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Prognostic relevance of combined IDH1 and NPM1 mutations in the intermediate cytogenetic *de novo* acute myeloid leukemia

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Abstract: Despite the great advance in treatment, cytogenetically normal Acute myeloid leukemia (CN-AML) is still a challenging entity. The discovery of IDH1 mutation in AML together with the frequent co-mutations; NPM1 and FLT3-ITD throughs a new insight into the pathogenesis and outcome of CN-AML. Recently, there has been an increasing number of recurring mutations in other genes for which the forecasting effect is still required. Despite the large number of risk variables established, there are relatively few prognostic indicators that can help in treatment decisions in AML patients. This study aimed at recording the frequency of IDH1 and NPM1 mutations in newly diagnosed AML and, dual clinicopathological significance. IDH1 and NPM1 mutations were analyzed using High-Resolution Melting curve analysis PCR in 78 newly diagnosed AML patients; 30 pediatric and 48 adult AML patients. IDH1 mutation was detected in 6 out of the 48 adult AML cases (12.5%) and all of them had intermediate cytogenetic prognostic stratification. 5/6 mutant IDH1 patients showed NPM1 co-mutation (P-value= 0.008). Mutant IDH1 patients showed significant resistance to induction therapy (P-value <0.001) and even those who achieved complete remission were relapsed later. Within the intermediate cytogenetic group, the IDH1 mutated patients had short overall survival (HR 12.9, 95% CI (3.1- 53.45) and event-free survival (HR 15.7, 95% CI (2.99-82.72) and P-value <0.001). IDH1 mutation is closely linked to the intermediate cytogenetic stratified group and in particular old age patients and has a great impact on their survival.

Key words: Acute myeloid leukemia (AML); IDH1; NPM1; Mutations; Cytogenetically normal.

Introduction

Acute myeloid leukemia (AML) is a clonal disease with a diverse subtype. AML constitutes about 80% of acute leukemia in the adult age group (1). The main pathology responsible for AML development is uncontrolled proliferation and blockade of differentiation (2). Despite the great advance in the therapeutic approaches of AML and the availability of stem cell transplants, only 40% of the patients younger than 60 years could achieve long-term remission (3). Cytogenetic abnormalities were nearly found in 50% of AML patients, and it is used for AML risk stratification for many years. According to the cytogenetic analysis, three prognostic AML groups have been identified: favorable, intermediate, and poor prognosis (4). Cytogenetically normal AML (CN-AML) falls in the intermediate-risk category which is quite heterogeneous. Molecular analysis of CN-AML lead to the discovery of new somatic mutations that changed the scientific understanding of AML pathogenesis, treatment selection and improved patient outcome (5).

Abnormalities in cellular metabolism can predispose to leukemogenesis. An example of enzymatic alterations that can enhance oncogenesis is isocitrate dehydrogenase (IDH) (6-9). There are 3 different isoforms of IDH, IDH1 is located in the cytoplasm and the other two are located in the mitochondria (10). In normal metabolism, IDHs use NAD and NADP as a cofactor in the oxidative decarboxylation of isocitrate to α-ketoglutarate (αKG) in the Krebs cycle and produce NADH or NADPH (11). IDH1 mutation will lead to the reduction of αKG to 2-hydroxyglutarate (2 HG) which has oncogenic properties (12). The high level of 2 HG will block hematopoietic differentiation and enhance leukemogenesis by competitive inhibition of the normal reaction that depends on α KG like TET2 mediated DNA hydroxymethylation and histone demethylation (13).

