Prognostic significance of DNMT3a gene expression and reactive nitrogen species in newly diagnosed Egyptian de novo adult acute myeloid leukemia patients

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Abstract

Background: DNA methyltransferase 3a (*DNMT3a*) gene is a frequently dysregulated epigenetic modifier gene involved in the process of carcinogenesis. Also, there is a dichotomous nature of nitric oxide action with the ability to both promote and repress cancers. There is a host of research work delineating the frequency of *DNMT3a* mutation in acute myeloid leukemia (AML), but little is known about its level of expression in AML patients or its probable relationship to nitrosative stress. This study aims at the assessment *DNMT3a* gene expression as well as nitric oxide levels in newly diagnosed adult patients with de novo AML. Moreover, it aims at relating these two variables to other disease features and prognostic indicators as well as treatment outcomes. The study included 45 adult de novo AML patients and 10 healthy control subjects. Measurement of *DNMT3a* messenger ribonucleic acid (mRNA) transcripts was done by real-time quantitative polymerase chain reaction (RQ-PCR) followed by Sanger sequencing to identify the presence or absence of *DNMT3a* arginine 882 (R882) mutation. This was followed by the assessment of serum nitrite level as a surrogate marker for nitric oxide radical (NO) using colorimetric methods.

Results: *DNMT3a* gene expression, as well as serum nitrite levels, were significantly higher among AML cases in relation to controls before chemotherapy with *P* values of < 0.001 and 0.035, respectively. Dividing patients into low and high expressors in relation to the hotspot mutation demonstrated no difference between the two groups in terms of demographic, clinical, and laboratory characteristics or treatment outcomes.

Conclusion: *DNMT3a* gene expression is increased among the AML population in relation to normal healthy controls. This may point out the need for the assessment of the influence of this gene expression on methylcytosine content of tumor samples with the subsequent implementation of hypomethylating agents as a line of therapy in cases exhibiting excessive hypermethylation.

Keywords: AML, APL, *DNMT3a*, RNS, NO

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Background

Acute myeloid leukemia (AML) is a disease with marked molecular as well as clinical diversity [1]. DNA methylation and histone tail modifications are the two fundamental epigenetic regulatory mechanisms modulating gene expression. They play a critical role in hematopoietic cellular differentiation with a contribution to leukemogenesis when dysregulated [2].

The process of DNA methylation is catalyzed by a set of enzymes called DNA methyltransferases (DNMTs) [3]. DNA methyltransferase 3a (DNMT3a) is an enzyme that catalyzes the addition of a methyl group to 5 cytosine residue of 5'Cytosine-phosphate-guanine3' (CpG) dinucleotides. This recruits methyl-binding proteins and other repressor complexes precluding the access of transcription factors to the gene with the subsequent gene silencing [4].

DNMT3a is one of the frequently mutated genes in myeloid malignancies such as acute myeloid leukemia, myelodysplasia, and myeloproliferative neoplasms [5]. The most common mutation of *DNMT3a* is the alteration of arginine 882 (R882) affecting the canonical methyltransferase domain with a substantial reduction of the enzymatic activity causing focal areas of DNA hypomethylation [6]. There is a controversy regarding the influence of this mutation on the clinical outcome in AML patients [7].

Reactive nitrogen species (RNS) is a collective term that includes nitric oxide (NO), peroxynitrite (ONOO–), nitrogen dioxide radical (NO2). NO is an essential molecule playing a role in cell signaling, immune responses as well as vasodilatation [8]. The role of nitric oxide in tumorigenesis is controversial with bimodal action. Some reports contend that it has a tumor promoting properties, whereas others claim that it has tumoricidal activity [9].

Methods

Subjects

This prospective case-control study was conducted in the Clinical Hematology and Oncology Unit, Internal Medicine Department at Center "X", City "Y", Country "Z". A total number of 55 adult subjects were enrolled. Participants of the study were divided into 2 groups.

Patient group (group 1): was made up of 45 patients of newly diagnosed de novo AML, who met the criteria of selection.

Inclusion criteria

- Adult patients of 18 years old or more.
- Newly diagnosed AML patients by the standard diagnostic criteria.
- Patients who were deemed fit for chemotherapy.

Exclusion criteria

- Secondary acute leukemia.
- Biphenotypic or bileaneage acute leukemia.
- Relapsed acute leukemia.
- Patients who were considered unfit to intensive chemotherapy of curative intent.

Control group (group 2): consisted of 10 healthy control subjects who were both age- and sex-matched.

Plan of treatment

All patients received chemotherapy protocols in accordance with the National Comprehensive Cancer Network (NCCN) guidelines 2016 [10]. Non-APL cases received standard 3 + 7 protocol [11]. APL cases were induced by PETHEMA protocol described elsewhere [12].

Non-APL cases received high-dose cytarabine (HiDAC) as consolidation chemotherapy every 28 days after the achievement of complete remission (CR) by the end of induction [13]. Good risk cases and cases having no matched sibling donor (MSD) received 4 cycles of HiDAC, whereas intermediate and poor-risk cases having MSD were allo-transplanted. Till their access to the transplantation facility, they were given HiDAC cycles with a maximum of 4 cycles.

Assessment of response

Recommendations published by the international working group in 2003 were used to standardize response criteria. Patients were classified into either responders who attained CR or refractory who failed to achieve CR. CR was defined as an absolute neutrophilic count ≥ 1,000/ ul, platelet count ≥ 100,000/ microliter (ul), and < 5% bone marrow blasts with no evidence of extramedullary disease. However; refractory disease was defined by patient surviving ≥ 7 days following the completion of the initial treatment course with persistent leukemia in the last peripheral blood smear and/or bone marrow, or with the persistent extramedullary disease [14].

Sample collection

For each subject, about 5 ml of venous blood was drawn under complete aseptic conditions and dispensed into a labeled vacutainer containing gel and clot activator, and serum was separated by centrifugation for 5 min, then stored at −20 °C till the assay for the measurement of *DNMT3a* expression as well as mutational status and NO levels. This was repeated after 1 month of induction therapy.

