Effect of Primary Systemic Therapy on PD-1, PD-L1, and PD-L2 mRNA Expression in Advanced Breast Cancer

Ramadhan Karsono1*, Muhammad Al Azhar2, Yulia Pratiwi3, Fahreza Saputra2, Siti Nadliroh2, Teguh Aryandono4

Abstract

Objective: The association between PD-1, PD-L1, and PD-L2 expression and prognosis has been extensively studied in various cancers but remained controversial in breast cancer. Besides, little is known about the prognostic value of PD-1, PD-L1, and PD-L2 upregulation or downregulation following systemic therapy (chemotherapy and hormonal therapy) in breast cancer. Therefore, we aim to investigate the change of PD-1, PD-L1, and PD-L2 expression in mRNA level after primary systemic therapy in breast cancer patients and its clinical implications. Methods: Expression of PD-1, PD-L1, and PD-L2 mRNA were measured before-after chemotherapy and hormonal therapy with real-time PCR in 80 advanced breast cancer patients. The correlation between alteration of PD-1, PD-L1, and PD-L2 expression and clinicopathological characteristics as well as overall survival was also statistically analyzed. Results: Chemotherapy and hormonal therapy altered PD-1, PD-L1, and PD-L2 expression in breast cancer with most patients have an increase expression. As much as 57.1%, 62.9% and 60% patients have an increase PD-1, PD-L1, and PD-L2 expression after chemotherapy, while 60%, 60%, and 64% patients have an increase PD-1, PD-L1, and PD-L2 expression after hormonal therapy. Alteration of PD-1, PD-L1, and PD-L2 expression was not correlated with all clinicopathological characteristics. Increase in PD-1, PD-L1, and PD-L2 expression was significantly associated with better OS (p=0.031, p=0.019, and p=0.019 for PD-1, PD-L1, and PD-L2, respectively), which remained significant in multivariate analysis including age, stage, primary systemic therapy, histology grade, subtype and primary tumor histology (HR PD-1 0.5 (95% CI 0.28-0.88) p=0.031; HR PD-L1 0.43 (95% CI 0.24-0.8) p=0.019; HR PD-L2 (95% CI 0.24-0.87) p=0.019). Conclusion: Expression of PD-1, PD-L1, and PD-L2 in breast cancer patients is mostly enhanced after chemotherapy and hormonal therapy, and the enhancement is associated with good OS. This result revealed the potential of measuring PD-1, PD-L1, and PD-L2 mRNA expression in predicting clinical outcome.

Keywords: PD-1- PD-L1- PD-L2- breast cancer- systemic therapy- immunotherapy

Introduction

Breast cancer has a high incidence and mortality rate and is estimated to be the most common malignancy in females worldwide. Every year incidences of breast cancer increase by more than 5%. In developing countries including Indonesia, most breast cancers were detected in advanced stages (3 and 4). Moreover, death is higher in low development countries (Ghoncheh et al., 2016; Youlden et al., 2014; Yuan et al., 2019). Types of therapies commonly used to treat breast cancer are radiotherapy, surgery, chemotherapy, hormone therapy, and targeted therapy. However, these therapies are not effective enough to treat breast cancer (Zhang et al., 2017).

Currently there is a growing interest of immunotherapy in cancer treatment. The use of immune checkpoint inhibitors especially anti PD-1/ PD-L1 becomes the most popular immunotherapeutic strategy in recent treatment (Esteva et al., 2019). Anti PD-1/ PD-L1 also has shown good clinical effect in treatment of breast cancer (Planes-Laine et al., 2019). Programmed death 1 (PD-1) is a protein receptor expressed by T cell, B cell, and other immune cells. It has 2 ligands including programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) (Pardoll, 2012; Topalian et al., 2016). Expression of PD-L1 is highly expressed in cancer patients (Azhar et al., 2020; Wang et al., 2016) and associated with poor prognosis in several cancer types (Zhang et al., 2017). However, PD-L1 expression in breast cancer is still controversial with some studies reporting conflicting results (Schalper et al., 2014; Zhang et al., 2017).

1Department of Surgical Oncology, Dharmais National Cancer Center Hospital, Jakarta, Indonesia. 2Department of Research and Development, Dharmais National Cancer Center Hospital, Jakarta, Indonesia. 3Department of Functional Medical Staff of Surgical Oncology, Dharmais National Cancer Center Hospital, Indonesia. 4Department of Surgery, Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada, Indonesia. *For Correspondence: ramadhan@dharmais-surgonc.com

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Positive PD-L1 expressions correlated with good response to immune checkpoint inhibitor therapy (anti-PD-1/PD-L1). Currently, measuring PD-L1 expression using immunohistochemistry (IHC) has been used to determine type of patients who respond immune checkpoint inhibitor therapy. However, the use of PD-L1 IHC has several limitations such as different cut offs, different scoring systems, variable detection antibodies, and processing variability (Bertucci et al., 2015; Patel and Kurzrock, 2015). This could affect the result which leads to conflicting data in several studies (Schalper et al., 2014). Therefore, the use of alternative method such as real-time PCR to assess PD-L1 expression may help to overcome such limitations. It has been shown that there is positive correlation between PD-L1 protein and mRNA expression, which indicated potential of measuring PD-L1 mRNA expression to assess response to anti-PD-1/PD-L1 therapy (Kim et al., 2018).

