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ORIGINAL RESEARCH

# Detection of IDH1, IDH2, and NPM1 Mutations in Acute Myeloid Leukemia by High-Resolution Melting Analysis in Comparison with Direct Sequencing and MRD Detection

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**Background:** Acute Myeloid Leukemia (AML) is a heterogeneous type of acute leukemia. Genetic mutations are the most important prognostic factors in AML. Mutations that affect isocitrate dehydrogenase 1 and 2 (IDH1 and 2) genes are associated with poor prognosis, but the nucleophosmin 1 (NPM1) mutation is favorable. In this study, we investigated the efficiency of the High-Resolution Melting (HRM) method for the detection of NPM1, IDH1, and IDH2 mutations, and used it for minimal residual disease detection.

**Methods:** Formalin-fixed, paraffin-embedded (FFPE) marrow biopsies of 78 patients with AML were analyzed. Mutation detection was performed using the HRM method and the results were compared with those obtained by direct sequencing. The melting temperatures (Tm) were analyzed using the blast cell percentage.

**Results:** NPM1, IDHs mutations were detected in 28 (35.8%) and 21 (26.9%) samples, respectively. Among all IDH mutations, 13 (16.6%) and eight (10.2%) samples were harboring the IDH-1 and −2 gene mutations, respectively. Based on the HRM results, samples with higher blast cells had a minimal difference in Tm compared to samples with higher blast cells.

**Conclusion:** Our study is generally distinguished from previous studies based on the desirable characteristics of the HRM method. Regarding mutation detection in low amounts of extracted DNA, the relationship between Tm and the blast percentage is an advantageous characteristic of the HRM method. As shown in a previous study, the use of NPM1 and IDHs for the detection of Minimal Residual Disease (MRD) by HRM is a sensitive method.

**Keywords:** NPM1, IDH1, IDH2, HRM, AML

## Introduction

Acute myeloid leukemia (AML) is a type of heterogeneous acute leukemia, it is the most common acute leukemia in adults and occurs in over 20000 cases per year in Europe and the United State.[1–4] AMLs have different genetic alterations and large chromosomal translocations; therefore, it is possible that they have considerable diversity in clinical signs and prognoses.[5] Balanced chromosomal translocations cause gene fusion, which plays a major role in leukemia, and is a diagnostic genetic marker.[6] However, translocations are rare in AML, and we classified AMLs to Normal Karyotype AML (NK-AMLs) or abnormal karyotype AML.[7]

In NK-AMLs, some gene mutations play biological and prognostic roles.[8,9] During the last decade, recurring mutations with prognostic significance have been identified FLT3,[10–12] NPM1,[13,14] CEBPA,[12,15,16] and MLL[17] genes involved in AML prognosis. In addition, isocitrate dehydrogenase (IDH) encodes the enzymes IDH1 and IDH2, Wilms' tumor gene (WT1), and some other genes, such as Janus-associated kinase 2 (JAK 2) and ten-eleven translocation 2 (TET2).[6]

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Isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) are encoded by *IDH1* and *IDH2* in the cytoplasmic/peroxisomes and mitochondria, respectively. The active forms of IDH1 and IDH2 are homodimers that depend on $NADP^+$. These enzymes catalyze the conversion of isocitrate to α-ketoglutarate (α-KG) by oxidative decarboxylation and NADPH generated from $NADP^+$. Mutations in IDH1 and IDH2 have been identified in several cancers. First, it reported in metastatic colon cancer and, approved by WHO for classification of gliomas in 2006.[18] Sequencing of IDH1 in different cancer patients revealed a single nucleotide variant (SNV) at R132 ($IDH1^{R132}$). Subsequent analyses of IDH2 identified two SNVs: $IDH2^{R140}$ and $IDH2^{R172}$. These mutations found in approximately 16–33% of normal karyotype patients.[19] In addition, in patients with myeloproliferative neoplasms and myelodysplastic syndromes the IDH1/2 SNVs are found at lower frequencies.[20,21] Mutations in IDH1 and IDH2 reduce the enzyme affinity for isocitrate and increase the affinity for α-KG and NADPH. These changes in enzyme activity (decarboxylation and oxidation), facilitating the reduction of α-KG and producing D-2-hydroxyglutarate (D-2HG). Accumulation of D-2HG has been observed in a subset of AML with IDH1 or IDH2 mutations.

