. It aims at investigating its role in AML pathogenesis and correlating its levels with clinicopathological, laboratory and prognostic markers.

Methods

A prospective, case–control study was carried out on 40 newly diagnosed AML patients recruited during the period from May 2019 to May 2020. In addition, 20 age- and sex-matched adult controls, free from any hematological or solid malignancy, were also included. The diagnosis of AML was established according to the recent 2016 World Health Organization (WHO), updated AML diagnostic criteria, based on morphology, immunophenotyping (IPT) and cytogenetic analysis.

A written informed consent was obtained from all enrolled patients. The approval of study was taken from the institutional Ethics Committee of Ain Shams University with approval No. FWA 000017585.

Sampling

Two milliliters of venous blood samples were collected under complete aseptic conditions from each patient into dipotassium ethylene diamine tetra-acetic acid (k2-EDTA) tube at a concentration of 1.2 mg/ml, for complete blood count (CBC) testing and preparation of Leishman-stained films. Aspiration of 4 mL bone marrow (BM) was performed and divided as follows: the initial 0.5 ml for Leishman-stained smears, 1 ml into heparin tube for cytogenetic fluorescence in situ hybridization (FISH) analysis and 3 ml were divided into two EDTA tubes for IPT and quantitative reverse transcription polymerase chain reaction (qRT-PCR). These tests were performed initially for the diagnosis of the patients and on scheduled intervals for follow-up. For optimal results, blood samples were processed within 2–3 h of collection and no stored samples were used.

All patients were subjected to full history taking, clinical examination, CBC testing using Sysmex XN-1000 (Sysmex Europe, GmbH) with examination of Leishman-stained peripheral blood (PB) films, BM aspiration with examination of Leishman-stained smears. IPT (for patients only) was carried on BM blasts/blast equivalent cells using a standard panel of monoclonal antibodies by 6-color Navios flow cytometer (Coulter, Electronics, Hialeah, FL, USA). Conventional karyotyping and FISH were performed in selected cases. qRT-PCR was done for all enrolled subjects (40 AML patients before starting chemotherapy and 20 controls) to detect *METTL3* mRNA expression level using; TaqMan Gene Expression assays (FAM-MGB) Hs00219820_m1 *METTL3*.

AML patients received induction therapy regimen consisting of Adriamycin (25 mg/m^2, day 1–3) and cytarabine (100 mg/m^2, every 12 h, day 1–7); however, PML / RARA positive acute promyelocytic leukemia (APL) was given another protocol of PETHEMA LPA 2005. The patients were followed up at day 28, 2nd, 4th and 6th month, post-induction, and resistant cases received re-induction of FLAG Adria chemotherapy (fludarabine, high dose cytarabine, filgrastim and Adriamycin). The morphological remission of AML patients was assessed depending on their BM blast counts at day 28, 2nd, 4th and 6th month (till either the end of the study or last contact with them). Accordingly, patients were classified as responders (identified by BM blasts $\leq 5\%$ at day 28 chemotherapy), non-responders (BM blasts $> 5\%$ at day 28). Moreover, during follow-up stages at time between 2nd and 4th month of chemotherapy, the *METTL3* gene

expression level was reanalyzed in 15 patients who exhibited higher gene expression before starting treatment to assess the effect of therapy on expression levels.

METTL3 expression level measurement by qRT-PCR

Total ribonucleic acid (RNA) was isolated by using the "QIAamp RNA Blood Mini Kit" (Qiagen, Hilden, Germany) following manufacturer instructions. A specialized high-salt buffering system allows RNA species longer than 200 bases to bind to the QIAamp membrane. During the QIAamp procedure for purification of RNA, erythrocytes are selectively lysed and leukocytes are recovered by centrifugation. The leukocytes are then lysed using highly denaturing conditions that immediately inactivate RNases, allowing the isolation of intact RNA. Homogenization of the lysate is done by a brief centrifugation through a QIAshredder spin column. Ethanol is added to adjust binding conditions and the sample is applied to the QIAamp spin column. RNA is bound to the silica membrane during a brief centrifugation step. Contaminants are washed away, and total RNA is eluted in 30 µl or more of RNase-free water for direct use.

mRNA of *METTL3* was reversibly transcribed into complementary deoxyribonucleic acid (cDNA) using QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). *METTL3* gene expression level was amplified from mRNA using *METTL3* TaqMan™ Gene Expression Assay, Thermo Fisher, cat. No: (4331182) with primer sequence (Forward 5′-CAAGCTGCACTTCAGACG AA-3′, Reverse5′ GCTTGGCGTGTGGTCTTT-3′) and Beta-Actin as housekeeping gene, which serves as internal control for cDNA quality.

As duplex real-time PCR requires the simultaneous detection of different fluorescent reporter dyes whose fluorescence spectra exhibit minimal spectral overlap, the two-reporter dye labeled the 5′ ends of the probes were FAM™ (for *METTL3* target gene 14q11.2) and VIC (for Beta actin reference gene). Both TaqMan Probes were labeled with a non-fluorescent quencher dye at the 3′ ends of the probe.

Real-time PCR was performed with real-time cycler (Applied Biosystems StepOne; Applied Biosystems by Life Technologies™, USA). The PCR reaction mix was prepared for a final volume of 20 µl per well reaction volume using the following: 4 µl cDNA, 10 µl 2 × TaqMan Gene expression master mix, 1 µl of 20 × TaqMan Gene expression primer assay, and de-ionized water up to 5 µl for each sample cup.

qRT-PCR was performed at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Results were analyzed, and the differences of expression level for the target gene *(METTL3)* were calculated using the ΔΔCT method for relative quantitation.

$$\begin{aligned}\Delta\text{CT (sample)} &= \text{CT target gene}(METTL3) \\ &\quad - \text{CT reference gene } (\beta - \text{actin}).\\ \Delta\text{CT (control)} &= \text{CT target gene}(METTL3) \\ &\quad - \text{CT reference gene } (\beta - \text{actin}).\end{aligned}$$

Next, the ΔΔCT value for each sample was determined as:

$$\Delta\Delta\text{CT} = \Delta\text{CT (sample)} - \Delta\text{CT (control)}.$$

Finally, the normalized level of target gene expression was calculated by using the formula: $2^{-\Delta\Delta CT}$ (Fig. 1).