Detection of *DNMT3a* gene expression and mutation

Measurement of DNMT3a gene expression by RQ-PCR

Using RQ-PCR, both groups were subjected to the measurement of *DNMT3a* mRNA transcripts and their

comparison to the expression of a reference housekeeping gene. For AML cohort, expression was assayed twice, once before induction chemotherapy and another after the end of induction. For that purpose, RNA was extracted from fresh peripheral blood samples with QIAamp RNA blood mini kit (Qiagen, Germany) according to the manufacturer's spin protocol. RNA quantification was done using Nanodrop at A230/A260 nanometer (nm) wavelength. High-Capacity DNA Reverse Transcription Kit (Applied Biosystems, USA) is designed for reverse transcription of RNA amounts greater than 20 nanogram per microliter (ng/ul). Reverse transcription reactions were performed using the High-Capacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems, USA) in a final volume of 20 μl (10 μl 2× RT master mix +10 μl RNA sample). Thermal cycler program utilized as follows: incubation was done for 30 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C then maintained at 4 °C. For real-time PCR, 2 μl of cDNA products were mixed with 10 μl of Taqman PCR master mixture (No UNG), 1 μl of Taqman gene expression assay and 7 μl of nuclease-free water in a final volume of 20 μl. All reactions were performed on a StepOne Real-Time PCR System (Applied Biosystems, USA) with the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Taqman gene expression assay for *DNMT3a* target gene was supplied ready to use by ThermoFisher Scientific (Cat no. 4351372). Relative quantitation was performed using ACTB TaqMan® Gene Expression Assays for *beta-actin* reference gene (*ACTB*) supplied ready to use by ThermoFisher Scientific (Cat no. 4448892). As RQ-PCR requires the simultaneous detection of different fluorescent reporter dyes whose fluorescence spectra exhibit minimal spectral overlap, the two reporter dye labels used were FAM™ (for *DNMT3a* target gene) and VIC (for *ACTB* gene). Both QuantiFast Probe Assays were labeled with a non-fluorescent quencher. Results were reported in relative quantification. Relative quantification was based on the expression levels of the target gene versus the reference one. To calculate the expression of the target gene in relation to the adequate reference gene, calculations were done based on the comparison of a distinct cycle in real-time PCR determined by cycle threshold (CT) values of thermal cyclers at a constant level of fluorescence [15]. After determining the CT, the ΔCT value for each sample was determined by calculating the difference between the CT value of the target gene and the CT value of the endogenous reference gene. This was determined for each unknown sample as well as for the calibrator sample (control subjects).

Assessment of DNMT3a mutations by Sanger sequencing
SigmaSpin™ Post-Reaction clean-up kit (Sigma-Aldrich, S5059) was used to clean up the PCR reactions. The sequencing reaction was set up using BigDye® Terminator v1.1 Cycle sequencing kit (Applied Biosystems, 4336774). A typical sequencing reaction consisted of 1 μL of cleaned Product, 4 μL of 2.5× ready reaction mix, 4 μL of 5× BigDye Sequencing Buffer, 0.5 μM of forward and reverse primers respectively in different tubes. The sequencing reactions were then run on ABI PRISM 310 Genetic Analyzer (POP6). The sequences were based, called, and analyzed using sequence analysis software SeqScape V 2.7 which is designed to detect mutations when aligned with reference sequences of the particular gene.

Primer sequences used for the analysis of *DNMT3a* mutations [16]

Forward: TGTGTGGTTAGACGGCTTCC
Reverse: CCCCCATGTCCCTTACACAC
Amplicon size (bp): 229 + tag

Detection of nitric oxide (NO)
At diagnosis, the measurement of C-reactive protein (CRP) was done to all the study participants as an index for the presence of infection. This was done to discriminate the elevation of NO secondary to infection from that which was related to the disease process. The assessment of NO level was impossible due to its very short half-life (only 15 s), and hence, serum nitrite was used as a surrogate marker. It was assayed in the serum of patients twice at diagnosis and on day 28, whereas it was assayed once in controls as seen in Table 1. The Biodiagnostic Nitrite Assay Kit number 2533, for nitric oxide assay, provides an accurate and convenient method for the measurement of endogenous nitrite concentration. The assessment of nitrite depends upon adding Griess reagent which converts nitrite into the deep purple azo compound, this is followed by photometric measurement of the absorbance resulting from this azo chromophore which accurately determines NO concentration. Blood sample centrifuged and stored at −200 °C to −800 °C. Prior to this, the sample was perfused with phosphate-buffered saline solution, then centrifuged at 4000 round per minute (rpm) for 15 min at 40 °C. Finally, the supernatant was removed and stored on ice as the assay was not conducted on the same day. The effect of media components on color development was assessed by making a nitrite standard curve in the presence of a fixed volume of blood sample and comparing it to a nitrite standard curve made in buffer alone. Results expected were as the following: if antioxidants were present in the blood, they would interfere with the color development when at a concentration as low as 100 micromole (μM), when using the maximum amount of sample for the nitrite assay (100 μl), the detection limit is 2.5 μM. With using reagents as follows: standard sodium nitrite (50 micromole/liter (μmol/l)), sulphanilamide (10 millimole/liter (mmol/l)), and *N*-(1-naphthyl) ethylenediamine (NEDA) (1 mmol/l) test was

Table 1 Demographic data and patient characteristics

Patients characteristics			Groups			
			Cases (*n* = 45)		Control (*n* = 10)	
			N	%	*N*	%
Sex		Male	24	53.3	5	50.0
		Female	21	46.7	5	50.0
Age		Range	20.0–78.0		33.0–50.0	
		Median (IQR)	50.0 (25)		41.50(10.5)	
Peripheral blood	**TLC**	Range	1.0–236.0		6.0–9.0	
		Median (IQR)	10.0 (38)		7.0(2)	
	HB	Range	4.0–12.0		10.0–14.0	
		Median (IQR)	8.0 (4)		12.0 (2.5)	
	PLT	Range	4.0–345.0		189.0–370.0	
		Median (IQR)	40.0 (61)		230.50 (101.28)	
	P.B	Range	0.0–87.0		–	
		Median (IQR)	32.0 (45)		–	
B.M asp		Range	30.0–99.0		-	
		Median (IQR)	70.0 (25)		-	
FAB. classification		M0	1	2.2	–	–
		M0–M1	1	2.2	–	–
		M1	3	6.7	–	–
		M1–M2	6	13.3	–	–
		M2	8	17.8	–	–
		M3	3	6.7	–	–
		M4	2	4.4	–	–
		M4–M5	16	35.6	–	–
		M5	3	6.7	–	–
		M7	2	4.4	–	–
Risk stratification		Poor risk	7	15.5	–	–
		Intermediate risk	25	55.5	–	–
		Good risk	13	28.2	–	–
Remission status after induction chemotherapy		Remitted	17	40.5	–	–
		Refractory	8	17.8	–	–
		Died	20	44.4	–	–
DNMT3A expression before treatment		Range	18.60–83.60		0.54–1.72	
		Median (IQR)	46.5 (33.9)		0.98 (0.57)	
DNMT3A expression after treatment		Range	2.96–71.20		–	
		Median (IQR)	17.05 (37.4)		–	
DNMT3A mutation		Mutant	12	26.7	–	–
		Wild	33	73.3	–	–

Table 1 Demographic data and patient characteristics *(Continued)*

Patients characteristics		Groups			
		Cases (**n** = 45)		Control (**n** = 10)	
		N	%	**N**	%
DNMT3A expression	Low expression	22	48.9	10	100.0
	High expression	23	51.1	0	0.0
NO level pre-treatment	Range	0.53–60.90		0.0–22.11	
	Median (IQR)	12.0 (16.78)		5.80(9.65)	
NO level post-treatment	Range	0.0–27.50		–	
	Median (IQR)	6.20(8.8)		–	

HgB hemoglobin, *NO* nitric oxide, *PB* peripheral blood blast, *PLT* platelets, *TLC* total leucocytic count
NB: the empty cells are for the unapplied points of comparison for the control grou

done by mixing the blood sample with reagent well, allowing it to stand for 5 min, then the absorbance of the sample examined was read against the absorbance of the standard blank sample at 540 nm (520–550 nm). The color was found to be stable for many hours with linearity up to 200 μmol/l.

