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The Clinical Impact of NPM1 Mutations and the Effect of Concurrent Mutations in Acute Myeloid Leukemia: Unraveling the Prognostic Significance

Faten Moassass[1] | Yahia Moualla[2] | Bassel AL-Halabi[1] | Atieh Khamis[2] | Walid Al-achkar[1]

[1]Department of Molecular Biology and Biotechnology, Human Genetics Division, Atomic Energy Commission, Damascus, Syria | [2]Department of Laboratory Diagnosis, Faculty of Pharmacy, Ministry of Higher Education, Tishreen University, Lattakia, Syria

Correspondence: Yahia Moualla (yahiamoualla@hotmail.com)

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ABSTRACT

Background and Aims: Nucleophosmin *(NPM1)* gene mutations occur in approximately 30%–35% of individuals with an initial diagnosis of acute myeloid leukemia (AML). Mutations in this gene have been reported in 50%–60% of AML patients with a normal karyotype. These mutations help to distinguish clinicopathological and molecular features, setting them apart as a unique subset within the heterogeneous landscape of AML. In the present study, we investigated the frequency and clinical impact of $NPM1^{mut}$ in 100 newly diagnosed adult Syrian patients with AML-normal karyotype (NK) using direct sequencing.

Methods: We analyzed 100 AML-NK patients using direct sequencing to assess the prevalence and clinical impact of *NPM1* mutations, as well as the co-occurrence of *FLT3*-ITD and *DNMT3A* mutations.

Results: Our results revealed that the prevalence of $NPM1^{mut}$ was 22% among the patients; 86.4% of these mutations were type A (NM_002520.5:c.860-863dupTCTG), while 13.6% were de novo mutations (c.863_864insCCTG, p.Trp288CysfsTer12), (c.861_862dup, p.Trp288SerfsTer13), and (c.863_864insCCGG, p.Trp288CysfsTer12). Among our patients, 22% exhibited $NPM1^{mut}$, with 7% also harboring $FLT3\text{-}ITD^{mut}$ and 2% having $DNMT3A^{mut}$. The presence of $NPM1^{mut}$ was correlated with a statistically significant increase in bone marrow blast percentage ($p = 0.017$). Notably, patients with $NPM1^{mut}$ displayed significantly higher mortality rates, with 72.7% succumbing to the disease compared to 29.5% of patients without $NPM1^{mut}$ ($p < 0.001$). Furthermore, our results showed that when the overall survival (OS) time exceeded 8.35 months, the likelihood of *NPM1* wild-type status was greater.

Conclusion: The evaluation of $NPM1^{mut}$ and co-mutation has consistently demonstrated remarkable prognostic significance in AML, suggesting the potential for improved response rates, extended disease-free periods, and OS. Our findings provide valuable insights for understanding molecular leukemogenesis in AML-NK patients and will aid in clinical diagnosis, prognostic implications, and the development of targeted therapy strategies for Syrian AML patients.

Faten Moassass and Yahia Moualla contributed equally to this article.

