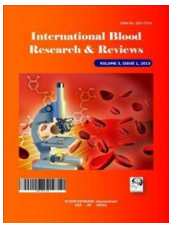

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Study of Isocitrate Dehydrogenase 1and 2 Mutations in Adult Egyptian Patients with Denovo Acute Myeloblastic Leukemia, Their Relation to Clinical Characteristics, FLT3/ITD and Nucleophosmin 1 Mutations and Impact on Treatment Outcome

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Acute myeloid leukaemia (AML) is a malignancy that is heterogeneous in nature characterized by genetic abnormalities some of which are established in the diagnosis and prognosis of the disease. An additional role for alterations in epigenetic mechanisms has been also highlighted in the pathogenesis of the disease. This may have a role in determining the disease outcome, impact the treatment decision and provide options for targeted therapies especially in patients who lack genetic aberrations. One of the modes of epigenetic dysregulation is mutation in genes encoding isocitrate dehydrogenase 1 and 2 that has been observed in AML with a higher incidence in patients with normal karyotype (NK).

Aim of the work: The aim of this work was to study the frequency of IDH1 (R132) and IDH2 (R140Q, R172K) mutations in adult Egyptian patients with de novo acute myeloblastic leukemia

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(AML), their relation with clinical characteristics, other molecular markers (the internal tandem duplication (ITD)) mutation of FLT3 gene and NPM1 gene mutation) and impact on treatment outcome.

Methods: Peripheral blood samples from 50 adult patients with denovo acute myeloid leukemia, admitted to the haematology unit at Alexandria Main University Hospital from February 2015 to February 2017, were used. The polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) was used for detection of IDH1 codon R132 and IDH2 codons (R140, R172) mutations on genomic DNA. PCR was used for detection of FLT3-ITD mutation on genomic DNA. PCR was used for detection of NPM1 mutation on RNA.

Results: IDH 1and 2 mutations occurred in 30% of newly diagnosed AML patients and 47.6% of NK patients. Both mutations did not co-occur except in one case. IDH positive patients were significantly older than IDH negative patients (p=0.003). There was no statistically significant correlation between any of the clinical parameters and the IDH mutations. FAB-M2 was the most common FAB subtype among IDH positive patients. No correlation between IDH mutations and NPM1 or FLT3 could be demonstrated. IDH positive patients had significantly lower CR rates after induction chemotherapy than IDH negative patients (p=0.021).

Conclusion: IDH1, 2 mutations are recurring genetic alterations in AML with a higher incidence in patients with normal karyotype and they may have an unfavorable impact on clinical outcome in adult AML patients.

Keywords: AML; IDH; epigenetics.

1. INTRODUCTION

Despite extensive research that has been carried out in search for prognostic biomarkers, AML is still a disease with a very variable prognosis. Cytogenetics and molecular markers at diagnosis are considered the most important prognostic factors [1].

According to these cytogenetic and molecular abnormalities, three risk categories have been identified in AML. These categories include favourable, intermediate and adverse risk groups. The intermediate risk group, representing approximately half of the patients, includes those with a normal karyotype, wild-type NPM1 without FLT3-ITD or with FLT3-ITDlow and cytogenetic abnormalities that are not classified as favourable or adverse [2].

For patients within the intermediate risk category, the optimal therapeutic strategies are still largely unclear and the outcome of treatment is heterogeneous. This led to the discoveries that have highlighted an additional important role of dysregulated epigenetic mechanisms in the pathogenesis of the disease. Among these epigenetic alterations are the mutations in genes encoding the isocitrate dehydrogenase enzyme 1 and 2 (IDH1 and IDH2) [3].

IDH1 and IDH2 are NADP+-dependent enzymes that play a major role in cellular respiration and defence against oxidative stress. IDH proteins catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate and carbon dioxide with the production of nicotinamide adenine dinucleotide phosphate (NADPH) [4].

