RESEARCH ARTICLE

Mutation Analysis of Isocitrate Dehydrogenase (IDH1/2) and DNA Methyltransferase 3A (DNMT3A) in Thai Patients with Newly Diagnosed Acute Myeloid Leukemia

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Abstract

Acute myeloid leukemia (AML) is a clonal hematopoietic stem/progenitor cell disorder which features several genetic mutations. Recurrent genetic alterations identified in AML are recognized as causes of the disease, finding application as diagnostic, prognostic and monitoring markers, with potential use as targets for cancer therapy. Here, we performed a pyrosequencing technique to investigate common mutations of IDH1, IDH2 and DNMT3A in 81 newly diagnosed AML patients. The prevalences of IDH1, IDH2 and DNMT3A mutations were 6.2%, 18.5%, and 7.4%, respectively. In addition, exclusive mutations in IDH1 codon 132 (R132H, R132C, R132G and R132S) were identified in all IDH1-mutated cases indicating that these are strongly associated with AML. Interestingly, higher median blast cell counts were significantly associated with IDH1/2 and DNMT3A mutations. In summary, we could establish a routine robust pyrosequencing method to detect common mutations in IDH1/2 and DNMT3A and demonstrate the frequency of those mutations in adult Thai AML patients.

Keywords: Acute myeloid leukemia- isocitrate dehydrogenase- DNA methyltransferase- pyrosequencing

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of clonal hematopoietic stem/progenitor cell disorders in which clinical represents, cell morphologies, immunophenotypes, disease progressions as well as treatment outcomes are important for diagnosis, prognosis, therapy response evaluation, and minimal residual disease monitoring (Zeisig et al., 2012). Aberrant proteins produced by genetic alterations are shown to be the therapeutic targets of effective molecules/compounds for treatment of a specific type of AML such as PML/RARα (targeted with ATRA) in acute promyelocytic leukaemia (APL) (Grimwade et al., 1998; Dohner et al., 2010; Grimwade et al., 2010). Thus, molecular identification of recurrent genetic/epigenetic alterations in AML is clearly important.

There were several leukemia association transcription factors (LATFs) involved in the establishment of AML including AML/ETO, PML/RARα, CBFβ/MYH11, NUP98/HOXA9, and MLL rearrangements which could be identified in different frequency (Look, 1997; Scandura et al., 2002). Additionally, different disease prognosis as well as clinical outcomes in each patient is defined by the presence of specific alterations which is critical for disease classification and treatment strategy (Vardiman et al., 2009; Grimwade et al., 2010). Although recurrent chromosomal translocations in some types of AML were identified and well characterized, the majority of patients (about 50% of de novo AML cases) harbored cytogenetic normal (CN) results (Mrozek et al., 2004). Several studies identified various somatic mutations in genes/pathways that are critical for normal hematopoiesis as well as in the development of AML. Those are including mutations in genes critical for cellular signaling such as c-Kit, N-RAS, K-RAS, FLT3, NPM1, and CEBPA (Estey and Dohner, 2006; Bacher et al., 2008; Schlenk et al., 2008; Estey, 2012; Allen et al., 2013; Ofran and Rowe, 2013). There are evidences indicating some genetic alterations representing a potential therapeutic target for AML treatment (Falini et al., 2015).

