p53/Surviving Ratio as a Parameter for Chemotherapy Induction Response in Children with Acute Myeloid Leukemia

Rinaldi Lenggana1, *, Susanto Nugroho2,4, Sri Winarsih3

1Biomedical Science Master Study Program, Faculty of Medicine, Brawijaya University, Malang, Indonesia
2Department of Paediatrics, Faculty of Medicine Brawijaya University, Malang, Indonesia
3Pharmacology Department, Faculty of Medicine, Brawijaya University, Malang, Indonesia
4Saiful Anwar Public Hospital, Malang, Indonesia

ABSTRACT

Acute myeloid leukemia (AML) is a malignancy that is often found in children. Many studies into the failure of apoptosis function, or programmed cell death, is one of the most important regulatory mechanisms of cellular hemostasis which is closely linked to the development of cancer, are important. Also, regulation of the apoptotic (p53) and anti-apoptotic (surviving) proteins influence treatment outcome. One role of p53 is to monitor cellular stress necessary to induce apoptosis. Surviving (BIRC5) is a group of proteins in the apoptosis inhibitor which works by inhibiting caspase-3. The role of surviving is considered very important in oncogenesis proliferation and cell growth regulation. Chemotherapy in childhood AML can inhibit cell growth and induce slowing as well as stopping the cell cycle. Thus, the aim of this study was to compare p53 and surviving before and after receiving induction chemotherapy in children with AML and also to determine the p53/surviving ratio. Peripheral blood mononuclear cells were collected from AML children before treatment and three months after starting their induction therapy. p53 and surviving were measured by flowcytometry using monoclonal antibodies. Data were analyzed by t-test for comparison between groups and Spearman’s test to find out the correlation between variables with a significant value of \( p < 0.05 \). A total of 8 children were evaluated. The intensity of p53 expression was not significantly increased after induction phase chemotherapy \( (p = 0.224) \), but surviving expression and the ratio of p53/surviving were significantly increased in the treatment group compared with the levels prior to chemotherapy \( (p = 0.002, p = 0.034) \), and there was a strong negative correlation between p53 and surviving after chemotherapy \( (r = -0.63, p = 0.049) \).

Keywords: p53, surviving, acute myeloid leukemia

INTRODUCTION

Leukemia is a malignant disease in children with the highest incidence between the ages of 2-5 years [1] and is the most malignancy in children with an incidence of 4-4.5 cases/year/100,000 [2]. In Europe, in the period between 1988 -1997, acute leukemia incidence was 22.6 per one million of which 15% was AML. In Germany, the proportion of AML was 13% while in the US, it was 16%. In Yogyakarta, the incidence of ALL was 20.8/1,000,000 while for AML it was 8/1,000,000. AML mainly occurs from 2-5 years [3]. But AML Treatment has made much progress in developed countries with success reaching 65%, whereas, in Indonesia, it is expected to remain below 10% [3].

The mechanism of apoptosis, which is a process of cell death, attempts to remove unwanted and excess cells. Thus, apoptosis is important for protection against pathogens and tissue homeostasis [4].

One role of p53 is to monitor for cellular stress and for its resulting damage which can be severe and irreparable. Tumor suppressors act to maintain tissue homeostasis and to control the number and behavior of cells.

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of cells in certain tissues and p53 is a suppressor that has been widely studied which acts in response to various forms of cellular stress and is involved in approximately 50% of human cancers [5]. The loss of p53 function causes genomic instability and increased mutagenesis, and it is thought that the mutation of p53 is one of the factors that cause cancer resistance to chemotherapy [6].

Surviving (BIRC5) is a therapeutic target because its expression occurs during the development of malignancy and its role is considered to be important both in oncogenesis and in cell proliferation and growth regulation [7]. Physiologically, surviving is detected in high levels in the fetal period, but declines as the fetus develops. When the surviving level was increased, it should be correlates with the incidence of relapse and treatment failure and the low life expectancy of patients with AML[7, 8].

The expression of surviving in solid tumors has seen many studies and in theory, there is a relationship between p53 as a pro-apoptosis and surviving as an inhibitor of apoptosis [8]. Thus, this research hoped it could be used as a reference for the ratio of p53/surviving that can be used as a prognostic marker for AML treatment in children. AML chemotherapy in children can result in DNA damage which can stimulate p53 to inhibit surviving expression [9].

Therefore, the aim of this study was to measure the ratio p53/surviving to investigate its use as a parameter in the induction phase chemotherapy response in children with acute myeloid leukemia.