Chemotherapy and hormonal therapy are type of systemic therapy that mostly used to treat breast cancer patients in advanced stages. The idea of combining immune checkpoint inhibitor therapy with chemotherapy or hormonal therapy has been proposed to enhance the response rate and duration and improve survival (Esteva et al., 2019; Hühn et al., 2019; Luo and Fu, 2016; Page et al., 2019). Several studies reported that some chemotherapy agents could change PD-L1 expression (Luo and Fu, 2016). On the other hand, the study of hormone therapy effect to PD-1, PD-L1, or PD-L2 expression is very rare. To investigate the potential benefit of combination therapy between hormonal therapy/chemotherapy and immunotherapy, it is crucial to understand the impact of chemotherapy or hormonal therapy to PD-L1 expression as well as PD-1 and PD-L2 expression. Besides, little is known about the prognostic value of PD-1, PD-L1, and PD-L2 upregulation or downregulation following systemic therapy in breast cancer. Therefore, in this study, we aimed to assess PD-1, PD-L1, PD-L2 mRNA expression before-after primary systemic therapy (chemotherapy and hormonal therapy) using real-time PCR and investigate the association with clinicopathological features and overall survival of advanced breast cancer patients.

Materials and Methods

Patients’ samples collection and therapy given

This study was a retrospective study conducted from 2011 to 2017 (n=80) at Dharmais National Cancer Center Hospital, Indonesia. Patients’ tumor tissues of advanced breast cancer (stages 3B and 4) patients were taken before and after primary systemic therapy (chemotherapy and hormonal therapy). Tissue samples were divided into two pieces, one piece for histological examination and another piece was directly put in cryotubes containing 1 mL of RNAlater then stored at -80°C to keep RNA integrity and quality. Criteria for stage 3B and 4 was determined based on American Joint Committee on Cancer 7th edition guideline (American Cancer Society, 2010).

From 80 samples, 35 patients were received primary chemotherapy and 45 patients received primary hormonal therapy. All patients were given systemic therapy before the patient undergone surgery. Hormonal therapy group received Aromatase Inhibitor, Tamoxifen or GnRH-analogue during 6 months of treatment. The chemotherapy group received FAC (5-Fluorouracil, Adriamycin, and Cyclophosphamide) which were given for 6 cycles.

Patients who have a mastectomy before primary systemic therapy, pregnant, and refuse to participate were excluded. The patient followed up was done continuously to obtain data of death, censored patients, and patients with new symptoms. All patients agreed to be involved in this study after signing informed consent. This study was approved by Ethical Committee at Dharmais Hospital-National Cancer Center, Indonesia (Number of Ethical Clearance: 9/KEPK/II/2019 and 10/KEPK/II/2019).

Extraction of total RNA and cDNA synthesis

Isolation of total RNA from tissue samples was done using RNA Tissue Mini Kit (Qiagen) according to manual instruction book provided by the kit. The total RNA was then measured for its concentration and purity using Nanodrop spectrophotometer. Maximum 2,000 ng of RNA was reverse transcribed to cDNA using High Capacity cDNA synthesis kit (Applied Biosystem). The process of cDNA synthesis was conducted based on standard procedure from the kit. Generated cDNA from all samples was then diluted to 100 ng to be used in real time PCR.

Primer and probes used in real time PCR

Primer and probes used in this study were designed to avoid genomic DNA amplification by spanning exon-exon junction. For PD-1, PD-L1, and GAPDH, each primer pair and its probe were formulated by Applied Biosystem into ready-to-use Custom TaqMan Gene Expression Assay. For PD-L2, pre-design TaqMan Gene Expression Assay Hs01057777_m1 was used. Sequences of PD-1 primers and probe are 5’-AGGCATGCAGATCCCAACA-3’ (forward), 5’-CCTGTCCTGGGAGTCTAAGA-3’ (reverse), 5’-CTGTGGCCGTGCTCAACT-3’ (probe). Sequences of PD-L1 primers and probe are 5’-GTGGCATCCAAGATACAAACTCAA-3’ (forward), 5’-TCTGGGCGGTGCTACAACT-3’ (reverse), 5’-TCAAGCAGGTATTCTACACC-3’ (probe). Sequences of GAPDH primers and probe are 5’-AGCCTCAAGATCATCAAACTCAA-3’ (forward), 5’-TACCTGATGTACAGACATCC-3’ (reverse), 5’-ACTGTTGGTCTAGAGTCTTC-3’ (probe). Sequences of PD-L2 primers and probe are 5’-CTGCACCACCAACTGCTTAG-3’ (probe).

