Correlation of DUSP4 mRNA Expression Postchemotherapy with a Chemotherapeutic Response

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Introduction
Breast cancer is the most common cancer and the second leading cause of cancer death in women worldwide, including Indonesia (Bray et al., 2013; Youlden et al., 2014). Advances in systemic therapy, such as chemotherapy, hormonal therapy, and targeting treatment, have improved the patient disease-free survival and overall survival, but some cancers are resistant to systemic therapy (Martin et al., 2014; Miller et al., 2016). Cancer patients with the same stage, grade, and histogenesis can have different treatment responses to various chemotherapy agents (Luqmani, 2005; Rouzier et al., 2005; Györffy et al., 2006). Some theories have proposed the mechanisms of therapeutic resistance. Biomarker and gene-specificity for chemotherapeutic resistance are challenges to be addressed (Holohan et al., 2013).

Neoadjuvant chemotherapy offers an estimation of the treatment response (Cortazar et al., 2014; Zardavas and Piccart, 2015). Chemotherapy induces upregulation or downregulation of most genes (Klintman et al., 2016). Residual disease after neoadjuvant chemotherapy may predict the prognosis and gene expression in residual disease, suggesting a biologic role in chemoresistant disease (Balko et al., 2012; Klintman et al., 2016) (Penault-Llorca and Radosevic-Robin, 2016).

Dual-specificity phosphatase 4 (DUSP4) is a protein responsible for dephosphorylating threonine/serine and tyrosine residues on their substrates (Boulding et al., 2016). DUSP4 selectively dephosphorylates signaling Mitogen-activated protein kinase’s (MAPKs), implicating them in signal transduction. Studies have found that DUSP4 is upregulated in malignant tissues, including breast cancer (Boulding et al., 2016).

Mazumdar et al., (2016) identified the low expression of the DUSP4 protein in ER-negative breast cancers. Overexpression of DUSP4 protein causes dephosphorylation of growth-promoting signaling proteins, hence inhibiting the growth and invasiveness of ER-negative breast cancer cells.

DUSP4 is significantly enriched in response to

Abstract

Objective: Evaluation of the neoadjuvant chemotherapy response can be performed by comparing the breast cancer burden and pathobiology before and after treatment. This study was aimed to investigate the pattern of dual-specific phosphatase 4 (DUSP4) mRNA expression in breast cancer cells before and after neoadjuvant chemotherapy.

Methods: This was a longitudinal study. Twenty samples of matched breast cancer tissue taken from biopsy before and after chemotherapy were subjected to qRT-PCR to detect DUSP4 mRNA expression.

Results: The mean value of DUSP4 mRNA expression in prechemotherapy breast cancer patients was 9.906±0.333 and that in breast cancer patients postchemotherapy was 10.016±1.062. In the responsive group, the rate of DUSP4 mRNA expression increased by 0.476 after chemotherapy. In the nonresponsive group, the proportion of DUSP4 mRNA expression likely decreased by 1.012. Statistical analysis found no significant correlation between DUSP4 mRNA expression prechemotherapy and the clinical chemotherapeutic response with p-value = 0.994 (p ≥0.05). A significant correlation was found between the postchemotherapy DUSP4 mRNA expression and the clinical chemotherapeutic response with p-value = 0.003 (p < 0.5).

Conclusion: No significant difference was found in the mRNA expression of DUSP4 in pre- and post-neoadjuvant chemotherapy specimens. High DUSP4 expression postchemotherapy shows a substantial correlation with the chemotherapeutic response.

Keywords: DUSP4- mRNA- qRT-PCR- Chemotherapy response
chemotherapy, and low levels of *DUSP4* in residual disease are associated with an impaired prognosis (Klintman et al., 2016). The loss of *DUSP4* activates the MAPK pathway, promoting a stem cell-like phenotype and decreasing the clinical response to neoadjuvant therapy in breast cancer (Balko et al., 2012; Balko et al., 2013). Some other studies also found that *DUSP4* expression is associated with resistance to cytotoxic chemotherapies such as doxorubicin and cisplatin chemoresistance (Liu et al., 2013; Boulding et al., 2016).

