RESEARCH ARTICLE

Prognostic Relevance of DNMT3A, FLT3 and NPM1 Mutations in Syrian Acute Myeloid Leukemia Patients

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Abstract

Objective: Among all types of hematological neoplasms, acute myeloid leukemia (AML) has the highest death rate. Recently, cytogenetic and molecular genetics are crucial in the management, as a consequence of their effect on AML pathogenesis, classification, risk-stratification, prognosis and treatment. **Methods:** 100 Syrian adults with Normal Karyotype (NK) newly diagnosed AML patients were included in this study, all cases confirmed histologically and immunohistochemically. Patients were divided into six subgroups using flow cytometry and cytological results. Polymerase chain reaction (PCR) was performed on exon 11-12 for FMS-like tyrosine kinase-3 internal tandem duplication (FLT3-ITD), exon 12 for Nucleophosmin1 (NPM1), and exon 23 for DNA methyltransferase 3A (DNMT3A) using target primers, the electropherograms were analyzed for gene mutations by comparing with the reference DNA sequence. Data were compared and aligned with different sequences using the NCBI BLAST Assembled Genomes tool. **Results:** FLT3-ITD, NPM1 and DNMT3A were detected in 24%, 22 % and 4% patients respectively. M2 subtype had the most frequent incidence of diagnosis in AML. FLT3-ITD mutation patients had the highest mean of death cases, while the DNMT3A mutation patients had the lowest. On the other hand, the highest mean of remission was in patients with NPM1 mutation and the lowest in the carriers of the FLT3-ITD mutation. It was observed that the mean relapsed patients with FLT3-ITD and DNMT3A mutation was 3.4 and 2 months respectively, with no significant differences between (FLT3-ITD and DNMT3A) carriers and non-carriers relapsed. On the contrary, the mean relapsed for NPM1 mutation carriers was 2.4 months with significant statistical differences. The mean survival time for patients with FLT3-ITD and NPM1 mutation was 5.9 months and 5.85 months respectively, with significant correlation. Between it was 5.88 months in DNMT3A patients with no significant differences. Finally, It was noted that the mean event free survival (EFS) of FLT3-ITD mutation patients was 4.818 months and the mean EFS of NPM1 mutation patients was 4.805 months, with significant statistical differences ($p<0.05$) between the mutation patients and non-mutated patients regarding to EFS, While this mean was not statistically significant in patients carrying DNMT3A mutation. **Conclusion:** Patients with FLT3-ITD and NPM1 mutations have the worst prognosis, where the presence of those mutations was significantly related to overall survival (OS) and EFS. Our study reflects that DNMT3A was not an extremely bad prognostic effect as an independent factor. We can declare according to this study that genetic mutation and variants detection could easily be incorporated into the regimen evaluation of AML patients.

Keywords: Acute myeloid leukemia (AML)- cytogenetic- prognostic factors- DNMT3A, FLT-ITD

Introduction

Acute Myeloid Leukemia is a genetically diverse clonal cancer that arises from clonal hematopoietic stem cells, characterized by chromosomal abnormalities, recurrent gene mutations, epigenetic chromatin alterations, and microRNA deregulations. Among the numerous variables that have an impact in therapy effectiveness for AML patients, genomic heterogeneity, patient individual variability, and gene alterations are a few key roadblocks. An tremendous diversity of genetic mutations has been uncovered due to the use of advanced molecular devices (Döhner et al., 2017). FLT3, NPM1, and DNMT3A are examples of such genes, patients with AML have been documented to have and incorporated into the risk categories proposed by the European Leukemia Net (ELN)

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(Döhner et al., 2017).