Apart from genetic aberrations in cellular signaling, recent studies demonstrated that gene mutation involving epigenetic regulations is strongly associated with the establishment of AML. The mutations disrupt normal function of HSC/progenitor cells in hematopoiesis resulting in AML transformation (Shih et al., 2012) including mutations of genes that function as histone modification and modify cytosine nucleotides on DNA
sequences such as TET2, IDH1/2, ASXL1, DNMT3A, MLL1, DOT1L, and EZH2 (Chung et al., 2012; Li and Zhu, 2014; Haladyna et al., 2015). Among those genes, mutation of IDH1, IDH2 and DNMT3A are frequently observed in AML (Abdel-Wahab and Levine, 2013; Li and Zhu, 2014). IDH1 and IDH2 (Isocitrate dehydrogenase 1 and 2) encode enzymes that convert isocitrate to α-ketoglutarate (α-KG) in Krebs cycle (NADP+ dependent) and may involve in oxidative damage response (Kon Kim et al., 2015). There were approximately 1% to 30% IDH1/2 mutations identified in de novo and secondary AML (Figueroa et al., 2010; Chotirat et al., 2012; DiNardo et al., 2013; Koszarska et al., 2013; Ahmad et al., 2014; Kon Kim et al., 2015; Ravendran et al., 2015). Mutations of IDH1 and IDH2 lead to conversion of α-ketoglutarate to oncometabolite 2-hydroxyglutarate (2-HG) (Dang et al., 2009; Ward et al., 2010). Moreover, DNA damage induced by reactive oxygen species may be occurred due to high level of 2-HG (Rakheja et al., 2012). Nevertheless, there is less clear about the prognostic significance of IDH2 mutation statuses in AML.

In addition, mutations in DNMT3A (DNA methyltransferase 3 A), a critical enzyme involving in DNA methylation are frequently identified in AML. DNMT3A is a member of DNA methyltransferase family that adds methyl group to cytosine in CpGs (Shih et al., 2012). The mutation frequency of hotspot R882 ranges from 3.9% to 13.3% in AML (Lu et al., 2013; Ahmad et al., 2014). Patients with DNMT3A mutations are associated with poor prognosis and increased risk of relapse (Ley et al., 2010; Marcucci et al., 2012). Furthermore, these patients exhibit high white blood cell counts in peripheral blood and predominantly represent in monocytic subtype. DNMT3A mutations are recurrently observed in cytogenetically intermediate-risk AML (Patel et al., 2012). Concurrent mutations of NPM1, FLT3 and IDH1 are frequently observed in DNMT3A mutant AML patients (Ley et al., 2010). Moreover, there is evidence indicating that mutations of DNMT3A are constant during relapsing state (Hou et al., 2012). Therefore, detection of DNMT3A mutations could be useful for disease monitoring especially in minimal residual disease (MRD) detection manner. Although genetic mutations of IDH1/2 and DNMT3A in AML have been widely studied, the mutation frequencies are different between regions. The mutational status in different countries is required for the investigation. The incidence of those mutations in Thai patients with AML is still limited. There is only one investigation of IDH1 and IDH2 mutations by Chotirat et al. in 2012. Here, we aimed to establish a highly sensitive and specific pyrosequencing technique to identify genetic mutations of IDH1, IDH2 and DNMT3A. Finally, we further investigated the clinical correlation of those mutations with other hematological parameters and clinical outcomes.

Materials and methods

Subjects and samples

Bone marrow samples were collected from 81 patients with newly diagnosed AML. These patients were treated at the Ramathibodi Hospital, Mahidol University during 2012-2015. AML patients were diagnosed and classified according to WHO criteria. Clinical data of the patients were reviewed by hematologists. The data including age, sex, complete blood count, cytogenetic results and gene mutation status (NPM1, CEBPA, FLT3-ITD and -TKD) were recorded. Genomic DNA was extracted using QIAamp DNA Blood Mini kit (Qiagen) and subsequently quantified using Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, USA). This work was approved by the Ethical Committee for Human Research, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand (ID 09-55-15).

