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Original research article

EZH2, new diagnosis and prognosis marker in acute myeloid leukemia patients

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ABSTRACT

Purpose: Acute myeloid leukemia (AML) is a heterogeneous disease. The discovery of novel discriminative biomarkers remains of utmost value for improving outcome predictions. Enhancer of zeste homolog 2 (*EZH2*) is a histone methyltransferase of H3K27me3. It is frequently up-regulated in human cancers and associated with silencing of differentiation genes. We aimed herein to investigate the prevalence and prognosis impact of somatic *EZH2* mutations and their potential associations with other prognostic markers *FLT3*, *NPM1*, *DNMT3A* and *IDH2*.
Materials and methods: Our study population was composed of 211 Tunisian patients with *de novo* AML and 14 healthy donors. The 11 last exons coding the set domain of *EZH2* were investigated by PCR and Sanger sequencing.
Results: *EZH2* mutations were identified in 66/211 (31%) patients with a sex ratio of 1.06. The presence of *EZH2* mutations was statistically significantly associated with failure consolidation therapy ($p = 0.004$). There were no differences in the incidence of *EZH2* mutations and *FLT3*-ITD, *NPM1*, *DNMT3A* and *IDH2* mutations. When *EZH2* mutations were associated with those *of FLT3* or *IDH2*, a short duration of progression free survival was observed ($p < 0.05$). Moreover, CD7 aberrant markers conferred a poor prognosis in *EZH2* mutated patients ($p < 0.05$).
Conclusions: Given these data we conclude that *EZH2* mutations are frequent in our patients, and can be used as a prognosis marker in combination with *FLT3*, *IDH2* mutations and CD7 marker, to stratify AML patients and to guide therapeutic decisions.

1. Introduction

Acute myeloid leukemia (AML) is the most commonly occurring acute hematological malignancy in adults. Despite advances in diagnosis, stratification and treatment, the disease remains largely incurable and overall 5 years survival rates remain poor at only 25% [1–5]. AML is a heterogeneous disorder characterized by the accumulation of complex genomic alterations that contribute to disease biology and prognosis. Although cytogenetic analysis can help diagnosis and provides powerful prognostic tool to risk stratify patients with AML, approximately 50% of patients with *de novo* AML have normal karyotype (NK) thus, the molecular screening of genetic defects is crucial in the stratification of the risk and the choice of the therapy protocol of acute myeloid leukemia. The discovery of novel discriminative biomarkers remains of utmost importance to provide new outcomes definitions and therapeutic targets.

EZH2 gene is located at 7q36.1, and encodes the catalytic component of the polycomb repressive complex 2 (PRC2) which is responsible for the methylation of H3K27. *EZH2* mutations could influence stem cell renewal by epigenetic dysregulation [6–10]. Mutations of this gene have been described in 10–13% of poor-prognosis myelodysplasia myeloproliferative neoplasms [11–14]. However, the prevalence and prognosis impact of somatic *EZH2* mutations remain largely unknown in patients with AML.

In this study, we investigated the prevalence and prognostic impact of somatic *EZH2* mutations and their potential associations with other prognostic markers in Tunisian AML patients. Patients were also analyzed for the presence of mutations in 4 other genes: *NPM1*, *FLT3*, *DNMT3A* and *IDH2*.

2. Materials and methods

2.1. Patient and control samples

Our study group included 211 *de novo* AML patients (110 males and 101 females) and 14 controls (8 males and 6 females; medium age

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Table 1
AML Patient demographics, clinical and cytogenetic data in correlation with *EZH2* mutational status.

Parameters	Total n or median ± SD [min-max]	*EZH2*+ mutated	*EZH2*- Wild type	P-value
Patients (n)	**211**	66	145	0.511
Female (n)	101	32	69	
Male (n)	110	34	76	
Age median (years)	**35** ± 16 [2–80]	33 ± 17 [2-64]	36 ± 15 [2-80]	0.127
Children (2-18 years)	36	15	21	
Younger adults(n) (19-40 years)	92	26	66	
Adults (n) (41-60 years)	73	22	51	
Older Adults(n) (> 60 years)	10	3	7	
WBC Median (x10^3/l)	47 ± 76 [0.6 -532]	93.5 ± 64 [0.6-262]	80 ± 80 [0.6-532)]	0.965
Hemoglobin median (g/dl)	7 ± 2 [3-14.8]	7 ± 1.7 [3.2-11.3]	7.5 ± 2.2 [3-14.8]	0.244
Platelets median (x10^3/l)	60 ± 64 [5-512]	61 ± 58 [6-300]	60 ± 67 [5-512]	0.859
LDH Median (U/ L)	1047 ± 721.8 [201-2875]	1188.4 ± 795 [475-2875]	952.9 ± 680 [201-2218]	0.436
Blasts Cells median (%)	61 ± 18.7 [12-100]	60 ± 18 [22-100)]	62 ± 19 [12-100]	0.565
FAB subtypes: M0 (n)	49	**15**	34	0.916
M1	26	5	21	
M2	30	11	19	
M3	7	3	4	
M4	33	10	23	
M5	52	**16**	36	
M6	6	3	3	
M7	7	2	5	
MPAL	1	1	0	
Abnormal Karyotype (n)	28	9	19	0.824
Normal Karyotype	173	**52**	121	
t (15;17)	7	3	4	0.652
t (8;21)	7	2	5	
t (9;11)	6	1	5	
t (9;22)	2	0	2	
Other translocations	4	1	3	
ND	10	5	5	
Monosomy 7	2	**2**	0	0.097
Response to induction treatment				
CR **(n)**	143	45	98	0.096
PR	42	17	25	
Therapeutic failure	26	4	22	
Response to consolidation treatment				
CR **(n)**	126	23	103	**0.0004** *RR = 5.860 [0.128-15.354]
PR	60	34*	26	
Therapeutic failure	25	9	16	
Progression; 6 months	163	48	115	0.113
Progression; 1 year	152	51	101	0.419
Number of death patients	31	10	21	1
Number of survival patients	180	56	124	