The objective of this study was to determine the pattern of *DUSP4* mRNA expression in locally advanced breast cancer patients pre- and post-neoadjuvant chemotherapy using anthracycline-based chemotherapy and the relationship with the clinical chemotherapeutic response.

Materials and Methods

Samples

This was an observational study. The samples were obtained from Wahidin Sudirohusodo Hospital Makassar, a top referral hospital in the east of Indonesia, from February to June 2016. Female patients with locally advanced breast cancer, invasive ductal carcinoma type, receiving neoadjuvant chemotherapy with a cyclophosphamide- Adriamycin-5-FU regimen, were included in the study. The *DUSP4* mRNA expression was detected using quantitative real-time polymerase chain reaction (qRT-PCR) from breast cancer tissue taken from biopsy and surgery.

Nucleic Acid Extraction

Samples of breast cancer tissue were subjected to nucleic acid extraction using the Boom method (diatom guanidinium isothiocyanate (GuSCN) method). Breast cancer tissue as much as 100 µg/ml was added to 900 mL “L6” solution containing 120 g GuSCN, 100 ml 0.1 mM Tris-HCl, pH 6.4, 22 ml 0.2 mM ethylenediaminetetraacetate (EDTA), pH 8.0, and 2.6 g Triton X-100 (final concentrations of 50 mM Tris- HCl, 5 M GuSCN, 20 mM EDTA, and 0.1% Triton X-100). Next, 20 ml diatom suspension was added consisting of 50 ml H2O and 500 mL 32% (w/v) Diatoms. This diatom suspension, which could bind 10 µg DNA tissue, was vortexed and centrifuged in a 1.5-ml Eppendorf tube at 13,000 rpm for 15 seconds. The supernatant was removed, and the sediment was washed with 1 ml “L2” solution (120 g GuSCN in 100 ml 0.1 M Tris-HCl, pH 6.4). Next, the sample was vortexed and then centrifuged at 13,000 rpm for 15 seconds. Next, the sediment was washed twice with “L2” solution and twice with 1 ml 70% ethanol and 1 ml acetone. The sample was then heated in a water bath at a temperature of 56°C for 10 minutes, followed by the addition of 60 mL “TE” solution (1 mM EDTA in 10 mM Tris-HCl, pH 8.0), vortexing and centrifugation at 13,000 rpm for 2 minutes. The sample was then incubated in an oven at 56°C for 10 minutes, followed by vortexing and centrifugation at 13,000 rpm for 30 seconds and collection of the supernatant. This supernatant was the result of nucleotide extraction and was stored at ~80°C before PCR analysis (Boom et al., 1990; Prihantono et al., 2017).

Expression of mRNA DUSP4 Genes by Real-Time PCR

The detection of *DUSP4* mRNA expression was performed according to real-time PCR as described by Liu et al. (Liu et al., 2013). The specific primers for *DUSP4* mRNA were as follows: forward, 5′-CCCACAGGCTATTAGGCTGAAG-3′; reverse, 5′-CAGCGTGAGTAGACACTGAA-3′. The primers for the reference gene (β-actin gene) were as follows: forward, 5′-GGAGATTACGCTCTCTTA-3′; reverse, 5′- GCATCTATCTCTCGTTGCTG-3′. Each sample required 1 µg of the template. Reverse transcription was performed using the RT reagent Kit with gDNA eraser. cDNA was synthesized using the cDNA Synthesis kit (Takara) following the instructions provided by the manufacturers. qRT-PCR was performed using an ABI7500 Sequence Detection System (PE Applied Biosystems) in the presence of SYBR-green I. Briefly, a 50-µl reaction mix containing 25 µl Premix ExTaq , Takara), 1 µl ROX reference Dye II (Takara), 1 µl PCR forward primer (10 µM), 1 µl PCR reverse primer (10 µM), 4 µl cDNA and 18 µl dH2O was premixed before reaction in 96-well plates. The reaction protocol was as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 34 s, followed by 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds. The relative gene expression profiles were determined by normalizing to the reference gene (β-actin) using the 2^ΔΔCt method. Each sample for this study was tested in triplicate (Liu et al., 2013).

