RESEARCH ARTICLE

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Unraveling Copy Number Alterations in Pediatric B-Cell Acute Lymphoblastic Leukemia: Correlation with Induction Phase Remission Using MLPA

Mururul Aisyi[1]*, Murti Andriastuti[2], Agus Susanto Kosasih[3], Ahmad Rusdan Handoyo Utomo[4], Fahreza Saputra[5], Teny Tjitra Sari[2], Hikari Ambara Sjakti[2], Fifi Dwijayanti[6], Kuntjoro Harimurti[7], Djajadiman Gatot[2]

Abstract

Objective: Acute Lymphoblastic Leukemia (ALL) is the most common malignancy occurring in children. Copy number alterations (CNA) like *PAX5, CDKN2A/2B, PAR1* Region, *ETV6, IKZF1, BTG1*, and *RB1* gene deletion are important genetic events that define and prognosticate B-cell ALL. Thus, this study aimed to evaluate associations of CNA with induction phase remission status in childhood B-cell ALL. **Methods:** This study was observational with a cross-sectional design at the Dharmais Cancer Hospital, Harapan Kita Mother and Children Hospital, and Tangerang Regional Public Hospital. We evaluated 74 pediatric B-cell ALL cases with 1-18-year-olds. Genomic DNA was analyzed by Multiplex Ligation Dependent Probe Amplification Assay (MLPA). This study used the *P335* ALL-IKZF1 panel kit, which contains several ALL-related genes. The patient's clinical and laboratory characteristics were collected from medical records from January to December 2019. **Result:** We observed gene copy number alteration in children with B-Cell ALL. *PAX5* was the most commonly observed gene deletion, followed by *CDKN21/2B, ETV6, IKZF1, BTG1*, *RB1*, and *PAR1* Region. Based on gene mutations, only the *PAX5* had a significant association with the remission status of pediatric B-cell ALL (p-value <0.05; OR = 3.91). It showed that patients with *PAX5* gene mutations have 3.9 times the risk of no remission and/or relapse compared to those without *PAX5* gene mutations. **Conclusion:** Patients with mutations in the PAX5 gene have a higher chance of not achieving remission and/or experiencing relapse than those without such mutations. The MLPA method can be utilized for examining copy number alterations, which is valuable for achieving more precise stratification in diagnosis.. Further research is needed to expand upon this finding.

Keywords: Acute Lymphoblastic Leukemia- Pediatrics- CNA- MLPA

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Introduction

Pediatric cancer is a growing global public health concern, with cases increasing each year [1]. While the incidence of cancer in children is relatively lower compared to that in adults, it remains the leading cause of disease-related deaths among children. The most common type of childhood cancer is Acute Lymphoblastic Leukemia (ALL), with an annual incidence rate of 3.5 per 100,000 children [2].

Acute Lymphoblastic Leukemia (ALL), which is the most common cancer in children, accounts for approximately 25% of all pediatric cancers. The overall 5-year survival rate for this disease is currently over 80% in developed countries due to risk-directed treatment and better supportive care [3-5]. Risk-directed treatment involves two main components: first, identifying the genetic alterations in leukemic cells at the time of diagnosis, and second, evaluating the initial treatment response by measuring minimal residual disease (MRD) after induction therapy. The interpretation of MRD levels varies depending on the ALL subtype [6 7].

During diagnosis, primary genetic abnormalities can be detected using karyotype and Fluorescence in Situ Hybridization (FISH) to determine the severity of the disease and decide on treatment options. However, due

*[1]Department of Pediatric Hematology-Oncology, Dharmais Cancer Center Hospital. Letjen S Parman Street Kav 84-86, Jakarta, 11420, Indonesia. [2]Department of Child Health, Faculty of Medicine Universitas Indonesia, Cipto Mangunkusumo Hospital, Jakarta, Indonesia. [3]Department of Clinical Pathology, Dharmais Cancer Hospital, Jakarta, Indonesia. [4]Graduate School of Biomedical Science, Universitas YARSI, Jakarta, Indonesia. [5]Research and Development Department, Dharmais National Cancer Center Hospital, Jakarta, Indonesia. [6]Department of Research and Development, Dharmais National Cancer Hospital, Jakarta, Indonesia. [7]Department of Internal Medicine, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital, Jakarta, Indonesia.*For Correspondence: aci97nabil@yahoo.com*

to the limited number of mitotic cells in bone marrow samples or the small specimen volume, almost 30% of ALL patients require accurate cytogenetic test results. This highlights the requirement for novel diagnostic methods as an alternative [8].