MATERIALS AND METHODS

The study used a study design with a pre and post-experiment approach (cohort study). Sampling was done by consecutive sampling and this research was conducted at the Department of Pediatrics Dr. Saiful Anwar Hospital, Malang and the Biomedical Laboratory of the Faculty of Medicine, Brawijaya University. Sampling was conducted from March 2015 to August 2015, and the subjects were patients under treatment in the Department of Pediatrics Dr. Saiful Anwar Malang Hospital. The criteria for the diagnosis of AML was based on morphological analysis of bone marrow punctures. The study subjects were then divided into complete remission, partial remission and no remission groups. Informed consent was obtained from all the relevant parents.

Criteria for the inclusion of cases was: 1. AML patients, 2. post induction phase chemotherapy 3. age below 14 years, 4. informed consent from relevant parents, and the exclusion criteria were: 1. patients with drug withdrawal during the induction phase of chemotherapy and 2. autoimmune diseases, such as SLE and myasthenia gravis. This study has been approved by the Ethics Committee of Saiful Anwar Hospital.

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA blood by Ficoll-Hypaque density gradient centrifugation. Intracellular staining of protein was performed according to the manufacturer’s instructions. Phycoerythrin (PE) labeled anti-surviving monoclonal antibodies were provided by R&D System Inc. (catalog #:IC6472P), and fluorescein isothiocyanate (FITC) labeled p53 monoclonal antibodies were obtained from Santa Cruz (catalog #:sc7272FITC). Briefly, cells were incubated in the dark at 18-240C for 15 minutes. Cells were treated with a cell staining buffer in each sample and centrifuged at 500 G for 5 minutes. Then, the Cells then were fixed by fixation buffer in the dark at 18-240C for 20 minutes and centrifuged at 500 G for 5 minutes. Cell pellets were washed with permeabilization buffer. They were then incubated with a cocktail of FITC p53 and PE anti-surviving in the dark at room temperature for 30 minutes. All the labeled cells were resuspended in 0.5 mL cell staining buffer and analyzed by flowcytometry using the FACSCalibur instrument (Becton Dickinson) and the Cell Quest software package (Becton Dickinson). In this study, data analysis techniques were calculated in three stages, respectively: (1) Normality test of data samples with Shapiro-Wilk test, (2) T-test was done for comparison between groups (3) Spearman’s Test to find out the correlation between variables. Statistical analysis of data was performed by SPSS software for Windows 22.0. The data are presented as mean ± standard deviations.

RESULTS AND DISCUSSION

All research subjects had been diagnosed with AML, then the expression levels of p53 and surviving were examined. After which, the subjects were administered the appropriate chemotherapy by TRAP/COAP/POMP protocols conducted by standard operating procedures of the Hematology-oncology Department in Saiful Anwar Hospital. The induction phase was carried out over three months, and then bone marrow puncture was re-done to see the results of chemotherapy response (complete remission, partial remission or no remission). Expressions of p53 and surviving were obtained by using flowcytometry conducted at the Biomedical Laboratory, Brawijaya University. All eight children received induction phase chemotherapy
and their levels of p53 and surviving were noted and before and after the chemotherapy.

The results are shown in Table 1. Study results indicated that 50% of subjects suffered from type M4 AML, 37.5% had type M2 AML, and 12.5% suffered from type M1 AML. After being given the induction phase chemotherapy, 7 out of 8 (88%) experienced complete remission and 1 out of 8 (12%) experienced partial remission.

Based on flowcytometry figures, the expressions of p53 and surviving were analyzed and the ratio of p53/surviving calculated (Table 2). The p53 expression was processed by descriptive analysis and paired T-test. In all 8 of the research subjects, comparative values obtained were p53 -> R2 = 3.24% so that it can be interpreted that chemotherapy affects approximately 3.24% of p53 expression. The significance level of the effect of chemotherapy on p53 was p = 0.224 (p > 0.05), the expression of surviving after the induction phase chemotherapy was not significantly increased.

Comparison of the results of paired T-test, found that surviving -> R2 = 62.25%, chemotherapy affected 62.25%. The significance level of the effect of chemotherapy on surviving was p = 0.02 (p < 0.05) and the expression of surviving after the induction phase chemotherapy was significantly decreased.

A paired T-test obtained a comparative ratio of p53/surviving before and after induction phase of chemotherapy with a value of p = 0.034 (p < 0.05). i.e. the p53/surviving ratio after induction phase chemotherapy was significantly increased.

Spearman correlation test obtained a correlation coefficient of -0.63, which means that this study’s results found a strong correlation with the direction of a negative correlation: the higher the variable of the p53 expression, the lower the surviving expression. The result obtained from the Spearman correlation test indicated a significance value of p=0.049 (p <0.05), i.e. a negative one.

