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The Role of FLT3-ITD Mutation, PI3K/AKT Pathway, and Leukemia Stem Cells in D3A7 Induction therapy – the Outcomes of Adult Indonesian Patients with Acute Myeloid Leukemia

Elly Yanah Arwanih[1, 2], Ikhwan Rinaldi[2], Septelia Inawati Wanandi[3, 4], Melva Louisa[5]

[1]Doctoral Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia, [2]Division of Hematology and Medical Oncology, Department of Internal Medicine, Cipto Mangunkusumo National General Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia, [3]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia, [4]Molecular Biology and Proteomics Core Facilities, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia, [5]Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

Correspondence: *ikhwanrinaldi@gmail.com*; Tel.: + 62 213 162497

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Abstract

Objective. This cohort study aimed to examine the impact of the FLT3-ITD mutation on the downstream signaling pathway of PI3K/AKT pathway, the percentage of leukemia stem cells, and the survival of patients receiving D3A7 induction therapy. **Method.** Bone marrow mononuclear cells were collected from 20 adult AML patients who had completed D3A7 induction therapy at Cipto Mangunkusumo National General Hospital and Dharmais Cancer Hospital. FLT3-ITD gene mutation was examined by the PCR-sequencing method. Expression of phosphorylated PI3K and AKT was detected using the sandwich ELISA method. Flow cytometry was used for detecting the number of apoptosis and proliferation cells, and biomarkers of leukemia stem cells. **Result.** The expression levels of PI3K and AKT proteins were higher in FLT3-ITD, both in the mutant group compared to the non-mutation group, and in the patient group with treatment failure outcomes compared to the patient group with treatment response. The percentage of the leukemia stem cell population did not differ significantly between the FLT3-ITD mutation group and the wild type group, and between the treatment failure outcome group and the response outcome group. **Conclusion.** This study presents the important role of FLT3-ITD mutation via its downstream signaling (PI3K/AKT) in the outcome of D3A7 induction therapy. The FLT3-ITD mutation plays an important role in the 12-month survival of AML patients after D3A7 therapy. However, the outcome of D3A7 therapy and FLT3-ITD mutation were not associated with leukemia stem cells.

Key Words: FMS-like Tyrosine Kinase ▪ Daunorubicin ▪ Cytarabine.

Introduction

The primary treatment for acute myeloid leukemia (AML) has been intensive induction therapy with the purpose of eliminating most leukemic cells to achieve remission. This typically involves a regimen known as D3A7, consisting of three days of an anthracycline (Daunorubicin) followed by seven days of Cytarabine (1-3). In young adults, the complete response (CR) rate for first-line treatment typically ranges from 60% to 80%, while in older adults aged 65 years and above, it ranges from 40% to 60%. However, the majority of patients subsequently experience relapse with poor survival. Recent studies have identified drug resistance as a critical factor leading to treatment failure, ultimately impacting short-term survival outcomes in AML. There are many factors that influence drug resistance, including gene mutations and the presence of leukemia stem cells (4).

The FMS-Like Tyrosine Kinase 3 internal tandem duplication (FLT3-ITD) mutation stands out as one of the prevalent mutations found in AML (5-7). The prevalence of this mutation is

around 20-30% and about 21.5% in Indonesia (8). The FLT3-ITD mutation is typically linked to unfavorable molecular prognostic outcomes in patients and a higher likelihood of relapse in AML. Patients with the FLT3-ITD mutation tend to have a lower one-year survival rate compared to those without the mutation (9, 10). This mutation occurs in the Juxtamebrane Domain, and activates the loop that abolishes the auto-inhibitory function, resulting in persistent activation of FLT3 kinase. Consequently, this activation triggers downstream proliferative signaling pathways, including the PI3K/AKT pathway. This pathway triggers the activation of anti-apoptotic mechanisms, and promotes cell proliferation in leukemia cells, and has become one of many resistance mechanisms in AML therapy (4, 11, 12).

Leukemia stem cells (LCSs), also sometimes referred to as leukemia initiating cells, display specific mutations, epigenetic modifications, and a specific metabolic profile compared to healthy hematopoietic stem cells (HSCs). Leukemia stem cells (LSCs) are typically regarded as resistant to chemotherapy, making them the primary instigators of relapse (13). The intracellular signaling pathways and the niche-driven mechanisms that control quiescence constitute the PI3K/AKT pathway (14). The identification and targeting of LSCs depends on membrane markers, such as CD34+CD38-CD123+, and specific metabolites, such as ALDH1 (13, 15). Both the NCCN (the National Comprehensive Cancer Network) and the ELN (European Leukemia Net) guidelines advocate the incorporation of FLT3 genetic testing into the diagnostic evaluation process. Specifically, the NCCN guidelines propose conducting FLT3 testing alongside cytogenetic testing at the time of AML diagnosis for all patients. This approach aims to identify individuals who could potentially benefit from targeted therapeutic interventions (16).