Abbreviations: WBC - white blood cell; HDL - high density lipoprotein; ND - None determined Karyotype data (absence of mitoses); t - translocations; CR - Complete remission; PR - Partial remission; * significant association.

13 ± 1 years). Bone marrow samples of consenting patients and healthy donors were collected between December 2013 and March 2017. The CD34+ immature cells were isolated from the bone marrow of healthy donors using a magnetic cell sorting method (Kit Miltenyi Biotec; 130-100-453).

The study cohort included pediatric (2–18 years old) and adult (19–80 years old) AML patients with median age of 35 ± 16 years. The demographic parameters and medical characteristics of AML patients are detailed in Table 1.

2.2. Analysis methods

AML diagnosis procedures included cytomorphology, immunophenotyping and molecular genetics of bone marrow blast cells.

2.2.1. Cytomorphology and immunophenotyping

For cytomorphology, May-Grünwald-Giemsa stained bone marrow smears were analyzed.

For immunophenotyping, bone marrow mononuclear blast cells were isolated with *Ficoll* density gradient and a panel of monoclonal antibodies was used to target myeloid-associated antigens including CD13, CD14, CD33, CD117, cMPO, as well as lymphoid associated antigens including CD2, CD5, CD7, CD19, CD20, CD22, CD19a, CD3 and stem cell antigens HLA-DR, CD34. To characterize leukemia phenotype, a flow cytometer (BD FACSCanto II) with Diva software was used.

The patients' clinical information and follow-up were obtained retrospectively from the AML database of different Tunisian hospitals. Patients were stratified according to the FAB (French American British) and ELN 2017 (European LeukemiaNet) classifications [15,16].

2.2.2. Molecular analysis

Genomic DNA and RNA were extracted from blast cells of AML patients and CD34+ cells using the Trizol method. The DNA was extracted following the manufacturer's instructions with modifications (Invitrogen Life Technologies).

The concentration of DNA was determined by spectrophotometry (NanoDrop Technologies, Thermo Scientific) at 260 nm.

The extracted DNA was amplified by PCR (S1000 thermal cycler) at the 11 *EZH2* exons (10–20) (NC_000002.12), all primers were designed by primer 3 tools (http://primer3.ut.ee/) (Table 2). PCR reaction products were further subjected to direct sequencing and the resulting sequences were compared to those of wild-type gene DNA.

Patients were also analyzed for the presence of *NPM1*, *FLT3*-ITD,

Table 2
The primer sequences of the target genes.

Genes	Sequences 5'→ 3'		DNA fragment bp
NPM1 NG_016018.1 Exon 12	12F	12R	600
	TTAACTCTCTGGTGGTAGAATGAA	CAAGACTATTTGCCATTCCTAAC	
FLT3 NG_007066.1 Exon 14-15	14F	15R	348
	GCAATTTAGGTATGAAAGCCAGC	CTTTCAGCATTTTGACGGCAACC	
IDH2 NG_023302.1 Exon 4	4F	4R	600
	TGGGCAATAGGAGCAAAACT	CTCGTCGGTGTTGTACATGC	
DNMT3A NG_029465.2	F	R	
Exon 18-19	CACCACTGTCCTATGCAGACA	ATTAGTGAGCTGGCCAAACC	561
Exon 20	CCTTGGCTCATCTTCAAACC	CACTATGGGTCATCCCACCT	579
Exon 21	CCGCTGTTATCCAGGTTTCT	CCCAGCAGAGGTTCTAGACG	279
Exon 22	TTTGGTAGACGCATGACCAG	AGCACAGCAATCAGAACAGC	285
Exon 23	TCCTGCTGTGTGGTTAGACG	ATGATGTCCAACCCTTTTCG	417
EZH2 NG_032043.1	F	R	
Exon 10	TTCTCTTCCATCAAAATGAGTTTTAG	TCCTCACAACACGAACTTTCAC	360
Exon 11	GAGTTGTCCTCATCTTTTCGC	CCAAGAATTTTCTTTGTTTGGAC	362
Exon 12	AAGAATGGTTTGCCTAAATAAGAC	CCTTGCCTGCAGTGTCTATC	212
Exon 13	TCTTGGCTTTAACGCATTCC	CAAATTGGTTTAACATACAGAAGGC	289
Exon 14	TGATCGTTTCCATCTCCCTG	AGGGAGTGCTCCCATGTTC	278
Exon 15	GAGAGTCAGTGAGATGCCCAG	TTTGCCCCAGCTAAATCATC	371
Exon 16	TTTTTGATGATGTGATTGTGTTTT	TGGCAATTCATTTCCAATCA	239
Exon 17	TTCTGTCAGGCTTGATCACC	CTCGTTTCTGAACACTCGGC	220
Exon 18	AGGCAAACCCTGAAGAACTG	TTCCAATTCTCACGTCAAAGGTA	217
Exon 19	CATTCGGTAAATCCAAACTGCT	AATGCTCATGGCAAAGTGACC	391
Exon 20	ACCCACTATCTTCAGCAGGCTTT	CTTCCACATATTCACAGGCAGTATTAGT	559