1 | Introduction

Nucleofosmin (*NPM1*), a phosphorylated protein frequently referred to as B23 or numatrin, is prominently expressed in the granulomatous region of the cell nucleus. This multifunctional protein participates in a diverse array of biological processes including cell growth inhibition and reproduction. Remarkably, *NPM1* is susceptible to a broad spectrum of genetic aberrations, which demonstrates its pivotal role in the molecular basis of tumorigenesis across multiple cancer manifestations. Consequently, the intricate association of the *NPM1* gene with cancer progression in individuals is highly notable [1]. The frequency of *NPM1* mutations within exon 12 is approximately 30% among newly diagnosed de novo acute myeloid leukemia (AML) patients, with approximately 50%–60% of these individuals exhibiting a normal karyotype (NK). This observation has provided a novel perspective on the role of *NPM1* mutations in scientific research and investigative endeavors [2, 3]. According to the most recent update of the World Health Organization (WHO) classification, the presence of *NPM1*-positive AML has been recognized as a distinct diagnostic parameter. Several researchers have observed a noteworthy association between *NPM1* genetic alterations and the clinical and biological features of acute myelogenous leukemia. This correlation has been substantiated through in-depth examination of the intracellular localization of *NPM1* [3, 4]. These genetic alterations correlated with Fms-like tyrosine kinase 3-internal tandem duplication (*FLT3*-ITD) and decreased CD34 and CD13 expression levels. Type A mutations, which account for approximately 70%–80% of all *NPM1* gene mutations, are characterized by the insertion of (TCTG) nucleotide base pairs at position 288. This insertion leads to frameshift changes in the downstream sequence [5, 6]. Individuals harboring *NPM1* mutations demonstrate notable heterogeneity in their response to pharmacotherapy. The presence of multiple concurrent mutational events, such as *FLT3*-ITD and DNA methyltransferase 3A (*DNMT3A)*, in addition to *NPM1* mutations, significantly influences treatment response and overall survival (OS), thereby contributing to the divergent outcomes observed. Patients with *NPM1* mutations but lacking *FLT3*-ITD mutations generally have a favorable outlook. Approximately 40% of the individuals in the cohort without *FLT3* mutations still experienced relapse, indicating inherent variability in outcomes. This discrepancy implies the presence of inter-tumoral molecular diversity within the subset of *NPM1*-mutant patients, which cannot be solely attributed to the presence of additional co-occurring mutations [7]. In this study, we conducted a comprehensive analysis and characterized the genetic diversity among 100 recently diagnosed Syrian patients with *NPM1* mutations using various molecular methodologies. We successfully identified distinct subcategories based on their mutational status, each exhibiting unique molecular features and displaying varying responses to conventional therapeutic approaches.

2 | Materials and Methods

2.1 | Subjects

A total of 100 newly Syrian patients diagnosed with AML, between November 2019 and December 2022, were included in this study. Males accounted for 51%, while females constituted 49% of the cohort, with a median age of 43.47 ± 18.19 years and an age spectrum was 17–86 years. Patients with NK, without previous history of exposure to chemotherapy/radiotherapy treatment, were selected for molecular analysis. AML diagnosis was evaluated by conducting complete blood count tests, blood smears, cytogenetic analyses, and flow cytometry. All patients underwent histological and immunohistochemical confirmation with a specific focus on the absence of prior treatment history. AML type's diagnosis was according to the French–American–British (FAB) classification system. Written informed consent was obtained from all patients or their families. Peripheral blood (PB) and bone marrow (BM) samples from each patient were collected. The study was approved by the Biosafety & Bioethics Committee of the Institutional Ethical Committee of the Syrian Atomic Energy Commission and was confirmed by the University Review Board under decision No. 2979 of 07/08/2018.

2.2 | Treatment Protocol

The patient underwent intensive treatment with cytotoxic chemotherapy. The induction therapy regimen included the administration of daunorubicin (at a daily dose of 60 or 90 mg/m^2 for 3 days) in combination with cytarabine (at a daily dose of 100 or 200 mg/m^2 for 7 days) or, alternatively, thioguanine, cytosine arabinoside, or daunorubicin [8]. This was followed by two or three cycles of consolidation therapy, which consisted of high-dose cytarabine (at a dose of 1.5 or 3 g/m^2 for 3 days). Complete remission (CR) was evaluated through BM examination conducted 28 days after each chemotherapy course. In patients for whom CR was not achieved after the first course of chemotherapy, a second course was administered.

2.3 | Cytogenetic and Molecular Analyses

Cytogenetic analysis of BM sample was conducted using for chromosomal banding (GTG) techniques, as described by Al-Achkar et al. [8]. Fluorescence in situ hybridization (FISH) using specific AML probes to detect translocations was performed to excluded patients with chromosomal abnormalities, as previously reported [9, 10].