Statistical analysis

The data were analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data with parametric distribution were expressed as mean ± standard deviation (SD), while those with nonparametric distribution were expressed as median and interquartile range (IQR). Qualitative data were expressed as frequency and percentage. For quantitative variables, independent *t* test was used in cases of two independent groups with normally distributed data, while Mann–Whitney *U* test was used in cases of two independent groups with non-normally distributed data. Chi-square (χ^2) test of significance was used to compare proportions between qualitative parameters. Spearman's rank correlation coefficient (r_s) was used to assess the degree of association between two sets of variables if one or both was skewed. Kaplan–Meier Survival Analysis was performed for examining the distribution of time-to-event variables. ROC curve was constructed to evaluate the prognostic performance of *METTL3* gene expression and assign a cutoff value that would best distinguish between different groups. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the Probability (*p* value) was considered significant as follows: p value ≤ 0.05 significant, p value ≤ 0.001 highly significant and p value > 0.05 insignificant.

Results

Our study included 40 newly diagnosed AML patients (22 males and 18 females with mean age of 41.93 ± 17.45 years and male/female ratio 1.2). Out of the 40 patients, 32 (80%) had hepatosplenomegaly (HSM). Twenty age- and sex-matched non-leukemic control subjects were also included in all laboratory assessments. Laboratory characteristics of the studied patients are illustrated in Table 1.

Morphological remission was assessed for all patients at day 28 to evaluate early response to chemotherapy. Moreover, follow-up of BM blasts was done for available

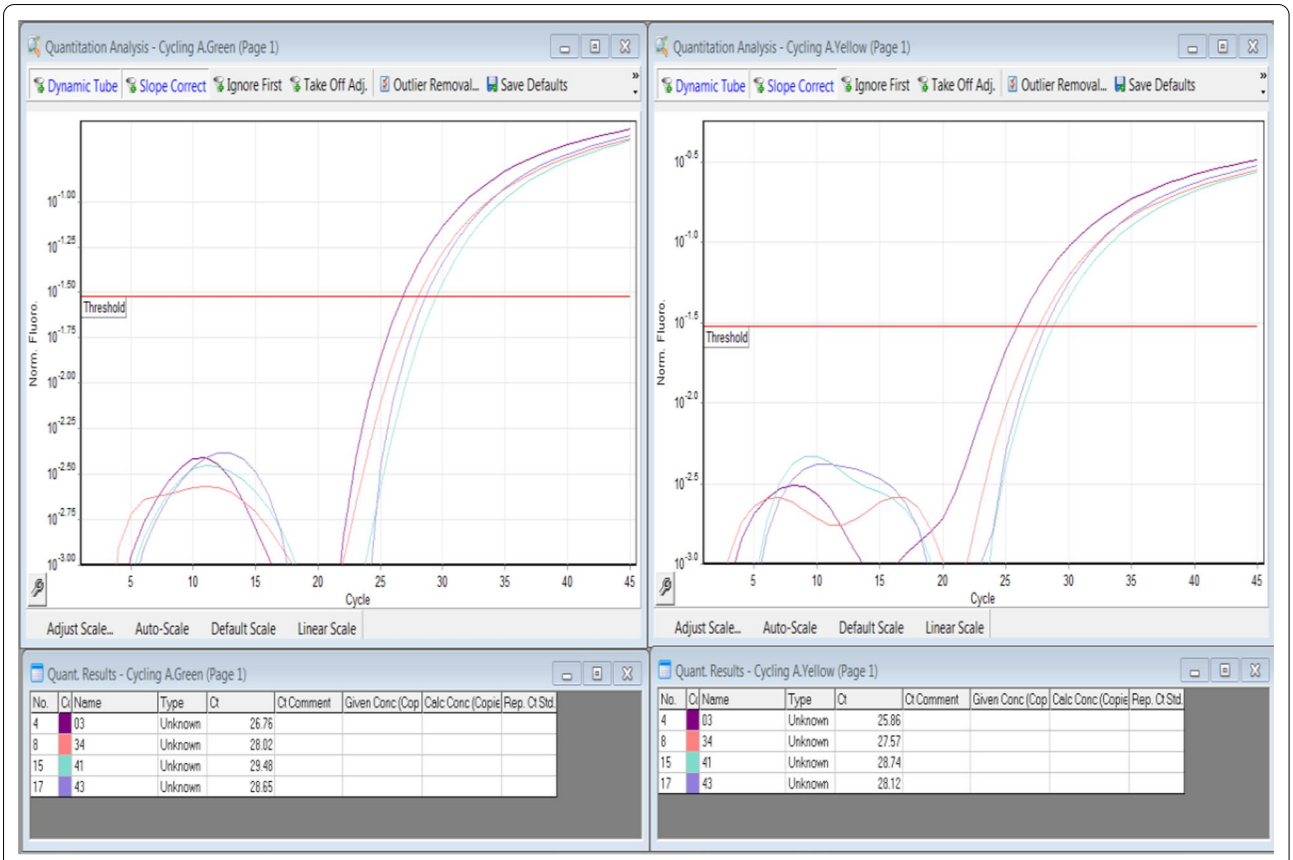

Fig. 1 AML cases with high normalized *METTL3* expression (high $2^{-\Delta\Delta CT}$ formula): the left side showing FAM™ (*METTL3* target gene CT), the right-side VIC (Beta actin reference gene CT)

AML patients at the 2nd, 4th and 6th month of chemotherapy to verify maintenance of complete remission (CR) (Table 2).

At baseline, pretreatment median *METTL3* gene expression level was statistically significantly higher in patients group compared to control group (6.95 vs 1.18; $p < 0.001$) (Table 3).