In Indonesia, testing for FLT3 mutation is not routinely conducted in the diagnostic process of AML patients due to technical limitations. Therefore, the first cohort study in Indonesia was conducted to understand the role of FLT3-ITD mutation and its downstream signaling (PI3K/AKT) in the outcome of D3A7 induction chemotherapy in AML patients, as well as its association with leukemia stem cells.

Methods

Patients

This prospective cohort study was conducted from July 2022 to March 2024 at Cipto Mangunkusumo National General Hospital and Dharmais Cancer Hospital in Jakarta. All patients enrolled in the study received treatment according to the clinical pathway established by the hospital. The inclusion criteria for the study involved de novo AML patients, except those with AML-M3, age above 18 years, and those who had completed induction chemotherapy with D3A7. On the 7th day following chemotherapy, 15 mL of bone marrow was extracted from these patients for laboratory analysis. The chemotherapy outcome criteria used were based on the guidelines of The International Working Group and the European Leukemia Network (ELN). After this procedure, the patients were followed up for one year to evaluate their survival outcomes. The exclusion criteria were: AML patients with a history of transformation from another hematological malignancy, such as myelodysplastic syndrome or chronic myeloid leukemia, and those who were scheduled for bone marrow transplantation. Additionally, individuals with incomplete medical records or who declined to provide informed consent for participation in the study were not included.

Bone marrow specimens obtained from post Induction chemotherapy D3A7 AML patients were analyzed for the presence of FLT3-ITD gene mutation and its downstream pathway (phosphorylated PI3K & AKT protein), the outcome of chemotherapy, early-stage cell apoptosis, late-stage cell apoptosis, proliferation cells, and expression of markers of leukemia stem cells (CD34+CD38-CD123+ & ALDH1).

Isolation of Mononuclear Cells

The isolation of mononuclear cells from the bone marrow blood of patients was performed by means of a gradient centrifugation method, using Ficoll-Paque Plus (Merck). After the mononuclear cells were isolated, the cell count was determined using 90 µL Turks Solution (Merck) in a 10 µL sample in a Neubauer chamber. The cells were then cryopreserved using 10% DMSO in a 1 mL sample, and stored in liquid nitrogen for further analysis.

Detection of FLT3-ITD Mutation

Mutation testing for FLT3-ITD in the mononuclear cells was performed using the PCR-Sequencing method. Genomic DNA was isolated from the mononuclear cells using the spin column method with the Quick-DNA™ Miniprep Kit (ZYMO RESEARCH). Genomic DNA amplification was carried out by PCR using the following primers: FLT3-ITD-F: 5'-GCAATTTAGGTATGAAAGCCAGC-3' FLT3-ITD-R:5'-CTTTCAGCATTTTGACGGCAACC-3' The primers were designed using GeneBank data NG_007066.1. The PCR mix comprised 100 ng/µL genomic DNA, 10 pmol FLT3-F and FLT3-R primers, 12.5 µL MYTAQ HS Ready Mix + Dye, and dH2O to make up the total volume of the mixture of 25 µL. All PCR reactions used the Thermal Cycler (Applied Biosystems 9700) for 35 cycles: pre-denaturation at 95°C for 1 minute, and cycles consisting of a 15-second denaturation step at 95°C, a 15-second annealing step at 56°C, and a 15-second extension step at 72°C, followed by final extension for 10 minutes at 72°C. The PCR products were subjected to electrophoresis on 3% agarose gel. The gel was prepared by dissolving 2% agarose in tris-acetic acid-ethylenediaminetetraacetic acid (EDTA) buffer (TAE) containing 40mM tris, 20mM acetic acid, and 1mM EDTA, supplemented with 0.5µg/mL ethidium bromide. Once the agarose solution was prepared, it was poured into a casting tray and left to solidify. The gels were then electrophoresed for 35 minutes at 100 volts. Following electrophoresis, the gel was visualized using a UV light transilluminator. PCR amplification of genomic DNA for FLT3-ITD yielded a band with a size of 330 base pairs (bp). If a mutation was present, additional bands would appear, indicating products larger than 330 bp. The PCR amplification products targeting the FLT3-ITD mutation were excised from the agarose gel and subsequently subjected to Sanger sequencing to identify and characterize the mutations. Before sequencing, PCR DNA fragments to be sequenced were purified using gel cut extraction. DNA sequencing was performed using the DNA sequencer 3130 × l (Applied Biosystems, USA) on the basis of capillary electrophoresis. The sequencing results were obtained in AB1 and SEQ file formats, which were then analyzed using BioEdit software to identify mutations in the nucleotide sequence of the target DNA.