Mutations in IDH1 and IDH2 result in impairment of this reaction leading to interference with normal cellular activity and also create a gain of function in the reverse reaction that reduces α-ketoglutarate to an aberrant oncogenic metabolite, 2-hydroxyglutarate (2HG). 2HG acts as a competitive inhibitor of α-ketoglutarate resulting in inhibition of multiple αKG-dependent dioxygenases including TET2. In addition, IDH1/IDH2 mutations are associated with global cytosine hypermethylation signatures that lead to impaired haematopoietic differentiation [5-7].

Common recurrent IDH mutations are located in codon 132 in exon 4 of the IDH1 gene and in codon 140 and codon 172 in exon 4 of the IDH2 gene. IDH1 encodes an enzyme that localizes to the cytosol and peroxisomes, while IDH2 encodes an enzyme that localizes to the mitochondria [7].

The prognostic implication of IDH mutations is complex and still under debate, as differing IDH-mutant enzymes, additional co-occurring mutations, and heterogeneous patient characteristics yield many possible clinical outcomes [8].

Since 2013, several mutant IDH-targeted inhibitors have been developed following the discovery of the active role of IDH mutations in tumorigenesis. In August 2017, the first mutant-targeted IDH2 inhibitor has been approved by the Food and Drug Administration (FDA) for patients with relapsed and/or refractory AML with IDH2 mutations [8].

1.1 Aim

In the present study, we aimed to detect the frequency of IDH 1 and 2 mutations in the Egyptian population, characterize clinical features of AML patients with IDH1 and 2 mutations, their correlation with NPM1and FLT3-ITD mutations and define their prognostic role as regards their relation to treatment outcome.

2. PATIENTS AND METHODS

This study was carried out on fifty adult patients with de novo acute myeloid leukemia, admitted to the haematology unit at Alexandria Main University Hospital, Egypt from February 2015 to February 2017. They included 23 males and 27 females with a median age of 38 years (range, 26-48 years). Patients older than 60 years and younger than 16 years were excluded. Patients with acute promyelocytic leukemia, secondary AML or insufficient samples to extract DNA were also excluded from the study. Written informed consents were obtained from all patients.

Pretreatment clinical and laboratory information [9,10] were obtained, bone marrow aspiration was done for diagnosis of AML according to the FAB classification [11] in addition to immunophenotyping [12] and conventional cytogenetics analysis performed.

The following assessments were done for each patient:

Whole blood genomic DNA extraction was done using thermo scientific gene JET whole blood genomic DNA purification mini kit [13].

2.1 FLT3-ITD Detection by Conventional PCR [14,15]

Extracted DNA from peripheral blood using thermo scientific gene JET whole blood genomic DNA purification mini kit (#K0781, #K0782) was subjected to PCR amplification of exons 14 and 15 of the FLT3 gene. The primers used were 14F (5´GCA ATT TAG GTA TGA AAG CCA GC-3´) and 15R (5´-CTT TCA GCA TTT TGA CGG CAA CC-3´). The total reaction volume of 50µl contained 300-500ng DNA and 20 pmol of each primer. ThermoScientific DreamTaq Green PCR Master Mix (2X) #K1081 was used. It was composed of dATP, dCTP, dGTPand Dttp, 0.4 mM each and 4mM magnesium chloride and 0.05 units/ml of Taq polymerase in a reaction buffer. Samples were amplified by DNA thermal cycler using the following protocol, initial denaturation step of 10 min at 94°C, followed by 35 cycles of PCR (each cycle consisted of denaturation at 94°C for one minute, annealing at 58°C for 1 minute, extension at 72°C for 2 minutes and a final extension step at 72°C of 10 minutes.

The PCR product was analyzed on a standard 2% agarose gel. The size-fractionated products were then visualized and photographed. A fragment of 328 bp, which is the normal FLT3 gene product, was produced from WT alleles. FLT3-ITD positive cases showed an extra PCR fragment that was larger than the wild-type fragment.