IDH1 mutation was firstly detected in patients with malignant gliomas and was associated with better patient outcomes compared to those with wild-type IDH1(14). IDH1 mutation was detected in AML patients especially those with normal cytogenetic (15). There is a frequent association between IDH1 mutation and FLT3-ITD, NPM1, TET2 and WT1 gene mutations (13). AML with mutant IDH1 has distinct pathophysiology, however, the prognostic significance of IDH1 mutation is still controversial. Some studies reported that the co-occurrence of IDH1 and NPM1 mutation in the absence of FLT3 –ITD have better outcomes in the

intermediate-risk AML subgroup (13,14). Meanwhile, other larger studies reported poor patients' outcomes in IDH1 mutated and coupled IDH1/ NPM1 mutated AML patients (15-18). Other studies denied any prognostic significance of IDH1 mutation in AML patients (19). This study aimed at recording the frequency of IDH1 and NPM1 mutations in newly diagnosed AML and, to figure out their dual clinicopathological significance.

Materials and Methods

In the period between January 2017 and December 2018, 78 newly diagnosed AML patients were selected from the internal medicine (48 cases) and pediatric (30 cases) departments, Tanta University Hospitals and enrolled in this study. Secondary and relapsed AML were excluded from the study. Also, patients who received bone marrow transplantation were excluded.

All the cases were diagnosed following the latest WHO guidelines after the performance of the initial laboratory investigations that included complete blood count, peripheral blood smear examination, Bone marrow examination, Immunophenotyping and cytogenetic study. The included cases were followed till the end of the study regarding the achievement of complete remission, development of relapse and their status at the last time seen in the clinic.

Molecular analysis

Peripheral blood and bone marrow EDTA samples were collected from all the included cases for molecular analysis. DNA was extracted using a QIAGEN DNA extraction kit (Qiagen, Hilden, Germany) and stored at -20^{O}C till the time of the PCR experiment. The quality of extracted DNA was evaluated by measuring the optical density of the sample at 260nm. IDH1 mutation and NPM1 mutations were detected by high resolution melting analysis using the Light Cycler 480, (Roche Diagnostics, Germany). Specific primers were used. One mutated and another wild-type samples for each gene were included as controls in each run. Patients' samples were tested in duplicate. Molecular detection of IDH1 mutation was repeated upon relapse for the initially recognized mutated cases.

IDH1 mutational analysis

DNA (20ng) were amplified in a total volume of 10 µl contain 0.2mM of IDH1 primers (Forward: 5'-CCATTTGTCTGAAAAACTTTGCTTCT-3'-Reverse: 5'-TCACATTATTGCCAACATGACTT-3') ,1X PCR Master Mix with 0.2Mm $MgCl_2$.

The PCR condition was as follows: initial 10 min. at 95 ºC, 10 sec. of 45 cycles at 95 ºC, 58 ºC for 10 sec. and 72 ºC for 20 sec. The melting program was denaturation at 95ºC for 1min., 45ºC for 1 min. with melting grade from 60 ºC to 95 ºC with a 0.02 ºC/sec. ramp rate and 25 acquisitions/ºC (20). The melting curve from different patients' samples was automatically normalized and analyzed by light cycler 480 software.

NPM1 mutational analysis

10 ng DNA was amplified in a total volume of 10 µl containing 1X high resolution melting PCR master mix (Roche diagnostics),0.4mM of NPM1 Primers (Forward: TGATGTCTATGAAGTGTTGTGGTTCC, Reverse: CTCTGCATTATAAAAAGGACAGCCAG) and 0.4 mM MgCl2 and (Roche Diagnostics). The reaction condition was done initially at 95°C for10 min, 10 cycles of 95°C for 10 sec., 56 °C for 15 sec. and 72°C for 30 sec. and final extension step for 5 min. at 72°C. the melting program was denaturation at 95°C for 1 min. ,45°C for 1 min. then 65°C -95°C with a ramp of 5 °C / sec. and acquisition 1/°C. Analysis was done by the software supplied with the LightCycler®480. The negative control was used as the baseline and the fluorescence of the tested samples was plotted relative to this negative control. Significant differences in fluorescence were used as an indication of NPM1 mutations (21).