A recurrent chromosomal translocation occurs in 30- 40% of AML cases, while the remaining 60-70 % have a normal karyotype. Because the genetic basis of AML in patients with a normal karyotype is still unknown, researchers have focused on finding particular gene alterations. Gene mutations have been found in 85% of AML cases with a normal karyotype, with FLT3 and NPM1 alterations being the most common (Moarii and Papaemmanuil, 2017). One of the most common genetic lesions seen in AML is NPM1 mutations, they found in 45-64% of instances of AML with a normal karyotype. Other human neoplasms consistently demonstrate nucleus-restricted NPM1 expression, indicating that NPM1 mutations are AML-specific. NPM1 and other recurrent genetic abnormalities are mutually exclusive. NPM1 mutations are typically heterozygous with the leukemic cells retaining a wild-type allele. They are most commonly seen in exon 12 of the NPM1 gene (Sami et al., 2020; Takahashi, 2011). NPM1 mutations have been linked to particular pathologic and clinical characteristics in AML patients with French-American-British (FAB) categories: M4 and M5. Multilineage involvement (myeloid, monocytic, erythroid, and megakaryocytic but not lymphoid), higher blast and platelet counts, a higher frequency of extramedullary involvement in the form of gingival hyperplasia, lymphadenopathy, and a female preponderance are other features of NPM1 mutated AML (Bhattacharyya et al., 2018; Bertoli et al., 2020). NPM1 analysis has substantial clinical consequences, since patients with normal karyotype AML who have NPM1 mutations had a higher complete remission rate after induction therapy than those who do not have NPM1 mutations (Grafone et al., 2012). FLT3 is a type III receptors tyrosine kinase involved in the differentiation and proliferation of hematopoietic stem cells. In most cases of AML, FLT3 is expressed on both progenitor and blast cells. FLT3 mutations can be divided into two major types: (FLT3-ITD) internal tandem duplication mutations within the juxtamembrane domain (JMD) (involving exon 14 and sometimes part of exon 15), which represents the most common type of FLT3 mutation, found in about 25% of all AML patients, and (FLT3-TKD) point mutations or deletion in the tyrosine kinase domain which affecting codons (835 or 836) occurring in approximately 7–10% of all cases with prognostic value uncertain (Schnittger et al.,2012). It was noticed that the occurrence of FLT3-ITD increases with age, where a study indicated that the incidence was lowest in pediatric AML patients and highest in elderly AML patients (Faiz and Rashid, 2019), besides, the mutation was linked to weak emergence and a poor prognosis (Sheikhi et al., 2017). AML patients with FLT-ITD mutation who had a poor prognosis were always with a lower survival rate, indicating that cytogenetic is a crucial prognostic factor in AML (Ait boujmia et al., 2021). FLT3 inhibitors have been licensed by the US Food and Drug Administration (FDA) and have demonstrated to increase clinical response in AML patients when used alone or in combination with chemotherapy (Schnittger et al., 2012). As a result, the European Leukemia Network recommends that FLT3 mutational test results should be available within 72 hours in all AML patients so that treatment options can be made based on the patient's mutation status (Kennedy and Smith, 2020). DNMT3A alterations were identified at various frequencies in multiple myeloid and lymphoid neoplasms, and are often associated with poor prognosis. DNMT3A is an important factor not only for the establishment, but also for maintenance of cellular methylation patterns. Researchers have declared that DNMT3A is often mutated in AML patients 18-23%. Most of the variants are located at R882 in exon 23 (Small, 2006). DNMT3A R882 mutation disturbs the normal ligation of methyltransferase protein subunits, creating a dominant negative influence on DNMT3A protein function, according to the findings. As an inferior prognostic marker for AML patients, DNMT3A has a unique function in hematopoiesis and AML pathogenesis. The prevalence of other non-R882 variants, including DNMT3A frameshift mutations, is substantially less common, and their prognostic relevance is not completly understood (Small, 2006; Choi et al., 2018).

Materials and Methods

Patients and Samples

A retrospective study included 100 newly diagnosed AML patients from division of Hematology and Oncology, in Tishreen Hospital University, Lattakia, Syria, during the period from November 2018 to May 2021, with a median follow-up period of 31 months. Diagnostic peripheral blood (PB) samples were analyzed from 100 adult patients 17 to 86 years, with AML newly diagnosed according to FAB Cooperative Group criteria. The specimens were cultured for karyotyping, patients who had normal karyotype were examined to identify gene mutations. The informed consents forms were signed by all patients who participated in this study according to the protocols of the ethics and research committee of Syrian ministry of higher education. All patients gave informed consent for both treatment and cryopreservation of bone marrow and peripheral blood according to the declaration of Helsinki. All AML patients received induction and consolidation chemotherapy consisting of 3 days doxorubicin 50 mg/m^2 and 200 mg/m^2 cytarabine for 7 days, followed by high dosages of a cytarabine-based consolidation phase (cytarabine at 3 mg/m^2 every 12 hours for three days, repeated for two to three cycles).

Cytogenetic

BM specimens were cultured overnight in RPMI 1640 medium with fetal bovine serum, antibiotics, and colchicine. After that, metaphase chromosomes were harvested by washing the cells in phosphate buffer saline (PBS), treating them with 0.075 M KCl, fixing cell membranes with a 3:1 mixture of methanol and acetic acid respectively, spreading the cells on slides, and coloring them with the G- or Q-banding methods. Under a light or fluorescence microscope, metaphase chromosomes were examined and karyotyped according to the international system for human cytogenetic nomenclature (2020).

Flow Cytometry

CD45, HLA-DR, CD34, CD25, FMC7, CD10, CD19, CD20, CD22, CD7, CD5, CD2, CD3, CD6, CD16, TDT, CD14, CD64, CD33, CD13, CD117 (c-kit), CD15, CD11b, CD11c, CD41, CD61, Cyto MPO and glicophorin A were studied using flow cytometry FACScalibur (Human genetic laboratory, atomic energy commission, Syria). The Cell Quest software suite was used to conduct the analysis. We divided patients into six subgroups using flow cytometry and cytological results, according to the FAB classification.