Chemotherapeutic Response Criteria

The response to neoadjuvant chemotherapy was classified according to RECIST criteria. The nonresponsive group displayed stable disease or progressive disease according to RECIST criteria if there is a reduction of the tumor size less than 30%, no change, an increase in the tumor size, or a new tumor. The responsive groups displayed a complete or partial response if there is a reduction of the tumor size >30%, no evidence of a tumor clinically or pathologically, or no further tumor found.

Ethical Clearance

This study has been approved by the Ethical Commission of Health Study, Medical Faculty, Hasanuddin University, with the registry number 799/H4.8.4.5.31/PP36-KOMETIK/2016 (Register: UH15060492).

Results

Characteristics

The twenty enrolled female patients with invasive breast carcinoma diagnosed and treated at Wahidin Sudirohusodo General Hospital met the inclusion criteria of the study. Their ages ranged from 28 to 64 years, with a mean age of 50.3 years. All twenty cases were invasive ductal carcinoma. The obtained histopathologic grading was a low grade in 1 case (5%), moderate grade in 15 cases (75%) and high grade in 4 cases (20%). The number of patients responsive to neoadjuvant chemotherapy was 15/20 (75%), and the number of nonresponsive patients was 5/20 (25%). No correlation was found between *DUSP4* mRNA expression and clinical data, including...
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Table 1. Characteristics of Samples

| Characteristic     | n (%)   | p*   |
|-------------------|---------|------|
| Age               |         |      |
| ≤ 50 years        | 11 (55.0%) | 0.617 |
| > 50 years        | 9 (45.0%)  |      |
| Grade             |         |      |
| Low Grade         | 1 (5.0%)  | 0.225 |
| Moderate Grade    | 15 (75.0%) |      |
| High Grade        | 4 (20.0%)  |      |
| Immunohistochemistry |      | 0.56  |
| ER                | 5 (25.0%)  |      |
| PR                | 6 (30.0%)  |      |
| HER2              | 13 (65.0%) |      |
| Ki-67             | 11 (55.0%) |      |
| Clinical response |         | 0.959 |
| Luminal A         | 3 (15.0%)  |      |
| Luminal B         | 6 (30.0%)  |      |
| HER2              | 7 (35.0%)  |      |
| Triple Negative   | 4 (20.0%)  |      |

*p, chi-squared test for the clinical chemotherapy response

Table 2. Comparison of the mRNA DUSP4 Expression Pre- and Postchemotherapy with the Clinical Response

| mRNA Expression       | Responsive (n=20) | Non Responsive (n=7) | Mean difference | p-value |
|-----------------------|-------------------|---------------------|-----------------|---------|
| DUSP4 (Prechemotherapy) | 9.902±0.336       | 9.917±0.378         | -0.015          | *       |
| DUSP4 (Postchemotherapy)| 10.378±0.785     | 8.905±1.082         | 1.472           | **      |
| Mean difference       | -0.476            |                     | 1.012           |         |

*p, Independent Samples T-test, **Mann-Whitney U test

Table 3. Correlation of the mRNA DUSP4 Expression and the Clinical Chemotherapeutic Response

| mRNA Expression                  | Correlation with the Chemotherapy response | p     |
|----------------------------------|-------------------------------------------|-------|
| DUSP4 mRNA (Prechemotherapy)    | -0.002                                    | 0.994*|
| DUSP4 mRNA (Postchemotherapy)   | 0.494                                     | 0.027**|
| Rate of DUSP4 mRNA Expression   | 0.24                                      | 0.307**|

*p, Pearson, **Spearman
response to chemotherapy (p-value = 0.003; p ≥0.05).

The relationship between the DUSP4 mRNA expression and the clinical response is demonstrated in Table 3. No significant correlation was found between the mRNA expression of DUSP4 prechemotherapy with the clinical response (p = 0.994; p>0.05). A definite correlation was found between DUSP4 mRNA expression postchemotherapy and the clinical response (r = 0.494); this association was significant with p = 0.027 (p<0.05). A definite correlation was found between the rate of DUSP4 mRNA expression and the clinical response with a value of r = 0.240; this association was insignificant with p = 0.307 (p>0.05).

**Discussion**

Breast cancer chemo-resistance influenced by several factors including drug inactivation, changes in drug targets, overexpression of ABC transporters, apoptotic dysregulation, epigenetic regulation, epithelial to mesenchymal transition, and cancer stem cells (Housman et al., 2015).