Nucleophosmin 1 (NPM1) is another gene that is mutated in AML. It is a protein in the nucleus and cytoplasm that functions as a molecular chaperone. Chaperones prevent protein aggregation and facilitate correct protein folding in the nucleolus. It is also necessary to regulate p53 levels.[22] A point mutation in the C-terminal region of NPM1 caused accumulation NPM1 in the cytoplasm.[22,23] This mutation is found in approximately 30% of AML and is associated with favorable outcome in treatments.[13,22] Mutations in IDH1, IDH2, and NPM1 have been detected using conventional diagnostic tests in clinical practice, such as PCR, TaqMan RT-PCR, and sequencing.[24–27] However, they are not routine clinical care because these tests are expensive and complicated. In addition, all of the mentioned tests had some defects, such as an open tube system with an increased possibility of carry-over contamination, resulting in false-positive PCR results. TaqMan RT-PCR is a closed-tube system, but it can detect mutations that are known and only match the designed probe.[28]

The High-Resolution Melt (HRM) curve characterizes DNA samples according to their melt behavior depending on the nucleotide sequence; therefore, mutations, deletions, insertions, and any changes in sequence can be identified.[29,30] HRM is a closed-tube, rapid post-PCR technique that is also a perfect tool for determining allele prevalence, screening for loss of heterozygosity, DNA fingerprinting, DNA methylation, species identification, and calculating the ratio of acquired somatic mutations.[28,29,31–33] The HRM method have some limitations like detection problem in little change in melting temperatures (Tm) between sample and wild type control.[28,29]

HRM analysis can help to identify mutations in IDH1, IDH2, and NPM1 genes, which are crucial for prognosis and treatment decisions in AML patients.[34–40] HRM can detect IDH1 and IDH2 mutations with sensitivities of 7.3% and 7.9%, respectively, against the background of wild-type transcripts. It also demonstrated near-perfect concordance with Sanger sequencing.[36,38]

In this study, we aimed to performed HRM to detect and Minimal Residual Disease (MRD) prediction in AML patients with IDH1, IDH2, and NPM1 genes mutation.

# Material and Methods

## Patients and Samples Preparation

Seventy-eight patients diagnosed with AML and referred to the hospital using molecular and clinical methods to confirm the diagnosis were recruited for this study. Formalin-fixed, paraffin-embedded (FFPE) marrow biopsy specimens were used in this study. All patients were newly diagnosed with AML of any subtype (M3 or non-M3) entered in our study. They did not receive any cancer-related medications (chemotherapy, etc.) or other types of cancer or medical complications (hepatosplenomegaly, lung cancer, etc).; therefore, these patients were excluded from our study. The patients' ages ranged from 24 to 86 years (mean 52 years). The demographic data of the remaining patients are summarized in Table 1. After obtaining written consent, FFPE BM biopsy specimens were obtained from the pathology center. The samples were transferred to the research laboratory to cut tissue sections, deparaffinized, and subjected to DNA extraction. In this way, 10 μm-thick tissue sections were prepared using a microtome. They were then incubated in xylene and deparaffinized at 37°C for 30 min. The tissue sections were sedimented, xylene was used as the supernatant fluid, and the xylene was

**Table 1** Patients' Demographic Data

| | | |
|---|---|---|
| WBC Count | Max | 19*106/L |
| | Min | 8*106/L |
| | Median | 11*106/L |
| Sex | Female | 37 (47.4%) |
| | Male | 41 (52.6%) |
| Age (year) | Max | 86 |
| | Min | 24 |
| | Median | 52 |
| Leukemic Blast (%) | Max | 65 |
| | Min | 32 |
| | Median | 46 |

removed after centrifugation. The latter step was repeated twice to remove all remaining paraffin. Subsequently, the samples were washed twice with ethanol and air dried for 30 min.