Real time PCR (qPCR)

The real time PCR reaction contained 20 µL reaction mixture consist of 10 µL TaqMan Gene Expression Master Mix, 1 µL Custom TaqMan Gene Expression assay (primer and probe), 5 µL nuclease free water, and 4 µL cDNA. PD-1, PD-L1, PD-L2, and GAPDH reactions of each sample before and after therapy were run together in Fast 7500 Real-Time PCR System (Applied Biosystem). After 50°C (2 minutes) and 95°C (10 minutes) hold stage, the qPCR reaction was continued with 40 cycles of 95°C (30 seconds) denaturation and 62°C (1 minute) annealing and extension. The real time PCR data was analyzed using
2^ΔΔCT method (Livak and Schmittgen, 2001) with GAPDH as reference gene (internal control). By using 2^ΔΔCT, we determine the fold changes of each sample. Fold changes more than 1 were categorized as increased PD-1, PD-L1, or PD-L2 expression, and less than 1 were categorized as decreased PD-1, PD-L1, or PD-L2 expression.

Statistical analysis
Statistical analysis was performed using IBM SPSS 21. Statistical comparisons between PD-1, PD-L1, and PD-L2 and clinic pathological were assessed by the Chi-Square test (χ² test). Correlation between fold change value of PD-1, PD-L1 and PD-L2 were assessed by Spearman Correlation. Analysis between effect therapy and fold change of PD-1, PD-L1 and PD-L2 were assessed by Independent T-test. Overall Survival (OS) were estimated using the Kaplan-Meier method. Cox proportional hazards model was used to estimate the prognostic factor of PD1, PD-L1, and PD-L2 on overall survival. All analyses were hypothesis-driven by P < 0.05 was considered statistically significant.

Results
Patients Characteristics
Among the 80 patients, mean of the age were 47.8 years old. A large portion of primary tumor histology was invasive ductal carcinoma, accounting for 91.3%. All patients’ histology grades were low grade (52.5%) or high grade (47.5%). Thirty-nine (48.7%) patients were in stage 3B and 41 patients (51.3%) were in stage 4. Most patients were estrogen receptor (ER) positive (68.7%), progesterone receptor (PR) positive (66.3%), and Her2 negative (70%). Breast cancer subtypes were mostly found in Luminal A (36.3%) and Luminal B types (36.3%)

Effect of Therapy to PD-1, PD-L1, and PD-L2 Expression
After undergoing chemotherapy, most breast cancer patients, 57.1% (20/35), 62.9% (22/35), and 60% (21/35) patients showed increased PD-1, PD-L1, and PD-L2 expression, respectively (mean fold change: 6.65, 2.93, and 5.88 for increased PD-1, PD-L1, and PD-L2). Meanwhile, 42.9% (15/35), 37.1% (13/35), and 40% (14/35) showed decreased PD-1, PD-L1, and PD-L2.

| Table 1. Characteristics of Patients |
|---------------------------------------|
| Variable                              | N  | %  |
| Age                                   | 47.8 ± 10.65 |
| Range (years)                         | 22 – 75    |
| Primary tumor histology               |     |
| Ductal                                | 73  | 91.3 |
| Lobular                               | 7   | 8.7  |
| Histology grade                       |     |
| Low (1-2 grade)                       | 42  | 52.5 |
| High (3 grade)                        | 38  | 47.5 |
| TNM stage                             |     |
| Stage 3B                              | 39  | 48.7 |
| Stage 4                               | 41  | 51.3 |
| ER status                             |     |
| Positive                              | 55  | 68.7 |
| Negative                              | 25  | 31.3 |
| PR status                             |     |
| Positive                              | 53  | 66.3 |
| Negative                              | 27  | 33.7 |
| Her2 status                           |     |
| Positive                              | 24  | 30.0 |
| Negative                              | 56  | 70.0 |
| Subtype                               |     |
| Luminal A                             | 29  | 36.3 |
| Luminal B                             | 29  | 36.3 |
| Her2 neu                              | 14  | 10.0 |
| Triple Negative                       | 8   | 17.5 |

Figure 1. Effect of Therapy to PD-1, PD-L1 and PD-L2 Expression. (A) PD-1, (B) PD-L1, and (C) PD-L2
expression, respectively (mean fold change: 9.44, 4.88, and 3.65 for decreased PD-1, PD-L1, and PD-L2). Similar to chemotherapy, hormone therapy also increased PD-1, PD-L1, and PD-L2 expression of most breast cancer patients. As many as 60% (27/45), 60% (27/45), and 64.4% (29/45) patients showed increased expression for PD-1, PD-L1, and PD-L2 expression, respectively (mean fold change: 8.48, 4.03, and 7.02 for increased PD-1, PD-L1, and PD-L2). Meanwhile, 40% (18/45), 40% (18/45), and 35.6% (16/45) showed decreased PD-1, PD-L1, and PD-L2 expression, respectively (mean fold change: 12.54, 4.03, and 7.02 for decreased PD-1, PD-L1, and PD-L2).

