CASPASE 3 AS PROGNOSTIC MARKER FOR TRIPLE NEGATIVE BREAST CANCER CHEMOTHERAPY

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ABSTRACT

Objectives: This study will examine the expression of caspase-3 and apoptotic index (AI) in triple negative breast cancer (TNBC). By knowing the non-responsiveness effect earlier, adverse effects of chemotherapy can be avoided.

Methods: This prospective cohort study has been approved by the local Ethics Committee. A total of 60 consent TNBC patients from Haji Adam Malik General Hospital and Bunda Thamrin Hospital were included in the study. Patients with heart, kidney, liver disease, history of surgery, chemotherapy, or hormonal therapy were excluded. Samples were analyzed immunohistochemically by monoclonal antibodies to assess caspase 3 and AI. Clinical chemotherapy response is determined as a positive or negative response based on Response Evaluation Criteria in Solid Tumors.

Results: The results of this study indicated that caspase 3 was increased post-chemotherapy but could not predict the clinical response of chemotherapy. Caspase-3 post-chemotherapy (5.27±1.27 pg/mL) compared to pre-chemotherapy (4.60±1.04 pg/mL) increased significantly (P=0.005) by 0.67±1.66 pg/mL, but no difference was found in AI score (P=0.819). Neither caspase 3 nor the AI were associated with a clinical chemotherapy response (P=0.514 and P=0.993, respectively).

Conclusion: Further research with larger samples is needed to determine the role and pathway of chemotherapy induced caspase 3 rise.

Keywords: Triple negative breast cancer, Chemotherapy, Caspase, Apoptosis.

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INTRODUCTION

Triple negative breast cancer (TNBC) accounts for 17-21% of breast cancer cases [1] but accounts for the worst prognosis of all breast cancers due to its complexity [2]. The absence of hormonal receptors makes chemotherapy an important role in the treatment of TNBC [3]. Multicenter studies showed that 36% of TNBC patients receiving neoadjuvant chemotherapy showed complete clinical response and a significant good prognosis for disease-free survival (p=0.001) [4]. In the EORTC study, it was reported that almost 23% of patients being enabled for breast-conserving surgery after neoadjuvant chemotherapy. However, there were also many chemoresistance cases making the neoadjuvant chemotherapy almost had no benefit but only yielded harmful side effects [5]. Biomarker for that can accurately identify a patient with sensitivity for chemotherapy is needed [6].

Although biologically breast cancer is one of the most heterogeneous cancers of genomics variation [7], the activation of apoptotic signaling in chemotherapy is demonstrated by activation of mitochondrial pathway apoptosis and signals through the death receptor (extrinsic pathway) that contribute to the sensitivity of tumor cells to cytotoxic therapy [8]. Both pathways will eventually activate the caspase and effector molecules resulting in cell death. Apoptosis assessment appears as an idea to determine the prognosis and predictive factors of the chemotherapy response [9]. This is reinforced as numerous studies have shown that conventional predictor factors such as tumor size and lymphatic status cannot be applied to patients as a whole [10].

This study will examine the expression of caspase-3 and apoptotic index (AI). Caspase-3 encoded by the CASP3 gene [11] is one of the apoptotic

excutants that activate endonucleases causing DNA fragmentation in apoptotic mechanisms. On the other hand, in histopathology, the AI is used as a measure of apoptotic or apoptotic cell count per 1000 tumor cells [12]. No research in Indonesia has been done about this topic before.

The aim of this study is to determine the relationship of either caspase-3 score or apoptosis index with clinical chemotherapy response of neoadjuvant chemotherapy in TNBC patients.

METHODS

Data selection

This prospective cohort was conducted from June 2015 to February 2017. As many as 60 TNBC subjects undergoing surgery in Haji Adam Malik General Hospital and Bunda Thamrin Hospital were included in this study. They must approve the informed consent and had a Karnofsky index >70. Exclusion criteria when there is morbidity of heart disease, kidney, liver, history of surgery, hormonal therapy, or previous chemotherapy.