Nitrite in the sample was calculated as follows:

Nitrite in micromoles (Umol/L) = examined sample/ standard sample × 50

Statistical methodology
Data were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Qualitative data were described as frequencies and percentages, whilst quantitative data were described using range (minimum and maximum), mean, standard deviation when they are parametric and median and interquartile range when they are non-parametric. The significance of the obtained results was judged at the 5% level.

Results
A total number of 55 adult subjects were enrolled. Participants were divided into 2 groups; the first group constitutes 45 patients of adult newly diagnosed de novo AML and the second one made of 10 adult healthy matched controls as per age and sex. Table 1 illustrates the demographic, clinical, and laboratory data of the whole cohort. AML group consisted of 24 males (53.3%) and 21 females (46.7%) whilst the control group included 5 males and 5 females. Age of AML patients ranged from 20 to 78 years with a median of 50 years whereas the control group age ranged from 33 to 50 years with a median of 41.5 years.

Bone marrow aspirates were done at diagnosis exhibiting a median blast percentage of 70%. Immunophenotyping segregated cases as per the French-American-British (FAB) classification. Twenty-one patients had

M4–M5 phenotype, eight patients belonged to M2 category making up 17.8% of the entire cohort. APL contributed by 6.7% of cases, i.e., 3 cases. AML (M4) and (M7) had comparable presentations of about 4.4%, whilst AML (M0) and AML (M0-M1) had the least contributions with 2.2% for each. Cytogenetic-based risk stratification of the study group was performed in congruence with the NCCN guidelines 2016 [11], twenty-five patients fitted into the intermediate-risk group constituting 55% of the cohort. Of them, 21 patients exhibited normal karyotype (i.e., cytogenetically normal AML (CN-AML)). The remaining 4 patients were divided into two cases with trisomy 8, one case with trisomy 6, and another case with monosomy X. The good risk was evident in 13 patients accounting for 28.9% of the cohort. Two patients of this group were belonging to APL with its hallmark cytogenetic abnormality t(15:17). Four patients had t(8:21) and 7 patients had inv(16) affecting the alpha and beta subunits of core-binding factor (CBF), respectively. Poor risk made the lowest contribution to the cohort making 15.5% (i.e., 7 cases). Four of them had complex karyotype with monosomal karyotype encountered in one of them. This patient had a karyotype of 40, XY with loss of chromosomes 2, 3, 7, 14, and 18. Two patients had monosomy 7 and 1 had deletion 5q. This information is well-delineated in Table 1.

Treatment outcome
Patients' cohort was followed up for 18 months from the start of the enrollment into the study, it was found that the median survival was 37 days. A total of 44.4% of cases enrolled died during the induction; of them, 95 % died out of infectious complications. When dealing with the patients who survived after the induction, they were 25 cases (55.5%) divided into three subgroups; complete responders, partial responders, and refractory. Patients who achieved CR (*n* = 13) were offered their consolidation in accordance with the cytogenetic risk category and the availability of an MSD. Out of our cohort, we

had 2 patients who were eligible for transplantation, the first patient was admitted in center "X" Bone Marrow Transplantation Unit to undergo the procedure; however, the other one died out of infection before accessing the transplant facility. By contrast, the patients who were not candidates for transplantation—either being good risk or lacking a potential donor-completed 4 cycles of HiDAC. Six patients out of the remaining eleven patients did not complete their 4 cycles due to the following reasons: four of them died out of infectious sequelae and the other two had disease recurrence and were deemed refractory. Four patients attained partial remission (PR) and hence they were re-induced with the same induction protocol. Eight patients did not achieve either PR or CR post-induction chemotherapy and were considered chemo-resistant. These patients were submitted to salvage chemotherapy.

DNMT3a expression

Upon the evaluation of *DNMT3a* expression among cases and controls, it was found that the median expression for AML group pre-chemotherapy was 46.5 with a range from 18.6 to 83.6 and IQR of 33.9. At the end of treatment, re-measurement of mRNA transcript levels yielded an evident decrease of the expression showing a median of 17 ranging from 2.9 to 71.2 with IQR of 37.4. This is in contrast to control group that showed markedly lower values in comparison to AML group in either time-points with a median of 0.98 ranging from 0.54 to 1.72 with IQR of 0.52. As shown in Fig. 1, when
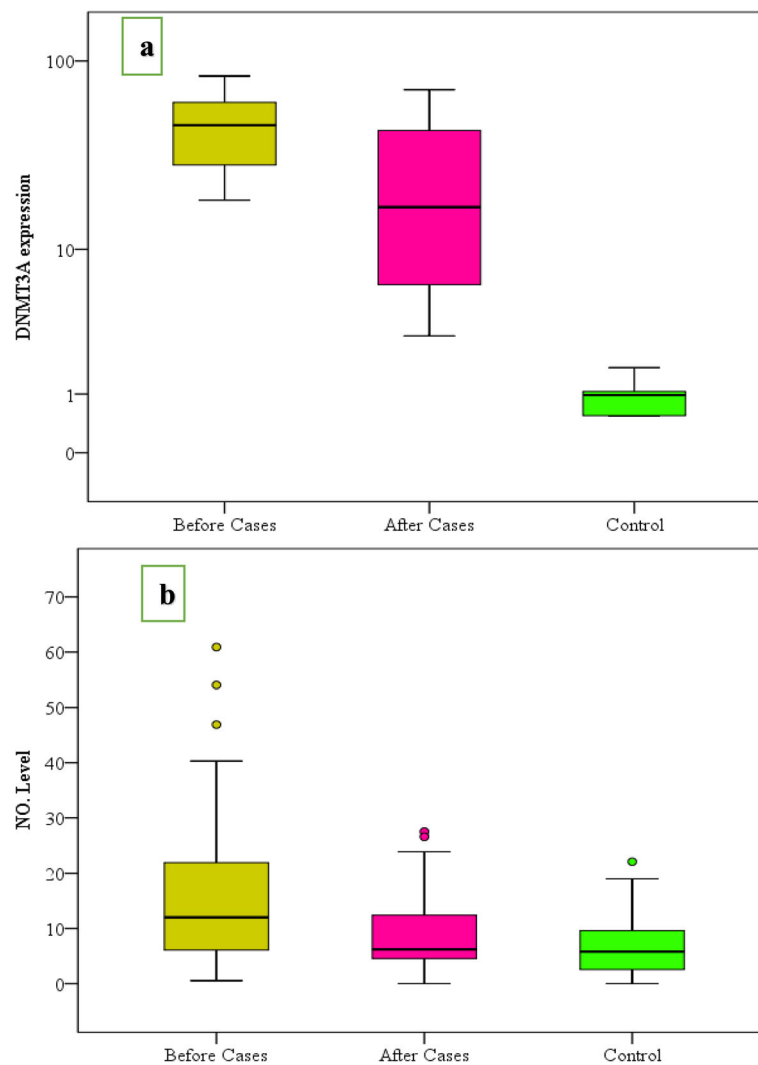

Fig. 1 a Comparison between mutated, un-mutated, and control groups as per DNMT3a expression before and after treatment. **b** Comparison between mutated, wild-type, and control groups as per NO levels before and after treatment

comparing the median expression levels of *DNMT3a* gene between AML group and control group, it was noticeable that AML group exhibited significantly higher expression levels in comparison to control group either before or after the administration of chemotherapy with *P* values < 0.001 for each. Moreover, there is a statistically discernible decline of *DNMT3a* expressions after chemotherapy when compared to before chemotherapy with a *P* value of 0.001 (Table 2). Figure 2 exhibits differential *DNMT3a* expression in patients and controls.