2.4 | Analysis of NPM1 Mutations

Genomic DNA was isolated from PB or BM samples using a QIAamp DNA Blood Mini Kit (Qiagen, Germany) in accordance with the guidelines provided by the manufacturer. The concentration of total DNA of each sample was measured by using a spectrophotometer followed by quantity ultraviolet light absorbance. *NPM1* and *FLT3* (ITD-TKD) mutations were detected using standard polymerase chain reaction (PCR) and direct sequencing as previously described protocols [6, 10]. *DNMT3A* gene mutations, specifically in Exon 23, with a focus on the hotspot R882 region was studied. Sequencing was performed using the following primer sequences: *DNMT3A*-EXON23 FW, 5′-GTGTGGTTAGACGGCTTCC-3′, and *DNMT3A*-EXON23 RV, 5′-CTCTCCCACC TTTCCTCTG-3′. PCR was conducted in a total volume of 50 μL, comprising

200 ng gDNA, 10× PCR buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl), 2 mM $MgCl_2$, 200 μM dNTPs, 10 pM of each primer, and one U Taq DNA polymerase. The PCR procedure involved an initial denaturation step at 95°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 60 s, and extension at 72°C for 75 s, and a final extension at 72°C for 5 min. Subsequently, the PCR products were purified and subjected to direct sequencing using a SeqStudio Genetic Analyzer (Applied Biosystems, USA). For mutational landscape visualization, the Oncoprinter tool, available at www.cbioportal.org, was used, and the obtained data were aligned and compared with different sequences using the National Center for Biotechnology Information (NCBI BLAST), as described previously [11].

2.5 | Ethical Approval and Informed Consent

This study was approved by the Biosafety & Bioethics Committee of the Syrian Atomic Energy Commission and confirmed by the University Review Board under decision No. 2979 of 07/08/2018. Written informed consent was obtained from all patients or their legal guardians before inclusion in the study. Participants were fully informed of the study's objectives, procedures, potential risks, and benefits.

2.6 | Statistical Analysis

Statistical analysis using SPSS computer software version 20 (BM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp; 2011). To compare the means between two independent groups, Student's independent *t* test was used. For comparisons among three or more independent groups, one-way analysis of variance (ANOVA) was employed. Receiver operating characteristic (ROC) curve analysis was performed to assess the diagnostic accuracy of various factors. Kaplan–Meier (K-M) curve analysis was used to estimate OS and event-free survival (EFS). The Cox proportional hazards regression model was used to identify risk factors and assess hazard ratios for OS. Spearman's rank correlation was applied to explore associations between continuous or ordinal markers. Multivariate statistical analyses, such as logistic regression or Cox regression, were used to identify factors influencing mortality and survival. Statistical significance was set at $p < 0.05$, with all tests being two-sided.

3 | Results

Data from 100 newly diagnosed AML patients with NK were recorded. The median age at diagnosis was 43.47 ± 18.19 years and an age spectrum spanning from 17 to 86 years. However, *NPM1* gene mutations were reported in 22 of the 100 AML patients (22%). According to FAB subtype of AML classification, *NPM1* mutations were found more frequently in AML-M2 than in the other subtypes. In addition, the frequency of $NPM1^{mut}$ was higher in older patients than in younger ones. Furthermore, no significant difference in the frequency of *NPM1* mutations between sexes was found. Of the 22% of patients with *NPM1* mutations; 86.4% of these mutations were type A. Type A mutations are commonly known as four-nucleotide insertion mutations (NM_002520.5:c.860-863dupTCTG), whereas 13.6% of these mutations were de novo as shown in Figure 1. In contrast, other mutations involve the insertion of different nucleotides, resulting in substitution of various amino acids. Therefore, in Table 1, the three nontraditional mutations are listed separately along with the age and sex of the carriers. Notably, all these mutations involve insertions in Exon 12, and the anticipated impact of these mutations will be included based on guidelines provided by the American College of Medical Genetics (ACMG). Sparingly, 7% of patient harboring *FLT3-ITD* mutations and 2% harboring *DNMT3A* mutations were associated with *NPM1* mutations. At the initial presentation, the median morphological aspirate blast count was 70%, ranging from 20% to 99%. In our cohort, the presence of *NPM1* mutations was correlated with a statistically significant increase in the percentage of BM blasts, as assessed using an unpaired *t* test with $p = 0.02$. Notably, patients with *NPM1* mutations had significantly higher mortality rates, with 72.7% of these individuals succumbing to the disease compared with only 29.5% of patients without *NPM1* mutations ($p < 0.001$), a 2.5-fold increased risk of mortality.