Abbreviations: R – reverse; F - forward.

IDH2 and *DNMT3A* mutations. The screening of the AML-related genes was carried out by classical PCR with the appropriate primers.

A standard protocol was used for each gene: PCR was performed in a final volume of 25 µl containing 100 ng of genomic DNA, $MgCl_2$ (50 mM), PCR buffer (10X), dNTP (25 mM) 10 pmol of each primer, 0.2 µl of Taq DNA polymerase (1U) (Invitrogen, Life Technologies, California, USA) and an adequate amount of H_2O. The PCR conditions were as follows: pre-denaturation step, followed by n cycles of denaturation, hybridization and elongation steps and an extension step. Details of the experimental protocols are provided in Supplement 1

The PCR products were sequenced bidirectionally with the same sets of primers used for the PCR amplification. Sequences were analyzed by Sequencer software version 4.10.

If any variation, in patients' sequences, was observed, a triple direct sequencing was done, to exclude any possible false positivity.

2.2.3. Treatment protocol and response interpretation

Pediatric patients were treated for *de novo* AML with the French multicenter ELAM02 protocol [17]. However, adult patients were treated with the United Kingdom Medical Research Council's AML protocol (MRC10) [18].

The treatment protocol was subdivided into 2 phases: the first phase is induction based mainly on Aracytin/Mitoxantron for pediatric AML and Idarubicin/Aracytin for adults. The second phase is a consolidation that consists on chemotherapy (Aracytin/Daunorubicin/VP16) or allogenic hematopoietic stem cell transplantation. The consolidation is indicated for pediatric and adult patients having a familial HLA-identical donor and pejorative prognosis. Details of the therapy protocols are provided in Supplement 2.

After each treatment phase, the achievement of complete remission (CR) was evaluated. CR is defined by blasts percentage count (< 5%). However, if blasts percentage count is between 5 and 25% patient is considered in partial remission (PR). If not, a treatment failure is considered [15,16]. Patient's clinical data are represented in Table 1.

There are some exceptions in the treatment protocol. In association to protocol therapy, patients with subtype AML-M3 are treated with all-trans retinoic acid (ATRA) while patients with t(9;22) are treated with Imatinib (400 mg–600 mg/day) [17,18].

In this study we did not explore the effect of *EZH2* mutations on patients who underwent bone marrow transplantation (BMT) and whether this influenced their outcome or not.

2.3. Statistical analysis and bioinformatics tools

Patient characteristics were analyzed by Chi-square (chi^2) or Fisher's exact test or Pearson chi-square to test proportion independence. Mann–Whitney U test or Student's *t*-test was used to compare numerical variable in two independent groups. The Kaplan–Meier method was used to estimate survival. The log-rank test was used to compare curves survival difference. Overall survival (OS) was defined as the period between confirmed diagnosis and death or the date of the last follow-up for patients that were still alive. Progression free survival (PFS) was defined as the time from the date of CR until relapse. OS and PFS were compared in the groups presenting or not *EZH2* mutations.

Relative risk (RR) was calculated to estimate the risk of progression in the mutated group compared to the group without mutation.

The relative risk interpretation is as follows:

RR = 1: no relation between the genotype and the concerned factor,

RR > 1: the genotype presents an adverse impact on concerned factor,

RR < 1: indicates that the genotype presents a favorable effect on concerned factor.

All data management was performed using SPSS package version 19.0. P-values less than 0.05 were considered statistically significant.

Bioinformatics tools used to predict the effects of new mutations are:

* PolyPhen-2: (http://genetics.bwh.harvard.edu/pph2/) uses structural information as well as physical data and homologies to make predictions.

* PROVEAN: (http://provean.jcvi.org/seq_submit.php) uses sequence homologies between species as well as the physicochemical properties of amino acids to make predictions.

* Mutation Taster: (http://www.mutationtaster.org/) tool for predicting the pathogenicity of mutations (missense and frameshift), it takes into account data of homologies, physical properties and also the impact mutations on the mRNA.