As the gene was universally expressed in all the study participants even the controls, Sanger sequencing of *DNMT3a* gene was performed so as to identify cases having a mutation in the hotspot region (R882) of *DNMT3a* gene (mutant) from those harboring no mutation (wild-type). There were 33 patients (73%) having a wild-type gene and 12 patients (27%) having mutant gene. In the 12 cases having a mutation, arginine residue was substituted for histidine in the majority of them (7/12), for cysteine in 3 cases and proline in 2 cases.

Patients were segregated in accordance with the magnitude of expression of the gene. A receiver operating characteristics (ROC) curve was plotted for that purpose to define a cut-off value in relation to the mutational status as seen in Table 3. The cut-off point identified was 46.5, with a sensitivity of 75% and a specificity of 60.6% with 40.9% PPV and 87% NPV. This subdivided AML group into two further subgroups: low expressors with *DNMT3a* expression below 46.5 and high expressors with expression at or above that figure. Low expressors included 22 patients (48.9%) and high expressors consisted of 23 patients (51.1%).

A comparison between both sub-groups was held regarding the different demographic, clinical data as well as clinical outcome as demonstrable in Table 4. There was no statistically significant difference between both sub-groups in regard to age or sex with *P* values of 0.46 and 0.87, respectively. Both sub-groups exhibited similarity in regard to their total leucocytic count (TLC), hemoglobin, platelets, peripheral blood, and bone marrow blast percentages with *P* values >0.05. There was no obvious predilection neither to any FAB subtype nor to a specific cytogenetic risk group.

Table 5 shows *DNMT3a* mRNA transcript levels as well as nitric oxide values in low and high expressors before and after treatment. Low expressors showed a median mRNA transcript levels of 27.8 before treatment versus 61.2 for high expressors. After treatment, these figures fell to 10.2 and 43.6 in low and high expressors, respectively.

Furthermore, both sub-groups had similar clinical outcomes concerning the response to chemotherapy and the overall survival with *P* values of 0.92 and 0.49, respectively. To illustrate, both sub-groups were similar in terms of the overall response with 40.9% response rate in low expressors versus 34.8% in high ones. Death was encountered in 40.9% in low expressors as compared to 47.8% in high expressors, whilst 18.2% of cases were refractory versus 17.4% in low and high expressors, respectively. This highlights that both sub-groups showed similar clinical outcomes despite the minor differences in favor of a better outcome in low expressors. As for the implication of *DNMT3a* gene expression on survival, it was demonstrable that both sub-groups had comparable median survival; being 37.5 and 37 days for low and high expressors, respectively. Figure 3 represents a Kaplan Meier curve of overall survival in relation to *DNMT3a* expression.

Table 2 Comparison between the two studied groups according to DNMT3A expression and NO level before and after treatment

	Cases		Control (**n** = 10)
	Before (**n** = 45)	After (**n** = 26)	
DNMT3A expression			
Min.–Max.	18.60–83.60	2.96–71.20	0.54–1.72
Mean ± SD	46.64 ± 19.90	24.88 ± 21.39	0.94 ± 0.36
Median	46.50	17.05	0.98
Significance between groups	p$_1$ < 0.001*, p$_2$ < 0.001*, p$_3$ < 0.001*		
NO level			
Min.–Max.	0.53–60.90	0.0–27.50	0.0–22.11
Mean ± SD	16.06 ± 13.90	8.81 ± 7.43	7.83 ± 7.35
Median	12.0	6.20	5.80
Significance between groups	p$_1$ = 0.008*, p$_2$ = 0.035*, p$_3$ = 0.538		

p$_1$: *P* value for Wilcoxon signed-ranks test for comparing between before and after
p$_2$: *P* value for Mann-Whitney test for comparing between before and control
p$_3$: *P* value for Mann-Whitney test for comparing between after and control
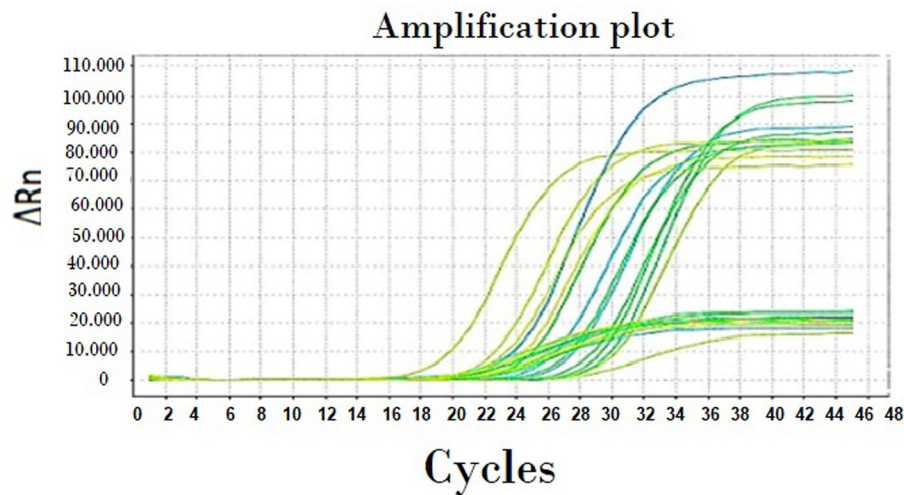
Fig. 2 DNMT3A mRNA expressions in patient and control groups by RQ-PCR

DNMT3a mutational status

Upon comparing the patient cohort having *DNMT3a* mutation to those having no mutation in terms of their age, sex, different full blood count parameters as TLC, hemoglobin and platelets, peripheral blood as well as bone marrow blast percentages, there were no prominent differences with *P* values of 0.85, 0.34, 0.45, 0.98, 0.30, 0.73, and 0.57, respectively. Also, there was no association to any FAB subtype or cytogenetic risk group.

There is no obvious difference between mutated and un-mutated cases with respect to *DNMT3a* gene expressions or NO levels either before or after treatments as demonstrable in Table 6. Although pre-chemotherapy *DNMT3a* gene expression is higher in mutated cases, being 54 compared to un-mutated cases, being 39, this did not reach a statistical significance with a *P* value of 0.063. However, post-chemotherapy figures are nearly the same for both un-mutated and mutated cases, being 14.05 and 18.25, respectively with a *P* value of 0.48.

