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Correlation of BCL-2 and ERα mRNA Expression with the Clinical Chemotherapeutic Response in Breast Cancer

[1]Prihantono Prihantono, [2]Christian Binekada, [3]Mochammad Hatta, [1]Daniel Sampepajung and [1]Andi Asadul Islam

Background and Objective: Estrogen Receptor (ER) expression promotes the resistance of breast cancer cells to chemotherapeutic agents via mechanism involving regulation of the B-cell lymphoma 2 (BCL-2) proto-oncogene. Overexpression of BCL-2 is commonly found in various types of cancers, including breast cancer. The BCL-2 expression might predict the patient's response to selected chemotherapies. The aim of this study was to investigate the association between Estrogen Receptor α (ERα) and BCL-2 mRNA expression and the clinical response to neoadjuvant chemotherapy in breast cancer. **Materials and Methods:** This was a longitudinal study of breast cancer patients who underwent chemotherapy using a cyclophosphamide-adriamycin-5-FU regimen. Detection of BCL-2 and ERα mRNA expression in tissue samples was conducted using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Evaluation of the clinical response to chemotherapy was assessed using Response Evaluation Criteria in Solid Tumor (RECIST). Statistical analysis was performed using t-test and Pearson correlation methods. **Results:** The mean value of BCL-2 mRNA expression in the responsive group was 9.887±2.731. The mean value of BCL-2 mRNA expression in the non-responsive group was 10.017±2.122. The mean value of the responsive group was lower than that in the non-responsive group, but there was no significant correlation between BCL-2 mRNA expression and the clinical response to chemotherapy with an r-value was 0.378 and a p-value = 0.223 ($p>0.05$). The mean value of ERα mRNA expression in the responsive group was 10.144±1.945. The mean value of ERα mRNA expression in the nonresponsive group was 12.433±0.801. The mean value of the responsive group was lower than that in the nonresponsive group and there was a significant difference between the baseline ERα mRNA expression and that of the group that exhibited a clinical response to chemotherapy with a p-value = 0.006 ($p>0.05$). There was a negative correlation between ERα mRNA expression and the clinical response to chemotherapy with an r-value = -0.260, but this correlation was insignificant with a p-value = 0.166 ($p>0.05$). **Conclusion:** These results suggest that BCL-2 mRNA expression has a minimal influence in the clinical response of breast cancer to neoadjuvant chemotherapy, while elevated mRNA expression of ERα has some association with a lack of responsiveness to neoadjuvant chemotherapy.

Key words: Breast cancer, chemotherapy, clinical response, mRNA, BCL-2, ERα, qRT-PCR

[1]Department of Surgery, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia
[2]Department of Surgery, Faculty of Medicine, Haluoleo University, Kendari, Indonesia
[3]Laboratory of Molecular Biology and Immunology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

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Prihantono Prihantono,
Department of Surgery,
Faculty of Medicine,
Hasanuddin University,
Makassar, Indonesia

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INTRODUCTION

Cancer develops due to disruption of the balance of cell growth and death[1]. Tumor cells tend to interfere with this balance by activating genes that either promote cell growth or inhibit apoptosis[2]. The B-cell lymphoma-2 (BCL-2) family plays a role in the regulation of apoptosis. Disrupted regulation of apoptosis is a causative event in many diseases. Since proteins in the BCL-2 family are key regulators of apoptosis, abnormalities in its function have been implicated in many diseases[3]. Tumor resistance to apoptosis is usually caused by either dysregulation of the expression of BCL-2 family proteins or mutation of the tumor suppressor gene p53[2,4]. Overexpression of BCL-2 is commonly found in various types of cancers, including breast cancer[5,6]. The BCL-2 is an important clinical prognostic marker in breast cancer and patients positive for BCL-2 expression tend to relapse and have a shorter overall survival[7,8]. Studies revealed that analyzing BCL-2 might predict the patient response to selected endocrine-based and other chemotherapies[5-7,9].

