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FMS-Like Tyrosine Kinase 3 (*FLT3*) and Nucleophosmin 1 (*NPM1*) in Iranian Adult Acute Myeloid Leukemia Patients with Normal Karyotypes: Mutation Status and Clinical and Laboratory Characteristics

Normal Karyotipli İran'lı Erişkin Akut Miyeloid Lösemi Hastalarında FMS-Benzeri Tirozin Kinaz 3 (*FLT3*) ve Nükleofosmin 1 (*NPM1*): Mutasyon Durumu ile Klinik ve Laboratuvar Karakteristikleri

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Abstract

Objective: In this study, we evaluated the frequency of FMS-like tyrosine kinase 3 (*FLT3-ITD* and *FLT3-TKD*) and nucleophosmin (*NPM1*) mutations in Iranian patients with cytogenetically normal acute myeloid leukemia (CN-AML). The clinical and laboratory characteristics were compared between wild-type and mutant cases.

Materials and Methods: Seventy newly diagnosed *de novo* AML patients were recruited at the time of diagnosis prior to chemotherapy; among them, 54 had CN-AML. For detecting mutations, the *FLT3* and *NPM1* genes were amplified by the polymerase chain reaction method, followed by direct sequencing.

Results: Our results showed that the frequencies of *FLT3-ITD*, *FLT3-TKD*, and *NPM1* mutations in CN-AML patients were 25.9%, 5.9%, and 20.8%, respectively. The most frequent NPM1 mutation type was the type A mutation. The *FLT3-ITD* mutation was seen more frequently in non-M3 patients compared with M3 patients. No mutation was observed in either the *FLT3-TKD* or the *NPM1* gene in patients in the M3 French-American-British group. There was no significant association between the presence of *FLT3-ITD* and *NPM1* mutations in CN-AML patients (p>0.05). The frequency of *FLT3-ITD, FLT3-TKD*, and *NPM1* mutation was higher in CN-AML patients in comparison with AML patients with cytogenetic aberrations, although the differences were not statistically significant (p>0.05). There were no significant differences in mean white blood cell and platelet counts, serum hemoglobin levels, and bone marrow blast percentages between patients with wild-type and mutant *FLT3-ITD* and *NPM1* genes

Öz

Amaç: Bu çalışmada, İran'lı normal sitogenetikli akut miyeloid lösemi (NS-AML) hastalarında FMS-benzeri tirozin kinaz 3 (*FLT3-ITD* ve *FLT3-TKD*) ile nükleofosmin 1 (NPM1) mutasyonlarının sıklığını değerlendirdik. Mutant olmayan (yabanıl-wild-type) ve mutant olgular klinik ve laboratuvar özellikler açısından mukayese edildi.

Gereç ve Yöntemler: Yetmiş yeni tanı *de novo* AML hastası kemoterapi uygulanması öncesinde çalışmaya dahil edildi; bunların 54'ü NS-AML idi. Mutasyonları tespit etmek için, *FLT3* ve *NPM1* genleri polimeraz zincir reaksiyonu ile amplifiye edildi ve bu işlemi direkt dizileme takip etti.

Bulgular: NS-AML hastalarında *FLT3-ITD, FLT3-TKD* ve *NPM1* mutasyonlarının sıklıkları sırasıyla %25,9; %5,9 ve %20,8 olarak bulunmuştur. En sık gözlenen NPM1 mutasyon tipi, tip A mutasyonuydu. *FLT3-ITD* mutasyonu M3 hastalarına göre M3-dışı olgularda daha sık görülmekteydi. Fransız-Amerikan-İngiliz M3 grubundaki hastalarda *FLT3-TKD* veya *NPM1* genine ait mutasyon tespit edilmedi. NS-AML hastalarında *FLT3-ITD* ve *NPM1* mutasyonlarının varlığı açısından anlamlı ilişki yoktu (p>0,05). *FLT3-ITD, FLT3-TKD* ve *NPM1* mutasyon sıklığı, her ne kadar istatistiksel olarak anlamlı farklılık saptanmasa da (p>0,05), NS-AML hastalarında sitogenetik aberasyonu olan AML olgularına göre daha fazlaydı. *FLT3-ITD* ve *NPM1* genleri açısından mutant olan ve olmayan hastalarda ortalama lökosit ve trombosit sayıları, serum hemoglobin düzeyleri ve kemik iliği blast yüzdeleri arasında anlamlı farklılık yoktu (p>0,05). Yaş ve cinsiyete göre *FLT3-ITD* veya *NPM1* mutasyonlarının sıklıkları açısından farklılık tespit edilmedi (p>0,05).