DNMT3a mutational status has not influenced therapy outcomes. Patients with wild-type gene had comparable chemotherapy response to those with the mutant gene. Despite slightly longer median survival observed among un-mutated group, being 45 days in comparison to 25 days for the mutated group, this did not have any statistical impact with a *P* value of 0.14. Both groups enjoy similar overall survival with *P* value >0.05 as well-illustrated in Fig. 4.

Nitric oxide

NO level in the serum of AML pre-chemotherapy group ranged from 0.5 Umol/l to 60.9 Umol/l with a median 12 Umol/l and IQR 16.77 Umol/l in comparison to control group, where the range was from 0 Umol/l to 22.11 Umol/l. Reassessment was performed for AML group after induction chemotherapy on day 28 to demonstrate a median level of 6.2 Umol/l ranging from 0 Umol/l to 27.5 Umol/l with IQR 8.8 Umol/l.

This demonstrates significantly higher NO values in patients' cohort in relation to control cohort before receiving chemotherapy with a *P* value of 0.035. However; after receiving chemotherapy, patients had a comparable median serum nitrite level to that of controls with a *P* value of 0.54. Interestingly, cases had a significantly lower median NO level after receiving chemotherapy when compared to themselves pre-chemotherapy with a *P* value of 0.01.

Another ROC curve was plotted for nitric oxide versus *DNMT3a* mutation. The cut-off point was ≤13 Umol/l with a sensitivity of 75% and a specificity of 51.52% with

Table 3 Agreement (sensitivity, specificity) for different parameters to diagnose mutated cases cut-off point as described in the ROC curve

	AUC	*P*	95% C.I	Cut-off	Sensitivity	Specificity	PPV	NPV
DNMT3A expression	0.683	0.063	0.50–0.87	>46.5	75.0	60.61	40.9	87.0
NO level	0.616	0.238	0.43–0.80	≤13.4	75.0	51.52	36.0	85.0

AUC area under a curve, *P* value probability value, *CI* confidence intervals, *NPV* negative predictive value, *PPV* positive predictive value

Table 4 Comparison between low and high DNMT3a gene expressors with respect to their demographics as well as clinical data

	Level of expression				Test of sig.	p
	Low expression (n = 22)		High expression (n = 23)			
	No.	%	No.	%		
Sex						
Male	12	54.5	12	52.2	χ^2 = 0.025	0.873
Female	10	45.5	11	47.8		
Age (years)						
Min.–Max.	20.0–72.0		20.0–78.0		t = 0.754	0.455
Mean ± SD	44.05 ± 14.85		47.70 ± 17.47			
Median	42.50		53.0			
Co-Morbidities						
None	16	72.7	14	60.9	χ^2 = 0.927	MCp = 0.743
Single	4	18.2	5	21.7		
Multiple	2	9.1	4	17.4		
Cytogenetic						
Poor risk	4	57.1	3	42.9	χ^2 = 3.012	MCp = 0.248
Intermediate risk	15	60	10	40		
Good risk	4	44.4	9	55.6		
Total leucocyte count						
Min.–Max.	1.0–152.0		1.0–236.0		U = 217.50	0.419
Mean ± SD.	27.59 ± 37.82		45.48 ± 76.04			
Median	11.0		6.0			
Hemoglobin						
Min.–Max.	4.0–12.0		5.0–12.0		t = 0.771	0.445
Mean ± SD	7.27 ± 1.98		7.74 ± 2.07			
Median	8.0		8.0			
Platelets						
Min.–Max	4.0–345.0		4.0–248.0		U = 242.50	0.812
Mean ± SD	68.18 ± 80.60		60.91 ± 64.05			
Median	42.0		38.0			
Peripheral blast						
Min.–Max.	10.0–82.0		0.0–87.0		U = 249.0	0.928
Mean ± SD	37.09 ± 24.16		36.35 ± 24.65			
Median	34.0		30.0			
Bone marrow blast						
Min.–Max.	40.0–92.0		30.0–99.0		t = 1.042	0.303
Mean ± SD	72.32 ± 16.59		66.61 ± 19.93			
Median	75.0		67.0			
NO level	2.79–46.9		3.2–60.9		0.931	0.352
	14.17 (16.30)		12.20 (12.27)			
Remission Status						
Responders (CR and PR)	9	40.9	8	34.8	χ^2 = 0.331	MCp = 0.924
Refractory	4	18.2	4	17.4		
Died	9	40.9	11	47.8		
Mortality						

Table 4 Comparison between low and high DNMT3a gene expressors with respect to their demographics as well as clinical data *(Continued)*

	Level of expression				Test of sig.	*p*
	Low expression (*n* = 22)		High expression (*n* = 23)			
	No.	%	No.	%		
Alive	7	31.8	7	30.4	$\chi^2 = 0.010$	0.920
Died	15	68.2	16	69.6		
Duration of survival						
Min.–Max.	10.0–90.0		10.0–90.0		$U = 223.0$	0.489
Mean ± SD	49.18 ± 31.53		45.0 ± 32.02			
Median	37.50		37.0			

χ^2, p: χ^2 and *P* values for chi-square test
^{MC}p: *P* value for Monte Carlo for chi-square test
U, p: U and p values for Mann-Whitney test
t, p: t and p values for Student *t* test

a PPV of 36% and an NPV value of 85%. Table 5 compares median serum nitrite levels in low and high *DNMT3a* expressor sub-groups. High expressors had higher serum nitrite values in relation to low expressors either before or after therapy. High expressors displayed a median serum nitrite of 13.4 Umol/l and 7.5 Umol/l before and after treatment, whereas low expressors had a median of 9.55 Umol/l and 6.2 Umol/l before and after induction chemotherapy, respectively. Despite these discrepancies, this has not reached a statistical significance with *P* values of 0.29 and 0.96 pre- and post-chemotherapy, respectively.

Discussion

The primary aim of the present study is to assess *DNMT3a* gene expression and nitric oxide levels in patients with newly diagnosed de novo AML. The secondary aim was to examine their relationships with other prognostic factors of the disease, responsiveness to intensive chemotherapy as well as patients' survival.

Table 5 Comparison between levels of DNMT3A gene expression and NO level before and after treatment

		Level of expression		*U*	*P*
		Low expression	High expression		
DNMT3A expression	**Before**	**(*n* = 22)**	**(*n* = 23)**		
	Min.–Max.	18.60–42.30	46.50–83.60	0.000[*]	<0.001[*]
	Mean ± SD	29.26 ± 8.26	63.27 ± 11.65		
	Median	27.80	61.20		
	After	**(*n* = 13)**	**(*n* = 13)**		
	Min.–Max.	2.96–23.40	5.69–71.20	17.00[*]	0.001[*]
	Mean ± SD	10.58 ± 6.68	39.18 ± 21.57		
	Median	10.20	43.60		
NO Level	**Before**	**(*n* = 22)**	**(*n* = 23)**		
	Min.–Max.	0.53–46.90	3.20–60.90	207.00	0.296
	Mean ± SD	14.14 ± 12.44	17.90 ± 15.21		
	Median	9.55	13.40		
	After	**(*n* = 13)**	**(*n* = 13)**		
	Min.–Max.	0.0–26.60	0.0–27.50	83.50	0.959
	Mean ± SD	8.47 ± 6.81	9.15 ± 8.26		
	Median	6.20	7.50		