To unveil the impact of *METTL3* gene expression on prognosis, AML patients were divided into 2 prognostic groups depending on prognostic factors adopted by the American Cancer Society (2021 guidelines), namely total leucocyte count (TLC), age and cytogenetic abnormalities.

Most of AML patients in our study exhibited good prognostic criteria; 32/40 (80%) were younger than 60 years old and their TLC was $< 100 \times 10^9$/L. Regarding cytogenetic abnormalities, two patients out of 19 studied cases revealed positive t(8;21), five patients out of ten studied cases were positive for t(15;17) and one patient out of four cases was positive for inv.16. Regarding the bad prognostic criteria, 8/40 (20%) were older than 60 years of age, of whom, 5 patients were associated with high TLC $> 100 \times 10^9$/L; unfavorable cytogenetics (11q23 & t(9;22)) were not detected in any case. *METTL3* gene expression was studied in each group; however, no statistically significant difference was found as regards *METTL3* gene expression level in both prognostic groups.

AML patients were classified into responders 15/40 (37.5%) and non-responders 25/40 (62.5%) according to morphological assessment of BM at day 28 post-therapy (Table 4). To evaluate the impact of *METTL3* gene expression on achievement of hematological remission, *METTL3* gene expression was studied among these 2 subgroups and responders revealed low normalized *METTL3* gene expression level (median 2.28; IQR 1.87–2.58), while non-responders exhibited higher gene expression level (median 9.58; IQR 7.7–14.6), and this difference was statistically highly significant ($p < 0.001$) (Table 4).

The prognostic performance of *METTL3* gene expression was assessed using ROC curve analysis to obtain best cutoff for predicting the poor outcome (failure of remission or death). A cutoff value of ≥ 4 with an area

Table 1 Laboratory characteristics of the studied patients

Variable		AML group ($n=40$)
TLC	Median (IQR)	36.05 (2.5–70)
HGB	Mean ± SD Range	7.37 (1.62) 3.3–10.4
PLT	Median (IQR)	22.5 (13.3–54.8)
BM blasts count	Mean ± SD	74.53(17. 76)
FAB classification	*N*	(%)
M0–M1	3	7.5%
M1–M2	19	47.5%
M3	10	25.0%
M4	6	15.0%
M5	2	5.0%
Cytogenetic findings		
t(8,21) (q22;q22)	2/19	5%
t(15;17) (q22;q21)	5/10	12.5%
Inv. 16/t(16;16) (p13;q22)	1/4	2.5%
11q23	0/1	0
t(9;22) (q34;q11)	0/7	0

TLC total leucocyte count, *HGB* hemoglobin, *PLTs* platelets, *BM* bone marrow, *FAB* French–American–British classification, *SD* standard deviation, *IQR* interquartile range

Table 2 Assessment of morphological remission and maintenance of complete remission (CR)

BM blasts count	No	%
Assessment of early response to chemotherapy	40	
Blasts% day 28		
≤ 5%	16	40
> 5%	23	57.5
Died	1	2.5
Assessment of maintenance of CR		
Blasts% 2 m		
≤ 5%	26/36	72.2
> 5%	8/36	22.2
Died	2/36	5.5
Blasts% 4 m		
≤ 5%	22/29	75.9
> 5%	7/29	24.1
Blasts% 6 m		
≤ 5%	20/29	68.9
> 5%	9/29	31.1

Table 3 Comparison between AML cases vs. control group regarding pretreatment *METTL3* gene expression level at diagnosis

***METTL3* gene expression (pretreatment level)**	**AML group ($n=40$)**	**Control group ($n=20$)**	***z* test**	***p* value**
Median (IQR)	6.95 (2.5–10.5)	1.18 (0.5–1.9)	18.544	< 0.001**

IQR interquartile range, *z* Mann–Whitney test, *HS* highly significant

**p value < 0.001

Table 4 Comparison between the two AML subgroups (responders and non-responders) according to their pretreatment *METTL3* gene expression level

***METTL3* gene expression (pretreatment)**	**Responders AML cases with low normalized *METTL3* gene expression ($n=15$)**	**Non-responders AML cases with high normalized *METTL3* gene expression ($n=25$)**	**Test**	***p* value**
Median (IQR)	2.28 (1.87–2.58)	9.58 (7.7–14.6)	$z=28.566$	< 0.001**

IQR interquartile range, *z* Mann–Whitney test, *HS* highly significant

**p value < 0.001

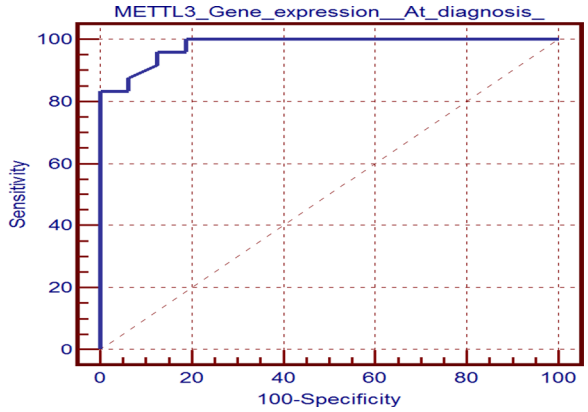

Fig. 2 Receiver operating characteristic (ROC) curve detect the best cutoff value of *METTL3* gene expression level predicating response to chemotherapy

under curve (AUC) 0.980 was found to have a sensitivity of 95.8%, specificity of 87.5%, PPV of 92%, NPV of 93.3% and a diagnostic accuracy of 98% (Fig. 2).

Comparison was done between AML subgroup with high normalized *METTL3* gene expression versus AML subgroup with low normalized gene expression regarding demographic, clinical data and hematological parameters at diagnosis and findings are illustrated in Table 5.