In this study, the median of WBC count was 43.96×10^9/L (range: 0.8×10^9/L–300×10^9/L).

Over the course of 34 months, a comprehensive evaluation was conducted to determine the median follow-up duration. Within this timeframe, the study revealed a median OS of 12.13 months, accompanied by a 95% confidence interval (CI) ranging from 0.1 to 36 months, indicating the variability in patient survival times. Among the 28 patients who achieved CR, 13 experienced relapse (46.4%). The median EFS was 8.69 months, with a 95% confidence interval ranging from 0.1 to 32 months. However, given that there is disagreement regarding whether the presence of these mutations can predict the OS of AML patients, we investigated the ability of *NPM1* mutations ($NPM1^{mut}$ vs. $NPM1^{wt}$) to predict OS using ROC analysis. The results show that the area under the curve was 0.707 and when the OS time was greater than 8.35 months, there was a greater probability of $NPM1^{wt}$, as shown in Figure 2A,B and Table 2.

4 | Discussion

Evaluation of genetic markers has enhanced the classification of cytogenetic profiles, and these factors helped in guiding treatment decisions, especially for allogeneic stem cell transplantation (SCT). According to the literature, mutations in the *NPM1* gene are detected in approximately 60% of AML patients with a normal chromosomal structure, and account for approximately one-third of all AML patients [12]. Generally, *NPM1* mutations are associated with a favorable outcome [6]. The prevalence rate of *NPM1* mutations in this study was 22%, which is consistent with the findings of several studies. For instance, the prevalence rate was 21% in India [13], whereas a separate study by Zidan et al. reported a comparable rate of 21.8% [14]. These results in different studies were in agreement with our results in the current study.

Our findings revealed that the *NPM1* mutation Type A (TCTG insertion) was common, and these findings align with global