Multiplex Ligation-dependent Probe Amplification (MLPA) has gained popularity as a tool for diagnosing various genetic diseases, including cancer. It is a cost-effective and efficient method for detecting large genomic rearrangements in patients. The technique is beneficial for identifying deletion and duplication patterns in genes linked with childhood acute lymphoblastic leukemia, making it a valuable tool for stratifying this disease in children [9]. Multiplex Ligation-dependent Probe Amplification (MLPA) is a sensitive method based on multiplex polymerase chain reaction and capillary electrophoresis that detects multiple copies of about 50 different genomic DNA targets. It has the advantages of lower cost and faster turnaround time than DNA sequences for identifying significant genetic changes. It is now widely used to detect copy number changes in ALL patients [10, 11]. Various research studies [9-11] have pointed out that MLPA is an effective technique for identifying mutations and potential biomarkers of B-cell Acute Lymphoblastic leukemia (ALL) in children. Consequently, this study aimed to investigate the relationship between Copy Number Alterations (CNA) and the remission status during the induction phase in B-cell ALL among children.

Materials and Methods

Study Design and Patients

This research is an observational study with a cross-sectional design. ALL cases of Acute Lymphoblastic Leukaemia (ALL) were collected from several hospitals, including Dharmais Cancer Hospital, Harapan Kita Mother and Children Hospital, and Tangerang Regional Public Hospital.. The diagnosis of leukemia is made by considering the patient's symptoms and conducting relevant tests and exams.. Supporting examinations include peripheral blood examination, bone marrow aspiration analysis (cell morphology), immunophenotyping, cytochemistry, and cytogenetics. Pediatric patients aged 1-18 years and diagnosed with ALL B-lineage were included in this study.

Immunophenotyping examination of B-cell ALL is a leukemia that expresses CD markers. The criteria for B cell ALL express at least one of the following 3 CDs, namely CD19, CD79a, and CD22. LLA PreB or common ALL expresses CD LLA B cells plus CD10 expression, immature ALL B expresses CD markers LLA B cells plus CD10 can be positive or negative and cIgM, and mature ALL B expresses CD LLA B cells cIgM or sIgM or Igκ or λ. The immature precursors of B lymphocytes are called pre-B cells and are expressed by CD19 and CD22 antigens on the cell surface [12]. All patients have been treated according to the Indonesia ALL National Protocol. The protocol categorizes the risk as either high or standard risk.

There were 74 pediatric patients included in this study with a diagnosis of Acute Lymphoblastic Leukemia (ALL) B-lineage in 2019. Patients older than 18 years were excluded from the study. The data collected are demographic, clinical, laboratory results, frequency and type of Copy Number Alteration (CNA), and gene mutations. Patient demographic data consists of age and gender. The patient's clinical data showed pallor, fever, bone pain, bleeding, lymphadenopathy, mediastinal mass, and clinical stratification. Copy Number Alteration (CNA) or gene mutation data looks at gene types of *PAX5*, *CDKN2A/2B*, *PAR1* Region, *ETV6, IKZF1, BTG1*, and *RB1.* In addition, this study also looked at the number of CNAs by age group (<5 years, 6-10 years, and >10 years).

Genomic DNA extraction

DNA was extracted from the peripheral blood of childhood patients using the QIAamp DNA mini-kit [Qiagen].

MLPA analysis

Genomic DNA was analyzed using MLPA, and some minor modifications were made. Several genes linked to ALL (*EBF1, IKZF1, JAK2, CDKN2A/B, PAX5, ETV6, BTG1, RB1, ZFY,* and *PAR1* Region (*SHOX, CRLF2, CSF2RA, IL3RA*, and *P2RY8*) were included in the P335 ALL-IKZF1 panel kit, which was employed in this study. Parallel with patient samples, three healthy samples were also included in the process as normalization data. Samples were processed by the Genetic Analyzer 3500 [Applied Biosystems] and analyzed using the Coffalyser.NET software [MRC Holland] to evaluate the copy number alteration. Peak height from each sample will be compared to healthy donors to obtain a ratio value; the cut-off ratio was set at ≥ 1.2 for duplication and ≤ 0.8 for deletion.

Statistical analysis

We performed statistical analysis to calculate relevant descriptive statistics for the patient characteristics. We used average values of descriptive methods and standard deviations for the continuous variables while we described the data with frequencies and percentages for the categorical variables. We conducted the chi-square test to identify an association between gene mutation and remission status. Additionally, we used the Mann-Whitney test to compare the laboratory results with the CNAs. All analyses were two-sided, and we set the significance level at $p < 0.05$.