DNA Extraction

Genomic DNA was isolated from PB or BM samples from AML patients using the QIAamp DNA Blood Mini kit (Qiagen, Germany) according to the manufactures instructions and was stored at -20°C. The total DNA of each sample was measured by using a spectrophotometer followed by quantity ultraviolet light absorbance.

Identification of Gene Mutations

PCR analysis were performed on exon 11 and 12 for FLT3-ITD, exon 12 for NPM1, and exon 23 for DNMT3A. The sequences of primers used in PCR were all described (table 1). PCR cycle was run according to MyTaq HS polymerase condition (Bioline, London, UK), with an initial denaturation step at 95°C for one minute, followed by 35 cycles at 95°C for 15 seconds, 58°C for 15 seconds, 72°C for one minute on a thermal cycler (Eppendorf Mastercycler, USA). The PCR products were purified using GeneJET PCR Purification Kits (Thermo scientific, USA), and were labeled with 0.5 µl of BigDye Terminator Version 3.1 (Applied Biosystems, Foster City, California, USA) in a final volume of 10 µl of the sequencing reaction. Sequencing was conducted in both directions, while only reverse sequence was performed for NPM1 by using the primers as PCR. Sequencing reactions were purified using the Ethanol/Ethylene di amine tetra acetic acid (EDTA)/Sodium acetate precipitation method and capillary electrophoresis was run on an ABI Prism® 3130 DNA Analyzer (Applied Biosystems, Foster City, California, USA) or Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA), and the electropherograms were analyzed for gene mutations by comparing with the reference DNA sequence using the SeqScape program V2.5 (Applied Biosystems, Foster City, California, USA). For all mutations, PCR and sequencing were performed again for confirmation. The reported mutation sequences were based on coding DNA

reference sequences of FLT3 (NM_004119.2), NPM1 (NM_002520.6), and DNMT3A (NM_022552.4) from GenBank. Data were compared and aligned with different sequences using the NCBI BLAST Assembled Genomes tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical Analysis

Statistical analysis was performed using SPSS computer software version 20 (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp; 2011). Student independent 't' test was performed to analyze the significance between different groups. Receiver's operating characteristic (Kaplan–Meier) curve analysis was performed to analyze the discriminatory efficacy between different components under study. Spearman's correlation analysis was performed to analyze the correlation between markers. Survival analysis was done using Kaplan Meier method and comparison between two survival curves was done using log- rank test. A p-value <0.05 was considered significant.

Criteria of Response and Survival Definitions

Complete remission (CR) required bone marrow blasts of <5.0%, absence of blasts with Auer rods, absence of extra medullary disease, absolute neutrophil count of >1.0x 109/L, and platelet count of >100x10⁹/L with independence from red cell transfusions. Relapse was defined by bone marrow blasts of ≥5.0%, reappearance of blasts in the blood, or development of extra medullary disease. OS was defined as the time from diagnosis to death, and EFS as the time from diagnosis to an event (either failure to achieve remission, death in first CR, or relapse), with patients not achieving remission being counted as having an event on day one.

Results

We identified 100 newly diagnosed AML patients. All patients were with normal conventional cytogenetics. The median age was 43.47 years (range, 17–86 years). FLT3-ITD, DNMT3A and NPM1 were detected in 24%, 4% and 22 % patients, respectively. Patient's data were taken in terms of: gender, age, smoking, alcohol, diagnosis according to FAB, leukocyte count, hemoglobin, presence of NPM1, FLT3-ITD, DNMT3A mutation as shown in the (Table 2). It was noted that subtype M2 had the highest rate of diagnosis in acute myeloid leukemia, and the other subtypes were something similar (Figure 1). After a median follow-up of 31 months 53% of patients relapsed

Table 1. Sequences of Primers Used in Polymerase Chain Reaction (PCR)

Gene	Primer	Sequences (5`-3`)
FLT3-ITD	Forward primer	5`-GCAATTTAGGTATGAAAGCCAGC-3`
	Reverse primer	5`-CTTTCAGCATTTTGACGGCAACC -3`
NPM1	Forward primer	5`-TTAACTCTCTGGTGGTAGAATGAA-3`
	Reverse primer	5`-CAAGACTATTTGCCATTCCTAAC -3`
DNMT3A	Forward primer	5`-TCCTGCTGTGTGGTTAGACG -3`
	Reverse primer	5`-ATGATGTCCAACCCTTTTCG -3`

FLT3-ITD, FMS-like tyrosine kinase-3 internal tandem duplication; *NPM1*, Nucleophosmin1; *DNMT3A*, DNA methyl transferase 3A

Table 2. Pathological Features of Syrian (CN) AML Patients with NPM1, FLT3-ITD and DNMT3A Mutations