Pretreatment *METTL3* gene expression level was statistically significantly higher in AML cases with hepatosplenomegaly (HSM) ($p=0.014$). There was no statistically

significant difference in *METTL3* gene expression level regarding laboratory parameters.

Comparison between pretreatment *METTL3*-based AML subgroups regarding hematological remission as evidenced by BM blast percentage was performed for follow-up samples from day 28 chemotherapy till the end of the 6th month of chemotherapy, the results are shown in Table 6. Statistically significant association was observed between high normalized *METTL3* gene expression pretreatment level and failure to maintain CR at 2nd month, 4th month and 6th month follow-up ($p=0.01$, 0.02 and 0.003, respectively). Also, among cases with high gene expression level, one case died at day 28 and another two cases died at the 2nd month (Table 6 and Fig. 3).

Among the 29 cases who could be followed till the end of the 6th month chemotherapy, 8/14 patients who expressed high pretreatment level of *METTL3* gene failed to achieve hematological remission (blast counts > 5%) till the end of the 6th m chemotherapy compared to only 1/15 patients who expressed low level of *METTL3* ($p=0.003$).

Within the short-term follow-up, between the 2nd and 4th month of chemotherapy, the *METTL3* gene expression level was reassessed (post-treatment) in 15 cases who expressed higher pretreatment *METTL3* gene levels. The initial mean value of the pretreatment gene levels of those 15 patients was 14 and became 24.62 post-treatment (range 0.36–131.59). Post-treatment *METTL3* gene level was elevated in 60% of the evaluated cases (9/15) with a mean value of 28.02, compared to 6/15 (40%) patients who revealed a decline in post-treatment *METTL3* gene expression level with mean value 11.02.

The frequency of maintaining CR based on BM blast percentage in follow-up samples was assessed for both groups (Table 7, Fig. 4) and statistically significant association was found between the elevated normalized *METTL3* gene expression level post-treatment and failure to maintain CR at 2nd month, 4th month and 6th month chemotherapy ($p=0.048$, 0.015, 0.015, respectively). Moreover, post-treatment *METTL3* gene expression level was positively correlated to BM blast percentage at 2nd month, 4th month and 6th month of chemotherapy, and this correlation was statistically highly significant ($p \leq 0.001$, 0.016, 0.002, respectively) (Table 8, Fig. 5).

Kaplan–Meier survival curve was drawn to evaluate the impact of higher *METTL3* expression on event free survival (EFS), yet the curve could not estimate statistical significance due to the few deaths number (only 3 cases). Interestingly, the curve revealed that all died cases were within the subgroup of AML with high *METTL3* expression level.

Table 5 Comparison between AML subgroups regarding demographic, clinical data and hematological parameters at diagnosis

Demographic data, clinical data and hematological data	Non-responder AML cases with high normalized *METTL3* gene expression ($n=25$)	Responder AML cases with low normalized *METTL3* gene expression ($n=15$)	Significance	
			Test	*p* value
Gender				
Female	13 (52.0%)	5 (33.3%)	$\chi^2=1.320$	0.251
Male	12 (48.0%)	10 (66.7%)		
HSM				
Yes	23 (92.0%)	9 (60.0%)	$\chi^2=6.000$	**0.014***
No	2 (8.0%)	6 (40.0%)		
Age (years)				
Mean ± SD	43.20 ± 18.44	39.80 ± 16.31	$t=0.347$	0.560
Range	18–76	20–63		
TLC				
Median (IQR)	40 (1.9–70)	31.9 (2–75)	$z=1.681$	0.203
HB				
Mean ± SD	7.69 ± 1.45	6.84 ± 1.81	$t=2.665$	0.111
Range	4.00–10.4	3.30–9		
PLTs				
Median (IQR)	19 (12–89)	34 (11–55)	$z=2.446$	0.126
BM blasts%				
Median (IQR)	75 (72–85)	85 (48–88)	$z=2.124$	0.153

HSM hepatosplenomegaly, *TLC* total leucocyte count, *HB* hemoglobin, PLTs platelets, χ^2 Chi-square test, *t* independent sample *t* test, *z* Mann–Whitney test

*p value < 0.05 S; p value > 0.05 NS

Bold value is the only statistically significant p value = 0.014

Table 6 Comparison between pretreatment *METTL3*-based AML regarding of BM blasts in follow-up samples from day 28 chemotherapy till the end of the 6th month of chemotherapy

BM blast count (%)	AML cases with high normalized *METTL3* gene expression ($n=25$)	AML cases with low normalized *METTL3* gene expression ($n=15$)	Significance	
			x^2	*p* value
Assessment of early response to chemotherapy	$N=25$	$N=15$		
Day 28 BM blast %				
CR	2 (8.0%)	14 (93.3%)	28.452	< 0.001**
Failure of CR	22 (88.0%)	1 (6.7%)		
Died	1 (4.0%)	0 (0.0%)		
Assessment of maintenance of CR	$N=23$	$N=13$		
2nd month BM blast %				
≤ 5%	13 (56.5%)	13 (100%)	–	0.01*
> 5%	8 (34.7%)	0 (0.0%)		
Died	2 (8.7%)	0 (0.0%)		
	$N=14$	$N=15$		
4th month BM blast %				
≤ 5%	8 (57.1%)	14 (93.3%)	5.18	0.023*
> 5%	6 (42.9%)	1 (6.7%)		
	N = 14	N = 15		
6th month BM blast%				
≤ 5%	6 (42.9%)	14 (93.3%)	8.62	0.003*
> 5%	8 (57.1%)	1 (6.7%)		

CR complete remission, *BM* bone marrow; follow-up samples from day 28 chemotherapy, χ^2 Chi-square test