U, p: *U* and *P* values for Mann-Whitney test
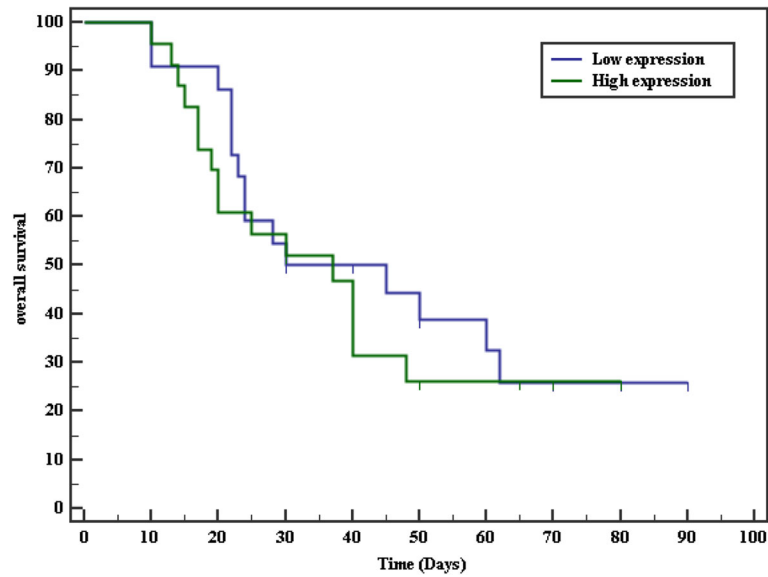*Signifies statistical significance

Fig. 3 Kaplan-Meier curve of the overall survival in relation to the level of gene expression

The present study included 45 adult newly diagnosed cases of de novo AML and 10 healthy control subjects matched as for age and sex.

The Cancer Genome Atlas has determined that 44% of patients with AML show recurrent mutations in the genes that control the methylation of genomic DNA. Moreover, significant changes in DNA methylation had been identified across AML samples at the abundance of CpG loci. These results indicate that DNA methylation could be important for leukemogenesis [17]. And hence, the assessment of *DNMT3a* expression using RQ-PCR was done in both AML group and control group. It was noticeable that all the participants either patients or controls were expressing *DNMT3a*. However; *DNMT3a*

Table 6 Comparison of DNMT3A expression and NO level before and after treatment in relation to DNMT3A mutational status

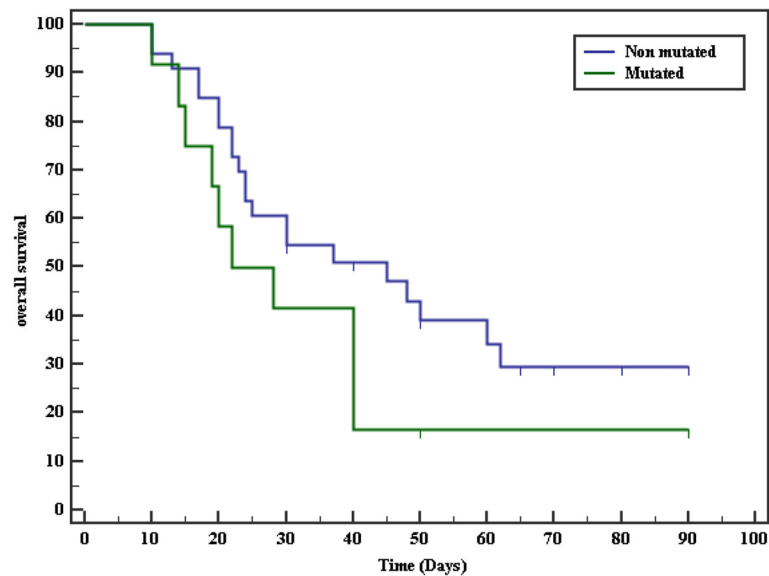|		DNMT3A mutation		*U*	*P*
		Mutated	Wild-type		
DNMT3A expression	**Before**	**(n = 12)**	**(n = 33)**		
	Min.–Max.	19.70–83.60	18.60–79.40	125.50	0.063
	Mean ± SD	56.43 ± 21.15	43.09 ± 18.49		
	Median	54.85	39.60		
	After	**(n = 6)**	**(n = 20)**		
	Min.–Max.	2.96–62.10	3.02–71.20	48.50	0.484
	Mean ± SD	20.16 ± 21.70	26.29 ± 21.65		
	Median	14.05	18.25		
NO level	**Before**	**(n = 12)**	**(n = 33)**		
	Min.–Max.	0.53–30.20	2.70–60.90	152.0	0.238
	Mean ± SD	11.49 ± 9.12	17.72 ± 15.04		
	Median	10.12	14.70		
	After	**(n = 6)**	**(n = 20)**		
	Min.–Max.	0.0–13.10	0.0–27.50	50.0	0.543
	Mean ± SD	6.25 ± 4.49	9.58 ± 8.03		
	Median	6.10	6.46		

U, p: *U* and *P* values for Mann-Whitney test

Fig. 4 Kaplan-Meier survival curve for overall survival in relation to DNMT3A mutation

expression in the control group was far below that of patients either before or after the institution of chemotherapy. In addition, patients after receiving treatment exhibited a statistically remarkable lowering of their readings in comparison to the same cohort before treatment.

This is in congruence with the study performed by Lin and colleagues [17] examining *DNMT3a* expressions among cases of AML and controls, also it assessed the expression of different splicing variants in AML. It was found that all patients expressed *DNMT3a* in that study as in ours, but they proved that *DNMT3a* variant 2 was significantly upregulated in comparison to *DNMT3a* variant 1 and that both variants were the main ones expressed in AML patients in comparison to other variants such as *DNMT3a* variant 3 or variant 4 [17]. In our study, the assessment of variable expression of *DNMT3a* splicing variants was not performed so as to be compared with that observation that needs further verification.

AML group was divided into high and low expressors of *DNMT3a* as per their mutational status with the comparison of both groups in terms of patients and disease characteristics. We failed to prove an association between *DNMT3a* expression status and any of the patients or disease aggressiveness features. But, high expressors exhibited slightly higher mortality during induction at 47.8% versus 40.9% for low expressors, slightly lower remission rates at 34.8% versus 40.9%, and almost comparable refractoriness rate at 17.4% versus 18.2%. Nonetheless, all of these were subtle differences that did not reach statistical significance with *P* values >0.05. This might indicate that

high expressors may be subjected to worse outcome in relation to low expressors and that a more ample sample size may be needed to prove or refute this hypothesis. Another point that can be raised is whether the use of hypomethylating agents may alter the outcome in such patients if used in conjunction with the standard induction chemotherapy.