## DNA Extraction

QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used for DNA extraction from ethanol-washed tissue sections. This step was performed in accordance with the manufacturer's instructions. DNA purity was determined by calculating the A260/A280ratio absorbance by using an ND-1000 Spectrophotometer (NanoDrop Technologies).

## HRM Analysis of the Mutations

HRM analysis was performed using a Qiagen Rotor-Gene Q thermal cycler (Hilden, Germany). Specific primers were designed using the vector NTI version11 software, used in the HRM pre-amplification step. The primer sequences used are listed in Table 2.

The HRM reaction was performed in a total volume of 20 µL. The reaction included 10 µL of Qiagen Type-It Master Mix (Hilden, Germany) and 2 µL and 0.7 µL of DNA and each of the primers (including about 10 ng). The remaining volume to 20µL (6.6 µL) was filled with nuclease-free water. The primer and DNA concentrations used in this reaction were 10 pmol and 80–100 ng per microliter of, respectively. Real-time PCR was carried out thermal cycling conditions included: 95 °C for 5 min as initial denaturation step, followed by 40 cycles of 20s at 94 °C and 30s at 60 °C for denaturation and extension, respectively.

**Table 2** Sequences for Primers Used for HRM Method

| Name | Sequence | Product Length (bp) |
|---|---|---|
| **IDH1$^{R132}$-F**<br>**IDH1$^{R132}$-R** | 5′-TCATAGAAGCCATTATCTGC-3′<br>5′-TTATTGCCAACACGACTTAC-3′ | 121 |
| **IDH2$^{R172}$-F**<br>**IDH2$^{R172}$-R** | 5′-CATCCCACGCCTAGTCCCTG-3′<br>5′-GATACCCTCTCCACCCTTGC-3′ | 91 |
| **IDH2$^{R140}$-F**<br>**IDH2$^{R140}$-R** | 5′-TGAAGAAGATGTGGAAAAG-3′<br>5′-TGTAGATGATGGGCTTACG-3′ | 98 |
| **NPM-1 F**<br>**NPM-1 R** | 5′-TATGAAGTGTTGTGGTGCCT-3′<br>5′-ACAGAAATGAAATAAGACGG-3′ | 141 |


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HRM analysis was performed followed by a pre-amplification step. In this step, the PCR products were amplified in the latter step, melted by gradual temperature increase from 40°C to 95°C with a ramp rate of 0.02°C/S. The detection of DNA melt was achieved using the Eva-green intercalating dye in preamplification step. This dye interacts with the double-stranded DNA and emits fluorescence. In the HRM method, by increasing the temperature of the DNA melt to single-stranded, the fluorescent dye dissociates and decreases fluorescent emission. Temperature-shifted difference and normalized graphs based on fluorescent emission during melting were analyzed using software linked to the Qiagen Rotor-Gene Q instrument (Hilden, Germany).

## Direct DNA Sequencing

After HRM analysis, the results were confirmed using a standard reference method. DNA sequencing was performed to confirm the SNV findings for the target gene. PCR products were amplified using the aforementioned primers in the HRM method and sequenced by the Bioneer Corporation (Daejeon, South Korea). The sequences were analyzed using Chromas v.2.1 (Conor McCarthy, Southport, Australia). In addition, they were compared with sequences obtained from the BLAST GenBank database.

## Statistical Analysis

Statistical analyses were performed using GraphPad Prism V9.0 software. Mann–Whitney and Kruskal–Wallis (P value <0.05 = Significant) statistical tests were performed, and the data were found to have no significant relationship between patients' demographic information and mutation rate. All data are presented as the mean ± standard deviation (SD) of three independent experiments. Also, the correlation between Absolute value of Tm difference and blast number of sample were performed.