*p value < 0.05 S; **p value < 0.001 HS

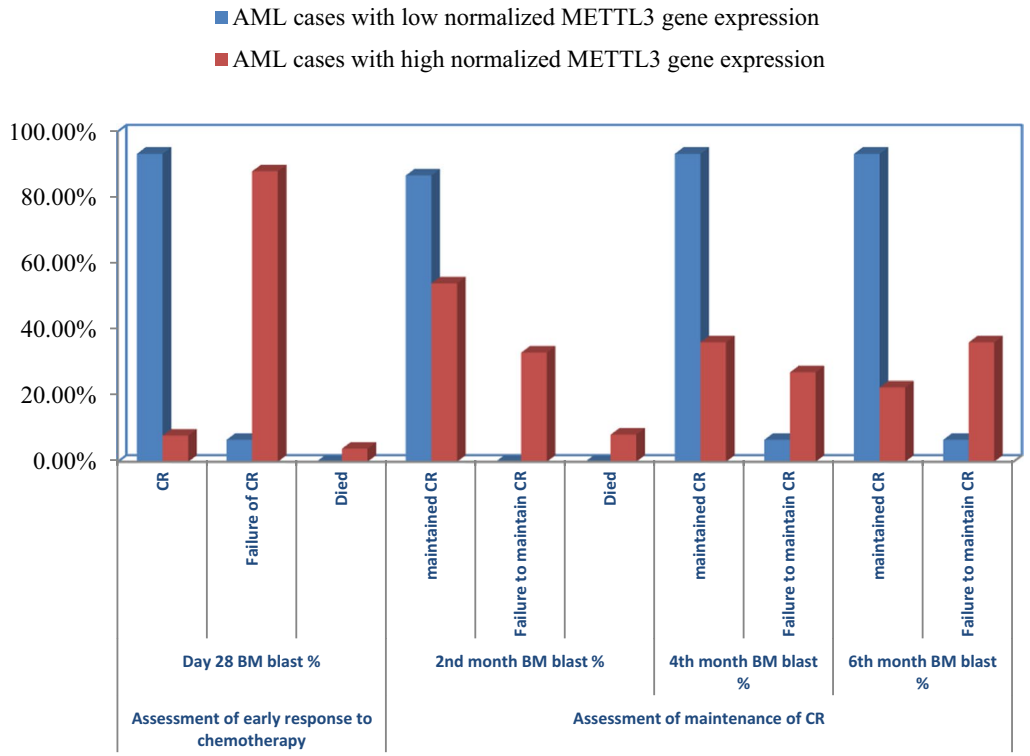

Fig. 3 Bar chart showing difference in BM blast counts during follow-up stages in both *METTL3*-based AML subtypes

Table 7 Comparison between patients with elevated post-treatment *METTL3* gene expression versus patients with lowered post-treatment gene levels regarding BM blast counts in follow-up samples

BM blast counts%	AML group with elevated normalized *METTL3* gene expression post-treatment ($n=9$)		AML group with reduced normalized *METTL3* gene expression post-treatment ($n=6$)		χ^2	*p* value
	No	%	No	%		
Assessment of maintenance of CR						
2nd month BM blast counts%						
≤ 5%	4	44.4%	6	100.0%	3.905	0.048*
> 5%	5	55.6%	0	0.0%		
4th month BM blast counts%						
≤ 5%	2	22.2%	6	100.0%	5.904	0.015*
> 5%	7	77.8%	0	0.0%		
6th month BM blast counts%						
≤ 5%	2	22.2%	6	100.0%	5.904	0.015*
> 5%	7	77.8%	0	0.0%		

CR complete remission, *BM* bone marrow, χ^2 Chi-square test

*p value < 0.05 S

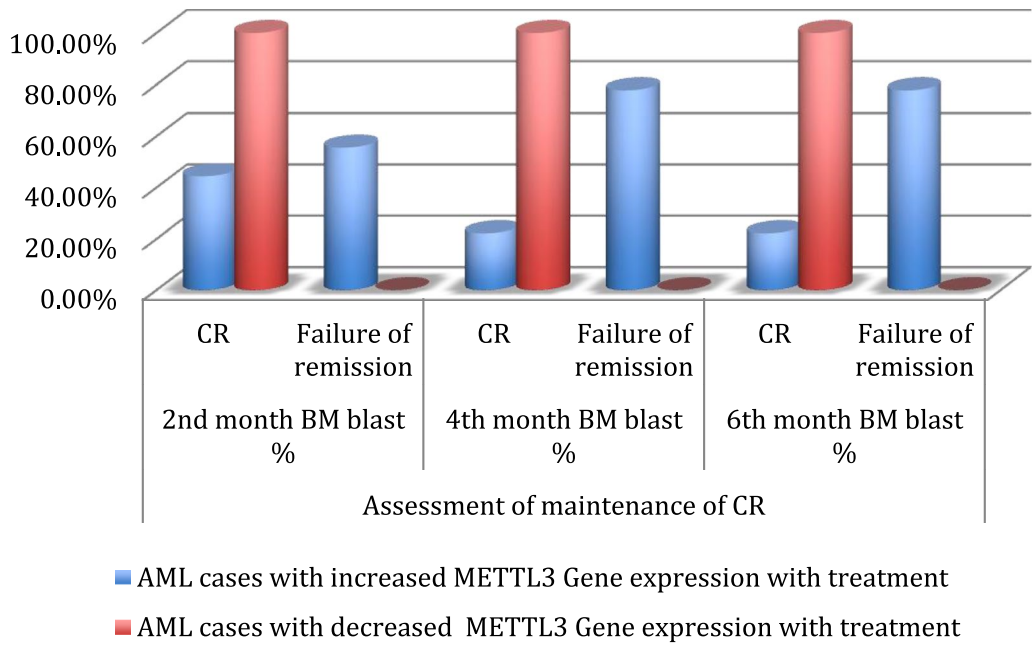

Fig. 4 Bar chart showing the follow-up response in both AML subgroups with elevated and reduced gene expression level with treatment

Discussion

In the past few years, the molecular characterization of AML has aroused the researchers' interest in better understanding of pathogenesis, prognosis prediction, treatment stratification, development of new targeted therapies and assistance in MRD detection.