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(p>0.05). No difference was observed in the frequency of *FLT3-ITD* or *NPM1* mutation regarding age or sex (p>0.05).

Conclusion: Given the high stability of *NPM1* during the disease course, it can be used in combination with *FLT3* as well as other known genetic markers to monitor patients, especially for minimal residual disease detection.

Keywords: Acute myeloid leukemia, Gene mutation, *FLT3, NPM1*

Sonuç: *NPM1* hastalık sürecindeki yüksek kararlılığı nedeniyle, özellikle minimal kalıntı hastalık tespiti açısından *FLT3* veya diğer bilinen genetik belirteçler ile kombine olarak hastaların izlenmesinde kullanılabilir.

Anahtar Sözcükler: Akut miyeloid lösemi, Gen mutasyonu, *FLT3, NPM1*

Introduction

Acute myeloid leukemia (AML) is the most common hematologic malignancy, characterized by uncontrolled proliferation of hematopoietic stem cells resulting in abnormal accumulation of myeloblasts [1]. Generally, based on the cytogenetic abnormalities, the prognosis of AML patients is categorized into three risk groups: good, intermediate, and poor [2]. However, about 50% of AML patients have the normal cytogenetic feature (CN-AML), which represents a diverse subset of patients who are usually classified into an intermediate risk group [3]. Recently, assessment of molecular abnormalities has proven to be a useful marker for risk stratification of these patients into good and poor risk subgroups [3,4,5,6]. In this regard, somatic mutations of the FMS-like tyrosine kinase 3 (*FLT3*), nucleophosmin 1 (*NPM1*), and Wilms' tumor 1 (*WT1*) genes have been well studied [3,7,8,9].

FLT3 is a member of the class III receptor tyrosine kinase (RTK) family, normally expressed in early bone marrow precursors and playing an important role in the regulation of hematopoietic cell proliferation and differentiation [10]. Binding of the FLT3 ligand to its receptor recruits and activates several signaling molecules affecting cell proliferation, differentiation, and survival [11]. The FLT3 receptor consists of five extracellular immunoglobulin-like domains (Ig1-Ig5), a transmembrane domain, a juxtamembrane domain (JM), and the two intracellular tyrosine kinase domains (TK1 and TK2) [12,13,14]. *FLT3* is one of the most frequently mutated genes as approximately 30% of all AML patients have a mutated form of it [15]. Two types of activating mutations have been identified in the *FLT3* gene: internal tandem duplication (*FLT3-ITD*) of the region between exon 11 and 12 in the JM domain (occurring in 20%-25% of AML patients), and a point mutation at codon 835 of exon 17 in the TK domain (*FLT3-TKD,* also known as *D835Y*, and occurring in 5%-7% of AML patients) [8,16]. Both mutations contribute to constitutive activation of the FLT3 receptor [8]. It has been shown that the *FLT3-ITD* mutation has an inverse correlation with patient survival and can be used as an important poor prognostic factor to predict clinical outcomes in AML patients, especially those with normal karyotypes. However, data on the correlation between *FLT3-TKD* and AML disease outcome are highly limited [3,4,7,17].

