Short Communications

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Comparison of High-Resolution Melting (HRM) Analysis with Direct Sequencing for the Detection of *DNMT3A* Mutations in AML Patients

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Abstract

Objective: Acute myeloid leukemia (AML) is caused by abnormal gene expression following mutations. Many of the mutations in AML lead to gene instability and poor response to treatment. Among these mutations, *DNMT3A* mutation is exceedingly important due to its major role in methylation and its effect on the expression of other genes. Aberrant methylation due to *DNMT3A* mutations that mostly occur in exon 23, affects the overall survival (OS) of patients with AML and myelodysplastic syndromes (MDS) showing the importance of identification of these mutations. According to the association of these mutations with short overall survival and disease progression in AML patients, we aimed to investigate *DNMT3A* gene exon 23 mutations using HRM. **Methods:** Fifty peripheral blood samples were taken from patients with AML. Mononuclear cells were isolated by ficoll method, and DNA was extracted. Then, mutation detection was detected using the HRM method. Efficacy of the HRM method in mutation detection was compared with direct sequencing method as gold standard. **Results:** Mutations in codon 23 of the *DNMT3A* gene were detected in 5 patients (10%). All of the detected mutations were missense type. A comparison between direct sequencing and HRM analysis demonstrated full concordance of mutation detection. **Conclusion:** According to the full consistency between the HRM and direct sequencing methods, HRM is suggested to be adopted as an alternative for the common time-consuming methods in detecting the gene mutations.

Keywords: HRM- AML- *DNMT3A*- leukemia

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Introduction

Acute myeloid leukemia (AML) is caused by abnormal gene expression following mutations, translocations, and epigenetic alterations. In recent years, scientists have discovered new mutations that can affect the progression and clinical outcome of AML via next generation sequencing (NGS) (Singh et al., 2012). Many mutations, such as mutation in *FLT3, WT1, NPM1, IDH, NRAS*, and *DNMT3A* genes, can cause gene instability and poor response to treatment in these patients. Among these mutations, *DNMT3A* mutation is exceedingly important due to its major role in methylation and its effect on the expression of other genes (Moonesi et al., 2021; Moradabadi et al., 2019). DNMT3A is an enzyme that belongs to a family of methyl transferases that transfer methyl groups from S-adenosyl-L-methionine to C-5 cythosine, resulting in DNA covalent changes. DNMT3A has a key role in epigenetic changes and regulation of gene expression (Faiz and Rashid, 2019; Lande-Diner et al., 2007; Miranda and Jones, 2007). DNMT1, DNMT3A, and DNMT3B encode a group of enzymes responsible for DNA methylation (Feinberg and Vogelstein, 1983; Jones and Baylin, 2007; Sheikhi et al., 2017). Aberrant methylation as a result of improper function of said enzymes can not only create various cancers but also influence cancer progression and prognosis as well. DNMT3A and DNMT3B perform primary methylations in embryogenesis and evolution (Al Azhar and Aisyi, 2021; Okano et al., 1999). DNMT1 subsequently stabilizes methylation performed by DNMT3A and DNMT3B (Jones and Liang, 2009; Khosravi et al., 2021). Aberrant

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methylation due to *DNMT3A* mutations affects the overall survival of patients with AML and myelodysplastic syndromes (MDS) (Figueroa et al., 2010; Figueroa et al., 2009; Zafari et al., 2019). Thus, it is important to identify these mutations in AML patients.

DNMT3A has 23 exons and sequencing by NGS is rather time-consuming and expensive. Alternatively, High Resolution Melting (HRM) technique can be utilized. It is a PCR-based method which can determine nucleotide changes in melting curves. This is done by comparing the melting curves of wild type (WT) and mutant samples (Khosravi et al., 2022). In this method, the desired gene fragment is replicated by PCR and fluorescent dyes lodge inside DNA strands. Then with a slight increase in temperature, the strands open and the fluorescent dye is released.

Because the increase in temperature is very small, the nucleotides are separated one by one in a sequence. The binding of opposite nucleotides in the gene is established by different covalent bonds, so the energy required to break these bonds varies. Therefore, the melting curve in the mutant sample and the WT at the mutation point is different. Evidently, most mutations occur in exon 23 of this gene AML patients. Because these mutations are associated with short overall survival and disease progression in these patients, here, we investigated mutations in exon 23 of the *DNMT3A* gene using HRM (Moradabadi et al., 2019; Zaka Khosravi et al., 2022).