Mutational analysis of *DNMT3a* gene has been performed. It was found that 12 out of 45 (i.e., 26% of cases) harbored *DNMT3a* R882 mutation. This is almost comparable to the incidence of mutations in a study conducted by Ley et al.in 2010. In that study, mutations were identified in 62/281 (i.e., 22%) [18]. But in our study, we evaluated only the mutation that affects the gene hotspot region R882, whereas Ley and coworkers assessed a wide range of mutations affecting different regions of the gene. They retrieved R882 mutation in only 37 patients, i.e., 13% of all cases enrolled. This is far below the incidence of that kind of mutation in our study which was double that figure. By sharp contrast to this, in a Brazilian study conducted by Pezzi et al, in 2012, it was found that incidence of *DNMT3a* mutations in AML patients was much lower than the incidence reported in our dataset as well as that noted by Ley et al. Of 82 AML samples collected, only 6 samples exhibited *DNMT3a* mutations with R882H identifiable in only 3 patients. Other mutations affecting other parts rather than the methyltransferase domain were also identifiable [19]. Another Taiwanese study by Hou et al. 2012 corroborated findings of the Brazilian study with a lower incidence of *DNMT3a* mutations in 14% of 500 patients recruited [5]. The wide range of variability among AML

patients' population concerning the prevalence of *DNMT3a* mutations might be largely explained by ethno-geographic variations.

Mutated and un-mutated cases were compared in terms of patients and disease features. It was noticeable that both groups were comparable except for a trend towards higher *DNMT3a* expression in mutant cases when compared to those having wild-type *DNMT3a* gene with a tendency towards statistical significance with a *P* value of 0.063. This is ascribed to the method used to define patients into high and low expressors based on mutational status. There was no discernible disparity among mutated and un-mutated cases in terms of response or survival data, though mutated cases had a trend towards shortened survival when compared to un-mutated cases (HR = 1.707 with 95% CI = 0.7–4). Tie et al. 2014 contradicts some of these findings in our study. In this meta-analysis, a total number of 12 studies, 5 American, 4 European ,and 3 Asian had been enrolled focusing on *DNMT3a* mutations in AML patients to display a total number of 1161 patients having mutations out of 6377 patients enrolled (i.e., 18%). It was found that patients with mutated *DNMT3a* had higher TLC and bone marrow blast percentages in comparison to those with the wild-type gene. In addition, they displayed lower overall as well as relapse-free survival in comparison to their un-mutated counterparts [7]. This may be partly attributed to the small sample size in our cohort. Also, only R882 mutations were identified in our study whereas, in Tie et al, *DNMT3a* mutations in any region of the gene were reported. Another study by Hou et al. 2012 showed that *DNMT3a* mutant cases exhibited higher TLC and platelets along with a tendency to cluster among cases of CN-AML (22.9%) in comparison to the overall prevalence in all risk categories (14%) [5].

Finally, serum nitrite has been assessed in patients before and after chemotherapy as well as in controls as an alternative to direct measurement of nitric oxide. It was found that patients before induction treatment showed higher serum nitrite levels when compared to themselves after treatment as well as to their controls with a *P* value of 0.008 and 0.035, respectively. Whereas serum nitrite values were comparable among patients post-chemotherapy and controls with *P* value of 0.538. These findings were concordant with those found by Olaniyi et al. where serum nitrate and nitrite levels were assessed in 25 patients with acute leukemia who were segregated into 14 cases of ALL and 11 cases of AML. Serum nitrite levels were compared with their values in healthy control subjects. That study culminated into the conclusion that acute leukemia patients had significantly higher oxidative/nitrosative stress that results in more nitric oxide production than their healthy control counterparts [20]. Another Indian study conducted by Sangwan et al. on 25 chronic myeloid leukemia patients showed that patients had significantly higher nitrite levels relative to their controls

as well as to themselves after the introduction of imatinib mesylate for six weeks at a dose of 400 mg twice daily [21].

Nowadays, there is an increasingly recognized dichotomous nature of NO in cancers. NO molecule has an unpaired single electron and so can donate it to act as an oxidant or accept an electron behaving like an antioxidant which might explain the discrepancy in its actions [22]. It may act as an oncogenic molecule promoting cellular malignant transformation, angiogenesis, tumor progression as well as spread and metastasis, but some studies emphasize its tumoricidal properties which make this area quite confusing with some trials working to develop medications to block the actions of NO to halt tumorous progression and others working to develop NO donors so as to serve the same purpose [19]. The final activity of NO in oncology is dependent on its working microenvironment, including the type of cell exposed to the compound, the redox state of the reaction, as well as the final intracellular concentration and the duration of intracellular exposure to NO [23]. At low levels, NO can lead to tumor formation. The mechanisms of action that lead to the proneoplastic activity of NO are numerous but include cell proliferation by the activation of oncogenes, stimulation of angiogenesis, and apoptosis inhibition by S-nitrosylation-inactivation of caspases [24]. The role of NO as an anti-oncogenic agent has also been well established to a comparable degree as its potent anticancer properties in other reports. Thus, the dual role of NO is well established and documented in the medical literature. Thus, it continues to be of crucial importance to investigate the system under study to evaluate the contribution of NO to the environment in which it is being released. In 2008, David Wink's group put much of this controversy to rest by reporting a specific concentration threshold where the bipartisan role of NO occurred. At high concentrations (>200 nM), NO had anticancer properties; whereas, below this threshold, cell survival and a pro-neoplastic function of NO was observed [24].

These data shed the light on different nitric oxide synthase isoforms and how they differ in terms of their location, amount, context, and duration of NO production by each. Of them, nitric oxide synthase isoenzyme 2 (NOS2) or the inducible nitric oxide synthase(iNOS) is the most important and its activity is associated with the release of larger micromolar amounts for extended periods that may last for hours or even days in comparison to the constitutively expressed isoforms nitric oxide synthase isoenzyme 1 (NOS1) (neuronal) and nitric oxide synthase isoenzyme 3 (NOS3) (endothelial) that produce smaller bursts which are nanomolar for shorter durations of minutes to hours. This NOS2 may give us a great insight about the relationship between chronic inflammation and cancers as it is abundantly expressed in macrophages and monocytes and is transcriptionally induced by

various pro-inflammatory stimuli as tumor necrosis factor-alpha, interleukin 1, and interferon-gamma [25].

In a study by Cianchi et al. 2003, the oncogenic role of NO was asserted by their findings that there was significantly more colorectal cancer specimens' expression of iNOS as compared to normal mucosa. Also, there was a significant positive correlation between iNOS expression from one side and vascular endothelial growth factor (VEGF) expression and microvessel density from the other side. Metastatic tumors expressed more iNOS than non-metastatic ones [26].

Conclusion

In conclusion; *DNMT3a* gene expression is increased among AML population in comparison to normal healthy control. Moreover, there is higher nitrosative stress in AML patients than controls; however, no association between *DNMT3a* expression and nitrosative status could be delineated in our study. This may point out to the need to assess the influence of *DNMT3a* gene expression on the methylcytosine content of tumor samples with the subsequent implementation of hypomethylating agents as a line of therapy in cases exhibiting excessive hypermethylation. Furthermore, the utilization of medications to halt the NO action may be experimented to reduce AML progression.