Breast cancer is usually a hormone-dependent tumor. Estrogens can regulate the growth of breast cells by binding to Estrogen Receptor (ER)[10,11]. Exposure to estrogen could increase the incidence and proliferation of breast cancer. Estrogen receptor also plays a role in the successful treatment of breast cancer. Estrogen has been implicated in breast cancer due to its pro-survival effects. The actions of estrogen are mediated by the estrogen receptor[12,13]. Estrogen Receptor α (ERα) is a nuclear receptor that functions as a ligand-activated transcription factor[8]. Estrogen E2 enhances cancer cell survival in part through its ability to upregulate BCL-2 expression. The ERα has been shown to play an integral role in regulating BCL-2 expression[14]. The objective of this study was to investigate the role of mRNA expression of BCL-2 and ERα prior initiating chemotherapy as predictor of the chemotherapeutic response in breast cancer.

MATERIALS AND METHODS

Sample collection: This study was conducted within a population of breast cancer patients who were clinically and histopathologically diagnosed with breast cancer and was treated at the Wahidin Sudiro Husodo Hospital in Makassar, South Sulawesi, Indonesia.

All the patients who fulfilled the inclusion criteria were willing to participate in the study and signed informed consent were recruited as research subjects. The cohort consisted of 30 patients with breast cancer who underwent a chemotherapeutic regimen comprising cyclophosphamide, adriamycin and 5-FU.

Nucleic acid isolation: Nucleic acid was extracted from breast cancer tissue using the diatom guanidinium isothiocyanate (GuSCN) method described by Boom *et al*[15]. The tissue samples were mixed with 500 µL of lysis buffer L6 (50 mM tris-HCl, 5.25 M GuSCN, 20 mM EDTA, 0.1% Triton X100), vigorously vortexed and centrifuged at 1,000 rpm for 5 min. After collecting the nucleic acid, the samples were lysed by incubating for 15 min at 18°C and 20 µL of diatom suspension was added. The diatom containing the bound nucleic acid was centrifuged at 12,000× g for 15 sec to obtain the diatom pellet. The diatom pellet was then washed with washing buffer L2 (5.25 M GuSCN in 0.1 M tris-HCl, pH 6.4), rinsed with 70% ethanol and acetone and dried at 56°C for 10 min. The pellet was resuspended in 60 µL of buffer comprising 10 mM tris-HCl (pH 8.0) and 1 mM EDTA buffer and the nucleic acid was eluted by incubating the samples at 56°C for 10 min. After sedimentation of the diatom by centrifugation, the supernatant was collected and stored at -20°C until real-time PCR was performed[15].

mRNA expression of BCL-2 genes by real-time PCR: Detection of mRNA expression of BCL-2 was performed using the real-time PCR method previously described by Martinez-Arribas[3]. Specific primers for the BCL-2 mRNA sequence are listed in Table 1[3]. Each sample was measured in triplicate.

Expression mRNA ERα by real time PCR: Detection of ERα mRNA expression was conducted using a real-time PCR. The following primers to detect ERα mRNA were used: forward: 5'-TGCTTCAGGCTACCATTATGGAGTCTG-3' and reverse: 5'-GTCAGGGACAAGGCCAGGCTG-3'. The reactions were run on a One-Step Quantitative RT-PCR system according to the manufacturer's instructions and the cycling conditions for ERα were as follows: 94°C for 3 min and 38 cycles of 94°C for 30 sec and 51°C for 30 sec. Each sample was measured in triplicate[16,17].

Data analysis: Data were analyzed using Statistical Package for Social Science (SPSS) version 22. The normality of the samples was analyzed using Shapiro-Wilk's test. The patient characteristics and clinical response were analyzed using the chi-square test. The mean difference of the BCL-2 mRNA expression levels between the responsive and non-responsive groups was assessed using the t-test and correlations were determined using the Pearson and Spearman tests[18].

Ethical clearance: Ethical approval for this study was obtained from the Research Ethics Committee at the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia (Number 1581/H4.8.4.5.31/PP36-KOMETIK/2015, Register UH15060492).

RESULTS

Samples were collected from 30 individuals with invasive breast carcinoma between July 2015 and August 2016 who were examined at Wahidin Sudirohusodo Hospital. The youngest subject was 28 years old and the oldest was 64 years old, the mean age of the subjects was 50.3 years (Table 2). The histopathological grading is summarized in Table 2.