Table 2. Association between PD-1, PD-L1, PD-L2 Expression after Therapy and Clinicopathology

| Variable                  | PD-1         |         | PD-L1         |         | PD-L2         |         |
|---------------------------|--------------|---------|--------------|---------|--------------|---------|
| Therapy                   | Decreased (%)| Increased (%)| Decreased (%)| Increased (%)| Decreased (%)| Increased (%)|Pv   |
| Chemo                     | 15 (42.9)    | 20 (57.1) | 0.797        | 13 (37.1) | 22 (62.9)    | 0.795    | 14 (40.0) | 21 (60.0) | 0.684 |
| Hormonal                  | 18 (40.0)    | 27 (60.0) | 18 (40.0)    | 27 (60.0) | 16 (35.6)    | 29 (64.4) |           |           |       |
| Age                       |              |         |              |         |              |         |           |           |       |
| < 40 years old            | 10 (50.0)    | 10 (50.0) | 0.359        | 8 (40.0) | 12 (60.0)    | 0.895    | 8 (40.0) | 12 (60.0) | 0.79  |
| > 40 years old            | 23 (38.3)    | 37 (61.7) | 23 (38.3)    | 37 (61.7) | 22 (36.7)    | 38 (63.3) |           |           |       |
| Primary tumor Histology   |              |         |              |         |              |         |           |           |       |
| Ductal                    | 29 (39.7)    | 44 (60.3) | 0.371        | 28 (38.4) | 45 (61.6)    | 0.815    | 27 (37.0) | 46 (63.0) | 0.759 |
| Lobular                   | 4 (57.1)     | 3 (42.9)  | 4 (57.1)     | 3 (42.9)  | 4 (57.1)     | 4 (57.1) |           |           |       |
| Histology grade           |              |         |              |         |              |         |           |           |       |
| Low                       | 19 (45.2)    | 23 (54.8) | 0.446        | 14 (33.3) | 28 (66.7)    | 0.296    | 12 (28.6) | 30 (71.4) | 0.083 |
| High                      | 14 (36.8)    | 24 (63.2) | 17 (44.7)    | 21 (55.3) | 18 (47.4)    | 20 (52.6) |           |           |       |
| TNM stage                 |              |         |              |         |              |         |           |           |       |
| Stage 3B                  | 16 (41.0)    | 23 (59.0) | 0.968        | 14 (35.9) | 25 (64.1)    | 0.61     | 14 (35.9) | 25 (64.1) | 0.773 |
| Stage 4                   | 17 (41.5)    | 24 (58.5) | 17 (41.5)    | 24 (58.5) | 16 (39.0)    | 25 (61.0) |           |           |       |
| ER status                 |              |         |              |         |              |         |           |           |       |
| Positive                  | 20 (36.4)    | 35 (63.6) | 0.188        | 19 (34.5) | 36 (65.5)    | 0.252    | 18 (32.7) | 37 (67.3) | 0.191 |
| Negative                  | 13 (52.0)    | 12 (48.0) | 12 (48.0)    | 13 (52.0) | 12 (48.0)    | 13 (52.0) |           |           |       |
| PR status                 |              |         |              |         |              |         |           |           |       |
| Positive                  | 20 (37.7)    | 33 (62.3) | 0.371        | 17 (32.1) | 36 (67.9)    | 0.086    | 18 (34.0) | 35 (66.0) | 0.36  |
| Negative                  | 13 (48.1)    | 14 (51.9) | 14 (51.9)    | 13 (48.1) | 12 (44.4)    | 15 (55.6) |           |           |       |
| Her2 status               |              |         |              |         |              |         |           |           |       |
| Positive                  | 11 (45.8)    | 13 (54.2) | 0.586        | 11 (45.8) | 13 (54.2)    | 0.395    | 11 (45.8) | 13 (54.2) | 0.313 |
| Negative                  | 22 (39.3)    | 34 (60.7) | 20 (35.7)    | 36 (64.3) | 19 (33.9)    | 37 (66.1) |           |           |       |
| Subtype                   |              |         |              |         |              |         |           |           |       |
| Luminal A                 | 10 (34.5)    | 19 (65.5) | 0.561        | 7 (24.1) | 22 (75.9)    | 0.167    | 8 (27.6) | 21 (72.4) | 0.11  |
| Luminal B                 | 12 (41.4)    | 17 (58.6) | 13 (44.8)    | 16 (55.2) | 11 (37.9)    | 18 (62.1) |           |           |       |
| Her2 neu                  | 8 (57.1)     | 6 (42.9)  | 8 (57.1)     | 6 (42.9)  | 9 (64.3)     | 5 (35.7) |           |           |       |
| Triple Negative           | 3 (37.5)     | 5 (62.5)  | 3 (37.5)     | 5 (62.5)  | 2 (25.0)     | 6 (75.0) |           |           |       |

Pv, Pearson Chi-Square
and PD-L2). However, the changes are not statistically significant (Figure 1).

**Relationship between PD-1, PD-L1 and PD-L2 expression**

The relationship between expression changes of PD-1 and PD-L1, PD-1 and PD-L2, PD-L1 and PD-L2 showed a significant positive correlation with a very strong close relationship (R-value: 0.762, 0.746, and 0.834 for PD-1, PD-L1, and PD-L2 respectively). We showed that samples with increased PD-1 expression also have increased PD-L1 and PD-L2 expression (Figure 2).