Abbreviations
ACTB: Beta-actin reference gene; AML: Acute myeloid leukemia; APL: Acute promyelocytic leukemia; ATRA: All-trans retinoic acid; CBF: Core-binding factor; cDNA: Complementary DNA; CN-AML: Cytogenetically normal acute myeloid leukemia; CpG: 5′Cytosine-phosphate-guanine3′; CR: Complete remission; CRP: C-reactive protein; CT: Cycle threshold; DNA: Deoxyribonucleic acid; DNMT1: DNA methyltransferase 1; DNMT3a: DNA methyltransferase 3a; DNMT3b: DNA methyltransferase 3b; DNMT3L: DNA methyltransferase 3 L; DNMTs: DNA methyltransferases; FAB: French-American-British; HiDAC: High-dose cytarabine; iNOS: Inducible nitric oxide synthase; IV: Intravenous; mmoll: Millimole/liter; mRNA: messenger ribonucleic acid; MSD: Matched sibling donor; NCCN: National Comprehensive Cancer Network; ng/ul: Nanogram per microliter; nm: Nanometer; NO: Nitric oxide radical; NO2: Nitrogen dioxide radical; NOS1: Nitric oxide synthase isoenzyme 1; NOS2: Nitric oxide synthase isoenzyme 2; NOS3: Nitric oxide synthase isoenzyme 3; ONOO −: Peroxynitrite; PCR: polymerase chain reaction; PR: Partial remission; R882: Arginine 882; RNA: Ribonucleic acid; RNS: Reactive nitrogen species; ROC: Receiver operating characteristics; rpm: Round per minute; RQ-PCR: Real-time polymerase chain reaction; TLC: Total leucocytic count; uL: Microliter; μM: Micromole; μmol/l: Micromole/liter

Ethical approval and consent to participate
This study was approved by the ethics committee of Ain Shams University with the reference number/approval number of 616.99419 22, and written consent was given by the study participant.
It was conducted according to the declaration of Helsinki.
Ain Shams University
Faculty Of Medicine
Ethical Committee Of Scientific Research
Informed consent form for patients who are invited to participate in the research

Research title Prognostic Significance of DNMT3a Gene Expression and Reactive Nitrogen Species in Newly Diagnosed Egyptian De-novo Adult Acute Myeloid Leukemia Patients

Introduction and Aim of work
Acute myeloid leukemia is a disease that shows remarkable heterogeneity in biological, clinical and molecular characteristics. DNMT3a gene is involved in the process of denovo DNA methylation which is a major epigenetic modifying mechanism for gene expression. There is accumulating evidence in the literature about the critical role of this mechanism in the process of carcinogenesis. Also, the dichotomous nature of nitric oxide action promoting or repressing cancers is increasingly realized. There is a host of research work delineating the frequency of DNMT3a mutation in AML, but little is known about its level of expression or a probable relationship with nitrosative stress.

The study aims at the assessment DNMT3a gene expression as well as nitric oxide levels in newly diagnosed adult patients with denovo AML. Moreover, it aims at relating these two variables to other disease features and prognostic indicators as well as treatment outcomes.

Place of work
Ain Shams University, Clinical Hematology and Oncology Unit, Department of Internal medicine.

Number and Selection of Participants
The study included 45 adult denovo AML patients and 10 healthy control subjects.

Inclusion Criteria
• Adult patients of 18 years old or more.
• Newly diagnosed AML patients by the standard diagnostic criteria.
• Patients who were deemed fit for chemotherapy.

Exclusion criteria
• Secondary acute leukemia.
• Biphenotypic or bileaneage Acute Leukemia.
• Relapsed Acute Leukemia.
• Patients who were considered unfit to intensive chemotherapy of curative intent.

Plan of work:
All cases enrolled in the study will be routinely subjected to
1-Full medical history.
2-Thorough clinical examination.
3-Measurement of DNMT3a gene expression by RT-PCR
4-Assessment of DNMT3A mutations by Sanger sequencing
5-Detection of nitric oxide (NO) by colorimetric methods

Benefits expected from the study
1-Benefits to the participants:
To find out a relationship between DNMT3a gene expression and RNS status in AML and disease aggressiveness. This can hold the gate open to future molecular as well as targeted therapies

Risks and complications:
This research will not expose you to any further risks or complications rather than the standard risks incurred by AML induction and consolidation chemotherapy protocols in Clinical Hematology and Oncology Unit of Ain Shams University Hospital.

The risk of blood sampling:
A single blood sample 5 ml will be obtained by trained professional nurse from antecubital fossa of the upper arm using sterile, disposable equipment. The risks of bleeding, bruising, or infection are small, and similar to having blood drawn at your doctor's office.
Some subjects report a feeling of dizziness or fainting upon withdrawing the blood sample. However, the volume of blood (5 milliliters) is small, and will be replaced quickly by your body.

Reimbursements in cases of risks and complications:
Should you get physically injured as a result of research related procedures, Doctor Dina Abdou Mansour will provide the first aid medical treatment.

Confidentiality:
All the data and information provided by you to the research will be private and will not be disclosed to anyone but the main researcher and supervisors of the research.

Certificate of consent:
I have read the former information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I had asked have been answered to my satisfaction. I consent voluntarily to participate in this research and I know that I have the choice to participate or not. Even if I

do not participate in that research, I will be offered the standard medical care given routinely in Ain Shams Clinical Hematology and Oncology Unit. I can make up my mind at any time throughout the research and stop participation even if I agreed earlier.

- Name of participant:
- Signature of participant:
- Identity number or finger print:
- Date: / /

I have accurately read or witnessed the accurate reading of the consent to the potential participant .The individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

- Name of researcher:
- Signature of researcher:
- Date: / /

This proposal has been reviewed and approved by Ethical Committee of Scientific research, which is a committee whose task is to make sure that research participants are protected from harm.

Authors' contributions

IAA is the principal investigator who had the research point, she created the study design, contributed to the analysis of the study data. HMH contributed to the analysis of data. WAE contributed to the analysis of data and contributed to writing and revising the manuscript. MTH contributed to the laboratory work including bone marrow aspirate histological examination and immunophenotypic evaluation and performing polymerase chain reaction for DNMT3a. DAM collected the clinical as well as the laboratory data. AMS analyzed the data, drafted the manuscript, and revised it. Furthermore, she is the corresponding author. All authors have read and approved the manuscript.

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Availability of data and materials

Data and materials are available upon request.

Consent for publication

The publication of the article is approved by all the study participants.
Patient Consent for Publication statement
The following information must be provided in order for this form to be processed accurately. Patients have the right to refuse to sign this consent form; refusal to sign this form will.

I hereby give my consent for images or other clinical information relating to my case to be reported in a medical publication.

I understand that my name and initials will not be published and that efforts will be made to conceal my identity, but that anonymity cannot be guaranteed.

I understand that the material may be published in a journal, Web site or other form of publication. As a result, I understand that the material may be seen by the general public.

I understand that the material may be included in medical books.
Name of the patient
Patient's date of birth
Signature of patient

Competing interests

The authors declare no competing financial interests or conflict of interest.

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