Materials and Methods

Sample selection

Fifty patients of Imam Reza Hospital who were diagnosed with AML using molecular and clinical methods were recruited into this study. All AML subtypes (M3 and non-M3) were included in our study, and the patients were all newly diagnosed. Any patient who had received cancer-related medications, such as chemotherapy, was excluded from our experiment. Patients suffering from other medical complications, such as hepatosplenomegaly, were also not entered in our study. Patients ranged in age from 24 to 56 years. After obtaining written consents, 5ml of peripheral blood samples were taken from patients in tubes containing K2-EDTA anticoagulant. The samples were transferred to the laboratory on ice and stored in a freezer until the test was performed. Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll hypaque and the DNA of these cells was extracted according to the manufacturer's instructions. Purity and concentration of the extracted DNA were measured in 260nm and 280nm wavelengths by ND-1000 spectrophotometer nanodrop. The quality of the samples was evaluated by electrophoresis on agarose gel. Samples were stored in -20 freezer until testing.

Primers

After reviewing previous articles, primers were designed via Vector NTI version 11.

Then the specific binding of these primers to the target DNA was authenticated by the BLAST website. The primers used in this study are listed in F:CAGGGTATTTGGTTTCGCAG and R:GACTGGCACGCTCTATGACC.

HRM

HRM was performed by ABI real-time thermal cycler in a total volume of 20 microliters, including 10 µl of Qiagen Master Mix, 2 µl of extracted DNA samples, 0.7 µl of forward and reverse primers and 6.6 µl of nuclease-free water. Thermal cycler conditions included an initial denaturation at 95 ° C for 5 minutes, 40 cycles at 94 ° C for 20 seconds, and a final expansion at 60 ° C for 30 seconds.

For HRM analysis, PCR products were melted at a temperature gradient of 0.02 ° C per second from 40 to 95 ° C. With the slight increase in temperature, separation of fluorescent dye from two strands of DNA occurs at nucleotide level. Because different energies are needed to break the bonds between different nucleotides, DNA melting curves differ in wild type and mutant samples at a specific point. The software connected to the ABI device was used to check the curves.

Sequencing

DNA sequencing was performed to confirm the HRM method. PCR products were sequenced by Codon Company. The sequences were compared with sequences obtained from *BLAST* Gene Bank and analyzed with Chromas v.2.1.

Statistical Analysis

Statistical calculations were performed with software Graphpad Prism V8.0. After performing statistical tests u mann whitney and kruskal wallis (P Value <0.05 = Significant), it was found that there was no significant relationship between patients' demographic information and mutation rate.

Results

Prevalence of mutations in codon 23 of *DNMT3A* gene in peripheral blood samples of 50 patients with AML detected by molecular and flow cytometry methods were evaluated by the HRM method. Mutations in codon 23 of this gene were detected in 5 patients (10%). The frequency of these mutations in male and female patients was 51% and 49%, respectively. The sensitivity and specificity of the HRM method compared to the direct sequence were 100% in both approaches.

The mutation detected in codon 23 of the *DNMT3A* gene is a missense type, and the change of nucleotide from G (WT form) to A (mutant form) causes a melting curve temperature difference of 4°C. Due to the significant temperature difference between the mutant samples and wild type in melting curve (1.18°C), samples with this mutation are easily identified in patients with AML (Figure 1).

As the HRM results show, the mutant samples were heterogeneous. Therefore, all samples PCR products sequenced. The results of their sequence were identified as CAC (His) (n = 5) and CGC (Arg) (n = 45) at nucleotide 882 of codon 23 of *DNMT3A* gene. On the other hand, the heterogeneity of HRM curves can be because of