Table 1: Primer sequences and conditions used[3]

Primers	Sequences (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
BCl-2a	CCCTGTGGATGACTGAGTAC		
BCl-2b	GCATGTTGACTTCACTTGTG	211	54
AC 1	GACCCAGATCATGTTTGAG		
AC 2	GAGTTGAAGGTAGTTTCGTG	486	55
Process		Time	Temperature (°C)
RT-PCR			
Inverse transcription		30 min	50
Activation prior to PCR		15 min	95
PCR			
Denaturation		1 min	95
Annealing		30 sec	55
Extension		1 min	72
No. of cycles		34 cycles	
Final extension		10 min	72

Table 2: Clinicopathological characteristics of the patients

Characteristics	Number	Percentage
Age		
≤50	14	46.7
>50	16	53.3
Grades		
Low grade	2	6.7
Moderate grade	19	63.3
High grade	9	30.0
Immunohistochemistrys		
ER+	8	26.7
PR+	11	36.6
HER2+	17	56.6
Clinical response		
Responsive	23	76.7
Nonresponsive	7	23.3

Comparisons of BCL-2 mRNA expression with clinical response to neoadjuvant chemotherapy is shown in Table 3. The mean value of BCL-2 mRNA expression in breast cancer patients was 9.917±2.568. The mean value of BCL-2 mRNA expression in the responsive group was 9.887±2.731. The mean value of BCL-2 mRNA expression in the nonresponsive group was 10.017±2.122. The mean value of the responsive group was lower than that in the nonresponsive group as seen in Fig. 1, but there was no significant difference between the baseline BCL-2 mRNA expression and the clinical response to chemotherapy with p-value = 0.862 (p>0.05).

Figure 1 shows the mean value of the responsive group was lower than that in the nonresponsive.

Comparisons of ERα mRNA expression with clinical response to neoadjuvant chemotherapy is shown in Table 3. The mean value of ERα mRNA expression in breast cancer patients was 10.678±1.993. The mean value of ERα mRNA expression in the responsive group was 10.144±1.945. The mean value of ERα mRNA expression in the nonresponsive group was 12.433±0.801. The mean value of the responsive group was lower than that in the nonresponsive group as seen in Fig. 2. There was a significant difference between the baseline ERα mRNA expression and the clinical response to chemotherapy with p-value = 0.006 (p>0.05).

Figure 2 shows the mean value of the responsive group was lower than that in the nonresponsive group.

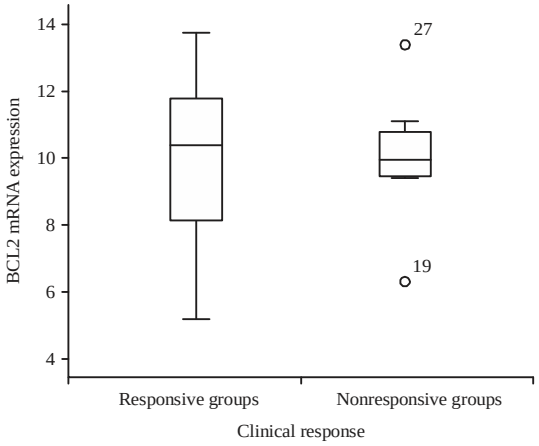

Fig. 1: Box plot comparing BCL-2 mRNA expression and the clinical response to chemotherapy

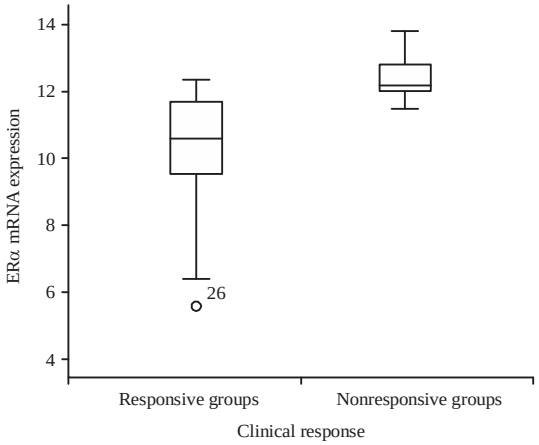

Fig. 2: Box plot comparing ERα mRNA expression and the clinical response to chemotherapy

Correlation of BCL-2 and ERα mRNA expression with the clinical response to chemotherapy is shown in Table 4. There was a slight positive correlation between BCL-2 mRNA expression and the clinical response to chemotherapy