# Results

The incidence of IDH-1, IDH-2, and NPM-1 mutations was determined in 78 formalin-fixed paraffin-embedded (FFPE) BM samples from patients' specimens. The genomic extraction was confirmed by nanodrop. Also, a simple PCR fragment was tested to investigate the quality of the extracted DNA (144bp product), which was then estimated by agarose gel electrophoresis (Figure 1).

Mutations were detected in 21 (26.92%) for both IDH genes and 28 (35.89%) for NPM-1. Among all mutations of IDH, 13 (16.6%) and eight (10.25%) samples detected the IDH-1 and-2 gene mutations, respectively. IDH2 mutations were investigated in $IDH2^{R172}$ and $IDH2^{R140}$ strains.

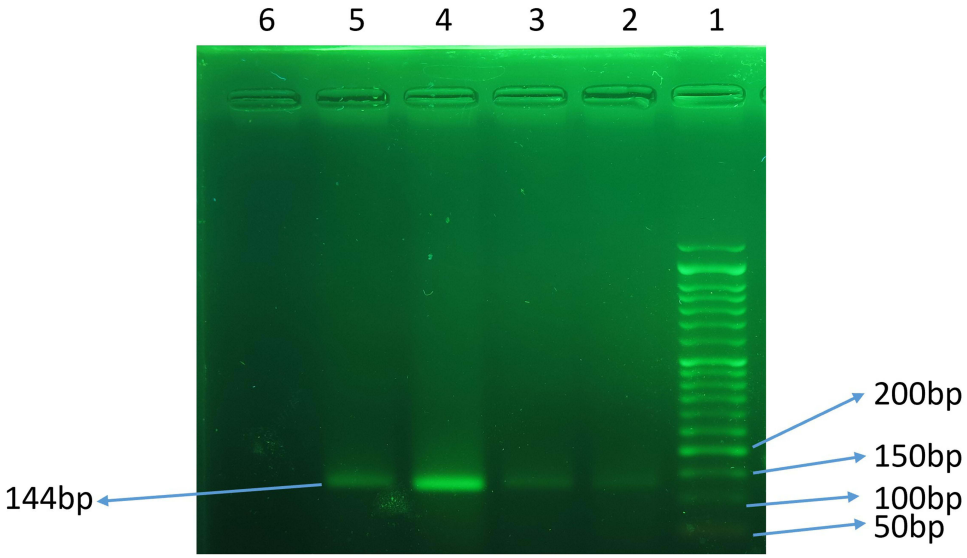


**Figure 1** PCR-amplified product electrophoresis on 1.5% agarose gel. 1: 50 bp ladder; 2, 3, 5: samples; 4: positive control; 6: non template control.

**Table 3** Mutation Number and Percentage Between All Patients and Subtype Patients

| | | |
|---|---|---|
| $IDH1^{R132}$ | | 13 (16.6%)* |
| IDH2 | $IDH2^{R172}$ | 6 (7.7%)* |
| | $IDH2^{R140}$ | 2 (2.5%)* |
| NPM1 | | 28 (35.8%)* |

**Notes**: * Percentage of mutations among all patients.

Frequency of the IDH-1 was higher in males (55%) than in females (45%). In contrast, IDH-2 was more common in females and was not significantly related to specific subtypes of patients. None of the patients had $IDH2^{R172}$ and $IDH2^{R140}$ point mutation. The NPM-1 mutations were more frequent in females (52%) and more frequent in non-M3 subtype of patients ($p<0.05$). NPM-1 mutations were found in 4 (14.7%) and 24 (54.54%) patients of the 34 patients with M3 subtype and 44 patients with non-M3 subtype, respectively. Differences in the sex distribution of NPM-1 and IDH mutations were not statistically significant (P = 0.832 and P = 0.762, respectively) (Table 3). Direct sequencing was used as the standard method to calculate the sensitivity and specificity of the HRM method. Nucleotide substitution IDH-1 ($IDH1^{R132}$), caused 1.1°C lower Tm compared to the wild type sequence (Figure 2A) (the mutant and wild type were detected based on sequencing method). The normalized fluorescence graph software output was used to differentiate mutant samples from the wild type. As shown in Figure 2B, the mutant samples had lower melting temperatures and were different from the wild-type samples.