The nucleophosmin gene encodes the NPM1 protein, which functions as a chaperone that shuttles between the nucleus and cytoplasm [3,5,7,8]. NPM1 regulates different intracellular processes such as transport of preribosomal particles, responses to stress stimuli, DNA repair, centromere duplications, and the activity and stability of tumor suppressor genes like *p53* [3]. Mutation within exon 12 of the *NPM1* gene, which is the most frequent mutation in AML patients (about 35% in all adult AML patients and 50%-60% of CN-AML cases), results in abnormal expression and localization of the protein within the cytoplasm [3]. The most common *NPM1* mutation (type A mutation, occurring in 75%-80% of cases) is the insertion of the TCTG tetranucleotide at position 956-959 in exon 12, but other less common mutations in exon 12 have also been described [18,19]. There are various reports describing that *NPM1* mutation is mostly associated with *FLT3-ITD* mutation and it has been shown that *NPM1* can be considered a favorable prognostic marker in the absence of *FLT3-ITD* mutation [3,4,7,17].

Accordingly, in this study, *FLT3* and *NPM1* mutations were evaluated in adult Iranian patients with *de novo* CN-AML and its correlations with clinical and laboratory parameters were also assessed.

Materials and Methods

Patient Selection

This study included 70 newly diagnosed adult patients with *de novo* AML who were referred to the Shiraz Namazi Hospital, affiliated to Shiraz University of Medical Sciences, from November 2014 to May 2016. All patients were recruited at the time of diagnosis prior to chemotherapy. AML was diagnosed using morphology, cytochemistry, and immunophenotyping. Clinical and laboratory data, including French-American-British (FAB) subclass, complete blood count, blast percentage, and hemoglobin (Hb) level, were also collected.

All patients received standard induction chemotherapy, which consisted of daunorubicin at 45 mg/m^2 on days 1 to 3 and cytarabine at 100-200 mg/m^2 on days 1 to 7, followed by high doses of a cytarabine-based consolidation phase (cytarabine at mg/m^2 3 every 12 h for 3 days, repeated for 2 to 3 cycles). This study was approved by the Ethics Committee of Shiraz University of Medical Sciences and written informed consent was obtained from all the participants.

Cytogenetic Analysis

Karyotypes were analyzed by standard G-banding technique [20]. Chromosomal abnormalities were tested by reverse transcriptase polymerase chain reaction (PCR) for AML1-ETO and CBFB-MYH11. Among the 70 AML patients, 16 had abnormal karyotypes: one patient had inv (16) translocation, one had both t (8;21) and inv (16), 12 had t (15;17), and the remaining two patients had other translocations. The 54 patients who were negative for these chromosomal abnormalities were considered as having CN-AML.

Sample Collection

Five milliliters of fresh peripheral blood and/or bone marrow samples was collected in ethylenediaminetetraacetic acid-containing tubes. DNA was extracted with a DNA extraction kit (GeNet Bio, Korea) and stored at -80 °C.

Detection of the *FLT3-ITD* Mutation

For detection of the *FLT3-ITD* mutation, the JM domain between exons 11 and 12 was amplified using specific forward primer FLT.11F 5'-GCAATTTAGGTATGAAAGCCAGC 3' and reverse primer FLT.12R 5'-CTTTCAGCATTTTGACGGCAACC-3'. The PCR reaction was performed in a total volume of 50 µL containing 200 ng of genomic DNA, 10X 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 1 U of Taq DNA polymerase. PCR conditions included initial denaturation at 95 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s with a final extension at 72 °C for 5 min. PCR reaction was conducted in a PCR T100 thermocycler (Applied Biosystems, USA). The 329-bp PCR products were run on 3% agarose gel stained with DNA SafeStain Dye and visualized under UV light. Samples with additional longer PCR products were identified as *FLT3-ITD*+. All mutant samples were verified by direct sequencing using the ABI Prism 3730XL DNA sequencing analyzer. The sequencing results were analyzed by Chromas software (version 2.4.3).