	FLT3-ITD	*NPM1*	*DNMT3A*	With mutation	without mutation
Total number	24	22	4	41	59
Mean age in years (range)	42.3 (17-64)	44.9 (18-81)	39.3 (18-65)	43.3 (17-81)	43.6 (18-86)
Number of patients <60 years	21	17	0	34	44
Gender (M/F)	10/14/22	13/9	02/02/22	20/21	31/28
Smoking (yes/no)	17/7	14/8	4/0	29/12	37/22
Alcohol (yes/no)	03/21/22	05/17/22	0/4	8/33	8/51
Mean WBC (range)	30.4 (1.3-100)	53 (0.80-300)	23.5 (1.3-46.1)	41.2 (0.8-300)	23.6 (0.2-165)
Mean Hb (range)	8 (2.4-12,5)	8.3 (3.2-16.7)	6.9 (6-7.9)	8.1 (2.4- 16.7)	8.6 (3.5-14)
FAB subtypes					
M1	4	2	1	5	10
M2	9	6	3	15	17
M3	2	4	0	5	13
M4	4	6	0	9	11
M5	5	4	0	7	8

FAB, French-American-British classifications; Hb, hemoglobin; WBC, White blood cells

after induction chemotherapy. The highest mean of relapse cases was in FLT3-ITD mutation patients 28.08% and the lowest was in DNMT3A mutation carriers 19.75%, and since (p >0.05), there are no statistically significant differences in the mean of relapse ranks. The results of the Kruskal Wallis test comparing the average ranks of relapses in the three mutations shown in (Figure 2). The FLT3-ITD mutation patients had the highest mean of death cases, while the DNMT3A mutation patients had the lowest, but this differences was not statistically significant. On the other hand, the highest mean of remission was in patients with NPM1 mutation and the lowest in the carriers of the FLT3-ITD mutation. It was observed that the mean relapsed patients with FLT3-ITD mutation was 3.4 months with a confidence interval of 95% (5.4-1.4) months, with no significant differences (p >0.05) between FLT3-ITD carriers and non-carriers relapsed: (Log rank=0.016, p-value=0.900), Figure 3 (A). The same result was identified with DNMT3A mutation, when no relation was found between the patients hold this

mutation and non- holder, in terms of relapse: the mean of relapse was two months, Figure 3 (C). On the contrary, It was observed that the mean relapsed for NPM1 mutation carriers is 2.4 months with a confidence interval of 95% (3.8-0.9) months, with significant statistical differences (p <.05) between the mean of relapse of mutation carriers and non-carriers, Figure 3 (B).

The mean overall survival time for patients with FLT3-ITD mutation was 5.9 months, with a 95% confidence interval (CI between: 3.7-8.2) months, with significant differences (p<0.05) between the mean overall survival of non-FLT3-ITD and exist FLT3-ITD patients as shown in Figure 4 (A), (Log rank=24.459, p=0). The mean overall survival time for NPM1 mutation carriers was 5.85 months, with a 95% confidence interval (CI between: 3.7-7.9) months, with significant differences (p<0.05) between the mean survival of non-NPM1 and NPM1 patients holder as shown in Figure 4 (B). where (Log rank=19.337, p-value=0). The mean overall survival time for patients with a DNMT3A mutation was 5.88
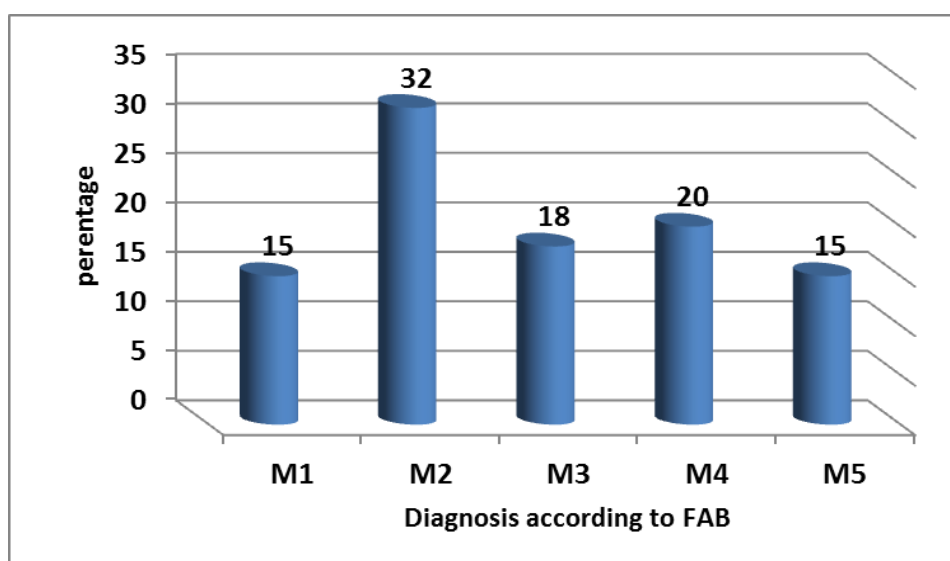