In the HRM curves for IDH-2 ($IDH2^{R172}$) and NPM-1 mutation the Nucleotide's substitution (G to A in both $IDH2^{R172}$ and NPM1) caused higher melting temperature in the mutant samples (1.6°C and 1.7°C respectively) (Figure 3A only $IDH2^{R172}$). As illustrated for the IDH-1 mutation for IDH-2 and NPM-1 mutations, the normalized fluorescence of the melting curve was used to detect mutant samples (Figures 3B and 4).

## HRM and MRD Detection

According to the AML detection based on FAB criteria, the bone marrow must be greater than 30%. In this study, the blast percentages in bone marrow were different. Therefore, FFPE DNA extraction of FFPE are a mixture of mutant leukemic blasts and wild-type cells. In addition, sequencing data showed heterogeneity of mutations in the samples. Based on these results, the mutation burden differs between samples and is related to the percentage of blast cells. The simple linear correlation test between absolute value of Tm difference of every mutant with wild type sample and percentage of blast cells (Figure 5).

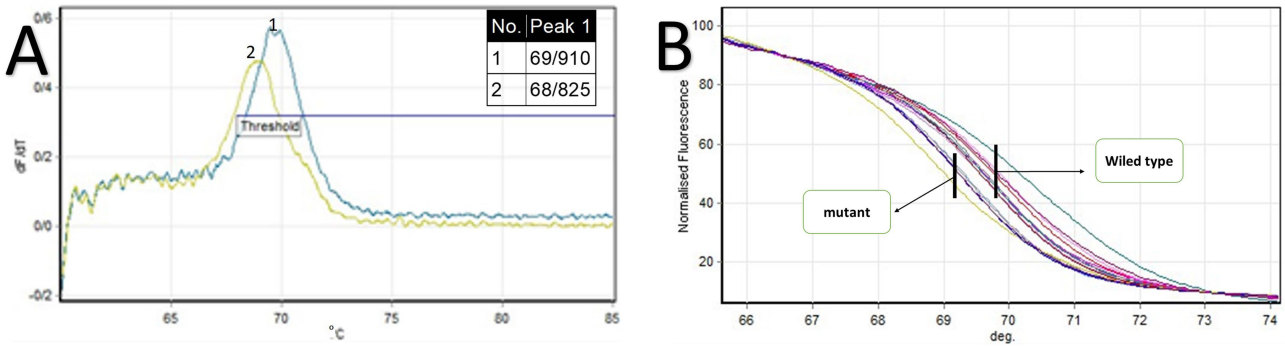


**Figure 2** $IDH1^{R132}$ mutation HRM curves: (**A**) different peak between mutant and wild type melting curve (**B**) melting curve were normalize. 1: Wild type (WT) sample; 2: Mutant sample.


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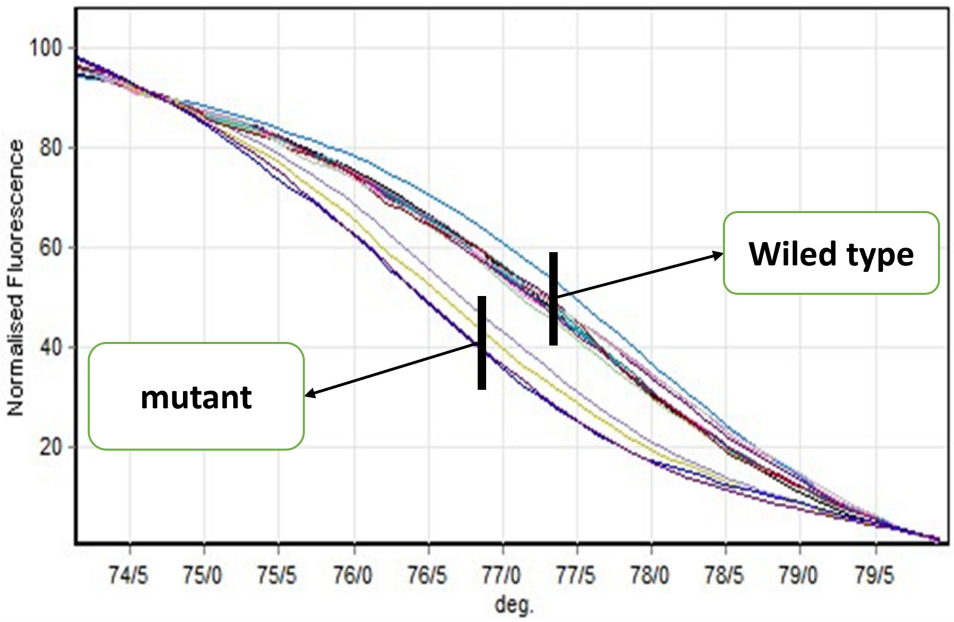