Detection of Phosphorylated PI3K/AKT Protein

Proteins were isolated from mononuclear cells using the protein isolation procedure with RIPA Buffer (SIGMA-ALDRICH) supplemented with 0.1% protease inhibitor cocktails (SIGMA-ALDRICH) and phosphatase inhibitor cocktails (SIGMA-ALDRICH). To determine the expression of phosphorylated PI3K and Akt proteins, a sandwich ELISA method was performed using the Phospho-PI 3 Kinase p85 + Total In-Cell ELISA Kit (ABCAM-ab207485) and Akt (pS473) + Total Akt ELISA Kit (ABCAM-ab126433) procedure. Finally, the concentrations of phosphorylated PI3K and AKT proteins were then compared to the concentrations of total PI3K and AKT proteins, resulting in the ratio of phosphorylated protein to total protein.

Detection of Proliferation and Apoptotic Cells

The proliferated and apoptotic cells were detected using flow cytometry. A total of 5 x 10^6 mononuclear cells were washed with 2 mL of PBS. After washing, 1 ml of mononuclear cell suspension was transferred and divided into tubes for each test, with 200 µl for the Ki-67 test and 400 µl for

the Annexin V-7AAD test. Each cell suspension for each test was divided into two tubes in equal amounts, one for the test tube and the other for the blank tube. Then, 10 µL (0.5 µg/µL) of Annexin V antibody (Annexin V Apoptosis Kit with 7-AAD, FITC, STEMCELL TECHNOLOGIES) and Ki-67 antibody (20Raj1), APC, eBioscience™ (INVITROGEN) were added to each test tube, followed by incubation for 10 minutes in the dark at 4°C. After the incubation was completed, all the test tubes, as well as the blank controls, were read on the BD FACSAria III flow cytometer machine.

Detection of Leukemia Stem Cells

The percentage calculation of the leukemia stem cell count was calculated using the markers CD34+CD38-CD123+ and ALDH1. A total of 5 x 10^6 cells were washed with 2 mL of PBS. After washing, 1 ml of mononuclear cell suspension was transferred and divided into tubes. Each cell suspension for each test was divided into two tubes in equal amounts, one for the test tube and the other for the blank control tube. Then, 10 µL (0.5 µg/µL) of the antibodies Anti-Human CD34 Antibody, Clone 563, PE, Anti-Human CD38 Antibody, Clone AT-1, FITC; Anti-Human CD123 (IL-3Rα) Antibody, Clone 6H6, APC (STEMCELL TECHNOLOGIES) were added to each test tube, followed by incubation for 10 minutes in the dark at 4°C. After the incubation was complete, all the test tubes, as well as the blank control tubes, were read on the BD FACSAria III flow cytometer machine. To determine the percentage of Leukemia Stem Cells expressing ALDH1, ALDEFLUOR™ assay was used following the ALDEFLUOR™ Kit (STEMCELL TECHNOLOGIES) procedure.

Ethics Statement

This study received ethical approval from the Institutional Review Committees of both Cipto Mangunkusumo National General Hospital and Dharmais Cancer Hospital, with approval number 105/UN2.F1/ETIK/PPM.00.02/2023. Written informed consent was obtained from all patients in compliance with the principles outlined in the Declaration of Helsinki.

Statistical Analysis

Data processing was performed using IBM SPSS Statistics 27 software, with a significance level set at $P<0.05$ (95% CI). Data normality was tested using the Shapiro-Wilk test, while data variance homogeneity was tested using Levene's test. All the obtained data were then subjected to non-parametric statistical testing. For bivariate analysis, unpaired t-tests and Mann-Whitney Tests were used. To examine the relationship between two categorical variable groups, Fisher's exact test was employed. A proportional hazard assumption test was conducted, involving the Kaplan-Meier method, the log-log –In(-ln) survival method test, and Schoenfeld's global test.

Results

Patient Characteristics and FLT3 Gene Mutation Prevalence

The total number of AML patients included 11 males and 9 females. The range of ages was 20-56 years (mean 38.50±SD 11.7). According to FAB classification for AML, two cases were diagnosed as AML-M1 (10%), 10 cases AML-M2 (50%), two cases AML-M4 (10%) and six cases AML-M5 (30%). In addition, 12 patients (60%) had a treatment failure outcome and eight patients (40%) had a response outcome following D3A7 chemotherapy.

Mutations in FLT3-ITD were found in four (20%) AML patients on the basis of the detectable amplicons at 330 bp and larger than 330 bp in 3% agarose gel electrophoresis. All of these patients were classified as FLT3-ITD mutants. The numbers of base pair insertion varied from sample to sample. The lowest insertion was 24 bp and the highest insertion was 84 bp. The results of gel electrophoresis are presented in Figure 1.