Mutation analysis of IDH1/2 and DNMT3A using pyrosequencing

Hot spot mutation regions in exon 4 of IDH1 (codon 132 and 140) and IDH2 (codon 172) and exon 23 of DNMT3A (codon 882) were amplified by polymerase chain reactions (PCR) using specific primer pairs including; IDH1-R132F (5’-GCTTTGAGTGATTGAGGTTA-3’), IDH1-R132R (5’-TTGCCAACATGACTCTTCTGAT-3’), IDH2-R140F (5’-AGAGTCAAGCTGAAGAAGATGTG-3’), IDH2-R140R (5’-CGTGGAGGTTTTTTCAGAT-3’), IDH2-R172F (5’-TCCGGGAGCCCCATCATCT-3’), IDH2-R172R (5’-CTTGGGCTACCTGGTCGC-3’) DNMT3A-R882F (5’-TACCCTCAGTTGTGGCCCATG-3’), and DNMT3A-R882R (5’-ATGCTCCGACACCTCCGTC-3’), respectively. PCR was performed in 25 μl of PyroMark PCR kit (Qiagen) with final concentrations of 1X PyroMark PCR Mastermix, 1X CollaLoad Concentrate, 0.2 μM of each primer and 40 ng of genomic DNA. The PCR was carried out in Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA). The optimal PCR condition was following; initial PCR activation at 95°C for 15 minutes, 45 cycles of denaturing at 94°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes, respectively. IDH1 (codon 132 and 140) and IDH2 (codon 172) and DNMT3A (codon 882) mutations were analyzed using a PyroMark Q24 pyrosequencer (Qiagen) according to the manufacturer instruction. Sequencing primers were following IDH1-R132S (5’-GGTGTAACCTCATCATCATA-3’), IDH2-R140S (5’-AATCCCAAATGGAACATA-3’), IDH2-R172S (5’-AGCCCCACCCATCATTG-3’), and DNMT3A-R882S (5’-AGCTCTGCTCGCC-3’). Pyrogram outputs were designed and analyzed using PyroMark Q24 software (Qiagen).

Control plasmids

Control plasmids including wild types and mutants of IDH1 codon 132, IDH2 codon 140 and 172 and DNMT3A codon 882 were customized using GeneArt™ Gene Synthesis (Thermo Fisher Scientific). Briefly, PCR products of known wild type and mutant DNA samples were purified and inserted into pMK-RQ (KanR) at the SfiI/SfiI cloning site. The plasmid DNAs were isolated after transformed into E. coli. Each control plasmid was verified by direct sequencing technique.
**Sanger sequencing**

The mutations of IDH1 codon 132, IDH2 codon 140 and 172 and DNMT3A codon 882 were confirmed by direct sequencing. In brief, PCR products were amplified with the same PCR conditions as in pyrosequencing analysis. Forward and reverse primers of each mutation were used as sequencing primers (as shown above). Sequencing data was performed by capillary electrophoresis on an ABI3730 Genetic Analyzer (Applied Biosystems) and analyzed using CodonCode Aligner software (CodonCode Corp., Inc).

**Statistical analysis**

The correlations between mutations and various patient characteristics such as age, hemoglobin level, WBC count, platelet count, and percentages of blasts were determined by the Mann Whitney test. The relationships between gender, cytogenetic results and mutation status were also tested by the Chi square test. For all analyses, p-value of less than 0.05 was considered as statistically significant.

**Results**

Establishment of pyrosequencing assay for detection of IDH1 codon 132, IDH2 codon 140 and 172 and DNMT3A codon 882

We firstly optimized pyrosequencing conditions to detect mutations of IDH1, IDH2 and DNMT3A. The limit of detections of the optimized pyrosequencing technique was further investigated. The plasmid DNAs of mutant genes were serially diluted with each wild type plasmid control (0:100, 1:99, 2:98, 5:95, 10:90, 15:85, 100:0, respectively) and subsequently assessed by pyrosequencing. The minimal ratios of mutant/wild type alleles that could be detected by pyrosequencing were 5% for IDH1 codon 132, IDH2 codon 140 and DNMT3A codon 882 and 10% for IDH2 codon 172 (Figure 1). In a total of 81 newly diagnosed AML patients, we could identify mutations of IDH1/2 and DNMT3A in 26 patients (32.1%) (Table 1). To further confirm these mutations, Sanger sequencing technique was performed in all mutation positive samples. In 19 cases of IDH1/2 mutations, 5 samples were positive for IDH1 including

![Figure 1. Threshold Evaluation of Limit of Detections. Mixtures of control plasmids between wild type and mutant alleles were analyzed. 5% mutant alleles of IDH1-R132 (a), IDH2-R140 (b) and DNMT3A-R882 (d) could be detected as peaks in the pyrograms. While 10% mutant alleles of IDH2-R172 (c) could be detected by higher “A” and lower “G” peak height. The linear correlations between measured mutation frequencies and actual mutation frequencies showed high concordance (p < 0.001). The arrows indicate mutant peaks](image)