**Figure 3** IDH2$^{R172}$ mutation HRM curves: melting curve were normalize. 1: Wild type (WT) sample; 2: Mutant sample.

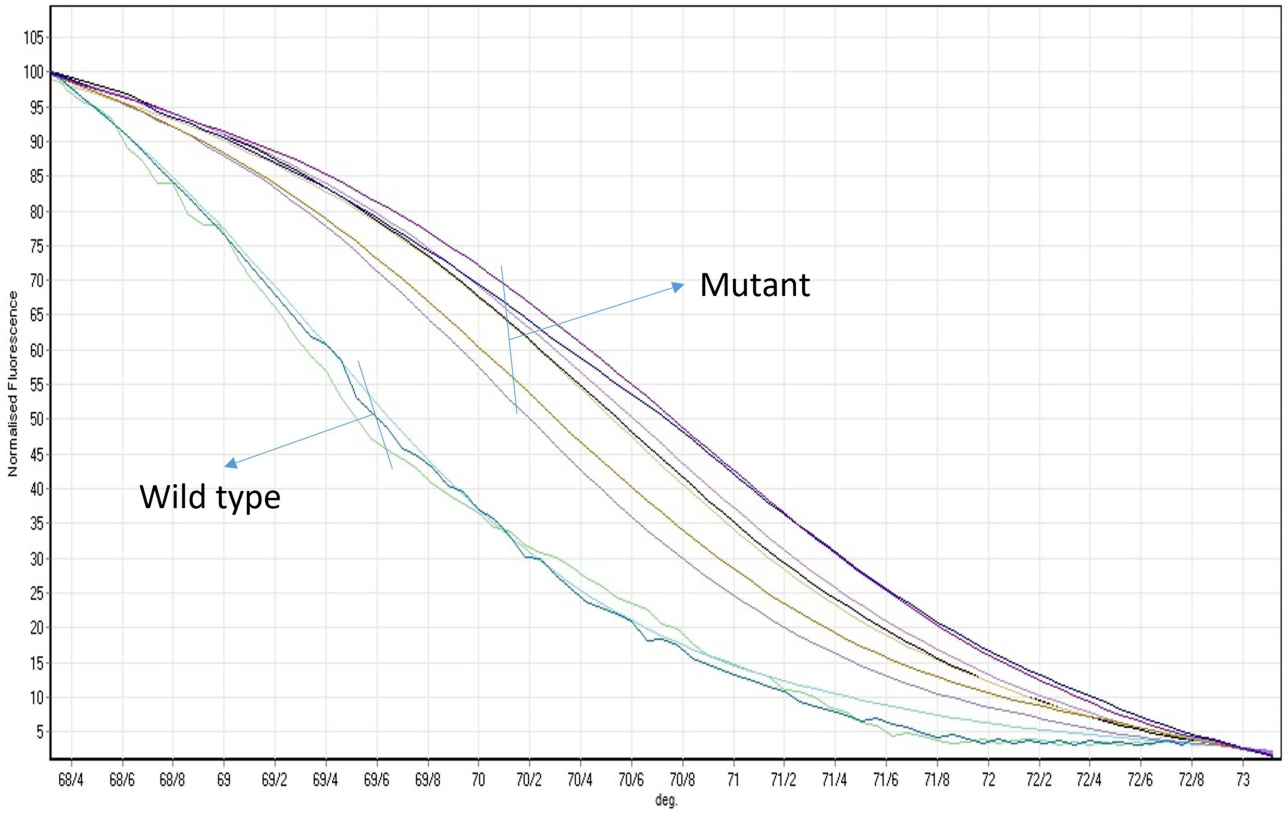


**Figure 4** NPM1 mutation HRM curves: melting curve were normalize. 1: Wild type (WT) sample; 2: Mutant samples. Every line in different color relates to specific sample.

Based on the HRM results, samples with higher blast cells had a higher mutant DNA burden; therefore, they had different melting temperatures. Our data in this study show that samples with higher blast cell percentages due to higher mutant DNA melt temperatures are different from mutant samples with lower blast cell percentages.

