ORIGINAL ARTICLE

Caspase 3 Expressions in Children with Acute Lymphoblastic Leukemia During Induction Phase Chemotherapy

Lukman Oktadianto¹, Mia Ratwita Andarsini¹, I Dewa Gede Ugrasena¹, Yetti Hernaningsih², Andi Cahyadi¹, Maria Christina Shanty Larasati¹

¹ Hematology-Oncology Division, Department of Pediatrics, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Academic Hospital, Surabaya 60285, Indonesia; ² Department of Clinical Pathology, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Academic Hospital, Surabaya 60285, Indonesia.

ABSTRACT

Introduction: Caspase-3 is a crucial mediator of the extrinsic apoptosis pathway. The role of caspase-3 for extrinsic apoptosis signalling is still a challenge and should be exploited in childhood ALL. This study aimed to compare the caspase-3 expression in the patient’s bone marrow before and after the induction phase of chemotherapy in childhood ALL. It will also to correlate the mean difference in caspase-3 expression between ALL standard-risk and ALL high-risk patients.

Methods: Seventeen newly diagnosed ALL subjects were enrolled in this study. Caspase-3 expression in bone marrow was assessed using flow cytometry and monoclonal antibodies. A T-test and a paired T-test were used to compare between groups. The correlation coefficient between ALL groups was evaluated using Spearman’s test and linear regression with a significant p-value of 0.05. Results: The caspase-3 expression is higher after induction therapy. However, it showed an insignificant difference (16.56±12.91% vs 27.71±12.33%; p = 0.08, p > 0.05). The mean difference of caspase-3 in ALL high-risk groups was significantly higher than in ALL standard-risk groups with a positive correlation (p = 0.007, r = 0.756). Conclusion: The caspase-3 expression after induction phase chemotherapy was increased in all standard-risk and high-risk patients; other lymphoblast apoptosis markers need to be confirmed alongside caspase-3.
and has not been well studied in ALL (13, 14). This study aimed to compare caspase-3 expression before and after receiving induction chemotherapy in pediatric ALL.

MATERIALS AND METHODS

This study was carried out in children with newly diagnosed childhood ALL who were admitted to the Pediatric Hematology and Oncology Department and were scheduled to receive the 2018 Indonesian ALL Chemotherapy Protocol. The diagnosis of ALL was based on morphological examination of bone marrow aspiration based on the French-American-British (FAB) classification and immunophenotyping (B-ALL and T-ALL). Caspase-3 expression was evaluated using flow cytometry and immunophenotyping at first diagnosis. The bone marrow specimens that met the inclusion and exclusion criteria were further divided into groups. The inclusion criteria include 1) children aged 1-18 years with newly diagnosed ALL; 2) the legal guardian’s approved and signed informed consent; and 3) patients who have completed the chemotherapy induction session. The exclusion criteria are 1) ALL L3 and 2) parents/guardians withdrawing from participating in the study. The drop-out criteria consist of 1) patients who have not completed the chemotherapy induction session and 2) incomplete research data.

The patients were divided into the following group based on stratified risk. The standard-risk group consists of ages 1 to 10 years with an initial leucocyte count of less than 50x10⁹/l; immunophenotype of B-ALL; absence of mediastinal mass, and no blasts identified in the CSF (15). The patients excluded from the standard-risk group belong to high-risk ALL.

This study is a prospective design conducted from March to June 2021. It has been approved by the ethical committee, reference number 0116/LOE/301.4.2/IIX/2020. Patient data were collected through anamnesis, physical examination, routine chemical investigations, complete blood count, morphological bone marrow examination, and immunophenotyping. A bone marrow aspiration was performed during the assessment to establish the ALL diagnosis and measure the caspase-3 expression before induction chemotherapy. These patients were followed up during induction therapy until remission or death for six months in 2021. A bone marrow aspiration was performed after the completion of induction chemotherapy. Caspase-3 expression was measured in 11 patients before and after chemotherapy induction using flow cytometry.