**Association between PD1, PDL1, and PDL2 expression after therapy and clinicopathology**

We found that the alteration of PD-1, PD-L1 and PD-L2 expression was not associated with age, primary tumor histology, histology grade, ER status, PR status, Her2 status, Ki67 status and molecular subtype. However, increased PD-1, PD-L1, and PD-L2 expression were found more on breast cancer patients with higher ages (>40

**Table 3. Overall Survival (OS) by PD-1, PDL-1, PDL-2 Expression and Multivariate Analysis**

| Group            | No. | Events | OS, Median (95% CI), days | Hazard Ratio (95% CI) | Univariate | Multivariatea | Pva |
|------------------|-----|--------|---------------------------|-----------------------|------------|---------------|------|
| PD-1 Increased   | 47  | 29     | 967 (511 – 1422)          | 0.55 (0.32 – 0.94)    | 0.50 (0.28 – 0.88) | 0.016          |
| PD-1 Decreased   | 33  | 27     | 587 (387 – 786)           | NA                    | NA         | NA             |     |
| PD-L1 Increased  | 49  | 33     | 951 (590 – 1311)          | 0.52 (0.30 – 0.90)    | 0.43 (0.24 – 0.80) | 0.007          |
| PD-L1 Decreased  | 31  | 23     | 490 (309 – 670)           | NA                    | NA         | NA             |     |
| PD-L2 Increased  | 50  | 33     | 967 (623 – 1310)          | 0.51 (0.29 – 0.89)    | 0.463 (0.24 – 0.87) | 0.018          |
| PD-L2 Decreased  | 30  | 23     | 566 (356 – 775)           | NA                    | NA         | NA             |     |

Abbreviation : NA, not applicable; a Multivariate analysis was performed to adjust for the potential effects of prior age, stage, primary systemic therapy, histology grade, subtype and primary tumor histology; a P value are from Multivariate Cox Regression analysis with adjusting for clinical covariates
years), ductal histology, higher grade, positive estrogen receptor (ER) status, negative HER2 status, and positive progesterone receptor (PR) status (Table 2).

**Association of PD-1, PD-L1, and PD-L2 expression with survival in breast cancer**

Further analysis was undertaken to explore the potential association of PD-1, PD-L1, and PD-L2 with patient prognosis and survival. Kaplan-Meier survival analysis indicated that increased PD-1, PD-L1, and PD-L2 expression after primary systemic therapy were associated with statistically significant better overall survival (Figure 3). Increased PD-1 expression was associated with longer OS than decreased PD-1 expression in advanced breast cancer (HR=0.55, 95% CI 0.32 – 0.94; p=0.031). Increased PD-L1 expression was associated with longer OS than decreased PD-L1 expression (HR=0.52, 95% CI 0.30 – 0.90; p=0.019). Increased PD-L2 expression was also associated with longer OS than decreased PD-L2 expression in advanced breast cancer (HR=0.52, 95% CI 0.29 – 0.89; p=0.019). Our data identified significant association between better overall survival and increased PD-1, PD-L1, and PD-L2 expression that was confirmed by multivariate analysis including prior age, stage, primary systemic therapy, histology grade, subtype and primary tumor histology (Table 3).

**Discussion**

In the present study, we have analyzed PD-1, PD-L1, and PD-L2 mRNA expression in breast cancer tissue from advanced stages patients. Our study found that expression of PD-1, PD-L1, and PD-L2 in breast cancer patients is mostly increased after chemotherapy with 57.1%, 62.9% and 60% patients have an increase in PD-1, PD-L1, and PD-L2 expression, respectively (Table 2). While 42.9%, 37.1%, and 40% breast cancer patients have their PD-1, PD-L1, and PD-L2 expression decreased after chemotherapy. It has been explained that chemotherapy can alter the expression of PD-1, PD-L1, and PD-L2 expression in several cancer types. However, the change depends on chemotherapeutic agents and cell line that were used in the experiment (Chacon et al., 2016; Ghebeh et al., 2010; Peng et al., 2015; Zhang et al., 2008).

Some chemotherapeutic agents that have been reported to increase PD-1, PD-L1, or PD-L2 expression are paclitaxel, etoposide, gemcitabine, dacarbazine, and cisplatin (Luo and Fu, 2016). Etoposide and paclitaxel induced PD-L1 expression in breast cancer cell line leading to the activation of co-inhibitory signals (Zhang et al., 2008). Carboplatin–paclitaxel treatment also induced PD-L1 expression in ovarian cancer cell lines (Peng et al., 2015). Both PD-L1 and PD-1 expression in leukemia cells were upregulated after decitabine treatment (Yang et al., 2013). Cisplatin could increase the expression of PD-L1 in hepatoma H22 cells when the concentration is less than IC$_{50}$ (Qin et al., 2010). Gemcitabine or paclitaxel was also enhanced PD-L1 expression in human pancreatic cell lines both in protein and mRNA level (Doi et al., 2017). Nonetheless, some chemotherapeutic drugs could downregulate PD-1, PD-L1, or PD-L2 expression. Oxaliplatin inhibit PD-L2 expression thus limiting immunosuppression by tumor cells and dendritic cells (Lesterhuis et al., 2011). Treatment with panobinostat suppresses PD-L1 expression in lymphoma (Oki et al., 2014). Research by Sheng et al. (Sheng et al., 2016) revealed the downregulation of PD-L1 expression in tumor cells of NSCLC patients after treatment with neoadjuvant chemotherapy (paclitaxel, pemetrex, and TKI). After chemotherapy, positive PD-L1 expression changed from 75% to 37.5% (Sheng et al., 2016).