Study parameters

The parameters of this study were caspase 3 pre-chemotherapy and post-chemotherapy, pre-chemotherapy and post-chemotherapy apoptotic indices, and clinical responses. Samples were analyzed immunohistochemically to assess caspase 3 and the AI in the Laboratory. Clinical chemotherapy response was classified based on Response Evaluation Criteria in Solid Tumors (RECIST) with or without response.
Analysis of caspase 3

Caspase 3 was checked with a monoclonal antibody kit. Breast tumor tissue was fixed with a 10% formalin buffer for 30 minutes and processed in paraffin block form for 48 hrs. The specimens were stored in room temperature, and glass object must be maintained in wet conditions to ensure well painted. The specimen then reacted with peroxidase block for 1-5 minutes, cleaning over buffer, application of antibody or negative control reagents, addition of peroxidase labeled polymer for 30 minutes, chromogen substrate for 5-10 minutes, counterstained with hematoxylin, and until the specimen was ready for mounting.

Analysis of AI

An AI examination was performed with a TUNEL assay. Endogenous peroxidase activity was deactivated with 3% hydrogen peroxide in phosphate-buffered saline (PBS) at pH 7.4 for 10 minutes. The nucleus was cut and washed or stained of the protein by incubation process with 0.5% pepsin at pH 2.0 for 30 minutes at 37°C and then washed with distilled water to remove the pepsin. Each of these pieces (nucleus) is ready to be processed through a TUNEL procedure that was incubated in Tris buffer (pH 7.6) for 5 minutes and then under temperature of 37°C in 100 µL. The mixture consists of 15 units of pure TdT (PharMingen, San Diego, CA), 0.5 nmol biotin-16-DUTP (Boehringer Mannheim, Mannheim, Germany), 50 µM dNTP mix, 2.5 mM MgCl2 (PH 6.6), and 0.25 mg/mL of bovine serum albumin were dissolved in distilled water. After rinsing or cleaning intensively then incubated 30 minutes at room temperature in a solution of 1: 400 ratio of horseradish peroxidase conjugated to streptavidin (Dako UK Ltd.) in PBS supplemented (1% PBS and 0.5% TWEEN 20). The color occurred 10 minutes, using a 0.05% solution of dianisobenzidine, 0.07% iodoxy, 0.5% hydrogen peroxidase, and 0.5% copper sulfate with 0.5% sodium chloride for 1 minute. Finally, the core pieces of the cell have been processed, performed counterstained in Mayer’s hematoxylin, dehydrated, cleared in xylene and mounted in DPX. The AI was assessed by counting cancer cells at 400 times magnification (Barcomb et al., 2008).

Clinical chemotherapy response interpretation

The clinical chemotherapy response assessment process is evaluated according to the RECIST (Clamarkova et al., 2010) by comparing tumor size before and after neoadjuvant chemotherapy. Breast tumors are determined locally and drawn on the surface of the breast skin, then ascertainment the longest and the shortest size of the tumor. Diameter is measured from the longest measure, the volume is determined from the longest size multiplied by the shortest size. The size of the tumor after the third series neoadjuvant chemotherapy will be used as the final measure that determines the tumor’s response rate. The tumor response rate was measured by comparing the initial size of the tumor.

RESULTS

A total of 60 TNBC patients followed the study until completion. Subjects ranged from 38 to 73 years old with the majority of premenopausal subjects (56.7%). As many as 75% of patients diagnosed in Stage III B and 43.3% had T3 tumor size, 80% ID histology type, and 41.7% were in Grade II. After 3 cycles of neoadjuvant chemotherapy, 31 subjects (51.7%) did not show clinical response while 29 subjects (48.3%) had a clinical response (Table 1).

Various biomarkers have been used in assessing apoptosis directly or indirectly. This study was conducted to determine the relationship of caspase-3 and AI with astrocytoma grade and clinical outcome. The value of caspase 3 pre-chemotherapy was 4.60±1.09 pg/mL and post-chemotherapy was 5.27±1.27 pg/mL. Analysis with t-paired test showed that there was a significant difference in mean score of caspase 3 post-chemotherapy compared to pre-chemotherapy as indicated by p=0.004. There was an increase of 1.15 times or by 0.67±1.66 pg/mL of caspase 3 post-chemotherapy (Table 2). On the other hand, the mean AI before chemotherapy was 5.47±1.38 pg/mL and after chemotherapy was 5.52±1.08 pg/mL. The result of t-paired test showed that there was no significant difference between the mean of AI post-chemotherapy and pre-chemotherapy (p=0.819) with only increase of 0.05±1.68 pg/mL (Table 3).