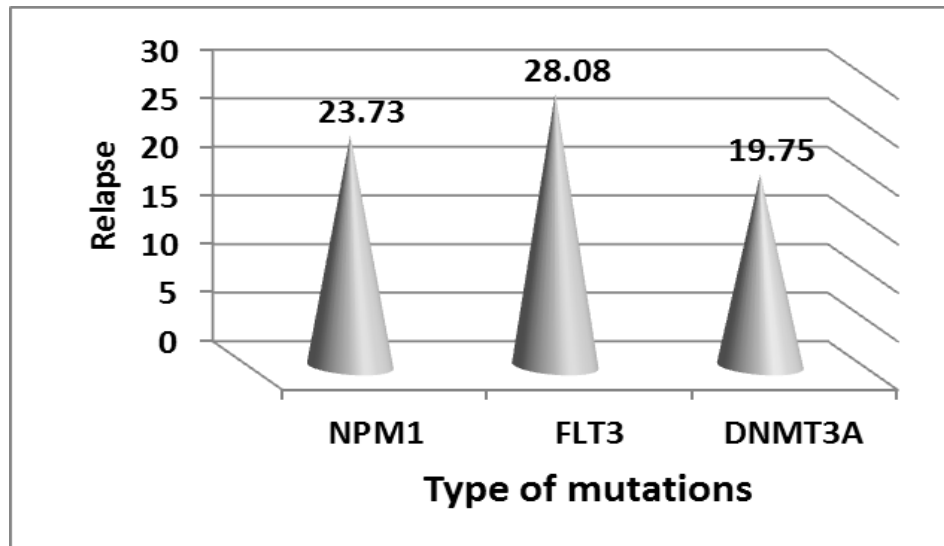Figure 1. Samples Diagnosis According to FAB Classification

Figure 2. Effect of Each Mutations on Relapse

months, with a 95% confidence interval (CI between: 0.8–10.9) months, with no significant differences (p>.05) between the mean survival for DNMT3A mutation patients and those who do not have it due to the smallness of the sample as shown in Figure 4 (C), where (Log rank=0.708, p-value=0.4). Finally, it was noted that the mean survival time for patients carrying the mutation of any kind is 6.46 months with a confidence interval of 95% (4.7-8.3) months, with significant differences (p <0.05) between the mean survival of the carriers of any kind of mutation and non-carriers. When using the cox regression method to determine the most important variables in the incidence of death, it was noted that the variables of age (β_1=0.026, p-value=0.01) and the presence of the mutation (β_2=2.201, p-value=0) are the most important variables in the incidence of death. Therefore The estimated model, which measures the relationship between the incidence of death, the variables of age and the presence of a mutation:

$$h=0.026 \, age+2.201 \, mutation$$

And that 98.99% where (R2 = 0.989) of the changes affecting the incidence of death are due to the variables of age and the presence of a mutation.

Regarding to the relation between EFS and the target mutations, our results demonstrate that the mean EFS of FLT3-ITD mutation patients is 4.818 months with a confidence interval of 95% (2.9-6.7) months, while the mean EFS of NPM1 mutation patients is 4.805 months and a confidence interval of 95% (1-2.8) months, with significant statistical differences (p<0.05) between the mean EFS of FLT3-ITD and NPM1 mutation patients and non-mutated patients (p<0.05), While this mean was not statistically significant (5.875 months) in patients carrying DNMT3A mutation (Figure 5).

Discussion

Many populations of AML have been examined for gene alterations, however in our community, this is the first study, to our knowledge, detected these three
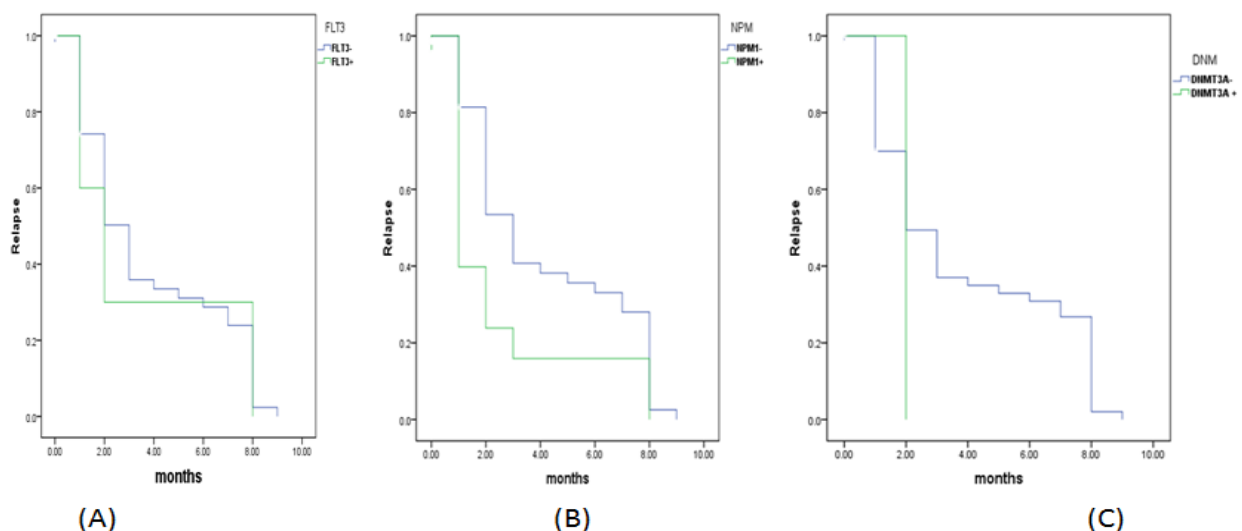