Table 3: Comparisons of BCL-2 mRNA expression and ERα mRNA expression with clinical response to neoadjuvant chemotherapy

mRNA expression	Responsive (Mean±SD) (n = 23)	Non-responsive (Mean±SD) (n = 7)	Mean difference	p*
BCL-2	9.887±2.731	10.017±2.122	0.134	0.862
ERα	10.144±1.945	12.433±0.801	2.289	0.006

*p = t-test

Table 4: Correlation of BCL-2 and ERα mRNA expression with the clinical response to neoaduvant chemotherapy

mRNA	mRNA expression level (Mean±SD) (n = 30)	Correlation with chemotherapy response (r)	p*
BCL-2	11.837±0.360	0.028	0.885
ERα	10.678±1.993	-0.260	0.166

*p = Pearson

(r-value = 0.028), but this correlation was insignificant (p-value = 0.885, p>0.05). There was a negative correlation between ERα mRNA expression and the clinical response to chemotherapy (r-value = -0.260, but this correlation was also insignificant (p-value = 0.166, p>0.05).

DISCUSSION

This study showed that there were neither significant differences nor a relationship between BCL-2 mRNA expression and the clinical response to chemotherapy. The results suggested that BCL-2 mRNA expression has a minimal influence on the chemotherapy response.

The BCL-2 protein localizes to the inner mitochondrial membrane and functions to inhibit apoptosis and promote survival[19,20]. The BCL-2 can inhibit apoptosis resulting from a variety of intracellular signals[19,20]. The BCL-2 has been shown into inhibiting apoptosis induced by chemotherapeutic drugs (including doxorubicin) in cancer cells[7].

Abdel fatah *et al*.[9] found that a lack of BCL-2 expression was associated with high proliferation rates and elevated levels of P-cadherin, E-cadherin and HER3, while cancers positive for BCL-2 were correlated with high levels of p27, MDM4 and SPAG5. The BCL-2 could provide both prognostic and predictive information to individuals with Triple Negative Breast Cancer (TNBC)[9,22]. Patients with TNBC negative for BCL-2 expression appear to benefit from anthracycline taxane combination chemotherapy (ATC-CT), whereas patients with TNBC positive for BCL-2 expression seem to be resistant to ATC-CT and may benefit from a different type of chemotherapy[9]. The elevated BCL-2 expression is a significant independent predictor of poor outcomes in TNBC patients who undergo anthracycline-based adjuvant chemotherapy and one study showed that BCL-2 could predict out comes in TNBC. Thus, a BCL-2 expression analysis could facilitate decision-making regarding adjuvant treatment in TNBC patients[21].

The BCL-2 expression has been associated with positive estrogen receptor expression and a favorable prognosis in breast cancer. Positive expression of BCL-2 predicts no benefit from adjuvant anthracycline-based chemotherapies patients with non-basal TNBC. The BCL-2 status showed both prognostic and predictive values in non-basal TNBCs, therefore, assessing the BCL-2 status and basal phenotype can provide information on the prognostic and therapeutic classifications of TNBCs[22].

Other studies found that BCL-2 expression was not significantly associated with complete pathological response in patients with triple negative breast cancer and patients in the BCL-2-negative group tended to be more chemosensitive than those in the BCL-2-positive group[7]. This finding is in agreement with our results showing that BCL-2 could not predict the response to neoadjuvantchemotherapy.

Dawson *et al*.[8] reviewed five studies comprising 11, 212 women with early-stage breast cancer concluded that BCL-2 is an advantageous independent prognostic indicator for all types of early-stage breast cancer. Those study sets the rationale for the introduction of BCL-2 immunohistochemistry to improve the prognostic stratification of breast cancers[8]. A study of 100 samples of breast cancer compared BCL-2 levels using IHC and RT-PCR techniques and found that measuring BCL-2 expression in breast cancer using either immunohistochemistry or RT-PCR produced very similar results[3]. These results also suggest an association between BCL-2 gene expression and favorable biological features and clinical tumor-small tumor size, low nuclear grade, hormone receptor expression, the absence of c-erb-B2 and mutant p53 expression and low proliferation rates[3]. Research on 2749 breast cancer cases concluded that BCL-2 and Ki-67 expression could be combined to produce an index that could independently predict survival in ER-positive breast cancer, thus increasing the potential prognostic utility of these expression markers[23]. The prognostic role of BCL-2 expression in breast cancer is subtype-specific. The BCL-2 expression differs according to the molecular subtype and is only a useful prognostic marker for luminal A breast cancer[24]. The prognostic influence of BCL-2 was also different across molecular subtypes of breast cancer and was dependent on HR, HER2 and Ki-67expression as well as tumor stage[25].