The analysis of FLT3 mutations based on the amino acid positions in the four samples showed

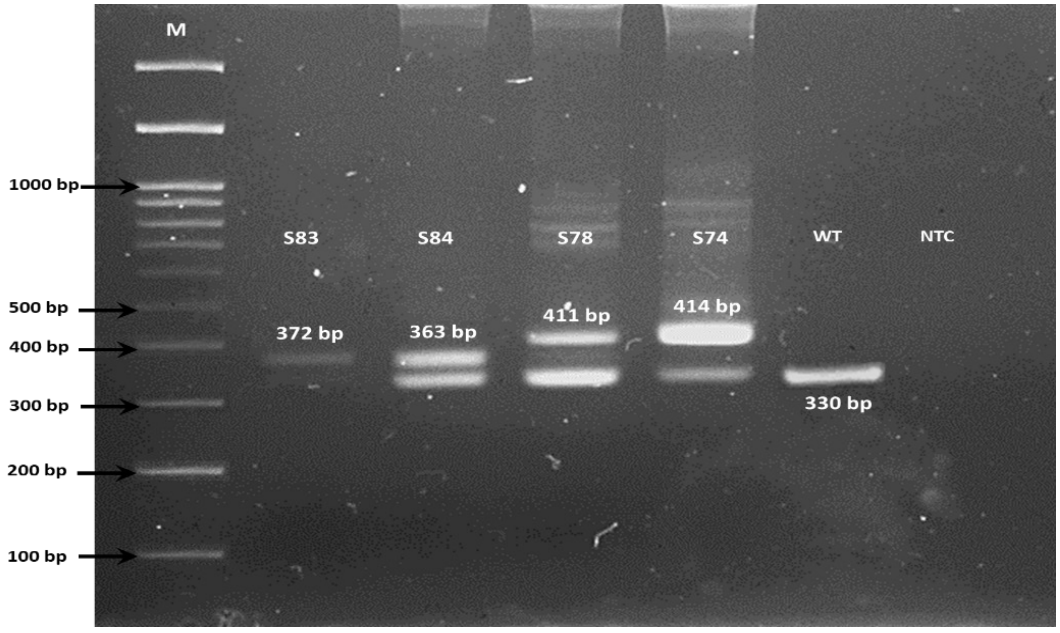

Figure 1. Electrophoresis of PCR products with agarose gel. Sample with FLT3-ITD homozygote allele mutation (S83), Sample with FLT3-ITD heterozygote allele mutation (S84, S78, S74), Wild type (WT) Sample, Non-template Control (NTC), DNA ladder 100bp (M).

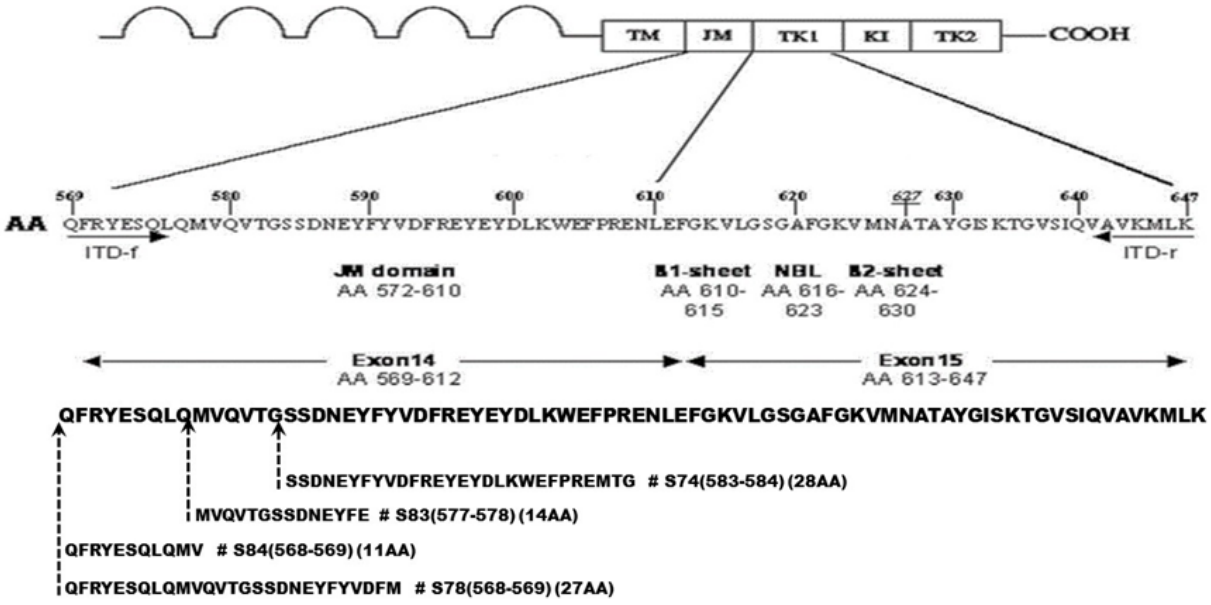

Figure 2. The position of amino acid insertions in samples with FLT3-ITD mutations.