Bone marrow mononuclear cells (BMNCs) were isolated by Ficoll-Hypaque density gradient centrifuge. Intracellular staining of protein was performed according to the manufacturer’s instructions. Phycoerythrin (PE) labeled anti-caspase-3 monoclonal antibodies were provided by BD Pharmingen (catalog #: C92-605-RUO). Flow cytometry analyzed all labeled cells using the FACS Calibur instrument (Becton Dickinson) and the Cell Quest software package (Becton Dickinson). The percentage of caspase-3 was determined using a flow cytometry gating strategy.

Statistical analysis was carried out using the IBM SPSS statistic software version 25.0. The normality of data was tested using the Shapiro-Wilk test. T-test and paired T-test were used to compare the groups. Spearman correlation test was used to determine the relationship between variables. Qualitative data were presented as frequency and percentage. The mean and standard deviation were used to present quantitative data. The result of the statistical examination is significant if p<0.05.

RESULTS

There were 37 children with suspicion of acute leukemia. A total of 19 children were excluded from this study; three children showed a hypoplastic marrow, seven children with myeloid lineage, and one child did not express a blast; eight children had no complete caspase-3 expression because the bone marrow aspiration sample volume was insufficient for the complete analysis. One child did not have parental consent (dropped out). Seventeen children had initial data for caspase-3 expression, and six children died during the induction phase of chemotherapy. Eleven children were thoroughly examined for the initial and final caspase-3 until the induction phase of chemotherapy was completed.

The characteristics of the primary data in 17 subjects found more boys than girls in a ratio of 15:2. The average age of the subjects was 7.56 ± 3.12 years which are 82.4% of children aged 1 to 10 years old. Statistical analysis showed no significant differences between sex, age, hemoglobin level, and leucocyte count in all groups. The baseline characteristics of the patients in this study are shown in Table I.

Seventeen children received induction phase chemotherapy, and only 11 children were able to complete the induction phase chemotherapy, while six children died while undergoing induction phase chemotherapy. Three children died of sepsis, and three children died of pneumonia, tumor lysis syndrome, and bleeding. Eleven subjects who completed the induction phase of chemotherapy showed complete remission and had less than 5% lymphoblast count in their bone marrow aspirates. The mean expression of caspase-3 before chemotherapy on survival was lower than the death outcome. However, the caspase-3 expression between the two groups of survival and death showed no significant differences (Table II).

Table III shows that the caspase-3 expression was higher
Table I: Patient characteristics at initial diagnosis based on chemotherapy induction phase outcome

<table>
<thead>
<tr>
<th>Characteristics (n=17)</th>
<th>Live (n=11 (64.7))</th>
<th>Died (n=6 (35.3))</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9 (52.9)</td>
<td>6 (35.3)</td>
<td>0.515*</td>
</tr>
<tr>
<td>Female</td>
<td>2 (11.8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-10</td>
<td>10 (58.8)</td>
<td>6 (35.3)</td>
<td>1.000*</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>2 (11.8)</td>
<td>1 (5.9)</td>
<td></td>
</tr>
<tr>
<td>Sign and symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>10 (58.8)</td>
<td>6 (35.3)</td>
<td>1.000*</td>
</tr>
<tr>
<td>Anemia</td>
<td>8 (47.1)</td>
<td>6 (35.3)</td>
<td>0.515*</td>
</tr>
<tr>
<td>Bone pain</td>
<td>10 (58.8)</td>
<td>4 (23.5)</td>
<td>0.515*</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>8 (47.1)</td>
<td>5 (29.4)</td>
<td>1.000*</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>2 (11.8)</td>
<td>4 (23.5)</td>
<td>0.109*</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>1 (5.9)</td>
<td>3 (17.6)</td>
<td>0.099*</td>
</tr>
<tr>
<td>FAB classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL L1</td>
<td>10 (58.8)</td>
<td>6 (35.3)</td>
<td>1.000*</td>
</tr>
<tr>
<td>ALL L2</td>
<td>1 (5.9)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Immunophenotyping result</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-ALL</td>
<td>8 (47.1)</td>
<td>5 (29.4)</td>
<td>1.000*</td>
</tr>
<tr>
<td>T-ALL</td>
<td>3 (17.6)</td>
<td>1 (5.9)</td>
<td></td>
</tr>
<tr>
<td>Risk stratification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard-risk</td>
<td>5 (29.4)</td>
<td>5 (29.4)</td>
<td>0.304*</td>
</tr>
<tr>
<td>High-risk</td>
<td>6 (35.3)</td>
<td>1 (5.9)</td>
<td></td>
</tr>
</tbody>
</table>