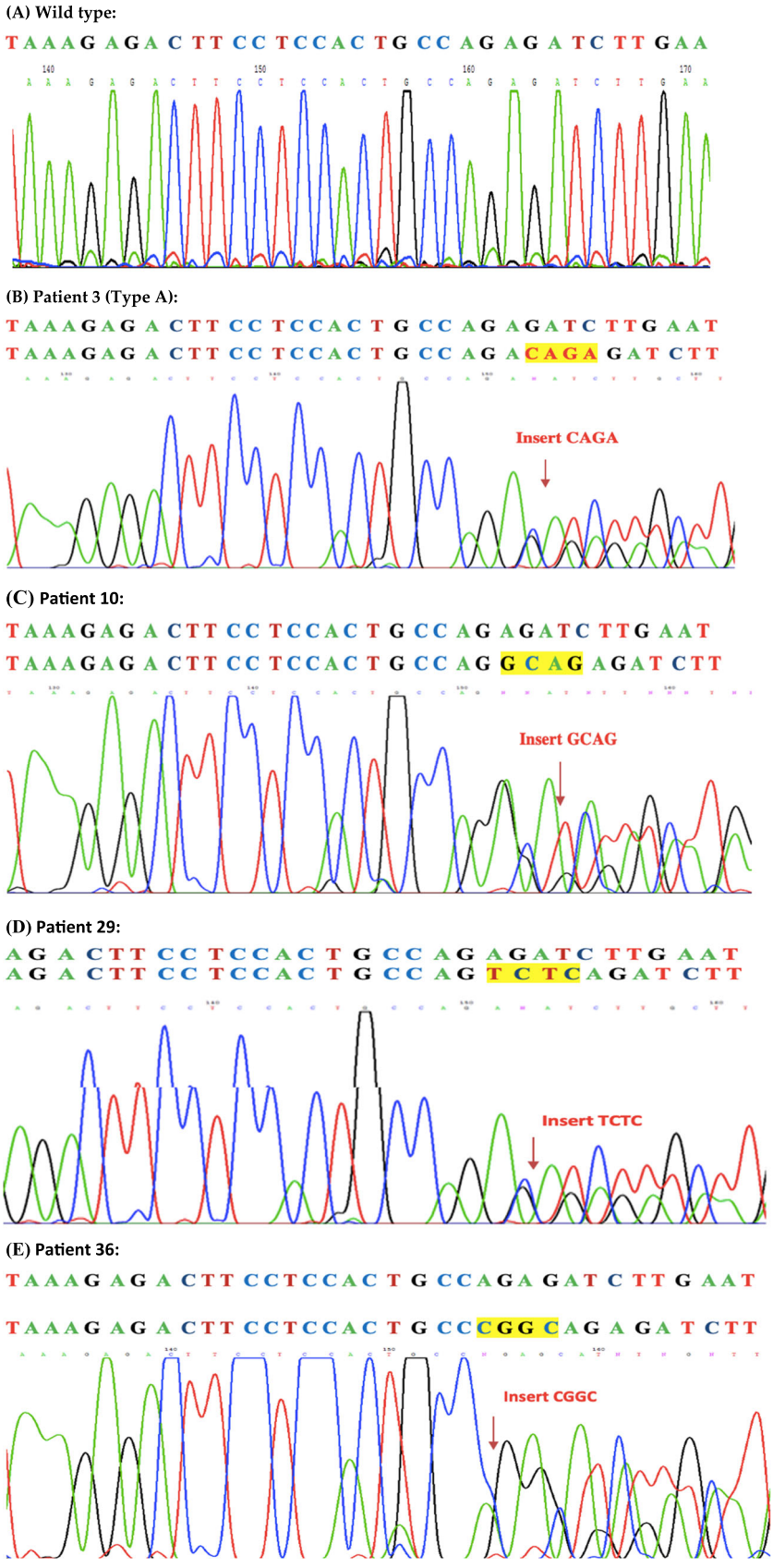

FIGURE 1 | Sanger sequencing (A) shows the normal sequence of the *NPM1* gene, (B) shows the most common type A mutation among the *NPM1* genes, and (C), (D), and (E) represent the sequences of the *NPM1* gene mutations in patients 10, 29, and 36, respectively, which are de novo mutations. (A) Wild type: (B) Patient 3 (Type A): (C) Patient 10: (D) Patient 29: (E) Patient 36.

TABLE 1 | The list of three nontraditional variants with ACMJ classification.

Patient number	Sex	Age	Genomic position (GRCh37)	Nucleotic alteration	Aminoacidic alteration	Coding impact	ACMJ classification
P10	F	20	g.171410541	c.863_864insCCTG	p.Trp288CysfsTer12	Frameshift	Uncertain Significance
P29	F	52	g.171410538	c.861_862dup	p.Trp288SerfsTer13	Frameshift	Uncertain Significance
P36	M	81	g.171410543	c.863_864insCCGG	p.Trp288CysfsTer12	Frameshift	Likely pathogenic

studies, indicating that *NPM1* Type A mutations are present in approximately 80% of AML patients. These mutations typically occur within the coding region of the C-terminus of the protein, leading to the disruption of tryptophan residues at positions 288 and 290 [15]. Importantly, Type A mutations were associated with inferior OS rates compared to other mutation types in the *NPM1* gene [16]. Given that most *NPM1* mutations in our study were Type A, we expected to observe lower OS rates among the study patients.

From a critical perspective, we investigated the mutational co-occurrence of *FLT3* and *DNMT3A* genes along with *NPM1* gene mutations. Notably, some patients simultaneously exhibited mutations in all three genes.

An intriguing aspect of our study was the identification of *FLT3*-TKD^{mut} patients, characterized by small point mutations of the common D835 type, in association with *NPM1*^{mut}. Notably, two patients (IDs: 16 and 68) who underwent conventional chemotherapy demonstrated an extended period of relapse-free survival and remained alive throughout the study and follow-up periods. This observation aligns with previous research indicating that TKD mutations, when occurring in conjunction with *NPM1*^{mut}, are associated with favorable prognosis [17]. These findings underscore the importance of determining *FLT3*-TKD status at diagnosis and raise the question of how to evaluate the prognostic relevance of *NPM1*^{mut} in the context of *FLT3-TKD* status.