that each mutant sample had mutations at different amino acid positions. In Figure 2, it was observed that samples S84 and S78 had insertions between amino acid positions 568 and 569, where the number of insertions were 11 and 27 amino acids, respectively. Meanwhile, in sample S83, there was an insertion of 14 amino acids between positions 577 and 588. In sample S74, there was an insertion of 28 amino acids between amino acid positions 583 and 584.

Proportional Hazard Assumption test of 12 Month Survival of AML Patients with FLT3-ITD Mutation

The Kaplan-Meier test showed that the survival curves in the Kaplan-Meier plot and the survival lines in the –ln(-ln) survival probability curve did not intersect. The global test (P=0.1) indicated that the assumption of proportional hazards (PH) met the requirements. This meant that the comparison of survival rates between the groups with a FLT3-ITD mutation and the wild type groups was consistent over time. The Cox regression analysis yielded a P-value of 0.03 and an HR: 6.027; 95% CI: 1.61-31.279.

Analysis Comparative of the FLT3-ITD Mutation and the Outcome of D3A7 Chemotherapy

The results of the comparison test between the FLT3-ITD group and the chemotherapy outcome group using Fisher's exact test, with a confidence interval of 95% and a standard deviation of 10%, show that there was no significant difference between the two groups, with P=0.5.

The results analysis in the group of patients with FLT3-ITD mutations and wild type showed that the average values of the variables PI3K, AKT, and late-stage apoptosis were higher in the mutant group compared to the wild type group (P=0.003, 0.009, and 0.023). Meanwhile, the variables early-stage apoptosis, cell proliferation, and leukemia stem cell markers CD34+CD38-CD123+ and ALDH1

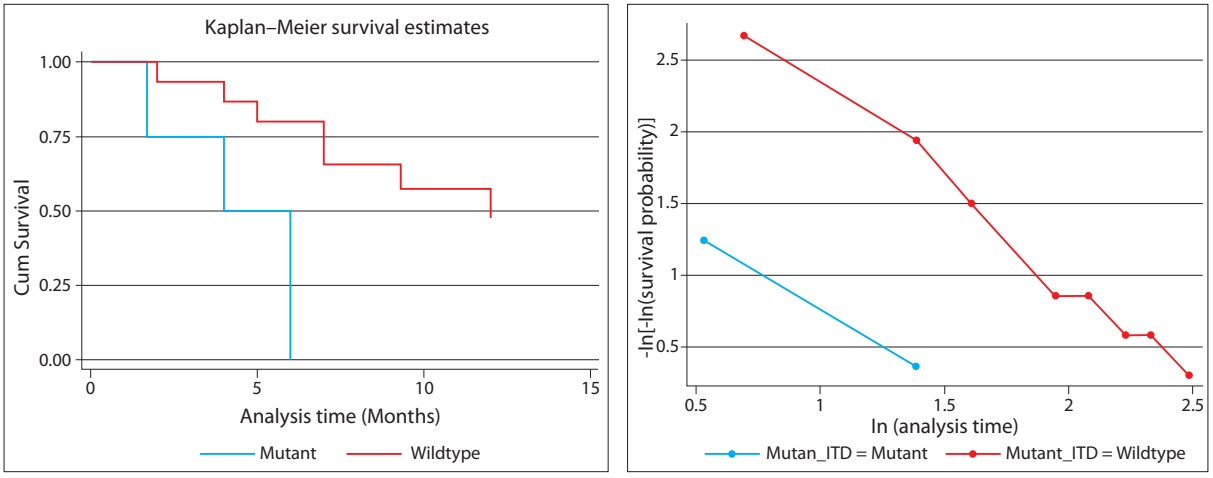

Figure 3. Kaplan-Meier Survival Curve and –ln (-ln) Survival Curve of Patients Based on FLT3-ITD Mutation Status.