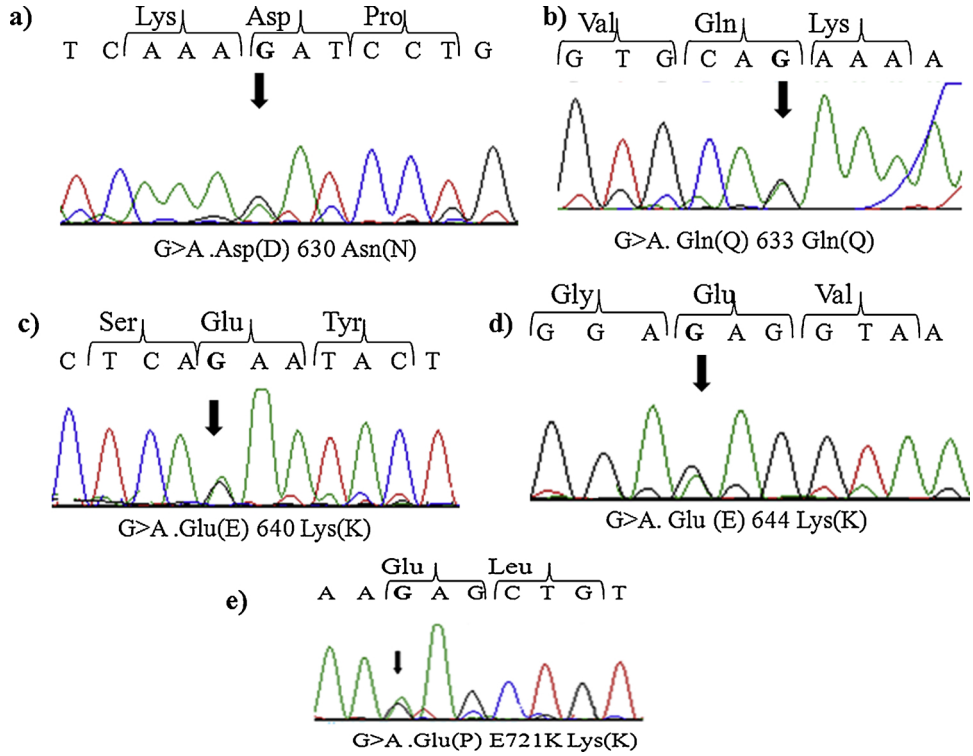

Fig. 1. *EZH2* mutations identified in AML patients. Heterozygous *EZH2* mutations (a) D630 N, (b) Q633Q, (c) E640 K, (d) E644 K, (e) E721 K.

2.4. Ethical issues

This study was approved by the Ethics Committee of Pasteur Institute of Tunis, Tunisia (code of ethics 2017/21/I/LR11IPT07) in accordance with the Code of Ethics of the World MEDICAL Association Declaration of Helsinki 1964.

3. Results

3.1. Prevalence of EZH2 mutations and the other gene markers

Among 211 AML patients, 66 patients (31.27%) harbored *EZH2* mutations. Five different mutations were found: 2 previously described mutations - E640 K, E644 K, and 3 newly described ones - D630 N, Q633Q and E721 K (Fig.1). All mutations observed in the region encoding the SET catalytic domain of *EZH2* protein (amino acid # 611-amino acid # 738) were heterozygous and were not found in the control population.

Newly detected mutations were analyzed by prediction software tools. According to PolyPhen-2, PROVEAN and Mutation Taster, they could cause damage to the protein affecting the catalytic site. This suggests that these mutations may play a role in leukemogenesis (Table 3).

Regarding the other gene prognostic markers, we recorded 4 different heterozygous mutations: 1) R140Q *IDH2* (32 patients, 15.16%), 2) *NPM1* gene insertion, 4bp 'TCTG' at position 960 (64 patients, 30%), 3) frame internal tandem duplication (ITD) in *FLT3* gene (68 patients, 32%) and 4) *DNMT3A* mutations (49 patients, 23.22%). All these mutations were not found in the control population. Moreover, some patients harbored mutated combined markers: *NPM1/EZH2* (15 cases), *FLT3*-ITD/*EZH2* (14 cases), *IDH2/EZH2* (13 cases) and *DNMT3A/EZH2* (5 cases).

3.2. Association of EZH2 mutations with clinical characteristics

Based on the FAB classification system, most *EZH2* mutated patients were classified as AML M0 and M5 (31/66), and M2, M4 (21/66), followed by M1 (5/66), M3 (3/66), M6 (3/66) and M7 (2/66).

No statistically significant association was found between the *EZH2* mutational molecular profile and gender, age, WBC count, platelets, LDH, percentage of blasts, karyotype, FAB subtypes and immunophenotype markers.

In the *EZH2* mutated group, 78.7% of patients had normal karyotype. Cytogenetic disorders harbored were: t (15; 17) in 4.5%, t (8; 21) in 3%, t (9; 11) in 1.5% and monosomy 7 in 3%.

During immunophenotypic analysis, an association of an aberrant marker CD7 with *EZH2* mutations was observed in 5 cases.

Table 3
Description of *EZH2* mutations.