Detecting of the *FLT3-TKD* Mutation

For detection of the *FLT3-TKD* mutation, the specific forward primer FLT.17F 5'-CCGCCAGGAACGTGCTTG-3' and reverse primer FLT.17R 5'-GCAGCCTCACATTGCCCC-3' were used. The PCR reaction was performed in a total volume of 15 µL with similar reagents as used for the *FLT3-ITD* mutation, except for the primers. PCR conditions were also the same, except for the annealing temperature, which was 65 °C for 30 s. The amplification reaction was conducted in a PCR T100 thermocycler (Applied Biosystems). The 119-bp PCR products were then digested with 2 U of EcoRV at 37 °C for 17 h, run on 3% agarose gel stained with DNA SafeStain Dye, and visualized under UV light. The presence of an undigested PCR product was an indication of a mutant sample.

Detection of the *NPM1* Mutation

Exon 12 of the *NPM1* gene was amplified using specific primer NPM1-F 5'-TTAACTCTCTGGTGGTAGAATGAA-3' and NPM1-R 5'-CAAGACTATTTGCCATTCCTAAC-3'. The PCR reaction was performed in a similar volume as was used for the *FLT3-ITD* mutation. PCR conditions included initial denaturation at 95 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 57 °C for 60 s, and 72 °C for 75 s with final extension at 72 °C for 5 min. The PCR products were purified and directly sequenced with reverse primer NPM1-R2 5'-GGCATTTTGGACAACACA-3' using the ABI Prism 3730XL DNA sequencing analyzer and analyzed by Chromas software (version 2.4.3).

Statistical Analysis

The statistical analysis of data was done using SPSS 18 (SPSS Inc., USA). For comparison of qualitative data between wild-type and mutant patients, chi-square and Fisher exact tests were performed. Independent sample t-tests and Mann-Whitney U tests were used to compare quantitative data between wild-type and mutant patients. A p-value of less than 0.05 was considered statistically significant.

Results

This study included 70 newly diagnosed adult patients with *de novo* AML (49 males and 21 females, mean age: 47.73±18.64 years, minimum - maximum: 17-87 years). The demographic and laboratory data of all the patients are shown in Table 1.

Screening for the Mutation of the *FLT3* and *NPM1* Genes in CN-AML

The chromatograms of *FLT3-ITD* and *NPM1* sequencing are shown in Figure 1.

Of all 54 CN-AML patients, 14 (25.9%) had the *FLT3-ITD* mutation, while 40 (74.1%) had the normal *FLT3* gene. In

Table 1. Demographic and laboratory data of acute myeloid leukemia patients.

Variables	Values
Sex, number	
Male (%)	49 (70%)
Female (%)	21 (30%)
Age, years, mean ± SD	47.73±18.64
Laboratory data, median (minimum - maximum)	
WBC count (x10^3/mL)	9 (0.3-164.6)
Platelet count (x10^3/mL)	49 (7-300)
Serum Hb (g/dL)	8.6 (4.4-13.4)
Blast count (%)	90 (50-99)

WBC: White blood cells, Hb: hemoglobin, SD: standard deviation.

addition, of the 52 patients genotyped for *FLT3-TKD* mutation status, 3 (5.9%) were mutant and 48 (94.1%) were normal. One patient had both *FLT3-ITD* and *FLT3-TKD* mutations.

Of the 53 CN-AML patients genotyped for the *NPM1* gene, 11 (20.8%) had *NPM1* mutation and 42 (79.2%) had wild-type *NPM1*. From the 11 patients with mutant *NPM1*, 8 (72.8%) had type A, 1 (9.1%) had type C, and 1 (9.1%) had type D mutation. One patient (AML-20) had a unique mutation pattern that did not belong to a typical *NPM1* mutation type. Of 11 patients with mutated *NPM1*, 5 (45.5%) were also positive for *FLT3-ITD*, while none had *FLT3-TKD* mutation. Thirty-three patients had the wild-type form of both the *FLT3-ITD* and *NPM1* genes. There was no significant correlation between the presence of the *FLT3-ITD* mutation and *NPM1* mutation in CN-AML patients (p>0.05).