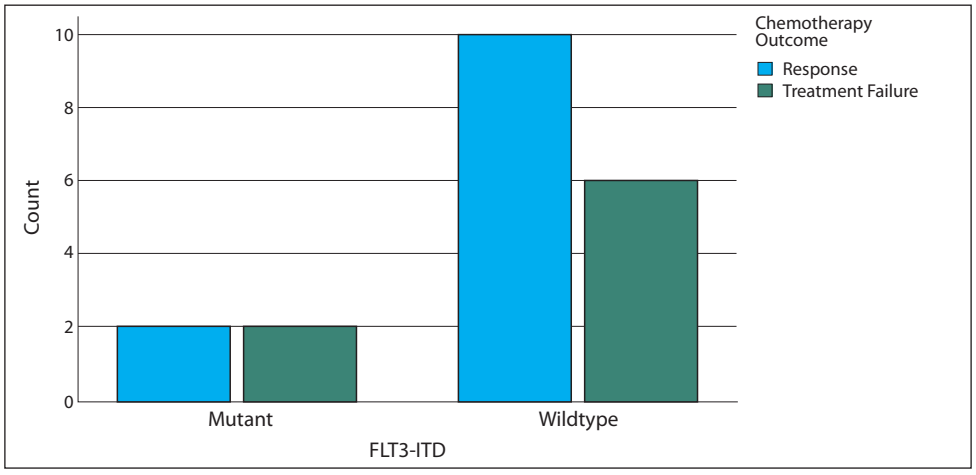

Figure 4. Comparison of the number of mutant and wildtype FLT3-ITD alleles in AML patients with chemotherapy D3A7 outcome using Fisher's exact test.

Table 1. The Results of Mann Whitney Test of the Variables PI3K, AKT, Early-Stage Cell Apoptosis, Late-Stage Cell Apoptosis, Proliferation Cells, CD34+CD38-CD123+, and ALDH1 in AML Patients with and without FLT3-ITD Gene Mutation

Variable	Wild-Type (N=16)	Mutant ITD (N=4)	P-value*
PI3K ratio mean ± SD	0.10±0.09	0.47±0.11	0.003*
AKT ratio mean ± SD	0.12±0.09	0.43±0.19	0.009*
Early-stage cell apoptosis (%) mean ± SD	3.66±5.64	18.35±23.65	0.256
Late-stage cell apoptosis (%) mean ± SD	87.46±20.70	51.62±39.72	0.023*
Proliferation cells (%) mean ± SD	24.56±12.33	32.67±21.80	0.126
CD34+CD38-CD123+ (%) mean ± SD	12.21±9.31	12.62±4.87	0.084
ALDH1 (%) mean ± SD	0.18±0.18	0.43±0.65	0.957

Mann Whitney test, *P-value < 0.05.

Table 2. The Results of Mann Whitney Test of the Variables PI3K, AKT, Early-Stage Cell Apoptosis, Late-Stage Cell Apoptosis, Proliferation Cells, CD34+CD38-CD123+, and ALDH1 in AML Patients with Outcome of D3A7 Chemotherapy

Variable	Response (N=8)	Treatment Failure (N=12)	P-value*
PI3K ratio mean±SD	0.11±0.15	0.28±0.17	0.016*
AKT ratio mean±SD	0.10±0.13	0.29±0.17	0.009*
Early-stage cell apoptosis (%) mean±SD	3.03±5.78	11.97±17.30	0.189
Late-stage cell apoptosis (%) mean±SD	95.63±6.70	57.30±33.23	0.001*
Proliferation cells (%) mean±SD	19.97±14.44	35.52±8.11	0.302
CD34+CD38-CD123+ (%) mean ± SD	9.61±6.96	14.09±9.23	0.352
ALDH1 (%) mean ± SD	0.17±0.09	0.33±0.49	0.571

Mann Whitney test, *P-value < 0.05.

showed no significant differences in average values between the ITD mutant and wild type groups. The analytical data are summarized in Table 1.

In terms of chemotherapy outcomes, the group with treatment failure had higher average values of PI3K, AKT, and late-stage cell apoptosis compared to the response group, with P-values of 0.016, 0.009, and 0.001, respectively.

Other variables in Table 2 do not show any significant differences in average values between the response and treatment failure groups.

Discussion

In this study, the FLT3-ITD gene mutation was most commonly found in patients with the AML-M2 subtype, with a prevalence of 75% (three out of four AML-M2 patients). Additionally, the FLT3-ITD mutation was also present in one patient with the AML-M5 subtype. This result is similar to a study conducted in Japan and Indonesia with AML-M2 as the most frequent subtype in AML patients with FLT3-ITD gene mutation (17, 18). In contrast, in a study in Germany, AML-M5 was the most common FAB subtype in AML with FLT3-ITD gene mutation, and a study on a Thai population showed that AML-M3 was the most frequent subtype (19, 20). Furthermore, we found 60% patients had treatment failure outcome and 40% a response to D3A7 therapy in this cohort. Compared to other studies, about 85% patients had treatment failure with induction therapy (8).