Figure 3. Kaplan-Meier Relapse of Patients with CN-AML (A): Relapse in FLT3-ITD patient, (B): Relapse in NPM1 patient, (C): Relapse in DNMT3A patient
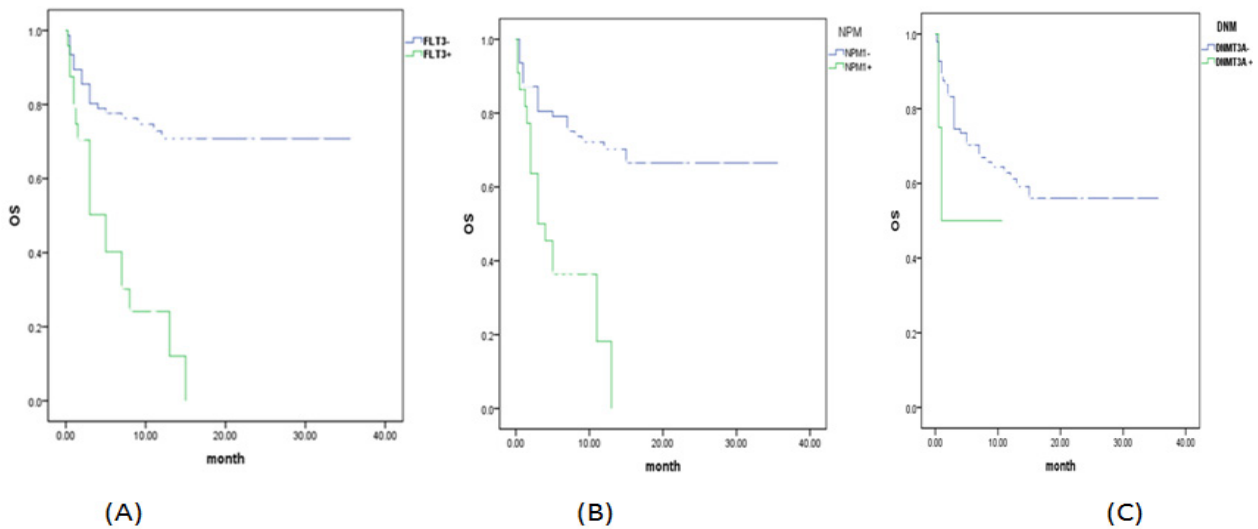
Figure 4. Kaplan-Meier Overall Survival of Patients with CN-AML, (A): OS of *FLT3*-ITD patients, (B): OS of *NPM1* patients, (C): OS of *DNMT3A* patients

gene mutations in adults, normal karyotype patients in our cohort. In each community, the frequency of gene mutations varied. All cytogenetically normal acute myeloid leukemia patients were sequenced for hotspot mutation exons in FLT3, NPM1, and DNMT3A genes. We have explored the incidence and type of FLT3 mutations. We observed that the JMD mutations (exons 14 and 15) were the most common type of alteration (24%) compared to TKD mutations at exon 20 (2%). When comparing the frequency of mutations in only normal karyotype of AML patients. Reports from Western countries have shown that the prevalence of FLT3-ITD mutations are found in approximately 25% of newly diagnosed AML cases (Daver et al., 2019), this percentage was similar to our study, between Ahmad et al., (2010) reported 19.1% of isolated ITD mutation, which was lower than our study. A decreased incidence of ITD was observed in the Korean populations 13% (Bang et al., 2008). NPM1 mutation was observed in 22% of CN-AML patients in the current study, a lower frequency than those of many past studies