Most of the subjects in the group that responded not responded clinically to chemotherapy showed an increased caspase 3 score (61.7%) and an increased AI (51.7%). However, Phil and Cramer’s V correlation analysis showed no significant difference of caspase 3 (p=0.514, Table 4) and apoptosis index (p=0.993, Table 5) with clinical response to neoadjuvant chemotherapy on TNBC. However, in the unresponsive group (14 out of 31 samples, 45.2%) showed a significantly lower caspase 3 score than the response group (9 of 29 samples, 31%).

DISCUSSION

The age-specific prevalence pattern for TNBC was not fully understood until SEER collected data from 1997 to 2002 [13]. In this study subjects aged 27-73 years old that mostly aged between 51 and 60 years (31.7%). Prevalence of subjects aged 35-50 years old was 76.7%. This result was in accordance with SEER survey that found peak incidence cases of TNBC from 35 to 60 years, and afterward not much different. The population based study by Ambrosone et al. showed that 2.9 times the increased risk of TNBC in women at this age was due to an unknown increase in the use of oral contraceptives [14]. However, there is a research controversy by Stark et al. who observed increased 1.9 times risk of TNBC occurred at younger ages. However, this can be due to the researchers comparing it with luminal breast cancer [15]. Phipps et al. in Americans showing that menopausal age was not associated with an increased risk of TNBC, as did other studies in China [16] and Poland [17] but increased the risk of luminal breast cancer. Phipps et al. even tried hormonal therapy on TNBC patients and found no improvement. The study also showed a nearly equal proportion between premenopausal and postmenopausal patients, 56.66% and 43.33%, respectively [18].

Based on Table 1, there were 31 samples (51.7%) that did not respond to neoadjuvant chemotherapy and 29 samples (48.3%) that respond to neoadjuvant chemotherapy. This proportion was higher than that of Varano et al., which showed only 15% of clinical responses [19] although
Table 2. Mean difference of caspase 3 postchemotherapy and prechemotherapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before chemotherapy (pg/mL)</th>
<th>After Kemoterapi (pg/mL)</th>
<th>Difference</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>4.60±1.09</td>
<td>5.27±1.27</td>
<td>Increase 0.67±1.66</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 3. Mean difference of apoptotic index postchemotherapy and prechemotherapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before chemotherapy (pg/mL)</th>
<th>After Kemoterapi (pg/mL)</th>
<th>Difference</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic index</td>
<td>5.47±1.38</td>
<td>5.52±1.08</td>
<td>Increase 0.05±1.68</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 4. The relationship between caspase 3 and neoadjuvant chemotherapy response

<table>
<thead>
<tr>
<th>Caspase 3 Clinical response</th>
<th>Total (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unchanged</td>
<td>7 (11.7)</td>
<td>4 (6.7)</td>
</tr>
<tr>
<td>Decrease</td>
<td>7 (11.7)</td>
<td>5 (8.3)</td>
</tr>
<tr>
<td>Increase</td>
<td>17 (28.3)</td>
<td>20 (33.3)</td>
</tr>
<tr>
<td>Total</td>
<td>31 (51.7)</td>
<td>29 (48.3)</td>
</tr>
</tbody>
</table>

Table 5. The relationship between apoptotic index and neoadjuvant chemotherapy response

<table>
<thead>
<tr>
<th>Apoptotic index Clinical response</th>
<th>Total (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unchanged</td>
<td>4 (12.9)</td>
<td>8 (13.3)</td>
</tr>
<tr>
<td>Decrease</td>
<td>11 (18.3)</td>
<td>21 (35.0)</td>
</tr>
<tr>
<td>Increase</td>
<td>16 (26.7)</td>
<td>31 (51.7)</td>
</tr>
<tr>
<td>Total</td>
<td>31 (51.7)</td>
<td>60 (100)</td>
</tr>
</tbody>
</table>