An increasing number of researches have focused on the crucial role of mRNA modifiers in progression of leukemia. *METTL3* is one of these mRNA modifications that have been associated with pathogenesis of AML [2]. The methylation process of the mRNA nucleotide by *METTL3* occurs regularly in approximately 20% of human cellular mRNAs. However, alteration of this methylation process in AML cells has been associated with high abnormal level of *METTL3* mRNA and protein

Table 8 Correlation of *METTL3* gene expression level to blast count during follow-up stages for assessment of early response to chemotherapy and maintenance of CR

	***METTL3* gene expression (during follow-up)**	
	R	***p* value**
Assessment of early response to chemotherapy		
Day 28 BM blast %	0.377	0.037*
Assessment of maintenance of CR		
2nd month BM blast %	0.872	< 0.001**
4th month BM blast %	0.649	0.016*
6th month BM blast %	0.833	0.002*

* mean statistically significant value (< 0.05) and ** mean highly statistically significant (< 0.001)

and leads to marked alteration in cell differentiation and developing of myeloid hematological malignancies [6].

In this work, we aimed to measure *METTL3* mRNA expression level by qRT-PCR in newly diagnosed AML cases and study this level in relation to clinical, laboratory and prognostic markers.

Forty newly diagnosed AML patients were enrolled in our study along with 20 age and sex-matched control subjects. We found that *METTL3* is significantly overexpressed in AML patients with median value 6.95 compared to median 1.18 in control group (p value < 0.001). Some recent studies have demonstrated similar results where overexpression of *METTL3* was shown in AML cells compared to the normal Hematopoietic Stem/Progenitor Cells (HSPCs) [6, 12].

Similarly, another study was done by Vu et al. [13] to assess the alteration of *METTL3* mRNA expression in leukemia in which they compared the *METTL3* mRNA expression in AML samples to other cancers based on the cancer genome atlas database. They found that *METTL3* mRNA expression is significantly higher in AML than in

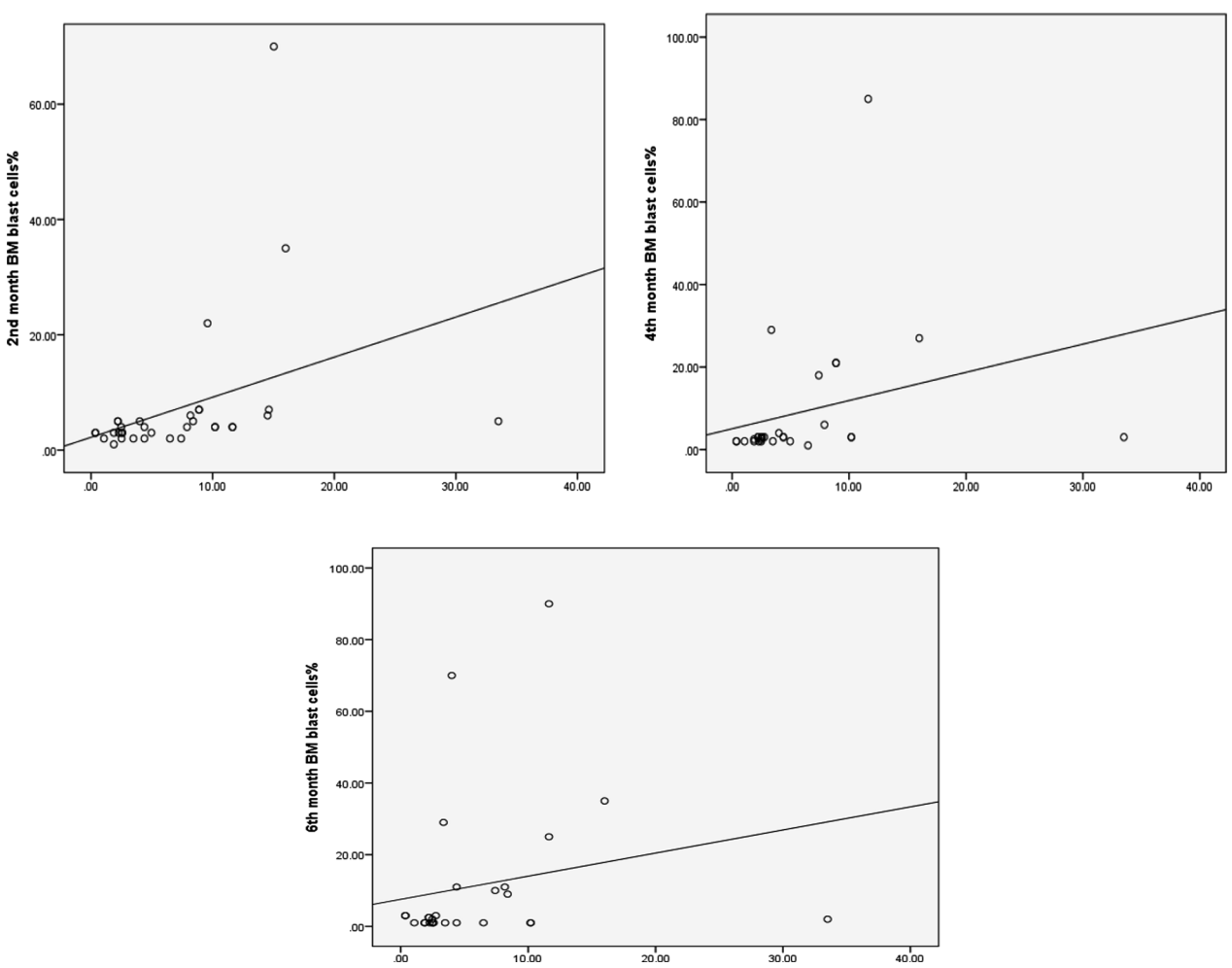

Fig. 5 Scatter plot between *METTL3* gene expression level during follow-up and 2nd, 4th and 6th month BM blast counts %

other cancer types with p value < 0.00001. They further assessed the relative abundance of *METTL3* in myeloid leukemia and examined both *METTL3* mRNA and protein levels in multiple leukemia cell lines in comparison with primary HSPCs cord blood-derived $CD34^+$ cells. They found that both *METTL3* mRNA and *METTL3* protein were more abundant in AML cell lines. However, there was no significant difference in *METTL3* expression across multiple subtypes of AML in the blood pool database [14].