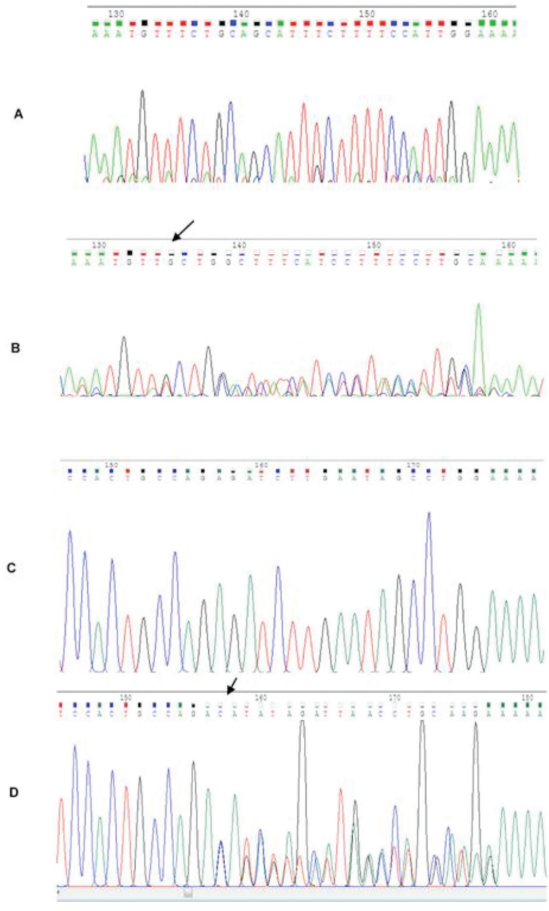

Figure 1. Sequencing results for *FLT3-ITD* and *NPM1* mutation: A and B are representative of patients with wild-type and mutant *FLT3-ITD* gene, respectively. C and D are representative of patients with wild-type and mutant *NPM1* gene, respectively. The arrows show the mutation site.

FLT3 and *NPM1* Mutations and Different Clinical and Laboratory Parameters in CN-AML

The mean white blood cell (WBC) and platelet counts, serum Hb level, and percentage of blasts in the bone marrow were compared between mutant and wild-type groups of CN-AML patients (Table 2).

As shown in Table 2, there were no significant differences in mean WBC and platelet counts, serum Hb level, or percentage of blasts in the bone marrow between patients with wild-type and mutant *FLT3-ITD* and *NPM1* genes. Moreover, the mean age of AML patients did not differ between wild-type and mutant patients for the *FLT3-ITD* and *NPM1* mutations (p=0.287 and p=0.387, respectively). No significant differences were observed between male and female patients in cases of *FLT3-ITD* and *NPM1* mutation frequency (p=0.450 and p=0.545, respectively).

FLT3 and *NPM1* Mutation in AML Patients with Different FAB Groups and Cytogenetic Aberrations

Of 70 *de novo* AML patients, 17 had *FLT3-ITD*, 3 had *FLT3-TKD*, and 12 had *NPM1* mutations. The frequencies of these mutations in patients with different cytogenetic abnormalities are shown in Table 3. Although the frequency of *FLT3-ITD*, *FLT3-TKD*, and *NPM1* mutation was higher in CN-AML patients in comparison with AML patients with cytogenetic aberrations, the differences were not statistically significant (p>0.05, data not shown).

Since the AML subtypes of some patients were not defined, AML patients were divided into M3 and non-M3 groups according to the FAB classification. As a result, 12 (17.1%) were M3 and 58 (82.9%) were non-M3. The *FLT3* and *NPM1* mutation status was analyzed in AML patients according to FAB groups. The results showed that there were no differences between the mutation status of the *FLT3-ITD*, *FLT3-TKD*, and *NPM1* genes in the M3 and non-M3 FAB subtypes (Table 4). No mutation was observed in either *FLT3-TKD* or *NPM1* genes in patients of the

Table 2. Comparison of baseline characteristics between wild-type and mutant groups.