In this study, 5-FAC (5-Fluorouracil, Adriamycin, and Cyclophosphamide) was used in the treatment of breast cancer patients. It has been reported that 5-Fluorouracil induce PD-L1 surface expression on breast cancer cell lines (Zhang et al., 2008). Doxorubicine (adriamycin) is reported to upregulate PD-L1 nuclear expression, although downregulate its surface expression in tumor. Thus, these previous finding supported our results that after 5-FAC treatment, PD-1, PD-L1, and PD-L2 expression in most breast cancer patients are increased. Meanwhile, the effect of cyclophosphamide on PD-1, PD-L1, or PD-L2 expression hasn’t been known. Moreover, it has been proposed that combination between 5-Fluorouracil, Adriamycin, or Cyclophosphamide with anti PD-1/ PD-L1 might give positive impact on cancer patients (Bailly et al., 2020).

The exact mechanism on how chemotherapy work on tumor microenvironment and affect PD-1, PD-L1, and PD-L2 expression is still not clear. However, some studies reported that some chemotherapeutic agents involved in several biological pathway. Chemotherapeutic agents through interferon (IFN)-γ-independent and IFN-γ-dependent may upregulate PD-L1 expression by activating different signal such as JAK/STAT3, PI3K/AKT, RAS/RAF, or release several immune suppression cytokine (Luo and Fu, 2016). In breast cancer, signaling through key proliferative pathways, like PI3K/AKT and MEK/ERK is known to induce PD-L1 expression (Crane et al., 2009; Hasan et al., 2011).

Similar with chemotherapy, the PD-1, PD-L1, and PD-L2 mRNA expression are mostly increased after patients underwent hormonal therapy. Percentages of increased PD-1, PD-L1, and PD-L2 expression after hormonal therapy are 60%, 60%, and 64% respectively (Table 2). It has been reported that some hormonal therapy could induce PD-L1 expression in several cancer. Expression of PD-L1 is increased in MCF7 cells (breast cancer cell line) after treatment with estrogen receptor (ER) antagonist. Treatment with tamoxifen is also increased PD-L1 expression in mouse mammary tumor virus-polyoma middle tumor-antigen (MMTV-PyMT) breast cancer mice models (Hühn et al., 2019). It also has been shown that aromatase inhibitor (AI) therapy might increase the expression of both PD-L1 and chemokine receptor CCR7 in tumors (Turnbull et al., 2020; West et al., 2018). In a prostate cancer trial, enzalutamide plus pembrolizumab was associated with increased PD-L1 expression in tumor and dendritic cells, and increased PD-1-positive in circulating T-cells (Bishop et al., 2015; Graff et al., 2016).

It is not clear that how hormone therapy affects PD-
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To the best of our knowledge, this study is the first that analyzed the relationship between alteration of PD-1, PD-L1, and PD-L2 mRNA expression after primary systemic therapy with survival rate. We have shown that PD-1, PD-L1, and PD-L2 expression in most samples are increased after chemo and hormonal therapy and the enhancement is associated with good survival. Since PD-L1 expression was used to assess response to PD-1/PD-L1 checkpoint inhibitor therapy, this finding indicated reassessment of PD-L1 expression after chemotherapy or hormonal therapy should be performed. Besides, because of high PD-L1 expression include expression in mRNA level is associated with good clinical outcome of anti-PD-1/PD-L1 therapy (Patel and Kurzrock, 2015; Schmid et al., 2016), we could suggest that PD-1/PD-L1 checkpoint inhibitor therapy might improve outcome of breast cancer patients who have an increased PD-L1 expression after completion of chemotherapy or hormonal therapy.

It has been shown that the combination of chemotherapy/hormone therapy and immunotherapy might provide effective and durable anti-tumor immune response and facilitate the clearance of the residual breast cancer cells, and reducing the percentage of patients that progress into metastatic disease (Hühn et al., 2019; Luo and Fu, 2016). Therefore, our finding may support the idea of combining chemo or hormone therapy with anti PD-1/PD-L1. This finding also revealed that PD-1, PD-L1, and PD-L2 mRNA expression potentially could be used to predict clinical outcome of breast cancer patients. However, the limitation of this study is mRNA expression is not the same with protein expression due to post transcriptional modifications. Thus, further study to compare PD-L1 mRNA expression with PD-L1 protein expression using IHC as gold standard is needed to confirm this finding.

