Original Article

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Detection of R882 Mutations in DNMT3A Gene in Acute Myeloid Leukemia: A Method Comparison Study

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Abstract

Background: Somatic mutations in the hotspot region of the DNA-methyltransferase 3A (DNMT3A) gene were recurrently identified in acute myeloid leukemia (AML). It is believed that DNMT3A mutations confer an adverse prognosis for AML patients. These lines of evidence support the need for a rapid and cost-efficient method for the detection of these mutations. The present study aimed to establish high resolution melting (HRM) curve analysis as a rapid and sensitive test to identify DNMT3A gene mutations in AML patients.

Materials and Methods: In this retrospective cohort study, a total of 220 AML patients who referred to hematology-oncology and stem cell transplantation centers (referral center) at Shariati hospital in Tehran, Iran, were included. AML-M3 and therapy-related AML patients were excluded. The HRM assay was used to identify R882 mutations in DNMT3A gene, and the results were compared with those of Sanger sequencing as the gold standard test for detection of such mutations.

Results: Among 220 samples from AML patients, Sanger sequencing detected 25 (11.4%) patients as having DNMT3A R882 mutations. HRM assay detected mutations in 23 (92%) samples and reported two false-negative results that were related to poor-quality DNA samples. There was an overall good agreement between direct sequencing and HRM assay (kappa value of 0.95) ($p<0.001$). Sensitivity assay showed that the analytical detection limits for HRM were 10% for the detection of R882H mutation compared with Sanger sequencing at 25%. Both Sanger sequencing and HRM assay reported no false-positive results.

Conclusion: HRM curve analysis can be considered as a sensitive, fast, and high-throughput method for the detection of DNMT3A R882 mutation in AML patients. These results validate HRM analysis as an alternative method to Sanger sequencing because of its simplicity along with the lower cost and less required time.

Keywords: Acute Myeloid Leukemia, DNA Methyltransferase 3A, Sequencing

Introduction

Acute myeloid leukemia (AML) is a genetically and epigenetically heterogeneous disease with clonal expansion of immature myeloid cells in bone marrow and peripheral blood. Gene fusion, cell signaling abnormalities, and epigenetic modification affect self-renewal and differentiation of hematopoietic stem cells which could lead to leukemogenesis (1, 2). Somatic mutations play an important role in the pathogenesis of AML. The prognostic risk stratification of AML depends on cytogenetic and increased detection of molecular aberrations. Almost half of AML patients have normal cytogenetic and the role of molecular biomarkers in these patients is tangible. FMS-related tyrosine kinase 3 (FLT3-ITD), nucleophosmine1 (NPM1), C/enhancer binding protein α (CEBPA), and DNA-methyltransferase 3A (DNMT3A) mutations play an important role in the pathogenesis of AML and act as predictive tools for prognosis (3).

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DNA methylation is a major mechanism of epigenetic regulation. Methyltransferase (MTase) is a key enzyme in methylation. DNMT3A is a member of the DNMTS family and plays an important role in the de novo methylation of cytosine residues in CpG islands. DNMT3A implements this process by adding a methyl group from S-adenosyl methionine to the fifth position of the cytosine residue in the CpG islands (4-6). DNMT3A gene comprises 23 exons for which various mutations have been described. More than 60% of mutations are localized on the R882 amino acid in the MTase domain. DNMT3A mutations are predominantly heterozygous and are strongly associated with FLT3-ITD, Isocitrate dehydrogenase 1(IDH1), and NPM-1 mutations.

The incidence of DNMT3A mutations detected in AML, ranges from 4.1% in a Japanese study to 25% in a Western study (7-9). Somatic mutations in the hotspot region of the DNMT3A gene (R882) were recurrently identified in AML. It is believed that DNMT3A mutations confer an adverse prognosis for AML patients. Clinically significant DNMT3A R882 mutations in AML patients support the need for a sensitive, rapid, and time-saving test for screening hotspot R882 mutations. In a comparative study to identify k-ras mutations, Jankik et al., showed that HRM analysis was superior to direct sequencing and pyrosequencing based on sensitivity basis and percentage of the detected mutations (10). In comparison with some advanced methods such as direct sequencing, high resolution melting (HRM) analysis has more sensitivity in detecting somatic mutations in tumoral tissues with identical sensitivity to that obtained by snapshot (11,12). HRM assay is a fast and dependable method for mutation scanning. It is a sensitive method for genotyping and mutation screening when technical considerations are observed carefully. The present study compared HRM assay with direct sequencing for the detection of DNMT3A R882 mutations in AML patients.