This study also showed that there were significant differences in ERα mRNA expression between the responsive and nonresponsive groups with p-value = 0.006 (p>0.05). However, in testing the correlation between ERα mRNA expression and the clinical response, the results were insignificant. It can be concluded from this study that ERα mRNA expression has little influence on the chemotherapeutic response.

The estrogen receptor mediates the effects of estrogen on the development and progression of breast cancer by binding to specific response elements within a target gene promoter and activating growth factor pathways via membrane-bound proteins[26]. Estrogen E2 predominantly binds to ERα, which leads to the transcriptional regulation of genes involved in cell growth and survival. Studies found that ERα knockdown remarkably impaired the induction of BCL-2 and cyclin D1 as well as survival via E2[27]. The ERα is essential for E2-dependent growth and its expression level is a crucial determinant of the response to endocrine therapy and prognosis in patients with ERα-positive breast cancer[27]. Clinical data suggest that the estrogen receptor contributes to the chemotherapeutic responsiveness. However, the estrogen receptor status alone does not consistently predict the chemotherapeutic response. Chen *et al*.[28] observed TFF1, ESR1, GATA3 and TFF3 were ER-related genes that were associated with a complete pathological response (pCR). Protein expression of ER may provide important predictive outcomes for responses to neoadjuvant chemotherapy and may allow for the identification of a subgroup of patients who could significantly benefit from chemotherapy[28]. The ER-positive and ER-negative cancers differ in the expression of specific genes and show distinct patterns of mutations and alterations in the DNA copy number. Different biological processes were associated with the prognosis and chemotherapy response in ER-positive and ER-negative breast cancers[29]. Resistance to chemotherapy treatment in breast cancer is multifactorial. Characterized mechanisms of resistance to chemotherapy treatment are related to the activities of estrogen receptor α, P-glycoprotein, multidrug resistance-related proteins and topoisomerase-II. In preclinical and clinical studies, positive ERα expression in breast cancer cells was correlated with decreased sensitivity to chemotherapy[30].

Studies found that ERα status may play a significant role in determining the sensitivity of breast tumors to chemotherapy. Studies have shown that some chemotherapeutic agents may beless efficient in patients with ERα+ tumors than those with ERα- tumors[31-33]. Other reports have indicated that ERα is an independent predictive factor for the pathologic response to neoadjuvant chemotherapy in primary breast tumors and that ERα negativity is associated with an improved chemotherapy response[34-36]. An *in vitro* study using ERα-transfected Bcap37 cells and ERα-positive T47D breast cancer cells that were treated with chemotherapeutic agents in the presence or absence of 17-beta estradiol (E2) pretreatment showed similar results. The ERα-positive breast cancer cells showed a decreased response to chemotherapeutic agents due to the influence of ERα on the growth of breast cancer cells[37].

CONCLUSION

This study showed that there was a significant difference in the ERα mRNA expression levels between responsive and nonresponsive groups to chemotherapy. However, the correlation was insignificant. This suggests that ERα mRNA expression has a reduced influence on the chemotherapy response. This study showed that there were neither significant differences nor a correlation between BCL-2 mRNA expression and the clinical response to neoadjuvant chemotherapy in breast cancer. This study suggested that BCL-2 mRNA expression exerts a minimal influence on the chemotherapy response.

SIGNIFICANCE STATEMENT

This study revealed that BCL-2 mRNA expression minimally influences the clinical response to neoadjuvant chemotherapy in patients with breast cancer. High mRNA expression of ERα tends to associate with a lack of responsiveness to neoadjuvant chemotherapy in breast cancer, although the correlation analysis was not significant. The results of this study suggest a reduced role of BCL-2 and ERα mRNA in the chemotherapeutic response.

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