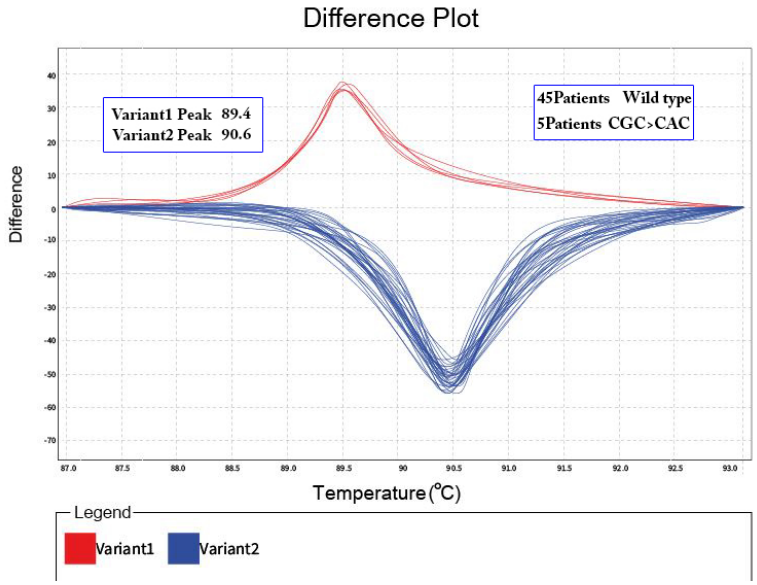

Figure 1. Difference Plot Melting Curve of Codon 23. The 5 patients with *DNMT3A* mutations (variant 1) exhibited lower DNA melting temperature compared to the wild type (variant 2).

Table 1. The Baseline Characteristics of the Patients

Variable	Value
Age (years) (mean ± SD)	42.8 ± 22.9
Sex (Male/Female)	1.3
Male (No. of patients)	28
Female (No. of patients)	22
BM Blasts (%) (mean ± SD)	67 ± 20
25%-50% (No of patients)	7
51%-75% (No of patients)	16
76%-100% (No of patients)	27
WBCs (x10^3/µl) (mean ± SD)	31.4 ± 40.2
Hb (g/dl) (mean ± SD)	13.2 ± 4.2
PLT (x10^3/µl) (mean ± SD)	65 ± 22
FAB Classification (Non M3/ M3)	4.0
M3	10
Non-M3	40

differences in the DNA ratio of mutant and wild-type samples (Figure 2).

Discussion

AML is one of the most well-known blood cancers. In this type of leukemia, an increase in the number of myeloid precursors in the bone marrow and their maturation arrest is observed. As a result of the unbridled proliferation of myeloid cells in the bone marrow and the use of hematopoietic space by these cells, the number of hematopoietic cells generally decreases. This type of leukemia can be with or without an increase in white blood cells count. Unfortunately, about 2.4 people per 100,000 people are diagnosed with AML each year in the United States. This number has increased in recent years to 12.6 per 100,000 adults aged 65 and over (Moonesi et al., 2021).

Various factors are involved in the development and

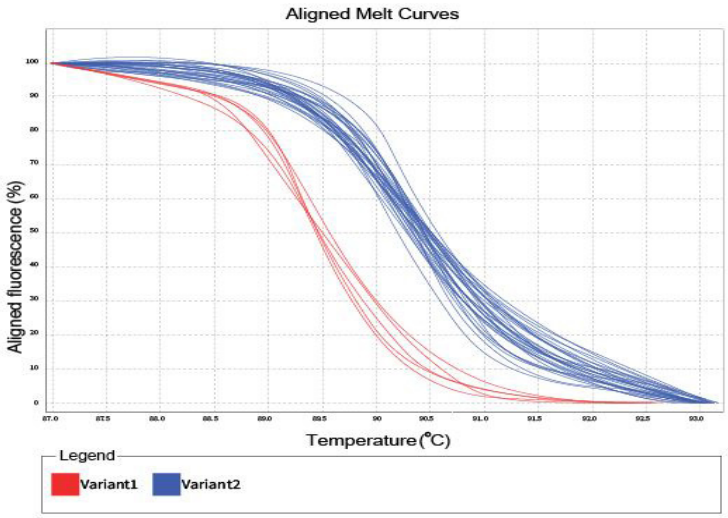

Figure 2. Normalized Fluorescence Melting Curve of Codon 23. The patients with *DNMT3A* mutations (variant 1) had lower fluorescence intensity compared to the wild type (variant 2).