Statistical analysis

Data were analyzed using SPSS (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL, USA) version 20 for Microsoft Windows. Numerical data were expressed as mean ± SD, median, and range as appropriate. Qualitative data were expressed as frequency and percentage. For quantitative data, comparison between two groups was done by Student *t*-test and nonparametric Mann–Whitney U as appropriate. Survival analysis was done using Kaplan– Meier method. Survival curves were compared with log-rank test. Univariate analysis using Cox proportional hazard regression model was conducted to calculate hazard ratios (HRs) with its 95% confidence intervals (CI). P-value <0.05 was considered significant and highly significant if P-value <0.001. All tests were two-tailed.

Results

This study included 78 newly diagnosed AML patients. The study group included 30 pediatric cases with a median age of 9.5(2-14) years and 48 adult cases with a median age of 50.0 (20-66) years. All the cases were diagnosed according to the WHO classification and followed the FAB morphological classifications. Demographic, initial laboratory and follow-up data of the studied group are presented in Table1.

IDH1 mutation

All the paediatric cases revealed wild-type IDH1 while in the adult group, 6 cases out of 48 (12.5%) showed the mutated form of IDH1. The initial laboratory data of the IDH1 mutated cases and their response to chemotherapy are presented in Table 2.

The mutated IDH1 group showed significantly low WBCs, high platelets count and high BM blast percentage when compared to the wild-type group (P-value <0.001). IDH1 mutation was associated with elderly patients (P-value =0.015), all the IDH1 mutated cases were above the age of 45 years. Detailed data are shown in Table 3

There was no association between IDH1 mutation and FAB subtype (P-value =0.245), however, 4 / 6 (66.7%) of the mutated cases were M1 i.e., undifferentiated morphology.

IDH1 mutation and Cytogenetics

Cytogenetic analysis of the studied group showed that 11 cases had favourable cytogenetics that included

Table 1. Demographic, initial laboratory and follow up data of the study group.

	Pediatric cases Number =30	**Adult cases Number =48**
Age (years)	9.5(2-14)	50.0(20-66)
Sex (no (%))		
Male	17 (56.7%)	29 (60.4%)
Female	13 (43.3%)	22 (39.6%)
WBCs count ($\times 10^9$/L)	21.6(3.2-91.5)	73.5(4.2-175)
Platelet count ($\times 10^9$/L)	26.0(10-85)	22.5(8-103)
% of BM blast	66(39-95)	58.0(30-98)
LDH (U/L)	303.9 (690-2015)	1205.0(480-2005)
Hgb (g/dl)	8.1 (6.2-10.4)	8.1(5.8-11.1)
FAB subtypes (no (%))		
M0	0(0%)	2 (4.2%)
M1	6(20%)	11 (22.9%)
M2	13 (43.3%)	12(25.0%)
M3	2(6.6%)	1 (2.1)
M4	5(16.6%)	8(16.7%)
M5	2(6.6 %)	12(27.1%)
M6	2(6.7%)	1(2.1%)
M7	0(0%)	1(2.1%)
NPM1 mutation (no (%))		
Wild type	27 (90%)	33(68.8%)
Mutated	3 (10%)	15 (31.3%)
IDH1 mutation (no (%))		
Wild type	30 (100%)	42(87.5%)
Mutated	0 (0%)	6 (12.5%)
Complete remission (no (%))		
Yes	25 (83.3%)	43 (89.6%)
no	5(16.7%)	5(10.4%)
relapse (no (%))		
Yes	6 (20%)	10 (20.8%)
no	24 (80%)	38(79.2%)
Status (no (%))		
Alive	19 (63.3%)	32 (66.7%)
Dead	11(36.7%)	16 (33.3%)
Overall survival (months)	22.2±11.5 24.9(1- 41.4)	10.9±5.5 10.1(1-25.4)
Event free survival	20.9 ±11.1 23.9(1-34.6)	9.4±6.0 8.7(0-24.4)

All the numerical data were presented as median (min-max).

Table 2. Initial laboratory and follow up data of the IDH1 mutated cases.