*Fisher exact test

Table II: The caspase-3 expression difference before induction phase chemotherapy

<table>
<thead>
<tr>
<th>Caspase 3</th>
<th>n (%)</th>
<th>Mean (SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive</td>
<td>11 (64.7)</td>
<td>16.56 (12.91)</td>
<td>0.491</td>
</tr>
<tr>
<td>Died</td>
<td>6 (35.3)</td>
<td>22.57 (22.58)</td>
<td></td>
</tr>
</tbody>
</table>

The statistical test used was the paired T-test.

Table III: Caspase-3 expression before and after induction phase chemotherapy

<table>
<thead>
<tr>
<th>Caspase-3</th>
<th>n (%)</th>
<th>Mean (SD)</th>
<th>Range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before chemotherapy</td>
<td>11 (100)</td>
<td>16.56 (12.91)</td>
<td>2.24-19.53</td>
<td>0.08</td>
</tr>
<tr>
<td>After chemotherapy</td>
<td>11 (100)</td>
<td>27.71 (12.33)</td>
<td>14.43-50.71</td>
<td></td>
</tr>
</tbody>
</table>

The statistical test used was the paired T-test.

Table IV: The caspase-3 expression difference between the stratified risk groups before and after initial chemotherapy

<table>
<thead>
<tr>
<th>Caspase-3</th>
<th>n (%)</th>
<th>Before Mean (SD)</th>
<th>After Mean (SD)</th>
<th>Delta Mean (SD)</th>
<th>p</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard-risk ALL</td>
<td>5 (45.5)</td>
<td>22.14 (6.99)</td>
<td>24.29 (18.99)</td>
<td>4.09 (15.26)</td>
<td>0.007</td>
<td>0.756</td>
</tr>
<tr>
<td>High-risk ALL</td>
<td>6 (54.5)</td>
<td>10.66 (7.63)</td>
<td>32.35 (14.43)</td>
<td>23.66 (11.55)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The statistical test used was the paired T-test and Spearman correlation test.

DISCUSSION

The characteristics data of 17 subjects found more males than females, with a ratio of 1:5.1. The percentage of the age group 1 to 10 years is higher than over 10 years old. ALL accounts for 25% of cancer diagnoses in children and the peak incidence occurs at the age of 2-5 years (16,17). Aisyi et al. (18) reported that 94 ALL children at Dharmais Hospital from January 2016 to December 2018 was male sex, with percentage of 60.6%, with a median age of 5 (1-8) years. Kakaje et al. (19) reported that the highest incidence of ALL occurred at the age of 5-9 years. Mairuhu et al. (20) also reported that there are more males than females with a ratio of 1.5:1. The molecular mechanisms underlying the sex prevalence of cancer are still unclear. Several genome-wide association studies (GWAS) attempted to identify male sex-specific genetic markers on the risk of childhood ALL (21). Healy et al. (22) reported that there was a statistically significant correlation between the specific effects of 2 SNPs on the ARID5B gene (AT-rich interactive domain 5b) and childhood acute lymphoblastic leukemia (rs10994982 P=0.01) and rs10740055 (P=0.03). The risk of ALL in boys on SNPs was rs10994982 with OR=3.79 and rs10740055 with an OR of 4.35 compared to girls. Gender differences in cancer prevalence arise from a complex combination of environmental, genetic, and epigenetic factors, as well as differences in gene regulation and expression (23).