In addition, we found one homozygous allele (S83) of the FLT3-ITD gene mutation and the others

were heterozygous alleles. This homozygous allele is the first reported in the population of AML patients in Indonesia. Previous studies in Indonesia have shown that only heterozygous alleles were present (8, 18, 21). FLT3-ITD is typically found in the heterozygous state, but there is evidence of a partial or complete loss of the wild-type allele in some cases. A hemizygous ITD/- genotype, present in 1% of pediatric patients and 5% of adult patients, is linked to a distinct phenotype that is associated with a significantly worse clinical outcome (22).

In this report, analysis comparative of the FLT3-ITD mutation and the outcome of D3A7 chemotherapy showed no significant difference (P=0.5) between patients with ITD mutation and without a mutation. It may be concluded that statistically, FLT3-ITD mutations have no impact on the outcome of induction chemotherapy, but the graph (Figure 4) shows the trend that patients with an FLT3-ITD mutations had a lower response to D3A7 therapy than patients without an FLT3-ITD mutation. Therefore, we paid more attention to all patients with the FLT3-ITD mutation. In contrast, all the patients with a FLT3-ITD mutation had a poor prognosis and high relapse rate. The sample S83 with 52 bp insertion and S74 with 84 bp insertion had treatment failure as their chemotherapy outcome. In sample S78, with an insertion length of 81 bp, although there was a response to induction therapy, treatment failure occurred one year after induction during consolidation treatment with high-dose cytarabine. Similarly, sample S84, with an FLT3-ITD mutation of 33 bp, showed a good response outcome in induction therapy, but experienced a relapse 12 months after therapy.

Furthermore, the survival analysis in this study showed that the survival rate of patients with the FLT3-ITD mutation 12 months after therapy was lower than patients without the FLT3-ITD mutation, with P=0.03 and HR: 6.027; 95% CI: 1.61-31.279. In other words, each month members of the group were 6.027 times more likely to die compared to the group of patients without the FLT3-ITD mutation. All the results suggest that the presence of FLT3-ITD mutation might affect the outcome of induction therapy, but also have a strong impact on the risk of relapse and survival after therapy. This cohort was similar to the findings of the studies by Grafone et al. and Liu et al. which explained that FLT3-ITD mutations were a significant independent prognostic factor that can influence outcome in terms of survival and duration of complete remission. The reports also stated that the length of base insertions in the FLT3-ITD mutation was associated with high FLT3 kinase activity. Patients with FLT3-ITD insertions >39 bp had worse overall survival and prognosis compared to patients with insertions <39 bp (22, 23). Other studies showed that the presence of an ITD in adults patients had no impact on achieving complete remission (CR) in induction therapy, but it was significantly correlated with an increased risk of relapse (RR), and reduced disease free and overall survival (OS). A systematic review by Rinaldi et.al. also presented the results that an FLT3-ITD mutation was associated with worse prognosis in adult, non-transplant patients with AML, both for overall survival and event-free survival (9).

The research conducted by Griffith et al. described that FLT3-ITD mutations longer than 15 bp in the juxtamembrane domain were known to alter the auto-inhibition conformation of the FLT3 kinase protein, thereby promoting ligand-independent FLT3 receptor dimerization, and resulting in autophosphorylation and activation of downstream signaling associated with cell proliferation and survival (23, 24). The average values of phosphorylated PI3K and AKT in this research were higher in patients with an FLT3-ITD mutation than in patients without the mutation, with a P value of 0.003 for PI3K and 0.009 for AKT. In addition, the average values of phosphorylated PI3K and AKT in the group of patients with treatment failure to D3A7 therapy were greater than in the group of patients with response. Moreover, a significant difference was also found in variable late-stage cell apoptosis, with a P value of 0.023, in patients with FLT3-ITD mutation +/- and a P value of 0.001 in relation to the patients' chemotherapy outcome. The results of this study indicated that the existence of FLT3-ITD gene mutations in AML patients had a significant role in activation

of downstream signaling, which affects the autophosphorylation of protein PI3K and AKT. The increase in phosphorylation of PI3K and AKT resulted in the higher possibility of cell survival and became a mechanism of drug resistance (25).

In the activated PI3K/AKT pathway, PI3K helps convert phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits AKT to the cell membrane, where AKT is then phosphorylated by phosphoinositide-dependent kinase-1 (PDK1) at residue Thr308 and residue Ser473 by the mTORC2 protein. The phosphorylated AKT is then in an active form and can activate mTORC1 by phosphorylating mTOR at Ser2448, which in turn phosphorylates proteins such as S6K1 (p70S6 Kinase 1) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1). Phosphorylation of 4EBP1 initiates the translation of mRNA coding for proteins such as hypoxia-inducible factor 1α (HIF-1α), Cyclin D1, and c-Myc, which can induce angiogenesis or increase cell cycle activity (12, 26, 27). FLT3-ITD mutations in leukemia cells are known to cause resistance to cytarabine (28). Jin et al. stated that myeloid K562 cells with FLT3-ITD mutations experience a reduction in ENT1 expression through an increase in HIF-1α expression. Equilibrative nucleoside transporter-1 (ENT1) is known to be a transporter protein that plays a role in the uptake of cytarabine into cells, so the decreased uptake of cytarabine due to reduced ENT1 function can lead to cells not responding to cytarabine treatment (29).