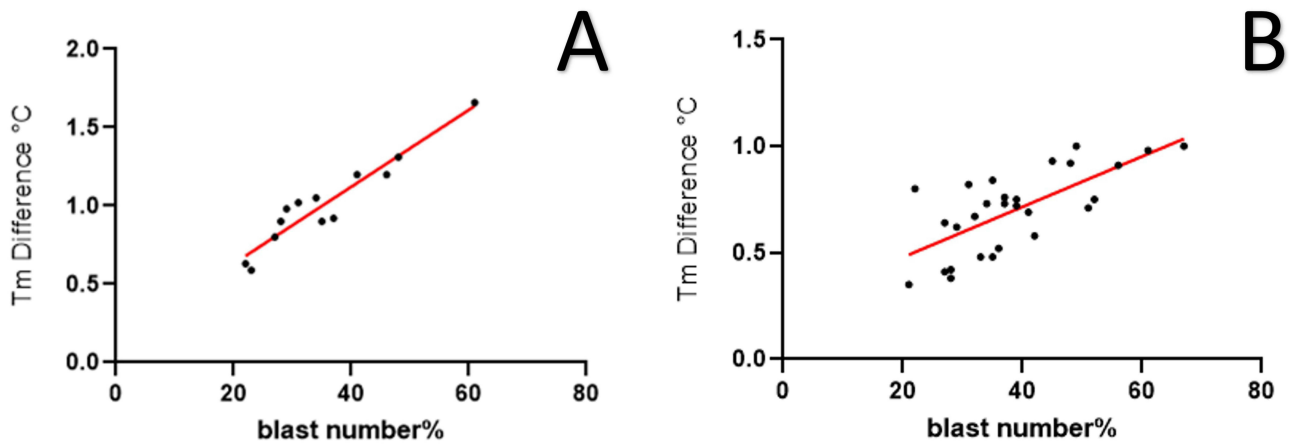


**Figure 5** The simple linear correlation between absolute value of Tm difference of every mutant with wild type sample and percentage of blast cells in (**A**) IDH1R132 and (**B**) NPM1.

Different mixtures of leukemic blasts and normal cells can produce different ratios of mutant and wild-type DNA in specimens. Therefore, the HRM curves can be related to the minimal difference in TM. These data can help us relate Tm to the blast cell percentage and MRD detection of samples after treatment. However, these minimal changes require a highly sensitive setup for Tm changes and the relationship between the blast percentages.