	Case 1	**Case 2**	**Case 3**	**Case 4**	**Case 5**	**Case 6**
Age (years)	62	66	59	58	61	48
FAB subtypes	M1	M2	M5	M1	M1	M1
WBCs count ($\times 10^9$/L)	14.2	4.2	43	35	12.7	16.1
Platelet count ($\times 10^9$/L)	100	71	103	61	96	98
% of BM blast	91	98	62	87	96	93
LDH (U/L)	1105	480	1450	1750	910	1105
Hgb (g/dl)	9.5	11.1	6.1	5.8	8.1	9.1
Cytogenetics	Normal	Normal	Normal	Normal	Normal	Trisomy8
NPM	Mutated	Mutated	Mutated	Mutated	Mutated	Wild type
CR	yes	NO	NO	NO	NO	yes
OS in months	8.1	3.1	2	1	2	8.2
EFS in months	6	NA	NA	NA	NA	6.1

t (8;21) (7 cases), inversion 16 (3 cases) and 1 case t (15;17).10 cases with unfavourable cytogenetics that included t (6;9) (one case), t (9;22) (2 cases), t (1;22) (one case) and 6 cases with complex cytogenetics. Most of the study group (25 cases) had intermediate cytogenetics; 22 had normal karyotype and 3 cases had trisomy 8. Two cases failed karyotyping. All the IDH1 mutated cases had an intermediate cytogenetic prognosis (5 cas-

Table 3. Comparison of the IDH1 mutated and wild type.

	Wild type IDH1 (42 cases)	Mutated IDH1 (6 cases)	P value
Age (years)	45.9±12.5	59.0±6.0	0.015
WBCs count (×10^9/L)	85.5±39.2	20.9 ± 14.8	< 0.001
Platelet count (×10^9/L)	24.3±12.2	88.2±17.6	< 0.001
% of BM blast	55.3±5.5	87.8±13.2	< 0.001
LDH (U/L)	1193.3±292.5	1133.3±437.9	0.661
Hgb (g/dl)	7.9±0.9	8.3±2.1	0.481
Overall survival (months)	11.9±5.2	4.0±3.2	0.001
Event free survival(months)	10.5±5.7	2.0±3.1	0.001

Table 4. The follow up status of the studied adult group in relation to IDH1 status.

		Mutated IDH1 (N=6)	Wild IDH1 (N=42)	P value
Response to induction therapy	CR (n=43)	2 (33.3%)	41(97.6%)	< 0.001
	No CR (n= 5)	4 (66.7%)	1 (20.0%)	
Relapse*	Yes (n=10)	2 (33.3%)	8(19 %)	0.048
	No (n=33)	0 (66.7%)	33(81%)	
Patient status	Alive (n=32)	0 (0%)	32 (76.2%)	0.001
	Died (n=16)	6 (100.0%)	10 (23.8%)	

*Relapse was applied only for the cases that achieved CR.

es were normal and one case had trisomy 8).

IDH1 mutations and NPM1 status

Fifteen (31.3%) cases of the adult study group revealed NPM1 mutation, and 33(68.8%) cases had wild-type NPM1. Among the NPM1 mutated group, 5/15 (33.3%) had dual IDH1 and NPM1 mutations versus 1/33 (3%) with IDH1 mutation in the wild type NPM1 group. This data showed a significantly high incidence of IDH1 mutation within the NPM1 mutated cases, P-value= 0.008.

IDH and response to therapy

Forty-three cases out of 48 (89.5%) of the studied adult group achieved CR after induction therapy. CR was identified morphologically by ≤5% blasts and normal cellularity of the BM, no blasts in the PB or evidence of extramedullary leukemia with the recovery of the granulocytes and platelets (22). The remaining 5 cases were refractory to induction therapy, 4 out of them had IDH1 mutation with a significantly higher failure percentage, P-value < 0.001.