Based on Table 3, the study subjects showed increased levels of p53 after their chemotherapy. However, the p53 expression had increased but not significantly because one subject had decreased levels of p53 after the induction phase chemotherapy. In networks where stressors produce severe damage which can not be repaired, p53 can initiate apoptosis so as to eliminate the damaged cells [10]. Also, tumor suppressors act to maintain tissue homeostasis to control the number and behavior of cells in the body. p53 is a much-studied tumor suppressor which acts in response to various forms of cellular stress to mediate antiproliferative processes. Therefore, p53 can be activated by DNA damage, hypoxia or aberrant expression of oncogenes that drive changes in the cell cycle, DNA repair, cellular senescence, and apoptosis. By the p53’s potential role in modulating cancer chemotherapy, the loss of p53 function is associated with chemotherapy resistance in certain tumor types [11]. Thus, for a complete understanding of the apoptotic program it is hoped that research into p53 will lead to better diagnoses and thus to better prognoses as well as to improvements in therapy [10, 12].

In this study there was one sample which was not consistent with the hypothesis, decreased levels of p53 after the induction phase chemotherapy. It was probably caused by the complexity of the apoptotic pathways themselves [10]. The inhibition of proteosome can cause decreased p53 levels together with the overexpression of surviving that is mediated by MDM-2 caspase. This explains that homologous of p53 such as DNp63, Tap73, dan DNp73 in mRNA are related to surviving levels [13]. There are two main pathways of apoptosis, first, the extrinsic pathway (death receptor pathways) and second, the intrinsic pathway (mitochondrial pathways). These two pathways end with the activation of caspase (the enzyme that effects cell death) [12, 14]. Loss of p53 function related to chemotherapy resistance in several tumor types.

The stimulation of receptor death cells will result in aggregation and the recruitment of adaptor Fas-associated protein with death domain (FADD) and caspase-8. After which, the caspase-8 will be activated and start the process of apoptosis by the caspase effector [15].
The Mitochondria is involved in intrinsic pathways through stress stimulation including UV radiation, C-radiation, heat, DNA damage, oncoprotein activators, chemotherapy and p53 [13, 16]. The permeability of mitochondrial membranes depends on the stability of the activity between pro-apoptoses such as sitokrom c, Smac/Diablo, Omi/HtrA2 (caspase-dependent), AIF and Endo G (non-caspase-dependent) and anti-apoptosis protein such as Bcl2 (Bax, Bak, Bcl2 dan Bcl-XL, Mcl-1) [17]. p53 plays a role in the regulation of pro-apoptosis in the nucleus.

The initiator caspase can directly activate pro-caspase-3 or other executor caspase that result in apoptosis. Also, the initiator caspase can activate Bid, a protein that can react with the mitochondrial membrane to stimulate cytochrome-c which activates caspase-9 and caspase-3 [18].

Chemotherapy initiates apoptosis pathways both extrinsically and intrinsically, and chemotherapeutic agents which cause DNA damage activate p53 from the inhibition of MDM-2 [19]. In the extrinsic pathway, p53 enables to capture of the death receptor signal, further inducing procaspase-8 to caspase-8 and procaspase-3 into caspase-3 to trigger apoptosis. In the intrinsic pathway, the mitochondrial membrane permeability is regulated by a balance between protein proapoptosis (Bid and Bax) and antiapoptosis proteins (Bcl-2) [16] [20]. The activation of Bax, caspase-2 tBid and leads to impaired mitochondrial membrane permeability and the release of cytochrome-c which then activates the apoptotic cascade. Cytochrome-c binds to procaspase-9 and Apaf-1 to activate caspase-9. Also, inhibition of the Bcl-2 action, also causes p53 to inhibit surviving (inhibitor of apoptosis proteins) so that the process of apoptosis via caspase-9 and caspase-3 continues. Caspase-12, which is activated by the endoplasmic reticulum stress, causes direct activation of caspase-9 and then activates caspase-3 as an effector caspase [21]. A study by Hui et al., 2010, found that the presence of a positive correlation between the levels of surviving and p53 in cases of liver malignancy [22], the results of which were also similar to those of a study by Baytekin et al., 2011, in kidney malignancies (renal cell ca) which discovered high levels of surviving in cases resistant to chemotherapy [23].

Based on Table 4, all study subjects showed decreased expressions of surviving after the induction phase chemotherapy, this is significant and conforms to the hypothesis of this study. Significant levels of surviving have been found in patients with such cancers like lung, genito-urinary, stomach, colon, liver, pancreatic and soft tissue cancers [24]. An increase in the expression of surviving, as can be seen in subjects 1-7, was also an indication of the presence of anti-apoptosis mechanisms to prevent cell damage.