	FLT3-ITD		p-value	*NPM1*		p-value
Clinical characteristics	**(-)**	**(+)**		**(-)**	**(+)**	
WBC count (x10^3/mL)	23.74±36.21	33.19±45.4	0.538	21.44±34.65	42.45±48.07	0.116
Platelets (x10^3/mL)	80.14±68.3	65.5±46	0.911	76.81±66.54	73±49.28	0.861
Serum Hb (g/dL)	8.69±2.02	8.92±2.02	0.719	8.6±1.75	9.31±2.74	0.429
Blast count (%)	83.17±9.36	82.64±13.22	0.912	81.59±11.9	86.67±8.16	0.347

WBC: White blood cells, Hb: hemoglobin.

Table 3. The frequency of *FLT3-ITD, FLT3-TKD*, and *NPM1* mutations in acute myeloid leukemia patients with different cytogenetic statuses.

Mutation type	*FLT3-ITD* (+) (n=17)	*FLT3-TKD* (+) (n=3)	*NPM1* (+) (n=12)
Normal cytogenetics	14	3	11
t(8;21)	-	-	-
t(15;17)	3	-	-
inv(16)	-	-	-
t(8;21) + inv(16)	-	-	1
Other mutation	-	-	-

Table 4. *FLT3* and *NPM1* mutation status in different French-American-British groups.

	FLT3-ITD		p-value	*FLT3-TKD*		p-value	*NPM1*		p-value
FAB subtypes	(-) (%)	(+) (%)		(-) (%)	(+) (%)		(-) (%)	(+) (%)	
Non-M3	44 (83%)	14 (82.4%)	0.604	52 (81.3%)	3 (100%)	0.548	45 (79%)	12 (100%)	0.080
M3	9 (17%)	3 (17.6%)		12 (18.7%)	0 (0%)		12 (21%)	0 (0%)	

FAB: French-American-British.

M3 FAB group. The *FLT3-ITD* mutation was more frequent in non-M3 patients compared to M3 patients (82.4% vs. 17.6%, respectively; Table 4).

Discussion

Genetic abnormalities are one of the most common features observed in AML patients, of which genetic variations of the *FLT3*, *NPM1*, *DNMT3A*, *IDH1/2,* and *WT1* genes have been given more attention [3,7].

In the current study, we analyzed the frequency of *FLT3* and *NPM1* mutation in 54 adult *de novo* AML patients with normal karyotypes (CN-AML). The results showed that the frequency of *FLT3-ITD, FLT3-TKD,* and *NPM1* mutations was 25.9%, 5.9%, and 20.8%, respectively. The most frequent *NPM1* mutant type was the type A mutation. Our results are consistent with previous studies that described the *FLT3-ITD* mutation in 25%-35%, *FLT3-TKD* mutation in 7%-10%, and NPM1 in 50%-60% of CN-AML cases [7,21]. In a study of 39 CN-AML patients by Aly et al. [22], the frequency of *FLT3-ITD* was reported to be 15.4%, while Fröhling et al. [23] and Kainz et al. [24] found that the frequency of *FLT3-ITD* was 32% and 30% in CN-AML patients, respectively. In addition, Falini et al. [18] showed that the frequency of *NPM1* mutation was 61.7%, while different mutation rates were reported by Zhang et al. [25] (14.3%), Döhner et al. [26] (48.3%), and Boissel et al. [27] (47%). The discrepancy in the frequency of *FLT3-ITD*, *FLT3-TKD,* and *NPM1* mutation between our study and others may be due to different population groups as well as the number of cases in the abovementioned studies.

Consistent with previous reports, our results also demonstrated that the frequency of *FLT3-ITD*, *FLT3-TKD,* and *NPM1* mutation was higher in CN-AML patients in comparison with AML patients with cytogenetic aberrations [3,7,28].