While 31% of the *NPM1*^{mut} patients had *FLT3*-ITD^{mut}, it was notable that the OS in this cohort did not exceed 2 months. This finding underscores the poor prognosis associated with the combination of *NPM1*^{mut} and *FLT3*-ITD^{mut}. Several studies support this observation, highlighting that *FLT3*-ITD^{mut}, particularly when accompanied by *NPM1*^{mut} Type A, is associated with a worse prognosis than other types of mutations in *NPM1* [16, 18]. This finding underscores the importance of distinguishing between specific mutation types in *NPM1*. However, this result was influenced by various genetic factors. Not all *FLT3*-ITD^{mut} patients present identically, and the allelic ratio (AR) is considered a crucial factor for determining prognosis. The guidelines from the European Leukemia Net (ELN) emphasize the significance of studying the association of *NPM1*^{mut} with *FLT3*-ITD^{mut} and categorizing risk groups based on the AR [19].

Hence, it is crucial to investigate *FLT3* and *NPM1* mutations in all study patients to assess their prognosis and make appropriate therapeutic decisions. The importance of this approach is underscored by Minetto et al., who demonstrated the efficacy of a treatment plan comprising fludarabine, high-dose cytarabine, and idarubicin in a retrospective study of AML patients [20]. In newly diagnosed AML patients under 55 years of age with *FLT3-ITD* and/or *NPM1* mutations, this treatment plan was found to overcome the negative effects of FLT3-ITD mutations associated with *NPM1*^{mut} [20]; consequently, this approach has the potential to prolong relapse-free survival in these patients. However, recent studies have indicated that patients with an *FLT3*-ITD AR < 0.5, an *NPM1*^{mut} mutation, and without other poor prognostic mutations, such as *DNMT3A*, *TP53*, or *TET2*, can be considered to constitute a good prognosis group given their response to a treatment plan consisting of pretreatment,

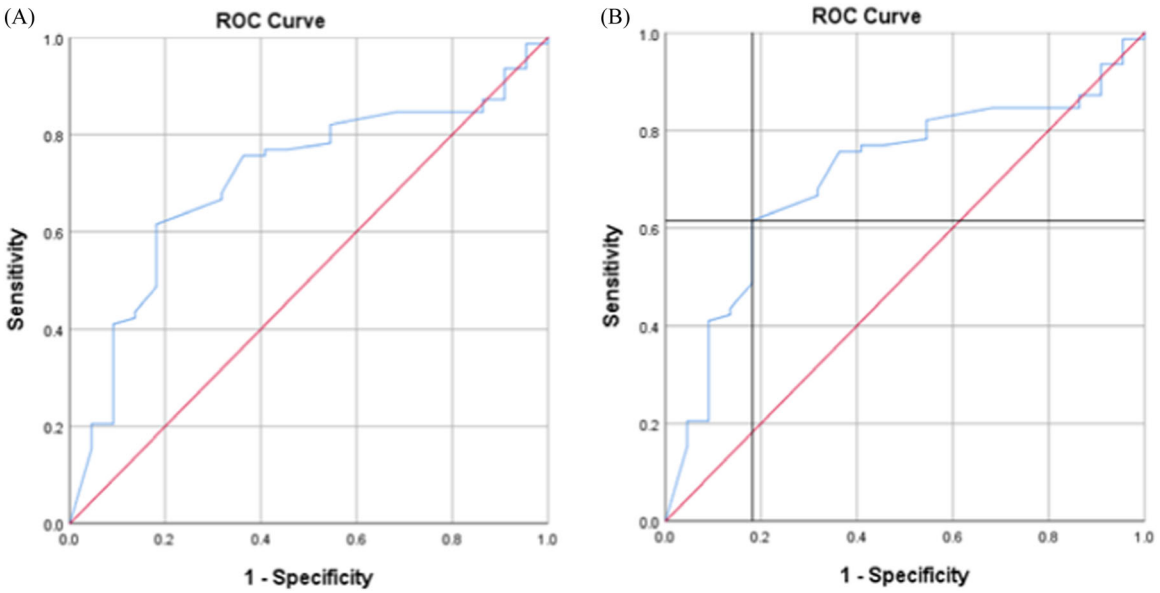

FIGURE 2 | ROC curve of OS time: (A) ROC curve of OS time, which represents sensitivity on the vertical axis and complement specificity on the horizontal axis according to the *NPM1*^{mut}; (B) ROC curve of OS time with the point closest to the left-north corner determined according to the *NPM1*^{mut}.