In our study, we divided the 40 AML patients into 2 prognostic subgroups according to the American Cancer Society (2021 guidelines). We found that most of our AML patients exhibited good prognostic criteria. However, we didn't find significant association between any prognostic criterion and the *METTL3* gene expression level.

In the same context, a cohort study was carried on 191 AML patients and detected mutations of m6A regulatory genes in 2.6% (5/191) and variation in gene copy number in 10.5% (20/191) of patients. They studied whether mutations and copy number variations (CNVs) of m6A regulatory genes were associated with clinical and molecular features (older age > 60 years, white blood cell count > median ($15{,}200/mm^3$), unfavorable cytogenetic risk and mutations of DNMT3A and TP53). They observed that mutations and/or CNVs of *METTL3, METTL14, YTHDF1, YTHDF2, FTO and ALKBH5* as a group were significantly associated with poorer cytogenetic risk in AML ($p < 0.0001$). Additionally, they detected a marked increase in TP53 mutations ($p < 0.0001$). However, these mutations and/or CNVs were not associated with older age (> 60 years) or high white blood cell count > median [15].

METTL3 gene expression pretreatment level was statistically significantly higher in AML cases with HSM (p value 0.014). There was no statistically significant relation between *METTL3* gene expression level and any other parameter (age, gender, initial TLC count, hemoglobin, platelet count or initial BM blast count).

Similarly, a study of *METTL3* and *METTL14* expressions in 37 ALL patients investigated the relation between *METTL3* and *METTL14* expressions with clinical features. They didn't find association between the expression level of *METTL3 and METTL14* with gender, age, initial TLC and blast percentage, indicating that these two genes may not be associated with tumor burden [16]. The association between *METTL3* expression level and clinical data in AML patients' needs further studies to be evaluated.

Another recent study of *METTL3* expression level in solid tumors was carried on 340 patients with oral squamous cell carcinoma reported that a higher *METTL3* expression level was significantly positively associated with advanced tumor stage, advanced clinical stage and lymph node metastasis, but no differences in other features, such as sex and age, were observed [17].

Our study revealed that no significant association was found between the higher *METTL3* expression levels and specific AML subtypes. The same finding was reported by Vu et al. [13] during their study of *METTL3* mRNA and protein levels in multiple leukemia cell lines. They found that *METTL3* mRNA was more abundant in AML cell lines with no significant difference in *METTL3* expression across multiple subtypes of AML.

As regards the cytogenetic abnormalities in our study, they were not statistically related to *METTL3* gene expression level. Contrarily, a recent study of the molecular function of m6A RNA methylation in cancer has reported that *METTL3* and *METTL14* are highly expressed in AML cells carrying t(11q23), t(15;17) or t(8;21) and are down-regulated during myeloid differentiation [18]. This controversy may be attributed to the few numbers of cases exhibiting these abnormalities in our study.

Another study by Weng et al. [10] found that *METTL14* is highly expressed in normal HSCs and AML and is down-regulated during myeloid differentiation. In particular, *METTL14* was found to be overexpressed in AML cells carrying 11q23 alterations, t(15;17) or t(8;21). Analysis of the Cancer Genome Atlas (TCGA) data revealed that AML blast cells expressed higher mRNA levels of both *METTL3* and *METTL14* than most cancer types, and genetic alteration of those genes has significantly correlated with poorer prognosis [19].

The associations between m6A and genetic alterations in AML were studied by Paris et al. [9] who reported that m6A promotes the translation of *c-MYC, BCL2* and *PTEN* mRNAs in the human AML cell line. Moreover, loss of *METTL3* leads to increased levels of phosphorylated AKT, which contributes to the differentiation-promoting effects of *METTL3* depletion. Overall, these results provide a rationale for the therapeutic targeting of *METTL3* in myeloid leukemia. In the same context, the molecular function of WTAP, which is a novel oncogenic protein in myeloid leukemia that acts as regulatory subunit of the m6A methylation complex, was evaluated by a group of researchers. Their results revealed a lack of association between WTAP levels and particular cytogenetic abnormalities, but a significant correlation was detected between some specific molecular mutations such as NPM1 and FLT3-ITD, and WTAP expression [20]. WTAP is commonly upregulated in myeloid leukemia, but this upregulation alone is not enough to induce cell proliferation in the absence of a functioning *METTL3* [21].

The patients enrolled in our preliminary study were de novo AML, with a mean age of 41.9 years and median TLC 36.05×10^9/L with the absence of unfavorable cytogenetic abnormalities (11q23 & t(9;22)). These data can collectively predict a good response to chemotherapy based on the updates of independent prognostic factors in AML [22]. However, during the initial stages of the follow-up, 23/40 (57.5%) failed to achieve CR with induction therapy and by the end of the 6th month chemotherapy, 9/29 (31.1%) failed to maintain their CR. This may suggest the adverse prognostic role of *METTL3* expression in AML.

In the current study, the initial response to chemotherapy was assessed morphologically at day 28 post-therapy and accordingly, based on the response to chemotherapy at day 28 post-therapy, AML patients were classified into responders (15/40; 37.5%) and non-responders (25/40; 62.5%). To evaluate the impact of *METTL3* gene expression on achievement of hematological remission, *METTL3* gene expression was studied in these 2 subgroups and surprisingly, responders revealed low normalized *METTL3* gene expression level (median 2.28; IQR 1.87–2.58) while non-responders exhibited higher median gene expression (median 9.58; IQR 7.7–14.6).