Exon	Normal Nucleotide	Allelic Forme	N° of Acide Amin	Frequency %	Prediction/Effets
16	c.1976G	G > A	p.D630N*	2% (4 cases)	According to prediction software; Polyphen-2, PROVEAN, Mutation Taster: * : New mutations: Affect catalytic domain, and could cause damage to the protein.
	c.1988G	G > A	p.Q633Q*	4.2% (9 cases)	
	c.2007G	G > A	p.E640K	12.3% (26 cases)	
	c.2019G	G > A rs766387427	p.E644K	12.3% (26 cases)	
19	c.2250G	G > A	p.E721K*	0.5% (1 case)	
Total				31.3% (66/211)	

Abbreviations: D - Aspartic acid; Q - Glutamine; E - Glutamic acid; K - Lysine; N - Asparagine; G - Guanine; C - Cytosine; T - Thymine; A - Adenine.

3.3. Prognostic relevance of EZH2 mutations

Our results reported a statistically significant association between *EZH2* mutations and non-achievement of CR after the consolidation phase (51.1% *vs* 17.9%: p = 0.004). Furthermore, we observed a statistically significant association between the most described mutations in our study cohort, E640 K (p = 0.049) and E644 K (p = 0.047), and chemotherapy resistance after the consolidation phase.

We found 8 patients presenting E640 K and E644 K mutations simultaneously. These patients had short period of progression and died within 6 months during the study.

In our cohort, *EZH2* mutations were not associated with any of the following gene mutations: *FLT3*-ITD, *NPM1*, *DNMT3A* and *IDH2* (p > 0.05). However, the combination of *EZH2* and *NPM1* mutations conferred a protective impact and improved achievement of CR after the induction therapy phase (n = 10/15; p = 0.049; RR < 1). By contrast, the combination of *EZH2* and *IDH2* mutations seemed to increase the risk of a PR after the consolidation phase (n = 8/13; p = 0.030; RR = 7.89).

Regarding the survival, the median OS was 35 months for *EZH2* mutated patients and 39 months in non-mutated patients. PFS were 13 months and 14 months for *EZH2* mutated and non-mutated patients, respectively. The Logrank statistic corresponds to a non-significant p value (p > 0.05). Results are presented in Supplement 3.

However, a statistically significantly shorter PFS was observed in the *EZH2* mutated patients with CD7 blast cells positive expression compared to *EZH2* mutated patients with CD7 negative expression (p = 0.01) (Fig.2f).

We noted, *EZH2*+ for mutated *EZH2*, *EZH2*- for non-mutated *EZH2*, CD7+ for expression, CD7- absence of expression; *FLT3*-ITD + for presence of internal tandem duplication in *FLT3* gene, *NPM1*+ for mutated *NPM1* gene; *IDH2*+ for mutated *IDH2* gene; *DNMT3A*+ for mutated *DNMT3A* gene.

Patient's OS with the following combinations: *EZH2*+/*FLT3*-ITD; *EZH2*+/*NPM1*+, *EZH2*+/*DNMT3A*+ and *EZH2*+/*IDH2*+ did not show any statistically significant difference. Results are presented in Supplement 3. However, a statistically significant difference in PFS, for *FLT3*-ITD+/*EZH2*+ (p = 0.016; 12 months *vs* 14 months), and for *EZH2*+/*IDH2*+ (p = 0.028 < 0.05; 5 months *vs* 14 months) was observed (Fig.2 (b, d)).

4. Discussion

Acute myeloid leukemia is the most commonly occurring acute hematological malignancy. Although the biological insight into AML has increased in the past few years, treatment algorithms have not changed substantially over the last 40 years.

The optimal post remission consolidation therapy has been debated on for years due to the clinical heterogeneity in outcomes of AML. Considering the insufficiency of cytogenetic data to distinguish the differences in outcomes, studies carried out in the last few years have provided strong and clear evidence that multiple dysregulations of epigenetic regulatory mechanisms have a central role in leukemia progression. Previous studies [8,19,20] hypothesized that *EZH2* mutation may have an effect in predicting outcome and aid in clinical decisions.

EZH2 is a member of the PRC2 coding for a histone methylation enzyme. *EZH2* controls the expression and regulation of genes to maintain the balance between self-renewal and differentiation of hematopoietic stem cell (HSC) by methylation of histone H3 lysine 27 (H3K27). Mutated *EZH2* functions as an oncogene and a tumor suppressor gene, depending on the context of gain or loss of function with a poor prognosis in *de novo* AMLs. The mutations lead to a loss-of-function type affecting the CH_3 group binding SET domain. Several studies [20–23] discussed the expression and methylation assay of H3K27 but the prognostic impact of *EZH2* mutated gene is poorly understood in terms of OS and PFS. Following this data, we carried out a mutational research at the last 11 exons of *EZH2*, by a conventional PCR followed by direct sequencing. We showed the presence of 5 different mutations in 66 patients. These mutations were not found in the control group.