In this study, Ki-67 expression as marker of cell proliferation was not statistically significant. However, the average Ki-67 expression values in the FLT3-ITD mutation group tended to be higher than in the wild type group. This is possibly due to the insufficient sample size. Research conducted by Kubota et al. gave the same result, showing that the activity of PI3K had an important role in cell proliferation (30). PI3K/AKT signaling also induces the expression of the BCL2 protein. BCL2 is a mitochondrial membrane protein that can alter membrane permeability, thereby preventing the release of cytochrome c into the cytoplasm. This can prevent apoptosis through the post-mitochondrial caspase cascade. AKT can phosphorylate Ser136 on the BAD protein. AKT can also activate PAK1, which phosphorylates BAD at Ser112, causing BAD to detach from the Bcl-xL complex and inhibit apoptosis. Active AKT can promote cell survival by activating BCL2 and inhibiting Bax (26).

Finally, the leukemia stem cell marker variables from the ITD mutant group had higher average values compared to the wild type group. Similarly, patients with chemotherapy treatment failure outcomes had higher average values of CD34+CD38−CD123+ and ALDH1 compared to the response group, although these differences were not statistically significant. Leukemia stem cells are highly adaptive, aided by a microenvironment that supports the stemness and survival of leukemia stem cells from various chemotherapeutic agents, leading to treatment failure and relapse. The ability of self-renewal is a complex process that involves multiple signal transduction cascades that regulate the balance between self-renewal and differentiation. One important signaling pathway that plays a role in the self-renewal ability in leukemia stem cells is the phosphatidylinositol-3-kinase (PI3K)/AKT pathway, whose activation is induced by ligand-receptor tyrosine kinase (14, 31-33). The expression of ALDH1A1 is known to be influenced by the activity of the transcription factor NF-κB (15). NF-κB activity can induce the expression of miRNA223-3p, which in turn can inhibit the expression of ARID1A. The inhibition of ARID1A expression can initiate histone acetylation at the promoter of the ALDH1A1 gene (11, 34). Meanwhile, in vitro studies have found that the overexpression of FLT3 can increase NF-κB transcriptional activity through the PI3K/AKT/mTOR pathway. FLT3-ITD activation induces NF-κB activity, and FLT3 knockdown or FLT3 inhibition reduces NF-κB activity in patients with MDS and AML (35).

Limitations of the Study

The inclusion of a relatively small sample size due to the strict inclusion and exclusion criteria is one of the limitations of this study. However, this study

provides novel data that shows FLT3-ITD mutation was not associated with leukemia stem cells and new analyses of CD34+CD38−CD123+ and ALDH1.

Conclusion

The presence of an FLT3-ITD mutation might impact the outcome of D3A7 therapy and cause the risk of relapse by the autophosphorylation of the downstream proteins PI3K and AKT. FLT3-ITD mutation also had a strong effect of survival after D3A7 therapy. The FLT3-ITD mutation also affects the downstream signaling associated with survival of the cell, that becomes a resistance mechanism to D3A7 therapy. Due to its prognostic relevance and being a good factor to predict survival in AML patients, we suggest including assessment of FLT3 mutation status for all AML patients before their treatment with D3A7 therapy, as well as the recommendations of the current World Health Organization (WHO) guidelines.

What Is Already Known on This Topic:

The high rates of relapse and refractoriness in relation to D3A7 therapy are classic issues in AML treatment. Several factors play a role in the mechanism of resistance to the D3A7 regimen, including the presence of FLT3-ITD mutations and the existence of leukemia stem cells.

Recent studies have shown FLT3-ITD mutation is associated with poor prognosis and the time of relapse, but the association with the survival of patients is still controversial. It also known that it had no impact on achieving complete remission (CR) with induction therapy but it is significantly correlated with increased risk of relapse (RR), and reduced disease-free time and overall survival (OS). Other studies explained that leukemia stem cells (LSCs) were typically regarded as resistant to chemotherapy, making them the primary instigators of relapse.

What This Study Adds:

This cohort study presents the important role of FLT3-ITD mutation via its downstream signaling (PI3K/AKT) in the outcome of D3A7 induction therapy, demonstrated by the number of cells undergoing apoptosis. The results also give the new insight that the FLT3-ITD mutation plays an important role in 12 month survival of AML patients after D3A7 therapy. However, the outcome of D3A7 therapy and FLT3-ITD mutation are not associated with leukemia stem cells.

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