TABLE 2 | The area under the curve and the test value of significance were determined by carrying or not carrying the *NPM1*^{mut}.

Test result variable(s) for the blast percentage values on the smear:				
			Asymptotic 95% confidence interval	
Area	Std. error[a]	Asymptotic sig.[b]	Lower bound	Upper bound
0.707	0.059	0.003 (< 0.05)[c]	0.591	0.824

Note: The test result variable(s): has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.
[a]Under the nonparametric assumption.
[b]Null hypothesis: true area = 0.5.
[c]ROC curve is significant.

aggressive treatment, and maintenance treatment if they are negative early in minimal residual disease (MRD), for which they should consider performing a high-sensitivity analysis to detect MRD [21].

Similarly, *DNMT3A* mutations were also associated with a very poor prognosis when associated with *NPM1*^{mut}, particularly, Type A. A study was also conducted in which the OS of AML patients positive for *NPM1*^{mut} Type A associated with *DNMT3A* was compared with the OS of a group of patients positive for *NPM1* but with different mutations. For type A patients and those positive for *DNMT3A* mutations, the OS rate was significantly lower [16]. Thus, we conclude that there is a need to investigate *NPM1* mutations, their different types, and their associations with *DNMT3A* and *FLT3-ITD* mutations. While some authors have shown that the presence of *NPM1* non-A mutants is associated with poor prognosis for survival [22, 23], several other studies have indicated that there are no statistically significant differences in prognosis, remission, or OS between patients with *NPM1*^{mut} type A and those with *NPM1*^{wt} [24, 25]. These results were similar to those of our study, in which there was no statistically significant difference between patients with *NPM1*^{mut} type A and those with *NPM1*^{wt}.

The effect of *NPM1*^{mut} on OS is still a matter of controversy, as many studies differ in terms of this effect because of the various factors that could play a role in this effect, and one of the most crucial factors is concomitant mutations. Many studies have demonstrated that independent *NPM1*^{muts} are associated with good prognosis in patients with AML [26]. Several studies have shown that *NPM1*^{mut} Type A does not appear to affect the risk stratification of cytogenetically normal AML patients, as evidenced by the fact that OS and relapse-free survival were not substantially different between individuals with *NPM1* Type A mutations [27].

In contrast, other studies have shown that these mutations negatively affect survival. Our finding agrees with these studies, in which patients carrying *NPM1* mutations had lower OS and a greater death rate [16, 28]. As shown in our results, when the OS time was greater than 8.35 months, the probability of being *NPM1*^{wt} was greater. These findings could help in assessing the genetic profiles of patients with AML in the early stages of the disease.

This study was conducted to support the value of molecular genetic screening in analyzing this diverse patient population, which may ultimately result in a more accurate risk assessment [11]. The prediction of treatment response and outcome depends on the discovery of novel molecular subgroups in *NPM1*-mutated AML. Currently, induction and consolidation chemotherapies are used to treat most patients with *NPM1* mutations but not those with *FLT3-ITD* mutations. Despite having a "higher" probability of long-term survival, these patients typically do not receive allogeneic hematopoietic SCT, and a sizable portion of them experience recurrence [29].

Author Contributions

Faten Moassass: investigation, funding acquisition, writing–original draft, writing–review and editing, visualization, validation. **Yahia Moualla:** investigation, funding acquisition, writing–original draft, writing–review and editing, visualization, validation. **Bassel AL-Halabi:** methodology. software. **Atieh Khamis:** supervision, conceptualization. **Walid Al-achkar:** supervision, project administration, writing–review and editing.

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Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board: Biosafety & Bioethics Committee of the Institutional Ethical Committee of the Syrian Atomic Energy Commission and Syrian Ministry of Higher Education (#2979/2018).

Consent

Before sample collection, signed written informed consent was obtained from all patients or their respective family members, according to the Declaration of Helsinki. Written informed consent was obtained from the patients for the publication of this paper.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author (Yahia Moualla) upon reasonable request. All relevant materials have been included in this publication.

Transparency Statement

The lead author Yahia Moualla affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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