CASPASE 3 AS PROGNOSTIC MARKER FOR TRIPLE NEGATIVE BREAST CANCER CHEMOTHERAPY

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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 biomolecular of breast cancer is one of the most heterogeneous cancers of genomic 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 executants 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 scale >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 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 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 remained 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 1% hydrogen peroxide in phosphate-buffered saline (PBS) at pH 7.4 for 10 minutes. The nucleus was cut and cleansed or stripped 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 for 1 hr temperature of 37°C in 100 µL. The mixture consists of 15 units of pure FPLC (Pharmacia, Windsor, Berkshire, UK), 0.5 nmol biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), 5 mM cobalt chloride, 0.2 M sodium cacodylate, 25 mM Tris HCl (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% mixture of diaminobezidine, 0.07% imidazole, 0.1% hydrogen peroxide, and 0.5% copper sulfate with 0.9% 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 (Burcombe et al., 2008).

Clinical chemotherapy response interpretation
The clinical chemotherapy response assessment process is evaluated according to the RECSI (Gizmarikova 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 ascertained 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 30 to 73 years old with the majority of premenopause subjects (56.7%). As many as 75% of patients diagnosed in Stage III B and 43.3% had T3 tumor size, 80% IDC 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=0.003. 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 nor responded clinically to chemotherapy showed an increased caspase 3 score (61.7%) and an increased AI (51.7%). However, Phi 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 [1.3]. 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 A. 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 43.3%, 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 response to neoadjuvant chemotherapy. This proportion was higher than that of Yarso et al., which showed only 15% of clinical responses [19] although
Caspase-3 as the executor or 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 [22]. Looking at the above-mentioned caspase-3 potential, Devarajan 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 [23]. 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=0.003) with an increase of 1.15 times (0.67±1.66 pg/mL) of caspase-3 post-chemotherapy compared to pre-chemotherapy. 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.


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</td>
<td>0.67±1.66</td>
</tr>
</tbody>
</table>

Table 3. Mean difference of apoptotic index postchemotherapy and prechemotherapy

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

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

<table>
<thead>
<tr>
<th>Caspase 3</th>
<th>Clinical response</th>
<th>Total (%)</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Unresponsive (%)</td>
<td>Unresponsive (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unchanged</td>
<td>7 (11.7)</td>
<td>4 (6.7)</td>
<td>11 (18.3)</td>
</tr>
<tr>
<td>Decrease</td>
<td>7 (11.7)</td>
<td>5 (8.3)</td>
<td>12 (20.0)</td>
</tr>
<tr>
<td>Increase</td>
<td>17 (28.3)</td>
<td>20 (33.3)</td>
<td>37 (61.7)</td>
</tr>
<tr>
<td>Total</td>
<td>31 (51.7)</td>
<td>29 (48.3)</td>
<td>60 (100)</td>
</tr>
</tbody>
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Table 5. The relationship between apoptotic index and neoadjuvant chemotherapy response

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<tr>
<th>Apoptotic index</th>
<th>Clinical response</th>
<th>Total (%)</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Unresponsive (%)</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unchanged</td>
<td>4 (12.9)</td>
<td>4 (6.7)</td>
<td>8 (13.3)</td>
</tr>
<tr>
<td>Decrease</td>
<td>11 (18.3)</td>
<td>10 (16.7)</td>
<td>21 (35.0)</td>
</tr>
<tr>
<td>Increase</td>
<td>16 (26.7)</td>
<td>15 (25.0)</td>
<td>31 (51.7)</td>
</tr>
<tr>
<td>Total</td>
<td>31 (51.7)</td>
<td>29 (48.3)</td>
<td>60 (100)</td>
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Torrisi et al. reported 77.5% [20]. von Minckwitz et al. found that the addition of neoadjuvant chemotherapy carboplatin to the regimen taxane, anthracycline, and targeted therapies significantly increase the proportion of patients achieving a complete response. This suggested that neoadjuvant chemotherapy could reduce the size of the tumor and eradicated almost half the TNBC cases [21]. Otherwise, some unresponsive patients were overtreated because of the unpredictability of TNBC. This condition should be prevented by the discovery of a prognostic factor that can predict neoadjuvant chemotherapy response earlier.

Caspase-3 as the executor or 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 [22]. Looking at the above-mentioned caspase-3 potential, Devarajan 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 [23]. 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=0.003) with an increase of 1.15 times (0.67±1.66 pg/mL) of caspase-3 post-chemotherapy compared to pre-chemotherapy. 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 Al 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 Al 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.

REFERENCES

5. Indran IR, Tufo G, Pervaz S, Brenner C. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochim Biophys