In our study, most patients presented with fever, pale skin, and hepatomegaly. ALL patients’ common signs and symptoms are fever (65.1-75.5%), anemia (42.8-79.2%), and bone pain (39.6%) (24). On physical examination, lymphadenopathy (62.6-69.2%), splenomegaly (59.4-60.8%), and hepatomegaly (50.1-59.5%) occur in most cases (25).

Approximately 35.3% of children with ALL die during the induction phase of chemotherapy. It consist of three deaths due to sepsis and the others due to pneumonia, tumor lysis syndrome, and hemorrhage. Kim Hao et al. (26) found that the mortality rate of pediatric ALL patients was 31.1%. Deaths were most frequent in the chemotherapy phase at 35.1% in maintenance phase, the induction phase at 24.3%, and 12.2% in the intensified phase. The most frequent cause of death is infection by 43.2%, bleeding 8.1%, and hyperleukocytosis by 1.35%. Another study in Islamabad stated that 20.8% of child ALL deaths occurred in the induction phase. The cause of death was predominantly bleeding 50%, 40% due to infection or sepsis, and 1% due to tumor lysis...
syndrome. Appropriate supportive therapy, availability of blood components, and a fine selection of antibiotics lower the risk of child ALL death in Islamabad (27).

Expression of caspase-3 before chemotherapy on survival was lower than in death subjects (16.56±12.91 vs. 22.57±22.58), p<0.05). Huang et al. (28) reported that low caspase-3 expression was associated with better disease-specific survival in patients in the early pathological phase compared to high expression. Caspase-3 could be used as a biomarker in tumorigenesis and a prognostic biomarker for specific stages in cancer patients.

The caspase-3 expression after induction phase chemotherapy was higher than before chemotherapy (16.56±12.91 vs. 27.7±12.33) yet statistically non-significant (p=0.08, p>0.05). Gebyaran et al. (2013) reported different results that the caspase-3 expression after chemotherapy was significantly elevated (p=0.03) in 12 children with ALL (29).

The expression of caspase-3 could cause the difference in the result in our study as the main effector of caspase of apoptosis in normal mammalian cells. The expression of caspase-3 was also found to increase the proliferation and differentiation of keratinocytes, B cells, myoblasts, osteoblasts, erythroblasts, and platelets. Another factor affecting the result of caspase-3 expression in our study is the difference in the time of examination of each sample after induction phase chemotherapy. Failure to detect increased expression may also be due to the half-life of caspase-3 in the circulation of blood (30).

Several studies reported the increasing activity level of caspase-3 after chemotherapeutic agent administration in ALL. Fortney et al. (31) reported that levels of caspase-3 increased up to 12 times in 8 hours after administration of cytarabine and etoposide in leukemia B cells (JML1). A specific caspase-3 inhibitor (DEVDFMK) was administered to confirm the role of the caspase cascade. After 48 hours of DEVDFMK administration, there was significant leukemic B cell viability compared with no caspase inhibitor administration. The decrease in the effectiveness of chemotherapy by cytarabine and etoposide suggests that the caspase cascade plays a vital role in the apoptosis in chemotherapy. Kuttikrishnan et al. (32) reported that caspase-3 expression increased after 24 hours of administration of chemotherapeutic agents. The administration of pan-caspase inhibitor (z-VADfmk) showed a decrease in cells undergoing DNA degradation using the Comet assay examination, which indicates that the caspase pathway cascade primarily mediates apoptosis due to the administration of cytostatic agents. Xia et al. (33) reported an increase in caspase-3 expression in an in vitro study using the anti-tumor ginsenoside Rh2(GRh2) in cultured B cells and T ALL cells. Mendivil-Perez et al. (34), in an ex vivo study, reported that pro-oxidant agents significantly induce apoptosis in CD34+/CD19+-expressing ALL B cells. Pro-oxidants significantly increase pro-apoptotic proteins, one of which is caspase-3 as an active indicator of the apoptotic pathway.