In conclusion, Expression of PD-1, PD-L1, and PD-L2 majorly increased after primary systemic therapy. Increase in PD-1, PD-L1, and PD-L2 expression after therapy was significantly associated with good OS. Strong positive correlation between PD-1, PD-L1, and PD-L2 alteration after systemic therapy suggested chemotherapy or hormonal therapy may affect the same pathway to alter PD-1, PD-L1, and PD-L2 expression. Our finding implied reassessment of PD-L1 expression and the potential benefit of anti-PD-1/PD-L1 therapy after completion of systemic therapy. This finding also revealed the potential to measure PD-1, PD-L1, and PD-L2 mRNA expression to predict clinical outcome of advanced stages breast cancer patients. However, subsequent study by comparing mRNA expression to PD-L1 IHC is needed to confirm the result.

Author Contribution Statement

R.K and M.A.A designed the study and took the lead in writing the manuscript. M.A.A carried the experiment and performed the measurement. F.S contributed to sample preparation. Y.P performed statistical analysis; All authors discussed the results and contributed to the final manuscript.
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Data Availability
The datasets are not publicly available due to ethical restrictions, but are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
This study was approved by Ethical Committee at Dharmais Hospital-National Cancer Center, Indonesia (Number of Ethical Clearance: 9/KEPK/II/2019 and 10/KEPK/II/2019).

Conflict of Interest
The authors declare no conflict of interest.

References
Ali HR, Glont, SE, Blows FM, et al (2015). PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes. Ann Oncol, 26, 1488–93.

American Cancer Society (2010). Breast Cancer Staging 7th Edition. American Joint Committee on Cancer. Available from: cancerstaging.org.

Azhar MA, Nadiroh S, Frameswari K, et al (2020). Profile of PD-1 and PD-L1 mRNA expression in peripheral blood of nasopharyngeal carcinoma. Mol Cell Biomed Sci, 4, 121-7.

Bailly C, Thuru X, Quesnel B (2020). Combined cytotoxic chemotherapy and immunotherapy of cancer: modern times. NAR Cancer, 2, 1–20.

Baptista MZ, Sarian LO, Derchain SFM, et al (2015). Prognostic significance of PD-L1 and PD-L2 in Hum Pathol, 47, 78-84.

Bertucci F, Finetti P, Colpaert C, et al (2015). PDL1 expression in inflammatory breast cancer is frequent and predicts for the pathological response to chemotherapy. Oncotarget, 6, 13506–19.

Bishop JL, Sio A, Angeles A, et al (2015). PD-L1 is highly expressed in Enzalutamide resistant prostate cancer. Oncotarget, 6, 234-42.

Chacon JA, Schutsky K, Powell DJ (2016). The impact of chemotherapy, radiation and epigenetic modifiers in cancer cell expression of immune inhibitory and stimulatory molecules and anti-tumor efficacy. Vaccines, 4, 1-28.

Crane CA, Panner A, Murray JC, et al (2009). PI(3) kinase is associated with a mechanism of immunoresistance in breast and prostate cancer. Oncogene, 28, 306–12.

Doi T, Ishikawa T, Okayama T, et al (2017). The JAK/STAT pathway is involved in the upregulation of PD-L1 expression in prostate cancer cell lines. Oncol Rep, 37, 1545–54.

Esteva FJ, Hubbard-Lucey VM, Tang J, Pusztai L (2019). Immunotherapy and targeted therapy combinations in metastatic breast cancer. Lancet Oncol, 20, 175–86.

Ghebeh H, Lehe C, Barhoush E, et al (2010). Doxorubicin downregulates cell surface B7-H1 expression and upregulates its nuclear expression in breast cancer cells: Role of B7-H1 as an anti-apoptotic molecule. Breast Cancer Res, 12, 1-12.

Ghonchel M, Pournamdar Z, Salehniya H (2016). Incidence and mortality and epidemiology of breast cancer in the world. Asian Pac J Cancer Prev, 17, 43–6.

Graff JN, Alumkal JJ, Drake CG, et al (2016). Early evidence of anti-PD-1 activity in enzalutamide-resistant prostate cancer. Oncotarget, 7, 52810–17.

Hasan A, Ghebeh H, Lehe C, Ahmad R, Dermime S (2011). Therapeutic targeting of B7-H1 in breast cancer. In Expert Opinion on Therapeutic Targets, 15, pp 1215–21.

Huerta-Reyes M, Maya-Núñez G, Pérez-Solís MA, et al (2019). Treatment of breast cancer with gonadotropin-releasing hormone analogs. Front Oncol, 9, 1-17.

Hühn D, Marti-Rodrigo P, Mournon S, et al (2019). Estrogen deprivation triggers an immunosuppressive phenotype in breast cancer cells. BioRxiv, 2019, 1-28.

Kim H, Kwon HJ, Park SY, et al (2018). Clinicopathological analysis and prognostic significance of programmed cell death-ligand 1 protein and mRNA expression in non-small cell lung cancer. PLoS One, 13, 1–14.