response to treatment of this disease, some of which are related to extracellular milieu and some related to intracellular conditions. Genetic abnormalities are the most important intracellular factors. Among these abnormalities, genetic mutations are especially crucial, resulting in poor prognosis and poor response to treatment in these patients (Döhner et al., 2015; Moradabadi et al., 2017; Sadek et al., 2020). Many mutations have been identified in correlation to this disease, including mutations in *FLT3, WT1, NPM1, IDH, NRAS and DNMT3A* genes (Moradabadi et al., 2019; Zafari et al., 2019). Ley et al. discovered the effect of NPM1 and FLT3 mutations in a patient with AML with a normal karyotype (Ley et al., 2010).The effect of the *TP53* and *BRCA2* gene mutations had previously been discovered in this disease. Yamashita et al., (2010) reported 11 gene mutations in AML patients, including mutations in *JAK3* and *DNMT3A* genes (Yazdani et al., 2020). Ley et al., (2010) examined 23 exons of the *DNMT3A* gene using the NGS technique and found that 22% of the patients had high-frequency mutations in exons 15 to 23. The identified mutations were missense, frameshift, nonsense and splice-site, which were associated with short survival of the patients (Thol et al., 2013). Considering the importance of DNMT3A function in the methylation and function of other genes, we investigated this gene for the presence of mutation by HRM method and sequence confirmation method (Singh et al., 2012).

DNMT3A is a methyltransferase that catalyzes the addition of the methyl group to the CpG dinucleotide, eventually producing 5-methyl cytosine. DNA methyltransferase plays an important role in the epigenetic regulation of gene expression or suppression (Park et al., 2020). Mutations in the gene that make up this enzyme occur in different forms and in different codons. One of the most important mutations detected in AML is the missense mutation in codon 23, in which nucleotide G (WT) is replaced by nucleotide A (mutant); thus, changing the amino acid made by this codon from Arg (WT) to His (mutant) (Döhner et al., 2015). This mutation is therefore called R882H. This change in the protein structure of DNMT3A reduces and disrupts the function of this enzyme. This mutation has been identified in both patients with AML and patients with MDS, with AML patients having poor prognosis and lower overall survival (Walter et al., 2011).

Recent studies have also shown that this mutation is involved in the development of leukemogenesis (Genovese et al., 2015; Jaiswal et al., 2014). Because the identification of this mutation is effective in the diagnosis, treatment and MRD diagnosis, there are various methods to study these mutations. The gold standard method for mutation detection is the sequencing method. Due to the high cost and time-consuming nature of this test, other molecular tests have been developed that among them, HRM is a fast, cost-effective and efficient method. This method can detect gene mutations even in a single nucleotide. This is done by comparing the melting curve of WT and mutant samples. Due to the slight increase in temperature, the bond between the nucleotides is opened one by one and the fluorescent dye is released and is detected by the detector. Due to the difference in energy required to break the bonds between the nucleotides, melting curves in mutant and normal samples will differ at the point of mutation (Wittwer et al., 2003). Therefore, considering the common mutations of genes involved in the development and stability of AML malignant cells and examining these mutations by HRM method and having melting curves of mutant samples, patient samples can be examined with this method. Thus, without sequencing a gene fragment, which is a costly and time-consuming procedure, important mutations were detected in these patients.

Given that in patients with AML, a number of malignant cells have these mutations, and since this method can differentiate heterozygous mutations from homozygous ones, this feature can be used to investigate the minimal residual disease of patients at the molecular level. In patients with a high level of mutation, the melting curve tends towards a homozygous melting curve, and in patients with a lower level, the melting curve tends toward a heterozygous melting curve.

This has two indications: 1) The patient had heterozygous mutations from the beginning; 2) The patient has homozygous mutations and responds to treatment and the mutations are normalized during treatment. Due to the importance of DNMT3A mutations in the development and response to treatment of AML disease and the effectiveness of the HRM method against other common molecular methods, this method can be used for the accurate and rapid diagnosis and reduce patient costs.

Author Contribution Statement

MM and SZK conducted the tests, AM and MR analyzed the data, MFH and ANA designed the study, GM and MM drafted the manuscript. All authors read and approved the final draft of the manuscript.

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Ethical approval

The study protocol was approved by the Ethics Committee of Aja University of Medical Sciences (Code No. 1399/1541).

Availability of data and material

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Conflicts of interest

We have no conflict of interest to declare.

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