Results

In 2019, from January to December, 74 pediatric B-Cell ALL patients were tested for CNA at Dharmais National Cancer Center, Harapan Kita Mother and Child Hospital, and Tangerang Hospital. Of the 74 patients, 51.4% were males, and 59.5% were aged five or younger. Patients' most common clinical characteristics were pallor (98.6%) and fever (90.5%). Moreover, 81.1% of patients were at high risk. Based on laboratory results, the average hemoglobin level was 8.75 g/dL, the average leukocyte count was 27079.47 /µL, and the average platelet count was 108660.81 /µL (Table 1).

The CNA examination results indicated that out of the 74 patients, 11 had deletions with the *PAX5* gene type

Table 1. Clinical and Laboratories Characteristics and Their Relation to Copy Number Alteration in Children with ALL

Characteristics Demographic	Total n (%)
Age	
≤ 5 years	44 (59.5%)
6 – 10 years	19 (25.7%)
≥ 10 years	11 (14.9%)
Gender	
Female	36 (48.6%)
Male	38 (51.4%)
Clinical Pallor	
No	1 (1.4%)
Yes	73 (98.6%)
Fever	
No	7 (9.5%)
Yes	67 (90.5%)
Bone Pain	
No	50 (67.6%)
Yes	24 (32.5%)
Bleeding	
No	51 (68.9%)
Yes	23 (31.1%)
Lymphadenopathy	
No	56 (75.7%)
Yes	18 (24.3)
Mediastinal mass	
No	74 (100%)
Clinical Stratification	
Standard Risk	14 (18.9%)
High Risk	60 (81.1%)
Laboratory	
Hemoglobin (g/dL) (mean ± SD*)	8.75 ± 2.99
Leukocyte (/µL) (mean ± SD*)	27079.47 ± 65352.32
Platelet (/µL) (mean ± SD*)	108660.81 ± 146016.39

*Standar deviation

(14.47%). Most of these deletions were found in exon 2-6 (2 people) and exon 7 (2 people). Additionally, only 1.32% of patients with the *PAX5* gene had duplications, while 84.21% had no *PAX5* gene abnormalities, either deletions or duplications (Table 2).

In the *CDKN2A/2B* gene type, eight patients experienced deletions (10.53%), with the most deletions being exon AB (7 people). Meanwhile, 1.32% of patients with the *CDKN2A/2B* gene had duplications, and 88.16% had no *CDKN2A/2B* gene abnormalities, either deletions or duplications (Table 2). In the *PAR1* Region gene type, three patients had deletions (3.49%), where the most deletions were exons *SHOX, CRLF2, CSF2RA, IL3RA*, and *P2RY8* (2 people). Meanwhile, 6.98% of patients with the *PAR1* Region gene had duplications, and 89.53% had no abnormalities in the *PAR1* Region gene, either deletions or duplications. The *ETV6, IKZF1, BTG1*, and *RB1* gene types have deletions below 10% and do not experience duplication. More than 90% of patients without ETV6, *IKZF1, BTG1*, and *RB1* gene abnormalities (Table 2). The distribution of CNAs by age group showed that the distribution of 3 or more CNAs was most significant in the B-Cell of ALL patients aged <5 years, as well as the number of CNAs, 1 CNA, and 2 CNAs (Table 3).

No relationship exists between the type and number of CNAs and the remission status of B-Cell ALL patients in children. Based on gene mutations, only the *PAX5*gene mutation had a significant association with the remission status of ALL patients in children (p-value <0.05; OR = 3.91). Patients with *PAX5* gene mutations have a 3.9 times higher risk of not remission or relapse than those without *PAX5* gene mutations (Table 4).

Discussion

Acute lymphocytic leukemia (ALL) is a malignancy of B or T lymphoblasts characterized by uncontrolled proliferation of abnormal, immature lymphocytes and their progenitors, which ultimately leads to the replacement of bone marrow elements and other lymphoid organs, resulting in a characteristic disease pattern. Patients typically present with symptoms related to anemia, thrombocytopenia, and neutropenia due to the replacement of the bone marrow with the tumor. Symptoms include fatigue, straightforward or spontaneous bruising and bleeding, and infections. Additionally, B-symptoms, such as fever, night sweats, and unintentional weight loss, are often present but may be mild, and hepatomegaly,