Materials and Methods

Patient selection

In this retrospective cohort study, from August 2012 to September 2014, 220AML patients who referred to hematology-oncology and stem cell transplantation center (referral center) at Shariati hospital with an available Pre-treatment diagnostic samples were enrolled. The sample size was determined according to prevalence of DNMT3A mutations obtained by previously published data. AML-M3 and therapy-related AML constituted exclusion criteria in the present study. Written informed consent was obtained from all patients in agreement with the Declaration of Helsinki and the ethical guidelines of the Iran University of medical sciences in Tehran, Iran, which approved this study (IR.IUMS.REC.1394.26066).

Sample preparation

Genomic DNA was extracted from the peripheral blood/bone marrow mononuclear cell via the standard salting-out extraction method. DNA concentration was adjusted to 10ng/µL. To determine sensitivity and do comparison between HRM and direct sequencing, a dilution series of DNA from patients with DNMT3A R882H mutation was used and mixed with wild-type DNA. Mutant DNA was serially diluted into wild-type DNA at ratios of 1:1, 1:3, 1:9 and 1:19 (mutant/WT) to obtain 50%, 25%, 10%, and 5% of mutant DNA; respectively. Both direct sequencing and HRM analysis were performed on serially diluted DNA samples.

Mutation screening

High Resolution Melting analysis

Primer sequences to amplify DNA fragments spanning the codon R882 were as the following: Forward 5′-TTTGGTTTCCCAGTCCACTATAC-3′ and Reverse 5′-CCAGCAGTCTCTGCTT-

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3' DNA was amplified in a final volume of 20µL containing 4µL of 1 ×HOT FIREPOL Eva Green QPCR mix plus (HOT FIREPOL DNA Polymerase, 5 × Eva Green QPCR buffer, 2.5 Mm $Mgcl_2$, Evagreen dye, ROX dye) 0.2 pmol/L of each primer. PCR and HRM were performed on a Light Cycler LC96 Real Time analyzer (Roche Diagnostics, Basel, Switzerland). PCR conditions were as the followings: initial activation 95 °C for 15 min followed by 45 cycles of 15 s at 95°C, 20 s at 60°C and 20 s at 72°C. After PCR amplification, the PCR product was denatured at 95°C for 1 min and cooled to 40°C for 1 min. A high-resolution melt was immediately performed from 65°C to 97°C rising at 0.04°C/S.

Bidirectional sequencing

Exon 23 DNMT3A gene was amplified by PCR using the following primers: Forward 5′-GTGTGGTTAGACGGCTTCC-3′ and Reverse 5′-CTCTCCCACCTTTCCTCTG-3′

Polymerase chain reaction (PCR) conditions were as follows: One cycle of 95 °C for 3 min; 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and a final extension of 72 °C for 10 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel, and then the PCR products were directly sequenced using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (Applied Biosystems® 3130 Genetic Analyzer). Positive mutations were identified by comparing bi-directional sequence data to a normal reference sequence.

Statistical analysis

Clinical characteristics of patients were compared using Mann-Whitney test for continuous variables and Pearson's chi-squared test for categorical variables. The level of agreement between HRM assay and direct sequencing was determined using Chi-square and Cohen's kappa coefficient test using SPSS.

Results

Out of 220 patients, 25 cases (11.4%) were detected to harbor a DNMT3A R882 mutation, including c.2645G>A p.(R882H) (n = 14), c.2644C>T p.(R882C) (n =9), and c.2645G>C p.(R882P) (n = 2). The clinical characteristics of AML patients are summarized in Table I. No correlation was observed between DNMT3A status and median age, gender, white blood cell (WBC) count, hemoglobin, and platelet count. Analysis of DNMT3A mutations was done with the FAB subtypes of AML. Although, according to FAB classification, no significant differences were found between patients with and without DNMT3A R882 mutations, these mutations were found more frequently in the M4 and M5 subtypes.

Response to induction therapy

Among 220 AML patients, 157 (71.3%) achieved complete remission (CR), 39 (17.7%) were resistant to chemotherapy, and 24 (10%) died before or during induction of chemotherapy. A total of 13 patients with DNMT3A mutations achieved CR, 9 were resistant, and 3 died during chemotherapy induction. There was a significant difference between the presence and absence of DNMT3A R882 mutation for achieving CR (p = 0.036). No significant correlation was found between NPM1 mutation status and response to induction chemotherapy (p=0.46). Regarding the FLT3-ITD mutation status and achieving CR, patients with FLT3-ITD mutation were more resistant to chemotherapy induction than those without mutations (p=0.008).