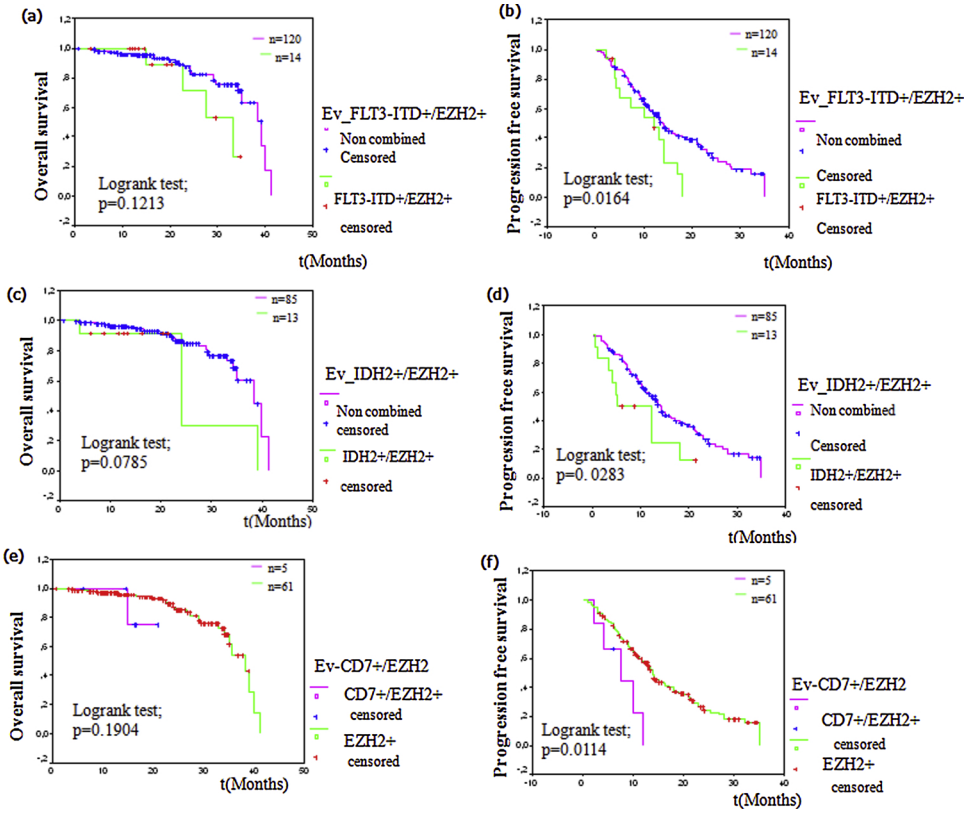

Fig. 2. Kaplan-Meier survival curves of *de novo* AML patients in comparison with patients without mutation. (a) OS *EZH2*+/*FLT3*-ITD+; Median survival 39 months CI95%: [34.63; 43.63] *vs* 33 months CI95%: [23.14; 42.97]. (b) PFS *EZH2*+/*FLT3*-ITD+; Median survival 14 months CI95%: [12.07; 15.93] *vs* 12 months CI95%: [5.10; 19.10]. (c) OS *EZH2*+/*IDH2*+; Median survival 38 months CI95%: [34.40; 42.21] *vs* 24 months CI95%: [24.04; 24.25]. (d) PFS *EZH2*+/*IDH2*+; Median survival 14 months CI95%: [12.74; 15.48] *vs* 5 months CI95%: [3.00; 14.84]. (e) OS *EZH2*+/CD7+;Median survival 38.3 months CI95%: [33.84; 42.78] *vs* 19 months CI95%: [16.93; 22.09]. (f) PFS *EZH2*+/CD7+; Median survival 14 months CI95%: [13.08; 14.92] *vs* 7.8 months CI95%: [4.78; 14.82] Abbreviations: OS - overall survival; PFS - progression free survival; CI - confidence intervals.

They were all in heterozygous form affecting the region encoding the catalytic *EZH2* SET domain and no Tyr641 alteration was observed.

We have shown that the frequency of *EZH2* mutations in our study population is 31.27%, it is higher than that mentioned in previous studies which is in order of 1–2% [24–27].

The difference in mutational frequency between these studies and our present study is probably due to an ethnic difference. The result can be explained also by the fact that our study population is younger compared to the study of Wang et al. [25]. Also, we excluded the hypothesis that *EZH2* mutations could be a simple polymorphism because they could have an adverse impact on pathology. Furthermore, *EZH2* mutations may represent germline variants frequent in the Tunisian population, this hypothesis could be poor because we did not find any *EZH2* mutations in the control population. Additionally, our study group was composed of blasts AML clone from patients who received allogenic stem cell transplant from non-related persons. This later hypothesis has to be investigated in AML families but, in our study group *EZH2* variations are somatic mutations. *EZH2* mutations might be depending on the differentiation degree of blast cells'. This can explain the high frequency of *EZH2* mutations observed in AML0 and AML5 subtypes.

We insist that the mutation frequency of *EZH2* is different from what is reported in the literature, where we find that *EZH2* mutation is frequent in myelodysplastic syndromes [11–14], suggesting that in our cohort we had many secondary AML.

In our study, we found that *EZH2* mutations were more common in the group of young adult AMLs (12.3%), we cannot compare our results to the study by Wang et al. [25] because we have not defined the same age range for the young adult patients. In addition, we noted a low frequency of pediatric AMLs carrying *EZH2*+ (7.3%) which showed that *EZH2*+ can reach the pediatric age.