from Western and Asia (Schlenk et al., 2008; Park et al., 2012). In addition, recurrence of NPM1 mutation in this study moreover lower than those of CN-AML in a ponder of Central Thailand where the frequency was 38.1% (Boonthimat et al., 2008). The contrasts in these change frequencies may be due to numerous diverse components counting the hereditary foundations of each populace, geographic locale and environmental components (Wangkumhang et al., 2013). DNMT3A is an enzyme that catalyzes the exchange of methyl groups to particular CpG structures in DNA, called DNA methylation, which controls gene expression by selecting proteins included in gene suppression or by inhibiting the binding of translation factor(s) to DNA. Transformations in DNMT3A quality change the gene's typical work and play an critical part in AML prognosis (Ley et al., 2010). DNMT3A mutation was found in 4% of CN-AML but other studies detailed the frequency run of 13.3-34.2% in CN-AML (Marcucci et al., 2012; Ahmad et al., 2014). In expansion, the recurrence of DNMT3Amut in CN-AML from Central
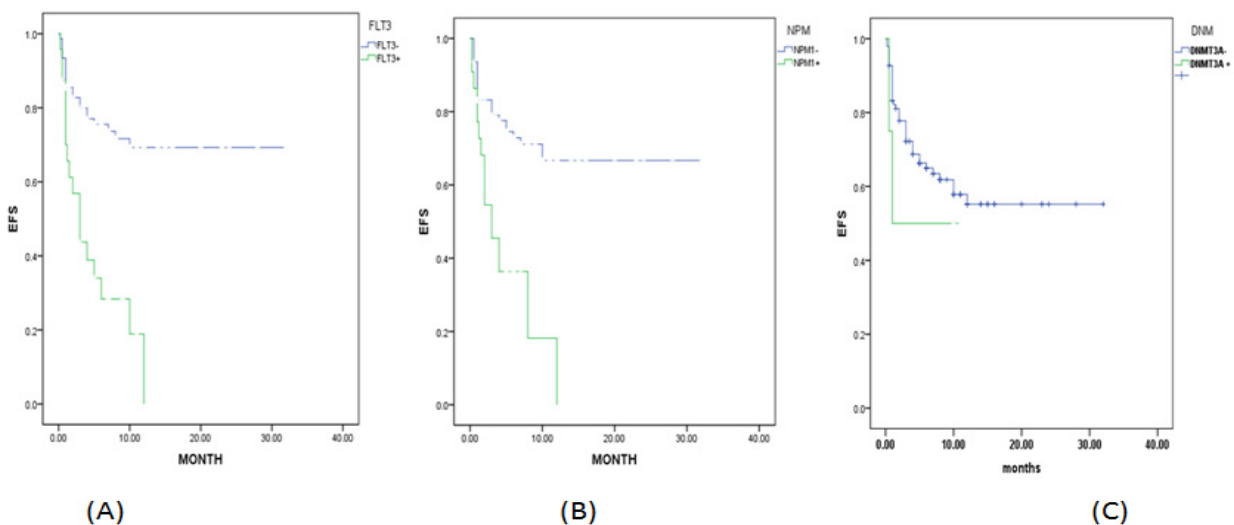

Figure 5. Kaplan-Meier Event Free Survival of Patients with CN-AML, (A): EFS of FLT3-ITD patients, (B): EFS of NPM1 patients, (C): EFS of DNMT3A patients

Thailand were 8.7% (Sirirat et al., 2017). In this study, we assess the clinical and outcome characteristics of AML patients with the three described mutations and there effect on relapse.This results inconsistent with other studies regarding to DNMT3A mutation, where no relation was found between it and relapse. Researches portrayed that DNMT3A mutations is one of the most crucial impact had a pronounced consequence on the clinical outcome causing a higher relapse rate (Marková et al.,2012). Apparently, DNMT3A mutations deserve more investigation and researches, as their detection paved the way to the possibilities of prognostic stratification of patients. Moreover, their examination may be of help in targeting therapy. Data from this study shed the light on FLT-ITD where variously did not play an obvious impact on relapse as common in most of studies, FLT3–ITD mutations cause an increase in reactive oxygen species production, which promotes DNA oxidative damage, DNA double-strand breaks, and repair mechanism errors, resulting in a cycle of genomic instability. The acquisition of novel structural chromosomal aberrations by leukemic cells between diagnosis and relapse supports the notion of genomic instability leading to cytogenetic evolution in AML (Kim et al.,2021). On the other hand, most studies reveal that NPM1 mutation is considered as a stable and reliable prognostic marker in AML and can be used to assess minimal residual disease(MRD) (Jain et al.,2014).