Comparison of HRM assay and Sanger sequencing

To evaluate the performance of both methods, DNA sample was prepared from

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an AML patient with DNMT3A R882H mutation with more than 90% blast in bone marrow. DNA sample revealed a robust signal in sequencing analysis. After preparing the dilution serial in the context of wild-type DNA, 50% dilution produced a robust peak, wherase 25% represented a weak signal. By contrast, HRM detected the R882H mutation with a detection limit of 10% in the context of wild-type DNA (Figure 1). There was an overall good agreement between direct sequencing and HRM assay (kappa value of 0.95) ($p<0.001$). In 218 cases (99%), complete agreement between assay findings was observed. The two cases (1%) with discordant findings resulted from the low quality of the DNA samples. The representative sequencing chromatograms and HRM curves for DNMT3A R882 mutations are shown in Figures 2 and 3.Samples containing of R882 mutations showed 2 peaks; whereas, wild-type sample showed only one peak at 82.8˚C.

Table I: Clinical characteristics of AML patients regarding DNMT3A mutation

Clinical characteristic	DNMT3A Mutation	DNMT3A Wild Type	P value
Age,years (median)	39	32	
Range	12-57	7-67	0.1
Gender			
Male	15	147	0. 4
Female	10	48	
FAB subtypes			
M0/M1/M2/M3/M4/M5/M6/M7	0/4/6/0/7/6/2/0	5/31/69/0/65/22/3/0	0.75
WBC at diagnosis			
Median	6200	13000	0.65
Range	700-151000	240-257000	
PLT at diagnosis			
Median	30500	61000	0.12
Range	6000-712000	6000-340000	
Molecular abnormalities			
NPM1 wild type	16	165	
NPM1 mutation	9	30	0.014
FLT3-ITD^{neg}	18	140	
FLT3-ITD^{pos}	7	55	0.28

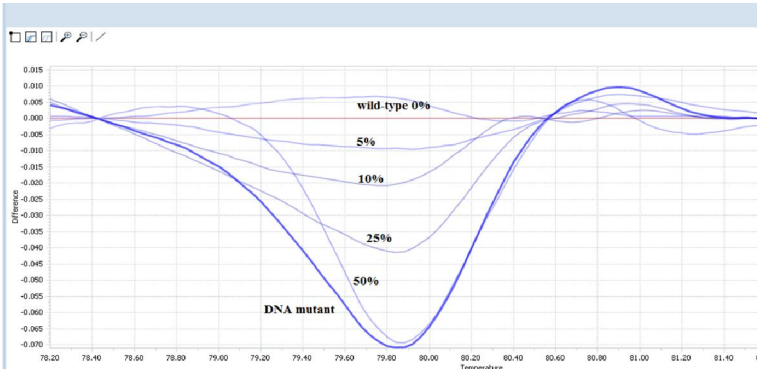


Figure1. The sensitivity analysis of a dilution series of DNMT3A R882 mutant in a background of wild-type DNA detected by HRM

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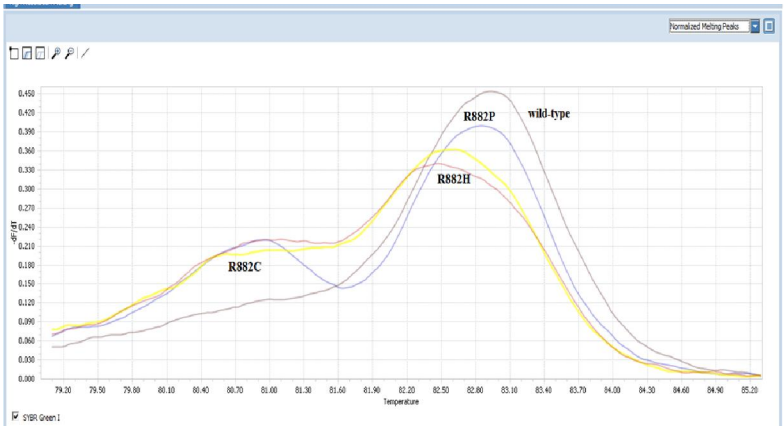