Table 2. Frequency and Type of Copy Number Alterations

Genes	Deletion		Duplication		Normal
	Exon number (patient with deleted genes)	N (%)	Exon number (patient with duplicated genes)	N (%)	N (%)
PAX5	1-10(2), 1-6(1), 2-6(2), 6-7(1), 7(2), 8(1), 10(1), 1(1)	11 (14.47)	1-10(1)	1 (1.32)	64 (84.21)
CDKN2A/2B	AB(7), B(1)	8 (10.53)	AB(1)	1 (1.32)	67 (88.16)
PAR1 Region	SHOX, CRLF2, CSF2RA, IL3RA, P2RY8 (2), CSF2RA, IL3RA, P2RY8(1)	3 (3.49)	SHOX, CRLF2, CSF2RA, IL3RA, P2RY8 (6)	6 (6.98)	77 (89,53)
ETV6	1-8(4), 5-8(1), 5(1)	6 (8.11)	-	0 (0.00)	68 (91.89)
IKZF1	1-8 (1), 2-8(2), 3-8(1), 4-7(1)	5 (6.76)	-	0 (0.00)	69 (93.24)
BTG1	1(1), 2(1), 2-3(1), 2-4(1)	4 (5.41)	-	0 (0.00)	70 (94.59)
RB1	6-26(3)	3 (4.05)	-	0 (0.00)	71 (95.95)

Table 3. Clinical and Laboratories Characteristics and Their Relation to Copy Number Alteration (CNA) in Children with ALL

No. of CNAs	<5 year (N= 44), n (%)	6-10 years (N= 19), n (%)	>10 years (N= 11), n (%)
3 or more CNAs	6 (60.0%)	2 (20.0%)	2 (20.0%)
2 CNAs	9 (69.2%)	4 (30.8%)	0 (0.0%)
1 CNA	4 (66.7%)	1 (16.7%)	1 (16.7%)
No CNA	25 (55.6%)	12 (26.7%)	8 (17.8%)

Table 4. Association of Copy Number Alterations and Gene Mutation with Remission Status

Variables	Remission Status		OR
	No Remission and relapse N (%)	Remission N (%)	
CNA			
Negative	19 (39.6%)	29 (60.4%)	1,048
Positive	10 (38.5%)	16 (61.5%)	
No. of CNAs			
3 or more CNAs	5 (50.0%)	5 (50.0%)	0.890
2 CNAs	5 (38.5%)	8 (61.5%)	
1 CNA	2 (33.3%)	4 (66.7%)	
No CNA	17 (37.8%)	28 (62.2%)	
Gene mutation PAX5			
Yes	8 (66.7%)	4 (33.3%)	3.906
No	21 (33.9%)	41 (66.1%)	
CDKN2A/2B			
No	23 (35.4%)	42 (64.6%)	0.274
Yes	6 (66.7%)	3 (33.3%)	
PAR1 Region			
No	27 (41.5%)	38 (58.5%)	2.487
Yes	2 (22.2%)	7 (77.8%)	
ETV6			
No	27 (39.7%)	41 (60.3%)	1.317
Yes	2 (33.3%)	4 (66.7%)	
IKZF1			
No	26 (37.7%)	43 (62.3%)	0.403
Yes	3 (60.0%)	2 (40.0%)	
BTG1			
No	27 (38.6%)	43 (61.4%)	0.628
Yes	2 (50.0%)	2 (50.0%)	
RB1			
No	29 (40.8%)	42 (59.2%)	0.275
Yes	0 (0.0%)	3 (100.0%)	

splenomegaly, and lymphadenopathy can be seen in up to half of adults on the presentation [13].

The present retrospective analysis was initiated to comprehensively investigate and analyze the prognostic role of CNAs in our cohort of pediatric B-ALL cases. The role of genetics in B-ALL has received much attention. The first report of genome-wide analysis in childhood ALL, integrating cytosine methylation profiling and DNA copy number alterations (CNA), was in 2013 [14]. Genomic assessments have identified numerous novel copy-number alterations (CNAs) that typically affect genes involved in lymphoid differentiation, proliferation, cell cycle regulation, and transcription [15]. Copy number alterations (CNAs) are constantly gaining relevance as potential risk stratification markers [16]. In contrast to cytogenetic chromosomal translocations, commonly initiating events, these CNAs usually cooperate with genomic aberrations that correlate with specific genomic subtypes and influence the ultimate patient outcome [15, 17].

Research in Mexico (2018) shows exciting findings in this study of over 200 children with a confirmed diagnosis of ALL. More frequently expected Complete Blood Count (CBC) findings, consisting of leucocytosis plus cytopenia, were found in only about 1 in 4 cases (27%); interestingly, anemia, present in 83% of the current cohort, was one of the most frequent CBC abnormalities. This was the same percentage as for thrombocytopenia, although in different patients. Leucocytosis, alone or combined with another abnormality, was considerably less frequent in only 37% of the children and almost the same as its opposite, leukopenia, found in 36.5% of the cases [18].