ROC curve analysis was used to evaluate the ability of *METTL3* gene expression level at diagnosis to anticipate response of AML patients to chemotherapy. A cutoff value of 4 was selected as discriminating point with sensitivity of 95.8%, specificity of 87.5%, PPV of 92%, NPV of 93.3% and a diagnostic accuracy of 98%. This cutoff value proves that AML patients with high gene expression levels have bad response to chemotherapy at day 28. Likewise, patients with low gene expression levels show good response to induction therapy.

Our study detected significant association between higher pretreatment level of *METTL3* gene expression in AML cases at time of diagnosis and failure to maintain CR at 2nd month, 4th month and 6th month follow-up ($p = 0.01$, 0.02 and 0.003, respectively). On monitoring patients with high gene expression level, we found that one case died at day 28 and another two cases died at the 2nd month. Out of the 14 patients who expressed higher *METTL3* level at diagnosis and could be followed till the end of the 6th month chemotherapy, eight patients (57.1%) did not achieve hematological remission and showed persistently high blast counts, compared to only 1/15 (6.67%) patients who expressed a low level of *METTL3*.

In the present study, we reassessed the *METTL3* gene expression level (between the 2nd and 4th month post-treatment) in 15 cases who expressed higher pretreatment gene levels. We intended to monitor the short-term effect of chemotherapy on the gene expression levels and determine possible correlations with patients' outcome. In comparison with the pretreatment levels, the gene level was found to increase after chemotherapy in 9/15 patients (60%). This elevation was associated with failure to maintain CR at 2nd m, 4th m and 6th m chemotherapy ($p = 0.048$, 0.015, 0.015, respectively). Moreover, 7/9 (77.8%) of AML cases with elevated gene level post-treatment failed to maintain hematological remission till the end of the 6th month; in contrast, none of the six AML cases with reduced gene expression level failed to maintain hematological remission. No related data in analogous studies are available in the literature. Nevertheless, these findings of gene levels monitoring comply with the poor prognostic effect obtained when analyzing the pretreatment gene levels. In addition, it is important to emphasize that despite the administration of chemotherapeutics, it can be speculated that this gene still has the ability to increase and exert its dismal prognostic effect.

Similarly, the poor prognosis of AML cases with m6A mutations was reported in a cohort study that was carried on 191 AML patients, where the authors found that mutation of any of the genes encoding m6A regulatory enzymes had a worse OS ($p = 0.007$) and EFS ($p < 0.0001$). Inferior OS and EFS were also evident in patients who had mutations and/or CNVs of these genes [15].

Recently, a therapeutic trial was done on small molecules that act as selective inhibitors of *METTL3* in AML. Their anti-tumor effects were evaluated in patient-derived xenotransplantation experiments as well as transplantation experiments using an MLL-AF9-driven primary murine AML model. They reported that daily dosing of 30 mg/kg significantly inhibited AML expansion and reduced spleen weight compared to control, indicating that inhibition of *METTL3* in vivo leads to strong anti-tumor effects in physiologically and clinically relevant models of AML [23].

Collectively, these studies highlight the prognostic role of both *METTL3* in malignant hematopoietic cells and will encourage further epigenetic studies of target therapies in AML. These upcoming studies will reveal new insights regarding the molecular mechanisms regulating normal and malignant hematopoiesis and offer better opportunities for AML patients to improve their clinical outcomes.

In conclusion, our results have proved an association between high pretreatment gene expression level and bad response to chemotherapy. In addition, patients with a further increase in gene expression during the course of the disease were more likely to show failure to maintain hematological remission. Accordingly, an adverse prognostic impact of *METTL3* expression on the outcome of AML adult patients can be concluded. However, since the small number of patients and the short follow-up time are two main limitations

of this study, we strongly recommend large studies with longer follow-up periods to verify the proposed role of *METTL3* gene expression in the pathogenesis and prognosis of AML.

Abbreviations

ALKBH5: Alkylated DNA repair protein alkB homolog 5; AML: Acute myeloid leukemia; BCL2: B-cell lymphoma 2; BM: Bone marrow; CBC: Complete blood count; cDNA: Complementary deoxyribonucleic acid; c-MYC: Cellular myelocytomatosis; CNVs: Copy number variations; CR: Complete remission; CT: Cycle threshold; DNA: Deoxyribonucleic acid; DNMT3A: DNA (cytosine-5)-methyltransferase 3A; EFS: Event free survival; EGFR: Epidermal growth factor receptor; FAB: French–American–British classification; FISH: Fluorescence in situ hybridization; FLT3: Famus-like tyrosine kinase-3; FTO: Fat mass and obesity-associated protein; HGB: Hemoglobin; HSM: Hepatosplenomegaly; IPT: Immunophenotyping; IQR: Interquartile range; k2-EDTA: Dipotassium ethylene diamine tetra-acetic acid ; m^6A: N^6-methyladenosine; METTL3: Methyltransferase-like 3; METTL14: Methyltransferase-like 14; mRNA: Messenger ribonucleic acid; NAOX: Nucleic acid oxygenase; ncRNAs: Noncoding RNAs; NPM1: Nucleophosmin-1; ROC: Receiver operating characteristic; 2OG: 2-Oxoglutarate; OS: Overall survival; PLTs: Platelets; PTEN: Phosphatase and tensin homolog; P value: Probability value; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; TLC: Total leucocyte count; TP53: Tumor protein P53; WHO: World Health Organization; WTAP: Wilms tumor 1-associating protein; YTHDF1: YTH N^6-methyladenosine RNA binding protein1; YTHDF2: YTH N^6-methyladenosine RNA binding protein2.

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Authors' contributions

All authors contributed to data interpretation and manuscript writing. AM conceptualized, designed the study and supervised laboratory analysis. RE, SP and SI contributed to study design and data interpretation. SP contributed to the conceptualization and the writing of the drafted manuscript. RN selected cases, collected clinical data and performed technical work. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

A written informed consent was obtained from all enrolled patients. The approval of study was taken from the institutional Ethics Committee of Ain Shams University with approval No. FWA 000017585 and was in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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