No mutation was detected in the *FLT3-TKD* or *NPM1* gene in patients in the M3 FAB group. *FLT3-ITD* mutation was more frequent in non-M3 patients compared to M3 ones. Consistent with our results, Falini et al. [18], Thiede et al. [19], and Suzuki et al. [29] reported no *NPM1* mutation in the M3 subtype. In addition, Verhaak et al. [30] reported a lower frequency of *NPM1* mutation in M3 and M0 in comparison with other subgroups. Therefore, it seems that both *FLT3* and *NPM1* mutations are generally mostly seen in AML patients with normal cytogenetics.

Evaluation of the clinical characteristics of the patients revealed that there were no significant differences in mean WBC and platelet counts, serum Hb level, or bone marrow blast percentage between patients with wild-type and mutant *FLT3-ITD* and *NPM1* genes. No difference was observed in the frequency of *FLT3-ITD* or *NPM1* mutation regarding age or sex. Consistent with our findings, Dehbi et al. [31] reported no significant association between *FLT3-ITD* mutation and WBC and platelet counts or blast percentage. Bao et al. [32] also did not observe any differences in *FLT3-ITD* mutation frequency according to age or sex. However, higher WBC counts and increased blast percentages in *FLT3-ITD*-positive patients were reported by Fröhling et al. [23]. Moreover, Haferlach et al. [33] showed a strong association of bone marrow blast percentage with *NPM1* and *FLT3-ITD* mutations. Gale et al. [28] and Döhner et al. [26] reported that a significant correlation existed between the presence of the *FLT3-ITD* mutation and the *NPM1* mutation. However, there was no significant correlation between the concomitant mutation of both the *FLT3-ITD* and the *NPM1* gene in our study, which might be due to the different sample sizes and also the type of AML (CN-AML in our study and unselected AML patients in the study by Gale et al. [28]).

It has been demonstrated that the *FLT3-ITD* mutation promotes constitutive activation of the FLT3 receptor, leading to ligand-

independent cell stimulation and subsequent uncontrolled proliferation of leukemic blasts [3,8]. Mutation in exon 12 of *NPM1* leads to aberrant cytoplasmic accumulation of the *NPM1*, which might contribute to leukemogenesis [21]. Association of the mutation in both of these genes with clinical outcome has been shown in various studies; *NPM1* has been shown to be associated with good prognosis, especially in the absence of the *FLT3-ITD* mutation, while *FLT3-ITD* has been independently considered as a worse prognostic factor that significantly reduces patients' survival [22,26,28,30,34,35].

According to our findings, the higher incidence of both the *FLT3* and the *NPM1* mutation in CN-AML patients underscores that both *FLT3* and *NPM1* can be used as candidate genetic markers for predicting the prognosis of CN-AML patients. In line with these genes, other known prognostic genetic markers like the *DNMT3A* and *IDH* genes should be considered, which are under further investigation by our group. Due to time limitations, it was not possible to follow our patients for a longer period of time in order to conduct survival analysis. However, further screening of patients for *FLT3* and *NPM1* mutations could be useful to verify the clinical significance of these genes for AML population prognosis, and especially for assessment of the presence of the remaining clones as minimal residual disease. In this regard, the value of increasing the number of patients in the studied population should be taken into account.

Conclusion

In conclusion, given the high stability of *NPM1* during the disease course, it can be used in combination with *FLT3* as well as other known genetic markers to monitor Iranian CN-AML patients, especially for minimal residual disease detection.

Acknowledgments

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Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of Shiraz University of Medical Sciences.

Informed Consent: Written informed consent was obtained from all the participants.

Authorship Contributions

Surgical and Medical Practices: M.R.; Concept: N.A.; Design: N.A.; Data Collection or Processing: N.R., B.V., M.K.; Analysis or Interpretation: S.H.; Literature Search: N.R.; Writing: N.A.

Conflict of Interest: The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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