Table 1. Types of IDH1, IDH2 and DNMT3A Mutations Identified in 81 Patients with AML

| Mutations | Nucleotide substitution | Amino acid substitution | No. of patients |
|-----------|-------------------------|-------------------------|-----------------|
| **IDH1**  |                         |                         |                 |
| c.C394A   | CGT-AGT                 | p.R132S                 | 1               |
| c.C394G   | CGT-GGT                 | p.R132G                 | 1               |
| c.C394T   | CGT-TGT                 | p.R132C                 | 1               |
| c.G395A   | CGT-CAT                 | p.R132H                 | 2               |
| **IDH2**  |                         |                         |                 |
| c.G419A   | CGG-CAG                 | p.R140Q                 | 8               |
| c.G515A   | AGG-AAG                 | p.R172K                 | 8               |
| **DNMT3A**|                         |                         |                 |
| c.G2645A  | CGC-CAC                 | p.R882H                 | 6               |

AML, acute myeloid leukemia; C, cysteine; G, glycine; H, histidine; K, lysine; Q, glutamine; S, serine
| Variable                      | All cases | IDHmt | IDHwt | P Value | IDH1mt | IDH1wt | P Value | IDH2mt | IDH2wt | P Value |
|-------------------------------|-----------|-------|-------|---------|--------|--------|---------|--------|--------|---------|
| No. of cases                  | 81        |       |       |         | 19     |        |         | 62     |        |         |
| No. of males/females          | 44/37     |       |       |         | 11/8   |        |         | 34/28  |        |         |
| Age, years                    |           |       |       | 0.447   |        |       |         | 0.555  |        | 0.526   |
| Median (range)                | 52.0 (15.0-80.0) | 52.0 (26.0-80.0) | 51.0 (15.0-77.0) | 53.0 (26-80) | 50.0 (15.0-77.0) | 51.0 (26.0-80.0) |
| Hemoglobin (g/dL)             |           |       |       | 0.867   |        |       |         | 0.347  |        | 0.62    |
| Median (range)                | 9.0 (9.0-13.0) | 8.0 (6.0-12.7) | 9.0 (3.0-13.0) | 9.0 (8.2-10.0) | 9.0 (3.0-13.0) | 8.0 (6.0-12.7) |
| Hematocrit (%)                |           |       |       | 0.967   |        |       |         | 0.185  |        | 0.429   |
| Median (range)                | 25.0 (13.1-41.5) | 26.0 (18.9-41.5) | 26.0 (13.1-41.5) | 29.0 (25.0-31.0) | 26.0 (13.1-41.5) | 24.0 (18.9-32.0) |
| White count, x10^9/L          |           |       |       | 0.167   |        |       |         | 0.872  |        | 0.333   |
| Median (range)                | 12.0 (0.5-444.0) | 29.0 (1.2-158.0) | 11.0 (0.5-444.0) | 7.0 (1.8-88.0) | 12.0 (0.5-444.0) | 25.0 (1.2-158.0) |
| Platelet, x10^9/L             |           |       |       | 0.509   |        |       |         | 0.012  |        | 0.727   |
| Median (range)                | 64.0 (6.0-380.0) | 89.0 (6.0-223.0) | 60.0 (7.0-380.0) | 181.5 (100.0-223.0) | 61.0 (6.0-380.0) | 71.0 (6.0-199.0) |
| Percentage of blasts          |           |       |       | 0.093   |        |       |         | 0.737  |        | 0.352   |
| Median (range)                | 24.0 (0.0-96.0) | 42.0 (0.0-90.0) | 17.0 (0.0-96.0) | 31 (0-54) | 24 (0-96) | 39 (0.0-90.0) |

**Table 2.** Characteristics of AML Patients with Wild Type or Mutations of IDH1 and IDH2

*Note: Cytogenetic risk groups (Grimwade et al., 1998); †, Combination with -7 cytogenetic abnormality．
The overall frequencies of these mutations in IDH1, IDH2, and DNMT3A were 6.2%, 18.5% and 7.4%, respectively.