Torrisi et al. reported 77.5% [20]. von Minowicz et al. found that the addition of neoadjuvant chemotherapy carboplatin to the regimen taxane, anthracycline, and targeted therapies significantly increases the proportion of patients achieving a complete response. This suggested that neoadjuvant chemotherapy could reduce the size of the tumor and eradicating almost half the TNBC cases [21]. Otherwise, some unresponsive patients to be overtreated because of the unpredictability of TNBC. This condition should be prevented by the discovery of prognostic factor that can predict neoadjuvant chemotherapy response earlier.

Caspase-3 as a executor and effector on the apoptotic process plays an important role in assessing the response of a cell to chemotherapy or cytotoxic substances. The killing of cancer cells by chemotherapeutic agents is based on apoptotic mechanisms, both intrinsic and extrinsic, with caspase 3 as the apoptotic executor. Looking at the above-mentioned caspase-3 potential, Devarawn et al. studied the regulation of caspase-3 using cell culture of breast cancer (MCF-7) administered doxorubicin. The results showed that there was an increase in caspase-3 and its receptor downregulation which confirmed the idea of caspase 3 as a promising marker of the effectiveness of chemotherapeutic agents. However, Salakou et al. showed that caspase 3 alone was less useful. The researchers proposed that the use of Bax/Bcl-2 ratio predicted better than caspase-3 alone [24]. In this study, there was a significant difference (p=0.005) with an increase of 1.15 times (0.67±1.66 pg/mL) of caspase-3 post-chemotherapy compared to pre-chemotherapy. This result suggested that neoadjuvant chemotherapy managed to trigger this apoptotic apoptosis mediator in the TNBC network. Although the assessment of the relationship between clinical response and caspase 3 after and before neoadjuvant chemotherapy was not statistically significant (p=0.514; Table 4), the clinical data analysis showed that only a few chemosensitive subjects had decreased or unchanged caspase 3 score (31%) compared to subjects with no chemotherapy response (45.2%). Caspase 3 in the group with positive chemotherapy response tended to increase (66%) compared to the group without clinical response (54.8%).

Reviewing the AI, there was no significant mean difference between post-operative and post-chemotherapy (p=0.819), and no significant differences were found between AI and TNBC chemotherapy response (p=0.97). The proportion of the increase in AI was almost comparable between those who responded and unresponsive to neoadjuvant TNBC. This result was similar to Yang et al. which showed that maybe the AI low due to the low concentration of doxorubicin given. In MCF-7 cells, 10 hrs doxorubicin was exposed that resulted in caspase activation and other apoptotic substrates in line with the addition of 2-10 microM [25]. Unlike O'Donovan et al. study in 103 breast tissue samples, they showed that caspase-3 precursor and active form were higher in breast cancer than normal tissue (p=0.0188; p=0.0002) [26]. Similarly, Sharma et al. showed that tumor biology markers (Bcl-2, AI and Caspase-3) change occurred 24-48 hrs after first neoadjuvant chemotherapy cycles. These markers could be as a factors to predict the response of chemotherapy but to prove them statistically need research with a larger sample size [27].

It has been shown in the previous data that there was a significant increase in caspase 3 after neoadjuvant chemotherapy but no significant increase in apoptosis index. The author concluded that there were other apoptotic pathways other than the normal extrinsic and intrinsic pathways that triggered caspase 3 so that the AI was normal but caspase 3 increased. On the other hand, the associated caspase 3 score may be a type 1 error in the study. The caspase 3 score that was found to be unrelated to clinical response should still be further investigated in larger samples because although statistically unrelated, samples with caspase 3 increased were found more in the group with than those without chemotherapy response.

CONCLUSION

There were no significant differences of either caspase 3 score or AI with clinical chemotherapy response to neoadjuvant chemotherapy on TNBC. Further research with larger samples is needed to determine the role and pathway of chemotherapy induced caspase 3 rise.

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5. Indran IR, Tufo G, Pervuz S, Breuer C. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochem Biophys