## Discussion

Acute Myeloid Leukemia (AML) is a heterogenous malignancy by genetic instability. The detection of point mutation - specifically IDH1, 2, and NPM1 - have a crucial role in diagnosis, risk stratification, and therapeutic decision-making. The IDH1/2 and NPM1 are detected in 16–32% and 30%, respectively, on NK-AML.[19] Our study also shows the mutation detected in 21 (26.92%) for both IDH genes and 28 (35.89%) for NPM-1. Our research aligns with international studies and demonstrates comparable outcomes across different regions. Advances in AML genomics demand molecular diagnostic tools that not only deliver high sensitivity and specificity but also remain rapid and affordable, enabling their integration into routine clinical workflows in resource-limited environments.[41–43]

High-Resolution Melting (HRM) analysis is a PCR-based method that identifies mutations, deletions, insertions, and any changes in sequence by examining the melting behavior of double-stranded DNA.[28,30,44,45] Following PCR amplification with saturating DNA dyes, a gradual rise in temperature induces DNA denaturation, and the resulting fluorescence changes are tracked in real time. Even single nucleotide mutations can shift the melting curve, allowing clear distinction between wild-type and mutant alleles.[45,46] In this method, DNA mixture of mutant and wild type have difference Tm so homozygotes and heterozygote status can be distinguished.[45] In hematological malignancy like Polycythemia Vera (PV) the JAK2 V617F point mutation detection in peripheral blood is different from zygosity status. The DNA mixture from mutant and wild type cells made different dilution of mutant and wild type of DNA so, difference in Tm in HRM analysis.[28,33] In our study, the detection of IDH1/2 and NPM1 in AML patients associated with differences in their blast counts. So, in this situation difference between mutant and wild type cells made different dilution of mutant DNA. Ultimately, this difference in concentration leads to variations in the melting temperature (Tm) observed in HRM analysis. This hypothesis is supported by the simple linear correlation analysis presented in this study.

IDH1, 2, and NPM1 gene burden in FFPE DNA extraction of newly diagnosed AML patients was correlated with the number of leukemic blasts seen in bone marrow biopsy slides, and the sensitivity of MRD detection depends on the method used. According to Dillon et al, the molecular MRD for patients with complete remission (CR) is less than 200 and 1000 copies per reference gene in the peripheral blood and bone marrow aspirate, respectively.[47] Therefore, using the mutant gene in AML, in addition to its prognostic and diagnostic features, can aid in sensitive molecular MRD detection.

Acute Myeloid Leukemia (AML) is a clonal hematological malignancy treated with conventional chemotherapy. Most AML cases after chemotherapy are not cured because the blast cells are not completely removed by chemotherapy.


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The remaining leukemia blasts after chemotherapy are residual diseases that are the most limiting factor of AML. Deletion of minimal residual disease (MRD) is a major goal in AML treatment. The accurate and sensitive detection of MRD makes individual treatment decisions such that those patients who require more aggressive approaches are treated promptly and to avoid toxic and expensive treatments for those patients who do not require them.

In the present study, the copy number of mutant recurrent cytogenetic genes (IDH1, 2, and NPM1) and clinical prognostic value of these genes in AML were first evaluated. IDH1, 2, and NPM1 gene burden in FFPE DNA extraction of newly diagnosed AML patients was correlated with the number of leukemic blasts seen in bone marrow biopsy slides, and the sensitivity of MRD detection depends on the method used. According to Dillon et al, the molecular MRD for patients with complete remission (CR) is less than 200 and 1000 copies per reference gene in the peripheral blood and bone marrow aspirate, respectively.[47] Therefore, using the mutant gene in AML, in addition to its prognostic and diagnostic features, can aid in sensitive molecular MRD detection.

## Conclusion

HRM is a sensitive method for the follow-up mutations in patients during therapy. In addition, the mutation burden is related to the blast percentage in the bone marrow; therefore, using the HRM method, can be related to the Tm change. In this regard, it is a good indicator for molecular MRD detection and prognosis evaluation, but its specificity needs to be further improved. We recommend that future studies investigate precise changes in Tm and their relationship with blast counts, in order to achieve a more accurate assessment of HRM efficiency in monitoring patients under treatment.

## Data Sharing Statement

Please contact corresponding author for data requests.

## Ethics Approval and Consent to Participate

The study approved in Research Ethics Committees of Khomein University of Medical Sciences and The Ethic Approval Cod is IR.KHOMEIN.REC.1404.001. al of study procedures are complies with the Declaration of Helsinki.

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## Disclosure

The authors declare that they have no conflicts of interest in this work.

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