DUSP4 expression has been found in various human cancers (Kidger and Keyse, 2016). Over-expression of DUSP4 is frequently observed in breast cancer and may play an essential role in cancer development and progression (Wang et al., 2003). DUSP4 is commonly upregulated in breast malignancy and may play a crucial role in cancer development and progression. DUSP4 may be a marker of adverse prognosis, especially in patients with early breast cancer (Kim et al., 2015).

Decreased expression of DUSP4, a negative regulator of extracellular signal-regulated kinases (ERK), is related to high RAS-ERK activation and has been recently identified as a mediator of resistance to neoadjuvant chemotherapy in triple-negative breast cancer, promoting to a shorter recurrence-free survival (Baglia et al., 2014). DUSP4 expression was also found to be responsible for the resistance to etoposide and mitoxantrone chemotherapy in breast cancer (Baglia et al., 2014).

We found that increased DUSP4 mRNA expression showed a better chemotherapy response than decreased DUSP4 mRNA expression, but the difference was not statistically significant. DUSP4 mRNA expression postchemotherapy was associated with chemotherapy response.

In our previous study on the expression of DUSP4 using immunohistochemistry, DUSP4 expression was found in 33% (21/63) of breast cancer samples. Analysis of DUSP4 expression with a chemotherapy response found no significant correlation, with p = 0.073 (> 0.05). However, stratification of DUSP4 expression based on the intrinsic subtype found that the Luminal B p-value = 0.02 (<0.05), the Luminal A p-value = 0.24 (> 0.05), and the Her2 p-value = 0.608 (> 0.05); the triple-negative subtype could not be analyzed because of the small number of samples. Furthermore, DUSP4 expression was correlated with the anthracycline-based chemotherapy response in the luminal B subtype (Prihantono et al., 2017).

Baglia et al., (2014) found that low DUSP4 expression was associated with increased recurrence and mortality in triple-negative breast cancer patients. Baglia concluded that low DUSP4 expression is a predictor of recurrence and death in triple-negative breast cancer patients.

Liu et al., (2013) demonstrated that DUSP4 expression affects the breast cancer cell response to chemotherapy. High DUSP4 expression requires higher doses of doxorubicin, whereas cells with low DUSP4 expression need lower doses of doxorubicin. Doxorubicin chemotherapy in breast cancer cells with high DUSP4 expression can lead to acquired chemoresistance by converting epithelial cells into mesenchymal (EMT) cells (epithelial-to-mesenchymal transition). With these EMT changes, cancer cells become more actively proliferating, invasion, migration, and apoptosis are reduced, and the cells become less sensitive to chemotherapy.

Hae Hyun Jung (2016), suggest that the loss of DUSP4, a potential biomarker of treatment-resistant TNBC, is associated with ets-1 overexpression via the PI3K and MAPK pathways. Statin, a small inhibitor of HMG-CoAR, is a likely therapeutic candidate for treatment-resistant TNBC because it can reverse ets-1 overexpression by restoring DUSP4 expression.

This study found DUSP4 mRNA expression postchemotherapy was associated with chemotherapy response. This finding is in line with the article of Rottenberg and Jonkers (2012). Cells with low DUSP4 expression show a high Ki-67 score, which is associated with a poor long-term outcome after neoadjuvant chemotherapy. Hence, the residual cells that show low DUSP4 expression are not quiescent, drug-tolerant cells. Instead, they appear to be genuinely drug-refractory and proliferate regardless of drug treatment. Residual cancer cells may still have another backup: entering a quiescence programme and lying low until the drug is eliminated (Rottenberg and Jonkers, 2012).

In conclusion, no significant difference was found in the DUSP4 mRNA expression of pre- and post-neoadjuvant chemotherapy specimens. Increased DUSP4 mRNA expression shows the tendency of better chemotherapy response, but it is not statistically significant. These results do not suggest that DUSP4 mRNA expression plays a role in conferring neoadjuvant chemotherapy resistance. DUSP4 expression postchemotherapy has a substantial correlation with the chemotherapy response. The findings warrant further research to observe the disease-free survival and overall survival with a larger sample size.

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Conflicts of Interest
We have no conflicts of interest.

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