We found no statistically significant difference in the occurrence of *EZH2* mutations and clinical-biological parameters. Our results are contrary to the study by Wang et al. [25] where the authors show a statistically significant association between blast cells decrease and *EZH2*+.

Cytogenetic and immunogenetic data showed that *EZH2* mutations are predominant in the AML5 and AML0 types (46.9% for both types). In addition, we recorded a statistically significant association of 5 *EZH2*+ cases and CD7 aberrant marker expression, that could have an adverse effect on patients' progression (PFS; $p < 0.05$). We did not find any studies investigating molecular and cytologic-immunophenotypic profile in AML *EZH2*+ patients.

In the group of patients with normal karyotype *EZH2* mutations are frequently encountered, however they are less observed in other groups with different cytogenetic abnormalities. Our findings are in agreement with the studies of LiHan et al. [11] and Wang et al. [25] that show the presence of *EZH2* in the group of intermediate cytogenetic risk and in adverse risk group by the presence of monosomy 7. We found no statistically significant difference while the study of Wang et al. [25] shows a statistically significant association of *EZH2*+ and the monosomy 7.

In our present study, we recorded the presence of 3 new mutations: D630 N, Q633Q and E721 K, as well as 2 mutations - E640 K, E644 K - which have already been described in the databases (dbSNP) and have not been associated with a specific pathology. The analysis of these mutations by prediction tools [31], indicated that they might alter their protein function as histone 3K27 methyltransferase. Indeed many studies have shown *in vitro* that SET domain modifications imply an overexpression of *EZH2*, and a decrease in expression of factor cell cycle regulator. It thus induces anarchic multiplication and blocks the myeloid lineage differentiation. All of these studies have shown that mutated *EZH2* could have oncogenic or tumor suppressor activity [23,27–30]. However, in our study we did not establish these criteria because no functional study of this gene was carried out.

We have shown that *EZH2* mutations have a poor impact on achieving CR after the consolidation phase. This is probably the effect of the E640 K and E644 K mutations. It shows that these mutations could have a poor prognosis on the response to consolidation treatment based on cytarabin. We suppose that they may have a functional impact inducing the increase in cytarabin efflux transporters and resistance in AML.

Also, we have shown that *EZH2* mutations by themselves have no effect on OS or PFS. This result is consistent with the study by Wang et al. [25]. Whereas, the coexistence of *EZH2* mutations with *FLT3* or *IDH2* mutations appears to be associated with an adverse OS and PFS [5,15]. On the contrary, the association of *EZH2* mutations with *NPM1* mutation is most likely in favor of CR which may be linked to the protective effect of *NPM1* in AML patients [15].

To the best of our knowledge, this is the first study conducted on *EZH2* mutations and frequencies among AML Tunisian patients. We hope to complete these results with a functional study and associate this gene in AML diagnosis with the other prognosis markers.

5. Conclusions

Our results provide evidence of clinical utility in considering *EZH2* mutation screening to stratify AML patients and to guide therapeutic decisions. A prospective validation of our findings is needed and we believe that our results may contribute to improving prognostication of patients with AML and the design of clinical trials.

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Conflict of interests

The authors declare no conflict of interest.

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The Author Contribution

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Data Collection: Amal Mechaal
Statistical Analysis: Amal Mechaal
Data Interpretation: Amal Mechaal
Manuscript Preparation: Amal Mechaal, Ines Safra, Samia Menif, Salem Abbes
Literature Search: Amal Mechaal
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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: 10.1016/j.advms.2019.07.002.

References

[1] Przybilla J, Hopp L, Lubbert M, Loeffler M, Galle J. Targeting DNA hypermethylation : computational modeling of DNA demethylation treatement of acute myeloid leukemia. Epigenetics 2017:1–11.
[2] Akalin A, Garrett-Bakelman FE, Kormaksson M, Busuttil J, Zhang L, Khrebtukova I,