Figure 2. HRM curves analysis of three DNMT3A R882 mutations and wild-type

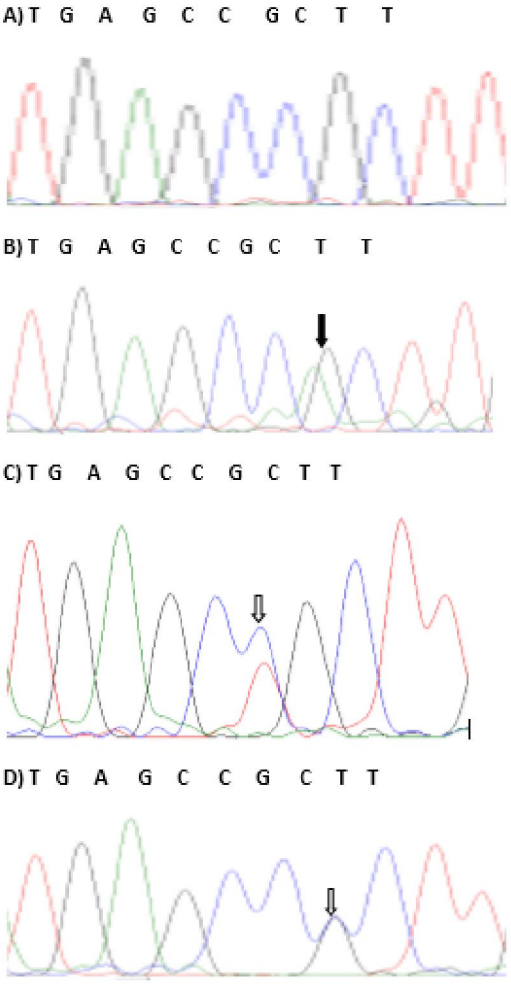


Figure3. Sanger sequencing analysis of DNMT3A R882 mutations (A: wild-type sequence; B: R882H mutation; C: R882C mutation; D: R882P mutation)

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Discussion

Molecular aberrations are important factors for risk-stratification of AML patients and molecularly targeted therapy. DNMT3A mutations have been attracting attention as markers for risk stratification in AML patients. Due to clinical significance and relatively high prevalence of DNMT3A R882 mutations in AML patients, progress in implementation of simple and rapid diagnostic methods is essential. HRM is a powerful technique for the detection of mutations and the scanning of epigenetic aberrations. It is cost-effective, time-saving, and more sensitive than Sanger sequencing (13).

Detection of mismatched duplexes which is formed after amplification of heterozygous DNA makes HRM operation possible. Mutation scanning via the current method relies on the melting of heteroduplexes that twists the shape of the melting curve. Sanger sequencing is considered to be the gold standard test for mutational analysis. It provides a high degree of accuracy and long-read capabilities; however, it is laborious and expensive (14). Sequencing requires extensive automation and expensive instrumentation, and is time-consuming according to the cost for service setup.

PCR amplification and mutation scanning can be performed in a single-step and closed-tube method by HRM technique and no other post-PCR processing is required. Therefore, limited sample handling reduces possible contamination.

The present study analyzed DNA samples from 220 AML patients for DNMT3A R882 mutations using HRM and Sanger sequencing. HRM was more sensitive than Sanger sequencing (10% and 25%, respectively), but two samples were missed by HRM due to the low quality of DNA. To avoid false-negative results, it is very important to observe technical considerations. No false-positive results were detected. The results of the HRM sensitivity limit for the detection of DNMT3A R882 mutations were in line with those of previous studies (15, 16).

However, some studies as what mentioned in the following, reported a lower limit of sensitivity for HRM analysis compared to the present study. Berenstein et al., performed a comparative study on various PCR-based methods for the detection of DNMT3A and IDH1/2 mutations and described the HRM assay as being time-saving, cost-efficient, and sensitive. Their HRM assay had a sensitivity of 5.9% for the detection of DNMT3A R882 mutations (17). In determining recurrent R882 mutations in chinese patients with AML and MDS, Lin et al., revealed that HRM analysis could easily distinguish DNMT3A R882H mutation with maximal sensitivity of 2% in the background of wild-type DNA, wherease mutated R882H was detected at the maximal sensitivity of 10% by sanger sequencing (18). Singh et al., designed HRM analysis to identify high frequency DNMT3A mutations in AML patients. Sensitivity studies for exon 23 were performed by using DNA sample with R882H mutation and a blast count of 68% and the sensitivity was determined in 6.7% of mutant DNA in the context of wild-type DNA. They also showed that the false-negative rate for HRM was 0% (19). The discrepancy in the sensitivity limit for HRM between the studies could be due to the reagents (Real Time master mix) and type of equipment used. Therefore, each laboratory should justify HRM technique with its equipments before application. To setup the HRM assay to detect mutations, the availability of wild-type control samples and those with specific mutations are essential. The high sensitivity of HRM compared with Sanger sequencing makes it beneficial for the detection of molecular aberrations in AML patients with low cellularity in bone marrow or low allele burden of mutations (20).

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Conclusion

HRM curve analysis was confirmed as a sensitive, fast, and high-throughput method for the detection of DNMT3A R882 mutations in AML patients. HRM analysis was also validated as an alternative to Sanger sequencing because it is simple, relatively quick, and cost-effective.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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