The 43 patients who achieved CR were followed during maintenance therapy. Interestingly, the two IDH1 mutated cases who achieved CR relapsed later compared to 8 relapsed cases from the wild type IDH1, P-value =0.048, Data are shown in Table 4. Furthermore, IDH1 mutation was detected in the originally mutated cases during relapse.

At the end of the study period, 32/48 (66.7%) patients were alive without disease and 16 (33.3%) cases died. All the IDH1 mutated cases were dead compared to 10 cases (23.8%) from the wild type IDH1 group, P-value= 0.001. Data are shown in Table 4.

Survival analysis

The overall survival (OS) was calculated from the first date of diagnosis till the last date seen alive in the hospital. The median (min-max) overall survival of the adult study group was 10.1(1-25.4) months. The strongest known cytogenetics prognostic parameter was estimated in this cohort and it showed a significant difference between the cytogenetics subgroups, P-value 0.02. Also, there was a significantly shorter mean survival in the mutated NPM1 cases, the mean ±SE was 10.9±2.1 versus 21.3 ±1.3 in the wild-type NPM1, P-value 0.004. Data are presented in Figure 1. However, our interest was the IDH1 mutation that was organized within the intermediate cytogenetics group, and it was more convenient to restrict the survival analysis to the intermediate cytogenetic group to get reliable data in relation to IDH1 mutation.

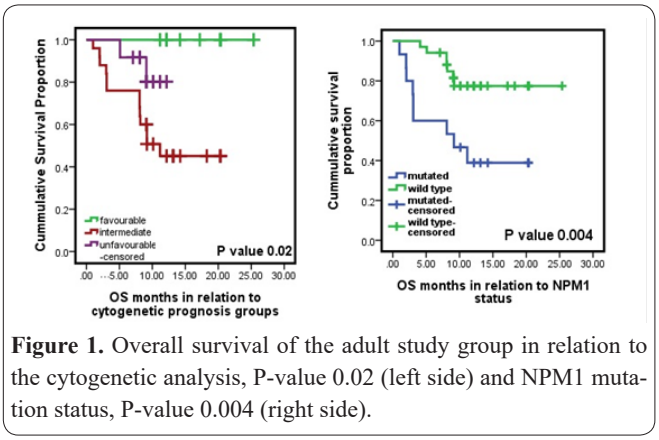

Figure 1. Overall survival of the adult study group in relation to the cytogenetic analysis, P-value 0.02 (left side) and NPM1 mutation status, P-value 0.004 (right side).

The mean OS ±SE was 4.0±1.3 for the IDH1 mutated cases within the intermediate cytogenetics group compared to 15.2±1.6 for the IDH1 wild type group that was significantly longer, P-value < 0.001. A comparable data could not be detected as regards NPM1 mutation where there was no significant difference between the mutated and the wild type NPM1 cases, P-value= 0.12. Data are shown in Table 5 and Figure 2. Univariate analysis of the intermediate cytogenetics group by Cox regression method showed that cases with IDH1 mutation had a significantly higher risk of death than cases without IDH1 mutation (HR 12.9, 95% CI (3.1-

Table 5. Overall Survival study of the intermediate cytogenetics group (25 cases) in relation to IDH1 and NPM1 status.

		One-year OS%	Mean OS time ±SE (Months)	95% confidence interval	P value
IDH1 status	Mutated (n= 6)	0%	4.0±1.3	(1.5-6.6)	< 0.001
	wild type (n=19)	59.4%	15.2±1.6	(12.1-18.3)	
NPM1 status	Mutated (n= 14)	34.3%	10.2±2.1	(6.0-14.3)	0.12
	wild type (n=11)	60.6%	15.7±1.9	(12.1-19.4)	

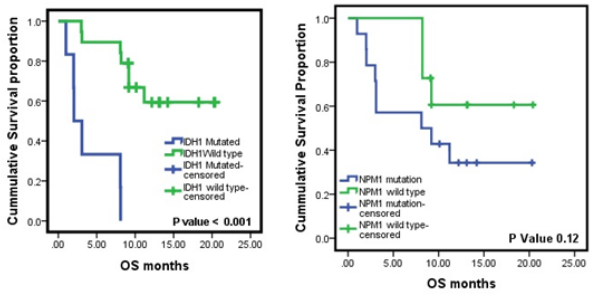

Figure 2. Overall Survival study of the intermediate cytogenetics group in relation to IDH1 status, P-value < 0.001 (left side) and NPM1 status, P-value 0.12 (right side).