et al. Base-pair resolution DNA methylation sequencing reveals profoundly divergent epigenetic landscapes in acute myeloid Leukemia. PLoS Genet 2012;6(8):e1002781.
[3] Deneberg S, Guardiola P, Lennartsson A, Qu Y, Gaidzik V, Blanchet O, et al. Prognostic DNA methylation patterns in cytogenetically normal acute myeloid leukemia are predefined by stem cell chromatin marks. Blood 2011;118(20):5573–82.
[4] Bullinger L, Ehrich M, Dohner K, Schlenk RF, Döhner H, Nelson MR, et al. Quantitative DNA methylation predicts survival in adult acute myeloid Leukemia. Blood 2010;115(3):636–42.
[5] Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 2010;18:553–67.
[6] Moritz LE, Trievel RC. Structure, mechanism, and regulation of polycomb repressive complex 2. J Biol Chem 2018;293(36):13805–14.
[7] Gao L, Sun J, Liu F, Zhang H, Ma Y. Higher expression levels of the HOXA9 gene, closely associated with MLL-PTD and EZH2 mutations, predict inferior outcome in acute myeloid leukemia. Onco Targets Ther 2016;9:711–22.
[8] Herviou L, Cavalli G, Cartron G, Klein B, Moreaux J. EZH2 in normal hematopoiesis and hematological malignancies. Oncotarget 2016;7(3):2284–96. https://doi.org/10.18632/oncotarget.6198.
[9] Lund K, Adams PD, Copland M. EZH2 in normal and malignant hematopoiesis. Leukemia 2014;28:44–9.
[10] Xie H, Xu J, Hsu JH, Nguyen M, Fujiwara Y, Peng C, et al. Polycomb repressive complex 2 regulates normal hematopoietic stem cell function in a developmental-stage-specific manner. Cell Stem Cell 2014;14:68–80.
[11] LiHan C, Chen Y. Targeting EZH2 for Cancer therapy: progress and perspective. Current Protein and Peptide Scienc 2015;16:559–70.
[12] Li B, Mascarenhas JO, Rampal RK. Leukemic transformation of myeloproliferative neoplasms: therapeutic and genomic considerations. Curr Hematol Malig Rep 2018;13(6):588–95. https://doi.org/10.1007/s11899-018-0491-5.
[13] McCabe MT, Graves AP, Ganji G, Diaz E, Halsey WS, Jiang Y, et al. Mutation of A677 in histone methyltransferase EZH2 in human B-cell lymphoma promotes hypertrimethylation of histone H3 on lysine 27 (H3K27). Proc Natl Acad Sci USA 2012;109:2989.
[14] Rinke J, Muller JP, Blaess MF, Chase A, Meggendorfer M, Schafer V, et al. Molecular characterization of EZH2 mutant patients with myelodysplastic/myéloprolifération neoplasms. Leukemia 2017;31(9):1936–43.
[15] Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood 2017;129(4):424–47.
[16] Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 2010;115:453–74.
[17] Alloin AL, Leverger G, Dalle JH, Galambrun C, Bertrand Y, Baruchel A, et al. Cytogenetics and outcome of allogeneic transplantation in first remission of acute myeloid leukemia: the French pediatric experience. Bone Marrow Transplant 2016;2017(52):516–21.
[18] Wheatley K, Burnett AK, Goldstone AH, Gray RG, Hann IM, Harrison CJ, et al. A simple robust, validated and highly predictive index for the determination of risk-directed therapy in acute myeloid leukemia derived from MRC AML 10 trial. United Kingdom Medical Resourch Council's Working Parties. Br J Haematol 1999;107:69–79.
[19] Skoda RC, Schwaller J. Dual roles of EZH2 in acute myeloid leukemia. J Exp Med 2019. https://doi.org/10.1084/jem.20190250.
[20] Gall Trošelj K, Novak Kujundzic R, Ugarkovic D. Polycomb repressive complex's evolutionary conserved function: the role of EZH2 status and cellular background. Clin Epigenetics 2016;8:55.
[21] Wen S, Wang J, Liu P, Li Y, Lu W, Hu Y, et al. Novel combination of histone methylation modulators with therapeutic synergy against acute myeloid leukemia in vitro and in vivo. Cancer Lett 2018;413:35–45. https://doi.org/10.1016/j.canlet.2017.10.015.
[22] Rush M, Appanah R, Lee S, Lam LL, Goyal P, Lorincz MC. Targeting of EZH2 to a defined genomic site is sufficient for recruitment of Dnmt3a but not de novo DNA methylation. Epigenetics 2009;4(6):404–14.
[23] Khan SN, Jankowska AM, Mahfouz R, Dunbar AJ, Sugimoto Y, Hosono N, et al. Multiple mechanisms deregulate EZH2 and histone H3 lysine 27 epigenetic changes in myeloid malignancies. Leukemia 2013;27(6):1301–9.
[24] Pastore F, Levine RL. Epigenetic regulators and their impact on therapy in acute myeloid leukemia. Haematologica 2016;101(3):269–78.
[25] Wang X, Dai H, Wang Q, Wang Q, Xu Y, Wang Y, et al. EZH2 mutations are related to low blast percentage in bone marrow and -7/del(7q) in de novo acute myeloid. PLoS One 2013;8:e61341.
[26] Ernst T, Pflug A, Rinke J, Ernst J, Bierbach U, Beck JF, et al. A somatic EZH2 mutation in childhood acute myeloid leukemia. Leukemia 2012;26(7):1701–3.
[27] Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet 2010;42(8):722–6.
[28] Simon C, Chargraoui J, Krosl J, Gendron P, Wilhelm B, Lemieux S, et al. A key role for EZH2 and associated genes in mouse and human adult T-Cell acute leukemia. Genes Dev 2012;26(7):651–6.
[29] Tanaka S, Miyagi S, Sashida G, Chiba T, Yuan J, Mochizuki-Kashio M, et al. Ezh2 augments leukemogenicity by reinforcing differentiation blockage in acute myeloid leukemia. Blood 2012;120(5):1107–17.
[30] Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic classification and prognosis in acute myeloid leukemia. N Engl J Med 2016;374:2209–21.
[31] Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7(4):248–9.