Researchers have described several *PAX5*-fusions so far, and these fusions involve various genes that encode proteins with diverse functions in signaling, transcription, chromatin remodeling, and cell structuring [19]. *PAX5* (9p13), a paired box gene family member, is a transcription factor necessary for normal hematopoietic development [20]. In childhood B-ALL, mutations in *PAX5* have been detected in 32% of cases by genome-wide analysis [21, 22]. *PAX5* rearrangements occur with an incidence of about 2.5%, with numerous reported rearrangements, including ETV6 (12p13) and *JAK2* (9p24) [23]. Recently, a heterozygous germline *PAX5* variant, c.547G > A, encoding p.Gly183Ser, was identified in two unrelated families with autosomal dominant B-ALL, suggesting that *PAX5* mutations may play a role in the inherited susceptibility of B-ALL [24].

Despite overall treatment progress, relapsed B-ALL has a dismal prognosis with an overall survival of 30% [25]. Several genetic subtypes and aberrations are associated with high treatment failure risk, including *CDKN2A/B, ETV6*, and *IKZF1* mutations. Relapse occurs across all B-ALL subtypes, with some cases demonstrating the acquisition of additional chromosomal abnormalities over time. According to Mullighan et al. (2008), around 50% of B-ALL have CNAs in genes that regulate B-lymphoid development, especially in *PAX5* and *IKZF1* genes [24]. In this study, *PAX5* gene mutation had a significant association with the remission status of ALL

patients in children. About 33.3% of B-ALL in pediatrics have the mutation *PAX5* and are in remission.

The transcription factor paired-box domain 5 (*PAX5*) located on the 9p13 chromosome is a crucial regulator of the early stages of B cell development [26]. At the molecular level, *PAX5* induces B-cell differentiation by activating B-cell-specific genes, crucial components of the pre-BCR signaling pathway [27]. Additionally, it is responsible for inhibiting progress toward other cell lineages through PD-1 and NOTCH1 transcription factors' negative regulation and M-CSFR inhibition [28]. Any change in *PAX5* expression, limited to B-cells only, can lead to leukemogenesis and trigger malignancy [29].

The *PAX5* gene constitutes the most critical target of somatic mutations in BCP-ALL in children, with its mutation being considered one of the most common genetic lesions in the B-ALL [30]. *PAX5* alterations include DNA copy number variations (CNVs), sequence mutations, and chromosomal translocations with an incidence of 30% in the case of CNVs, 5–9% in non-silence sequence mutations, 5–7% in children, and 2–4% in adults with chromosomal translocations [31-34].

Identification of ALL biomarkers and a better understanding their molecular basis may lead to better monitoring of the disease course. In-depth identification of the genetic aberrations in these neoplasms is essential for assessing the risk of recurrence and implementing molecular-targeted therapy to reduce this risk [35]. A more accurate risk calculation will allow for better treatment of ALL with fewer side effects [36]. However, extensive screening for genetic susceptibility to leukemia is not recommended because of the potentially significant false predictive value. Many children with genetic variants peculiar to ALL will never develop it [37].

Based on gene mutation, only *PAX5* had a significant association with the remission status of children with B-Cell ALL. Patients with *PAX5* gene mutation have a higher chance of no remission or relapse than those without *PAX5* gene mutations. *PAX5*gene mutations often occur in leukemia patients, so more appropriate protocols are needed. The MLPA method can be used for CNA examination, which is helpful in seeing more precise stratification in diagnosis. Further research is required to investigate the other factor of remission status in childhood B-cell ALL.

Author Contribution Statement

The authors declare that there are no specific contributions to report for this manuscript.

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Approval

No approvals were required for the research conducted or data used in this manuscript.

Ethical Declaration

The ethical aspects of this research were carefully considered and addressed. The Dharmais Cancer Center Hospital No.244/KEPK/X/2022 and Faculty of Medicine Universitas Indonesia KET-1201/UN2.F1/ETIK/PPM.00.02/2022 approved all human participant procedures. Informed consent was obtained from all participants before their involvement in the study. Additionally, appropriate measures were taken to ensure the confidentiality and anonymity of participants' data.

Study Registration

This study was not registered in any registering dataset for clinical trials, guidelines, or meta-analysis.

Conflict of Interest

The authors declare no conflict of interest.

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