In AML, relapse remains the principal cause of treatment failure for the majority of patients. Therefore the ability to identify those patients with a high risk of relapse would represent a valuable advance, which may lead to the introduction of alternative forms of therapy in this group of patients. The first deliberate of the ELN genetic categories was to standardize announcing of hereditary abnormalities especially for correlations with clinical characteristics and result, so we focused in this study on the correlation between the genetic mutations and clinical features, and what was clearly obvious that patients with DNMT3A mutations had different results than patients with FLT3-ITD and NPM1 mutations. Different studies shown that DNMT3A mutations reflects independent prognostic factor of shorter OS in patients with de novo AML (Hou et al.,2014). Moreover, other study (Shirarov et al.,2013) also illustrated that DNMT3A mutations were related with more regrettable result counting essentially shorter OS and EFS and that is totally different from the ELN risk classification for AML, due significance is put on for NPM1 and FLT3 but not for DNMT3A mutations in CN-AML. In expansion, later information illustrate that DNMT3A mutations status has an effect on treatment choice; in one ponder, patients with DNMT3A mutations who were treated with high-dose daunorubicin compared to standard-dose treatment were appeared to have longer OS (Patel et al., 2012). However, results showed that the FLT3-ITD mutation has been identified as a marker of poor prognosis in AML, and that what most of studies agree, and the clinical outcomes of patients with FLT3-ITD-mutated AML combined with favorable cytogenetics was improved, when compared with those of patients with FLT3-ITD mono-mutated AML (Lenzi et al., 2020). Our findings showed that NPM1 mutation

had a bad prognosis in AML patients and that in contrast with many studies, which they declared that OS were significantly longer for patients with NPM1 mutations. Likewise they demonstrated a favorable impact of NPM1 mutations on outcome (Martelli et al., 2010; Wang et al., 2010). This discrepancy could be explained by ethnic differences, various inclusion criteria, and different sample sizes. Continuing to investigate the clinical associations of the three mutations, we evaluate their effect on EFS, and it was revealed that the EFS rates in 24 patients harboring FLT3-ITD mutations were shorter (4.818 months), compared with those in patients without FLT3-ITD mutations, with significant differences were observed between these two groups. This study agrees with several studies, which they illustrated that FLT3-ITD mutations become one of the most important factor predicting for EFS (Kottaridis et al., 2001). Schnittger et al., (2011) evaluated that, the FLT3-ITD status per se is predictive for OS and EFS in mutated AML. The authors thus proposed that cells with FLT3-ITD confer a greater growth advantage when compared to cells having FLT3 wild type or a heterozygous genotype. To date, recent studies in NPM1-positive AML have focused on the prognostic impact of genetic mutations such as FLT3-ITD. In our results, NPM1-positive AML had a poor prognostic impact in EFS as independent factor. Our results are same to a study where they showed that high NPM1-mutant allele burden at diagnosis is an independent predictor of unfavorable clinical outcomes, and they also indicate that the prognostic effect of NPM1 mutation in de novo AML may be influenced by the relative abundance of the mutated allele (Patel et al., 2018). however, we are limited in our study to validate this finding by studying the variant allele frequency (VAF). Patel etal., (2018) and most of studies, showed that NPM1 mutations alone were associated with significantly longer EFS, but those studies should take in consideration the VAF to improve their findings. Our reports showed that the presence of DNMT3A mutation was not associated with significant adverse outcomes among CN-AML patients, where the status has no impact on EFS in the presence of DNMT3A mutation, this results agree with a study made by Gaidzik et al., (2013), when he concluded that DNMT3A mutation represents a frequent genetic lesion in adults with AML but has no significant impact on survival end points. However, our study does not agree with many studies refer to the bad prognosis of DNMT3A mutation and it is related with shorter EFS among AML patients, They demonstrated that DNMT3A mutation was associated with poor AML outcomes, especially in cytogenetically normal-AML (Park et al., 2020; Hou et al., 2012). The DNMT3A mutation remained as the independent unfavorable prognostic factor after multivariate analysis, that is could be caused by CDKN2B which plays a crucial role in the regulation of hematopoietic progenitor cells and DNMT3A mutation is associated with CDKN2B promoter methylation (Jang et al., 2019). Our study has limitations because it is a retrospective study, was limited to a small sample size, and did not examine a controlled clinical trial setting. Confirming our findings in larger groups and with a longer follow-up period will be important for

establishing the truth of our findings. On the other hand, numerous studies showed the prevalence and prognosis of these mutations in AML, but this was the first report of the prognostic significance of these mutations in Syria. The present study revealed the effects of these mutations in single terms of OS, EFS and relapse. We demonstrate that mutations in NPM1, FLT3-ITD, and DNMT3A are among the most important predictors for outcome. Our results further emphasize the value of comprehensive molecular genetic screening for the dissection of this heterogeneous patient subgroup, which may ultimately lead to improved risk stratification.

Author Contribution Statement

Authors: Yahia Moualla (MY), Faten Moassass (MF), Bassel AL-Halabi (HB), Walid Al-achkar (AW), Michael Georgeos (GM), Haissam Yazigi (YH), Atieh Khamis (KA). MF, KA and AW performed banding cytogenetics and molecular genetics, MY and HB performed the molecular genetic analysis, MY, YH, and GM provided the clinical data and the chemotherapy plan. MY, MF, and AW, drafted the paper. All authors worked on the final version of the paper. All authors read and approved the final manuscript.

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