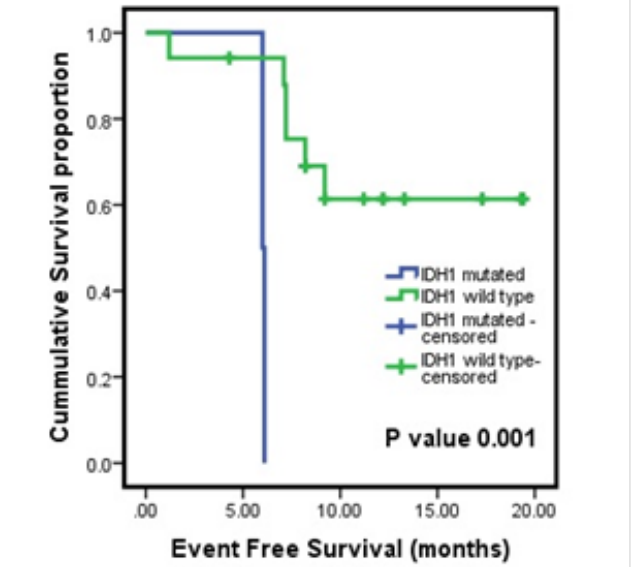

Figure 3. Event free survival study of the intermediate cytogenetics group in relation to IDH1 status.

53.45) and P-value < 0.001).

Event-free survival

It is calculated from the date of complete remission till the occurrence of either death, relapse, or the end of the follow-up period. The median (min-max) was 8.7(0-24.4) months for the adult study group. Like the analysis of the OS, only the cases with an intermediate cytogenetic group who achieved CR (19 cases) will be analysed for the EFS. Only 2 cases with IDH1 mutation could achieve CR, both were dead by 6.1 months. Despite being only 2 cases but this was significantly shorter EFS compared to the wild type IDH1that had a mean ± SE of 14.5±1.6 months, 95% CI (11.4-17.6) and one-year EFS 61.4%, P-value 0.001 (Figure 3). Univariate Cox regression analysis showed that IDH1 mutation had a significantly higher risk of poor prognosis (HR 15.7, 95% CI (2.99-82.72) and P-value = 0.001).

Discussion

Despite the recent advances in the treatment modalities for malignancy, still, AML patients have a relatively poor outcome. The AML patients are classified into different prognostic categories according to many clinical and laboratory parameters. Cytogenetic profile classifies the patients into good, intermediate, and poor prognosis subgroups and it is considered now as the most important factor (23). However, 50% of AML have normal cytogenetic and prognostically fall in the intermediate-risk category. This subcategory still needs further studies for a better understanding of the disease progress.

Cytomorphology, immunophenotyping and cytogenetics are the basis for diagnosing AML. Stratification models for therapy are based mostly in combination with age on these pre-therapeutic characteristics. In each grouping, however, the prognosis of patients remains variable. Several more pre-therapeutic factors were examined for their forecast importance, such as cytogenetic, molecular testing and, disease history. However, it was demonstrated that the cytogenetically based designation separates patients more precisely by their forecasts.