Another in vitro study reported that administration of anticancer agents to ALL T cells (Jurkat and Molt4) showed an increase in cell apoptosis up to 79-83% expressed by apoptotic markers (Annexin V-FITC/7-AAD). The increase in apoptosis is linear to the elevated level of caspase-3 activity, and ALL T cells stop growing in the cell cycle’s S and G0/G1 phases (35). Fu (36) reported that after administration of Lithospermum erythrorhizon extract as a cytotoxic agent on Human leukemic lymphoblast (CCRF-CEM cell line) cells resulted in inhibition of cell viability and downregulation of CDK2, CDK4, CDK6, cyclin D1, and cyclin E1 but also increased caspase-3 expression and apoptosis.

There is an elevated CD95 (FAS) level, caspases-3, 8, and 9 after 48 hours of anti-tumor administration in leukemic cell cultures (37). A similar result was reported by Lonetti et al. (38) in an in vitro study that showed the cytotoxic effect of nelarabine on chemotherapy-sensitive ALL T cells (Jurkat and MOLT4) was correlated with the induction of apoptosis indicated by the cleavage of caspase-3 as an effector caspase. The administration of nelarabine showed apoptosis activation through the extrinsic pathway characterized by caspase-8 cleavage and intrinsic, which was characterized by caspase-9 cleavage.

Analysis of the mean difference of caspase-3 expression between the ALL stratified risk groups showed a higher trend in the standard-risk group and the high-risk group (4.1±15.26 vs. 23.86±11.55) with a statistically significant difference and a positive correlation (p=0.007, p<0.05; r=0.756). However, the clear evidence for caspase-3 expression between stratified risk groups was limited and not fully understood. Salama et al. (40) reported that caspase-3 levels in B-ALL cases showed a higher difference before and after induction chemotherapy compared to T-ALL patients (p=0.025). Gebyaran et al. (41) reported different results that the mean expression of caspase-3 after chemotherapy was higher than before chemotherapy with a statistically significant difference (p=0.03). Faderl et al. (11) suggested possibility to complete remission was significantly higher in the cut-off of caspase-3 > 0.37. Another study also stated that patients with a greater caspase-3 level after induction therapy have more remarkable survival (37). T-lymphocytes, which belong to the high-risk group, are revealed to express a lower level of caspase (39).

Fortney et al. (31) reported an in vitro study that 8 hours after administration of cytarabine and etoposide in leukemia B cells (JML1) there was an increase in caspase-3 expression up to 12 times, and it disappeared.
after 24. A specific caspase-3 inhibitor (DEVDFMK) was administered to confirm the role of the caspase cascade. After 48 hours of DVEDDFMK administration, there was significant leukemic B cell viability compared with no caspase inhibitor administration. The decrease in the effectiveness of chemotherapy by cytarabine and etoposide after administration of caspase inhibitors suggests that the caspase cascade plays a vital role in the apoptosis in chemotherapy. Another study reported that the intrinsic apoptotic pathway proapoptotic protein also increased fourfold after induction phase chemotherapy, especially in acute lymphoblastic leukemia patients who were in remission (41).

CONCLUSION

In this study, we found that caspase-3 expression before and after induction phase chemotherapy is insignificant. Meanwhile, the mean difference of caspase-3 between the stratified risk groups was significantly increased with a positive correlation, especially in high-risk groups. Due to the limited sample, short duration, and single-center study, the significance of caspase-3 and its correlation with cancer cell apoptosis and overall survival should be validated in further studies. We recommend that future research use a larger sample size, a more extended follow-up period, and collaboration with multiple centers to achieve a more remarkable result. Investigating caspase-3 during the maintenance phase of chemotherapy and other lymphoblast apoptosis markers is also essential to determine the role of caspase-3 in pediatric ALL.

ACKNOWLEDGEMENTS

The author would like to thank all the staff in the Hematology-Oncology Division, the doctors, nurses, and the administrator at the Faculty of Medicine, Universitas Airlangga/ Dr. Soetomo General Academic Hospital Surabaya for granting us the permission and necessary support to conduct our research.

REFERENCES