The correlation of IDH1/2 and DNMT3A mutation and clinical parameters and cytogenetic risk group in AML patients

We further investigated the relationship between the observed mutant alleles of IDH1/2 and DNMT3A and clinical data of each patient (Table 2). We found that patients with IDH1 codon 132 mutations had no significant difference in all variables including age, gender, hemoglobin level, hematocrit level, WBC counts and percentages of blasts when compared with wild-type. However, higher platelet count (p = 0.01) was significantly observed in patients with IDH1 mutations (Table 3). Similar to IDH1, patients with IDH2 mutations had no significant difference in all compared parameters except percentage of blast cells which was significantly increase in patients with IDH2 codon 172 mutation (p = 0.04) (Table 4 and Fig 4). While all patients with DNMT3A mutations had no significant difference in almost all parameters when compared with wild-type, DNMT3A mutation was significantly higher in the elderly group (p = 0.01) (Table 5). Analyzing the association between IDH1/2 and DNMT3A mutation statuses and cytogenetic risk groups, we observed that patients with no IDH1/2 mutation were associated with poor cytogenetic risk group (p = 0.048). Interestingly, patients with inv(16) were associated with IDH2 (p = 0.035) and IDH2-R172 (p = 0.0002) mutations. Moreover, IDH2-R172 mutations were significantly associated with FLT3-TKD (p = 0.008). Taken together, we demonstrated the associations between IDH1/2 and DNMT3A mutations and clinical data of AML patients.

Discussion

We developed pyrosequencing assay with high sensitivity and specificity for the detection of common mutations in IDH1/2 and DNMT3A in adult AML. To standardize pyrosequencing technique, we performed mixing assay in which mutant control plasmid DNAs were serially diluted in a wild type DNA and subsequently analysed using established method (Setty et al., 2010; Arita et al., 2015). The established pyrosequencing technique demonstrates a greater potential tool to detect
a tiny proportion (5%) of mutant IDH1 (codon 132), IDH2 (codon 140), and DNMT3A (codon 882) in normal DNA counterpart when compared to Sanger sequencing method (limit of detection are approximately to 20-25%).

The sensitivity of the method to correctly discriminate IDH2 codon 172 mutant allele required minimum 10% of tumor DNA samples which was comparable to the previous report of IDH1/2 mutation detection in gliomas (Arita et al., 2015). This pyrosequencing technique had greater potential to detect IDH2 (codon 172) mutation in a wild type DNA background when compared to the high-resolution melting curve analysis (sensitivity was 7.3%) (Patel et al., 2011). Moreover, pyrosequencing technique could quantify the number of mutation alleles in normal DNA background suggesting its potential role for the detection/monitoring of MRD in AML.

Although pyrosequencing technique demonstrates several advantages to detect IDH1/2 and DNMT3A mutations such as routine robust, fast, not expensive, high specificity and sensitivity, the assay was not a suitable screening test due to its limited by the selected mutations.

Using the established method, we analysed 81 DNA samples isolated from newly diagnosed AML including 46 of 81 (56.8%) with normal karyotype and 35 of 81 (43.2%) with aberrant karyotypes. We observed that 23.5% of IDH1 and IDH2 mutations were predominantly positive in patients who harbour intermediate-cytogenetic risk group (22.2%) whereas 1 patient (1.2%) positive for IDH2 mutation had favourable-cytogenetics. Nevertheless, IDH1 and IDH2 mutations could not be identified in patients with adverse-cytogenetic risk group.

In contrary, Paschka et al. (2010), analysed 805 adult AML patients and demonstrated that IDH1 and IDH2 mutations were positive in both patients with intermediate (19%) and adverse-cytogenetics (13%) risk groups. This suggested that different observed frequency of IDH1/2 mutations may be influenced by the number and selected subtype of AML. Interestingly, mutations of IDH1/2 were predominant in patients who harbour cytogenetically normal AML (Table 2). This finding was similar to previous reports and suggested the tumorigenicity of cyogenetically normal AML by the aberrant of IDH1/2 coding sequences (Paschka et al., 2010; Chotirat et al., 2012; Nomdedeu et al., 2012; Ahmad et al., 2014; Raveendran et al., 2015).