In the last five years, interrelated mutations were detected in AML by next-generation sequencers, like IDH1, NPM1 and FLT3. The prognostic significance of these combined mutations is still a point of discussion (24). Therefore, the current study attempted to define a new risk score by combining IDH1 and NPM1 analysis. In the present study, IDH1 132 and NPM1 mutations were analyzed in 78 newly diagnosed AML patients and evaluated in relation to the clinical parameters of the study group and their treatment response. IDH1 mutation was detected in 12.5% of the adult AML group. On the other hand, none of the studied pediatric patients was carrying the studied IDH1 mutation. Our results were in agreement with previously published studies that reported a range of IDH1 mutation detection 14-15% (20,23, 25-28). In contrast to our data, Raveendran et al.,2015 reported IDH1 mutation in only 4% of AML patients. This variability in the frequency of IDH1 mutation may be related to other factors such as age and cytogenetics (29, 30). These two factors are highly addressed in this study where all the cases that had IDH1 mutation were above 45 years and all of them had an intermediate cytogenetic risk. With regards to the hematological data at presentation, the studied IDH1 mutated cases revealed significantly higher platelets and BM blasts (P-value < 0.001). Our results are in the hands of Yasser et al., 2020 and DiNardo et al., 2016 (28, 33)

Unexpectedly, the TLC was significantly lower in the IDH1 mutated patients compared to the wild IDH1 cases. Although the same data was not previously reported, DiNardo et al., 2015 reported a significantly

lower absolute neutrophilic count in IDH1 mutated patients (24). In the current study, no significant relation could be stated between IDH1 mutation and FAB subtype. Although, 66% of the mutated IDH1 cases were AML-M1. This result is in accordance with Schnittger et al 2010, who reported a more common IDH1 mutation in AML /M1 followed by M2 and M0 (32). The studied cohort showed intermediate cytogenetics risk in around half of the cases (52.1%) denoting the predominance of this group. The IDH1 mutated cases were only located in this group where 5 cases had normal cytogenetic, and one case had trisomy 8. These findings are comparable to the previous reports (28, 33-35). Furthermore, 83.3% of the IDH1 mutation had NPM1 mutated status as well, which agreed with previous studies (33, 23). Extensive genetic testing is required in this regard (37, 38).

The poor response to therapy was obvious in the IDH1 mutated cases as shown by a significantly higher failure rate of CR achievement (P-value <0.001), the higher relapse rate (P-value 0.04) and the death rate (P-value 0.001). These findings agree with other reports (17,27, 36)**.** The survival studies of the IDH1 mutation within the intermediate cytogenetic group reveal poor survival where the OS and EFS were significantly shorter, P-value < 0.001 and 0.001 respectively. Furthermore, the HR in univariate analysis was significantly high in OS and EFS (HR 12.9 and HR 15.7 respectively) These findings are analogues to previously reported data (25,27,36). However, we could not address such a relation with NPM1 mutation in the intermediate cytogenetics group.

From the presented data we can address that analysis of IDH1 mutation is of great prognostic significance in de novo AML patients that could help in further classifying the intermediate cytogenetic groups. IDH1 mutation testing would be of value if the patient has AML without maturation morphology (M1). Furthermore, IDH1 mutation could be a target for directed therapy, especially it showed stability during relapse. The use of high-resolution melting curve analysis to detect IDH1 and NPM1 mutations offered rapid and less expensive methods especially in areas with limited resources.

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Conflicts of interest/Competing interests
The author does not have any competing interest to declare

Ethics approval
The study was reviewed and approved by the Ethics Committee functioning in the hospital under Tanta University Hospitals.

Consent to Participate (Ethics)
The ethical approval board has given consent to Publish the result from the study and all participants were informed above consent to enroll for study which was obtained in writing from participants.

Authors' contributions
Amal Ezzat Abd El-Lateef- Concepts, Design, Data Analysis, Statistical analysis, Manuscript preparation, Manuscript review, Guarantor
Manar M. Ismail & Mohammed Almohammadi- Concepts, Design, Data Analysis, Statistical analysis, Manuscript preparation, Manuscript review, Guarantor
Amr M Gawaly- Concepts, Design, Data Analysis, Statistical analysis, Manuscript preparation, Manuscript review, Guarantor

Availability of data and material
Data is available in the manuscript and raw data can be requested from the corresponding author.

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