2.2 NPM1 Detection by Semi-nested Allele Specific RT-PCR [16]

Extracted whole blood genomic RNA was done using Thermo Scientific GeneJet Whole Blood RNA purification mini kit was reverse transcribed into cDNA using random hexamer primers. cDNA was amplified by PCR using the following primers : forward primer NPM-A (5´CCA AGA GGC TAT TCA AGA TCT CTC TC-3´) and reverse primer NPM-REV-6 (5´ACC ATT TCC ATG TCT GAG CAC C-3´). The total reaction volume of 25 µl contained 50-100 ng cDNA and 20 pmol of each primer. PCR master mix composed of: dNTPs(dATP, dCTP, dGTP, dTTP) 200 µM of each, Mgcl2 (1.5 mM) and 2.5 units/ml of Taq polymerase in a reaction buffer. Samples were amplified by DNA thermal cycler using the following protocol: initial denaturation step at 95ºC for 7 min followed by 35 cycles of PCR (each cycle consisted of 95ºC for 30 sec, 67ºC for 45 sec, 72ºC for 45 sec), then a final extension of 7 minutes was performed at 72ºC.

PCR products were resolved on 2% agarose gel, detected by fluorescence under ultraviolet light and photographed using a Dolphin-Doc Wheal Tech. A 320 bp fragment was detected in NPM1 mutation positive cases while no amplification band was detected in the wild type NPM.

A semi nested ASO-PCR strategy was designed to increase the sensitivity of the assay. 5µl aliquot of the first step ASO-RT-PCR product was amplified in a total volume of 25µl of the reaction mixture containing 20pmol of a forward primer (NPM-AN) with the sequence 5´CAA GAG GCT ATT CAA GAT CTC TGT CTG-3´ and 20pmol of a reverse primer (D4NPM-REV-6) with the sequence 5´CCATTTCCATGTCTGAGCACC-3´. PCR mastermix were also used (200 mmol/LdNTPs, Taq polymerase, Mgcl2). Samples were amplified by DNA thermal cycler using the same conditions as that of ASO-RT-PCR. A 319 fragment was visualized on 2% agarose gel electrophoresis.

2.3 IDH1 R132 Detection by PCR-RFLP [17]

Extracted DNA was subjected to PCR amplification of exon 4 codon 132. PCR was performed in a 50 µl reaction volume, containing 10 pmol of each of the following primers: IDH1f-R132 5′- TGG GTA A AA CCT ATC ATC ATC GAT-3′ , IDH1r-132 5′- TGT GTT GAG ATG GAC GCC TA-3′.

The PCR conditions included initial denaturation at 94°C for 2 min, followed by 35 cycles of 30 sec each at 94°C, annealing for 30 sec at 55°C and extension for 40 s at 72°C. A final extension step at 72°C for 5 min creating a 261 bp PCR product.

After amplification, 20 µl of the PCR product was digested using a FastDigest PvuI #FD0624 (Thermo scientific) enzyme for 5 min at 37°C.

Complete restriction of this PCR product resulting in a single band upon electrophoretic separation confirmed wild type sequence. Detection of two bands indicated a mutation.

2.4 IDH2 (R140, R172) Detection [18]

Extracted DNA was subjected to PCR amplification of IDH2 (R140Q) and IDH2 (R172K) mutations on exon 4. ABL was used as an internal control using ABL-ENF as forward and ABL-ENR as a reverse primer (IDH2 M140F: 5′-GAA AAG TCC CAA TGG AAC TGT CCA-3′, IDH2 R: 5′-CAG CCT CAC CTC GTC GGT GTT-3′, IDH2 M172F: 5′-CAA GCC CAT CAC CAT TGC CAA -3′, IDH2 R: 5′-CAG CCT CAC CTC GTC GGT GTT-3′, ABL-ENF 5′-GGAGATAACACTCTAAGCATAACTAAAGGT-3′, ABL-ENR 5′-GAT GTA GTT GCT TGG GAC CCA-3′). The PCR conditions included the following: preheating of the mixture at 95°C for 5 min for initial denaturation. followed by 35 cycles for 30 s at 95°C, annealing for 1 min at 62°C for IDH2 (R140Q), and 1 min at 60°C for IDH2 (R172K), elongation for 45 s at 72°C and a final extension of 5 min was carried out at 72°C.