| Variable                      | All cases | DNMT3Amt | DNMT3Aw | P Value |
|-------------------------------|-----------|----------|---------|---------|
| No. of cases                  | 81        | 6        | 75      |         |
| No. of males/females          | 44/37     | 02-Apr   | 40/35   | 0.528   |
| Age, years                    |           |          |         | 0.016   |
| Median (range)                |           | 52.0(15.0-80.0) | 70.0 (54.0-75.0) | 49.0 (15.0-80.0) |            |
| Hemoglobin (g/dL)             |           | 9.0 (9.0-13.0) | 7.0 (3.0-10.5) | 9.0 (4.6-13.0) | 0.369   |
| Hematocrit (%)                |           | 25 (13.1-41.5) | 25 (16.0-41.5) | 26.0 (13.1-39.5) | 0.882   |
| White count, x10⁹/L (median)  |           | 12.0 (0.5-444.0) | 41.0 (0.5-74.0) | 12.0 (0.5-444.0) | 0.745   |
| Platelet, x10⁹/L (median)     |           | 64.0 (6.0-380.0) | 64.5 (48.0-199.0) | 64.0 (6.0-380.0) | 0.605   |
| Percentage of blasts (median) |           | 24.0 (0.0-96.0) | 39.0 (0.0-80.0) | 20.0 (0.0-96.0) | 0.253   |

* 0.000001, Cytogenetic risk groups (Grimwade et al., 1998); †, Combination with -7 cytogenetic abnormality
We observed that the frequency of DNMT3A at R882 mutation s 7.4% (6 of 81) and the majority of DNMT3A mutated cases were cytogenetic normal (4 of 6). Likewise, DNMT3A are predominantly positive in AML patients with normal karyotype which the observed frequency is approximately to 30-35% (Ley et al., 2010; Marucci et al., 2012; Ahmad et al., 2014). Interestingly, there are evidences indicating that DNMT3A mutations are co-persistence with other common genetic alterations (FLT3, NPM1, IDH1/2, TET2, SF3B1, and U2AF1) in AML and other hematological malignancies such as MDS and MPN. This suggested the critical role of DNMT3A in the establishment of pre-leukemic initiating cells (Celik et al., 2016). However, the molecular underlying DNMT3A mutations in the establishment of AML are still under investigated. To gather, we demonstrated the distributions of IDH1/2 and DNMT3A mutations in Thai patients with AML. The similarity and discrepancy of the observed frequencies may be affected by the variability in inclusion criteria for selected AML cases, sample size, the genotyping methods and ethnic backgrounds.

Prospective for the clinical correlations of IDH1/2 and DNMT3A mutations and clinical representations of pretreatment AML, we further analyzed individual patient’s hematological data and their harboring genetic mutations. While patients with IDH1 and IDH2 mutations had no significant difference in age-onset of AML, DNMT3A was predominantly mutated in patients with older age (Im et al., 2014). This data suggested the risk of AML with DNMT3A mutations could increase in the elderly group. Furthermore, patients with IDH1-R132 mutations exhibited high platelet counts when compare with patients who have wild type IDH1 (Marcucci et al., 2010; Chotirat et al., 2012). Although we observed that patients with IDH2-R172 mutations have significantly increased in numbers of blast cells, several reports demonstrated no significant difference in blast cell counts between mutant and wild type IDH1/2 patients (Marcucci et al., 2010; Nomdedeu et al., 2012). Moreover, unlike Pløen et al. (Pløen et al., 2014), which reported significantly increasing in leukocyte and thrombocyte counts in DNMT3A mutated patients, similar in the number of those parameters were found in this study. Taken together, we proposed a high sensitive and accuracy pyrosequencing technique to detect somatic mutations of IDH1/2 and DNMT3A in pretreatment AML patients. We also reported the frequency of those genetic mutations in AML patients with Thai ethnic background. Moreover, we analyzed the patients’ specific mutations with demographics and hematological findings. This work could help to clearer understanding and emphasized the role of IDH1/2 and DNMT3A in the pathogenesis of adult AML.

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