### Table 2. p53 and surviving expression results before and after the induction phase chemotherapy.

| Subject | p53 before chemotherapy (%) | p53 after chemotherapy (%) | Surviving before chemotherapy (%) | Surviving after chemotherapy (%) | p53/Surviving Before chemotherapy | p53/Surviving after chemotherapy |
|---------|-----------------------------|-----------------------------|-----------------------------------|----------------------------------|----------------------------------|---------------------------------|
| 1       | 56.38                       | 99.28                       | 96.16                             | 55.26                            | 0.58                             | 1.79                            |
| 2       | 62.21                       | 86.74                       | 59.41                             | 38.20                            | 1.04                             | 2.27                            |
| 3       | 45.14                       | 79.85                       | 44.23                             | 22.01                            | 1.02                             | 3.62                            |
| 4       | 56.18                       | 77.61                       | 58.20                             | 48.49                            | 0.96                             | 1.6                             |
| 5       | 60.53                       | 63.75                       | 80.45                             | 68.57                            | 0.75                             | 0.93                            |
| 6       | 72.71                       | 79.54                       | 99.86                             | 63.54                            | 0.73                             | 1.25                            |
| 7       | 99.92                       | 55.92                       | 89.04                             | 58.05                            | 1.12                             | 0.96                            |
| 8       | 24.38                       | 35.40                       | 60.20                             | 53.54                            | 0.4                              | 0.66                            |

### Table 3. p53 expression before and after induction phase chemotherapy.

| Chemotherapy | p53 expression (%) | p-value | CI 95% |
|--------------|--------------------|---------|--------|
| Before       | 59.7 ± 21.6        | 0.224   | 41.6   |
| After        | 72.3 ± 19.9        |         |        |

### Table 4. Surviving expression before and after the induction phase chemotherapy.

| Chemotherapy | Surviving expression (%) | p-value | CI 0.9% |
|--------------|--------------------------|---------|---------|
| Before       | 73.4 ± 20.6              | 0.002   | 56.2    |
| After        | 50.6 ± 14.9              |         |         |

### Table 5. The p53/surviving ratio before and after the induction phase chemotherapy.

| Chemotherapy | p53/surviving ratio (%) | p-value | CI 0.9% |
|--------------|-------------------------|---------|---------|
| Before       | 0.83 ± 0.25             | 0.034   | 0.62    |
| After        | 1.64 ± 0.96             |         |         |
pression of surviving in various malignancies correlates with the incidence of relapse and treatment failure and the low life expectancy of patients with AML [25].

Surviving is a protein that can be expressed on a regular or cell neoplasms and controls deregulation of tumorigenicity. The regulation of surviving itself is usually dependent on cell cycles. At the time of cell proliferation, surviving can be expressed at the G2/M phase and then rapidly decreases to a resting phase (G1 phase) [13, 26]. Furthermore, surviving is modulated to promote the cycle-dependent elements (CDE) and the cycle homology region (CHR) where the promoter is located in the transcription of G2 phase [13] [26, 27]. Moreover, surviving can be affected by changes in RNA (siRNA) or by a suppressor surviving (YM155) where surviving will increase cell death through the p53 dependent apoptosis pathway [28]. Thus, the early detection of surviving levels will be very beneficial to both a treatment and prognosis.

Table 5 shows that the ratio of p53/surviving after the induction phase chemotherapy in AML patients increased significantly which was confirmed the hypothe-
sis of the study.

All AML patients’ response to the treatment were very varied due to prognostic factors and characteristics of AML malignancy, patients (age, clinical course and karyotype), etc. The treatment of AML has made much progress and in developed countries treatment success has reached 65% while in Indonesia is expected to remain below 10% [3, 29]. Apoptosis abnormalities accelerate the transformation and lead to the proliferation of tumors toward malignancy. Also, changes in the pathway of tumor suppressor genes and activation of proto-oncogenes that can play a role in cancer development especially with the deregulation of programmed cell death [30, 31].

Surviving levels can identify the presence of suppression by p53 (wild-type), either directly or indirectly [32] and low levels of surviving caused by the role of p53 (wild-type) dependent apoptosis. Thus, both the expression of surviving and p53 can describe a synergism regarding determining the prognosis in the case of AML in children [33, 34].

The problems in this study were when patients did not turn up for scheduled chemotherapy and when patients did not come for follow-up check-ups on time, then some patients dropped out while undergoing the induction phase chemotherapy. Another problem was fatalities while undergoing chemotherapy as well as patients with absolute neutrophil counts (ANC) of less than 500 so that the necessitating improvements in their general health first. However, none of the obstacles and constraints above affected the results. The limited number of samples in this study may have affected the results statistically, but with it is hoped that they will be of use as a reference for similar studies in the future and it is also hoped that these findings will contribute to prove that the expression of p53, surviving and the P53/surviving ratio could be used as a marker in the prognosis for chemotherapy in children with AML.

CONCLUSION

Expressions of p53 did not increased significantly after the induction phase chemotherapy in children with AML, whereas the expressions of surviving decreased significantly after the induction phase. The p53/surviving ratio increased significantly after induction phase of chemotherapy in children with AML. A negative correlation was found between the p53 and surviving expressions in children with AML after induction phase chemotherapy. In this case, p53/surviving ratio can be use as parameter for induction phase chemotherapy in children with AML.

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