PCR products were visualized by electrophoresis on a 2% agarose gel by loading 5 µl of PCR product. The size-fractionated products were then visualized on an ultraviolet transilluminator (UVP Dual intensity transilluminator) and photographed using a using a Dolphin-Doc Wheal Tech. A fragment of 686 bp, which is the normal IDH2 gene product, was produced from WT alleles. The 376 bp band represented IDH2 R140Q mutation and the 277 bp band represented the IDH2 R172K mutation.

2.5 Treatment

All patients received the 3 and 7 protocol which consisted of doxorubicin 25 mg/m^2 for 3 days and ARA-C 100 mg/m^2 by continuous infusion for 7 days.

Assessment of the patients was done after 2 cycles of chemotherapy. Complete remission (CR) was defined as a normocellular BM containing less than 5% blasts and showing evidence of normal maturation of other marrow elements with peripheral blood regeneration with a neutrophil count of ≥1 × 10^9/L and a platelet count of ≥100 × 10^9/L.

2.6 Statistical Analysis

SPSS package version 15.0 was used for data management. Mann–Whitney test was used to compare numerical variable in two independent groups. The Chi-square or Fisher's exact test was used to test proportion independence. *P* values ⩽0.05 were considered significant.

3. RESULTS

In a total group of 50 AML cases, 15 cases (30%) of IDH mutations were identified. IDH2 mutation was more common than IDH1 mutation (nine patients versus five) and one patient had both mutations. Thirty four patients had results for cytogenetics, 21(61.76%) patients were normal karyotype. The frequency of IDH

mutations was higher in the normal karyotype group (47.6%).

IDH positive patients were significantly older (p=0.003).

There was no association between IDH mutations neither with the patients' gender nor with clinical manifestations.

IDH positive patients had higher peripheral white cell counts and higher blast percentage in the bone marrow but this was not statistically significant. Nearly half of the IDH positive patients (7 patients) belonged to the M2-FAB subtype.

There was no significant association between IDH mutations and either FLT3-ITD or NPM1 mutations. IDH mutations were higher among FLT3-ITD negative patients however this was not statistically significant.

Complete remission after induction chemotherapy was significantly lower among IDH positive patients (p=0.021).

4. DISCUSSION

The past years have shown improvements in the risk stratification of AML with karyotyping and molecular mutations being the major determinants of the patient prognosis, response to therapy and outcome besides their role in the pathogenesis of the disease. Despite this, nearly half of the patients have intermediate-risk disease that has a heterogeneous outcome, mandating the need for more prognostic markers.

Alterations in genes responsible for epigenetic regulation, including mutations in genes responsible for DNA methylation like IDH1 and 2 mutations, have emerged as a third class of mutations involved in the pathogenesis of AML in attempt to further refine disease stratification [19,20].

A recent sophisticated genomic classification of AML revealed that IDH2-R172 may represent a distinct genomic subgroup in AML, with different patterns of mutational co-occurrence than IDH2-R140 [21].

Table 1. The basic clinical and biologic data of all patients

	IDH_{mut}	**IDH_{wild}**	**p value**
Age (years) Median(IQR)	48(39 - 49.50)	31(25 - 40.50)	p=0.003*
TLC ($x10^9$/L) Median(IQR)	80(45 - 106)	42(30.5 - 95)	p=0.175
Plt ($x10^9$/L) Median(IQR)	32(22- 49.5)	26(12- 64)	p=0.711
Hb (gm/dl) Median(IQR)	6.9 (5.9 - 8.35)	7(6 - 8.35)	p=0.759
BM Blasts (%) Median(IQR)	85 (70.5 - 92)	73 (63 - 87)	p= 0.165
Sex			
Males	5(33.3%)	18(51.4%)	p=0.239
Females	10(66.7%)	17(48.6%)	
FAB (N)			
M0	0	5	p=0.540
M1	3	5	
M2	7	9	
M4	2	6	
M5	3	8	
M6	0	2	
FLT3-ITD (N)	3	10	p=0.527
NPM1mut (N)	7	10	p=0.216
Response			
CR (N)	5	24	p = 0.021*
No CR (N)	10	11	

IQR: Inter-quartile range
N: number of patients

In this work, we confirmed the occurrence of IDH1 (R132) and IDH2 (R140, R172) mutations as recurrent genetic alterations in the Egyptian adult de novo AML patients. Using RFLP-PCR and AS-PCR, both mutations were detected at a percentage that was higher (30%) than almost all published data concerning the frequency of IDH mutations in de novo acute myeloid leukemia to date. The incidence reported in several studies ranged from 15-20% [22]. Only Parkin et al. reported (27.2%) [23] but sequencing was used for detection of IDH mutations in their cohort which expectedly yield higher results than RT-PCR used in our study.

IDH2 mutations were more common in our study than IDH1 (9 vs 5), only one patient had both mutations. IDH2 R140 was more common than R172 (7 vs 3). IDH-140 and 172 were mutually exclusive.

In the normal karyotype group, frequency of IDH mutations was even higher with 47.6% of the patients being positive. Other authors reported higher frequency of IDH mutations in the group of AML patients with normal karyotype than when cytogenetic analysis was not taken into consideration (Marcucci et al. reported 33%, [24] Emadi et al. reported 25%, [25] Ma et al. reported 21.56%) [26] but still the frequencies reported in their studies were less than that detected in our study.

Ethnic differences were previously reported in the literature as regards the variation in the frequency of IDH mutations in the different populations. The most remarkable of these variations was the lower frequency of IDH mutations in the Chinese patients (3.6-6.3%) in comparison to patients in the Western World (up to 19%) [20].

IDH positive patients were significantly older than IDH negative patients (p=0.003). This was similar to the findings by Dinardo et al. who stated that IDH1 and IDH2 mutated patients in their cohort were older [27]. This was also reported by Paschka et al who had older median age in their IDH positive patients [28].

As regards the response after two cycles of induction chemotherapy, IDH positive patients had significantly lower CR rates than IDH negative patients in our study (p=0.021). In NK patients with IDH mutation, CR was lower than patients without the mutation.

Patients with (NK/FLT3-neg/NPM1-pos) with either IDH1 or IDH2 did not achieve CR suggestive of the negative impact of IDH mutations on this group of patients who are expectedly of favorable outcome. Ravandi et al reported that in the group of patients with normal karyotype and the favorable genotype of NPM1 positive/FLT3 negative, patients with either IDH1 or IDH2 mutations had a significantly worse survival than those without the mutations but there was no association with achievement of complete response [29].

A subtle but significantly inferior event-free survival and possible adverse overall survival for IDH1 mutation in patients with AML, with mutated NPM1 but without FLT3-ITD mutation, was also reported in a meta-analysis by Zhou et al. [30].

In a meta-analysis by Feng et al, the presence of IDH1 mutations did not impact CR rates in AML patients but IDH1 mutations were found to be associated with a lower CR rate in NK-AML patients [31].

Controversial results were reported by Dinardo et al who found improved OS of patients with AML with FLT3-ITD negative, NPM1-mutated, and IDH1/2-mutated intermediate-risk disease [27].

Differences in sizes of patient cohorts analyzed, varying inclusion criteria, age, and treatment administered might contribute to these discrepancies among studies.

5. CONCLUSION

IDH mutations are recurrent genetic abnormalities in Egyptian patients with de novo AML and confer poor prognosis in the form of lower rates of CR. Testing for these mutations could help in modifying risk stratification especially in patients with intermediate risk disease and may change treatment decision. PCR is an easy, rapid and convenient method for detection of IDH mutations in countries with limited resources.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard written ethical permission has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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