Lesterhuis WJ, Punt CJ, Hato SV, et al (2011). Platinum-based drugs disrupt STAT6-mediated suppression of immune responses against cancer in humans and mice J Clin Invest, 121, 3100-8.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25, 402–8.

Luo M, Fu L (2016). The effect of chemotherapy on programmed cell death 1/ programmed cell death 1 ligand axis: Some chemotherapeutic drugs may finally work through immune response. Oncotarget, 7, 29794–803.

Oki Y, Buglio, D, Zhang J, et al (2014). Immune regulatory effects of panobinostat in patients with Hodgkin lymphoma through modulation of serum cytokine levels and T-cell PD1 expression. Blood Cancer J, 4, 1-4.

Page DB, Bear H, Prabhakaran S, et al (2019). Two may be better than one: PD-1/PD-L1 blockade combination approaches in metastatic breast cancer. NPJ Breast Cancer, 5, 1–9.

Pardoll DM (2012). The blockade of immune checkpoints in cancer immunotherapy. Nat Publishing Group, 12, 252–64.

Patel SP, Kurzrock R (2015). PD-L1 expression as a predictive biomarker in cancer immunotherapy. Mol Cancer Ther, 14, 847–56.

Peng J, Hamanishi J, Matsumura N, et al (2015). Chemotherapy induces programmed cell death-ligand 1 overexpression via the nuclear factor-xBto foster an immunosuppressive tumor microenvironment in Ovarian Cancer. Cancer Res, 75, 5034–45.

Planes-Laine G, Rochigneux P, Bertucci F, et al (2019). PD-1/ PD-L1 targeting in breast cancer: The first clinical evidences are emerging. A literature review. Cancers, 11, 1-25.

Qin X, Liu C, Zhou Y, Wang G (2010). Cisplatin induces programmed death-1-ligand 1(PD-L1) over-expression in hepatoma H22 cells via Erk/Mapk signaling pathway. Cell Mol Biol, 56, 1366–72.

Rothenberger NJ, Somasundaram A, Stabile LP (2018). The role of the estrogen pathway in the tumor microenvironment. Int J Mol Sci, 19, 611.

Sabatier R, Finetti P, Mammessier E, et al (2015). Prognostic and predictive value of PDL1 expression in breast cancer. Oncotarget, 6, 5449-64.

Schafer KA, Velchti V, Carvajal D, et al (2014). In situ tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas. Clin Cancer Res,
Effect of Primary Systemic Therapy on PD-1, PD-L1, and PD-L2 mRNA Expression in Advanced Breast Cancer

Schmid P, Hegde PS, Zou W, et al (2016). Association of PD-L2 expression in human tumors with atezolizumab activity. *J Clin Oncol*, 34, 11506.

Sheng J, Fang W, Yu J, et al (2016). Expression of programmed death ligand-1 on tumor cells varies pre and post chemotherapy in non-small cell lung cancer. *Sci Rep*, 6, 1–10.

Topalian SL, Taube JM, Anders RA, Pardoll DM (2016). Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. *Nat Rev Cancer*, 16, 275–87.

Turnbull AK, Arthur LM, Renshaw L, et al (2020). Accurate prediction and validation of response to endocrine therapy in breast cancer. *J Clin Oncol*, 33, 2270-8.

Ulcer M, Sanders AJ, Owen S, et al (2017). Clinical significance of PD1 and PDL1 in human breast cancer. *Anticancer Res*, 37, 4249–54.

Wang X, Teng F, Kong L, Yu J (2016). PD-L1 expression in human cancers and its association with clinical outcomes. *Oncotargets Ther*, 9, 5023–39.

West J, Park D, Harmon C, et al (2018). Evolutionary exploitation of PD - L1 expression in hormone receptor positive breast cancer. *bioRxiv*, 2018, 1-3.

Yang H, Dinardo C, Davanlou M, et al (2013). Expression of PD-L1, PD-L2, PD-1 and CTLA4 in myelodysplastic syndromes is enhanced by treatment with hypomethylating agents. *Leukemia*, 28, 1280-8.

Yearley JH, Gibson C, Yu N, et al (2017). PD-L2 expression in human tumors: Relevance to anti-PD-1 therapy in cancer. *Clin Cancer Res*, 23, 3158–67.

Yearden DR, Cramb SM, Yip CH, Baade PD (2014). Incidence and mortality of female breast cancer in the Asia-Pacific region. *Cancer Biol Med*, 11, 101–15.

Yuan C, Liu Z, Yu Q, et al (2019). Expression of PD-1/PD-L1 in primary breast tumours and metastatic axillary lymph nodes and its correlation with clinicopathological parameters. *Sci Rep*, 9, 1–8.

Zhang M, Sun H, Zhao S, et al (2017). Expression of PD-L1 and prognosis in breast cancer: A metaanalysis. *Oncotarget*, 8, 31347–54.

Zhang P, Su DM, Liang M, Fu J (2008). Chemopreventive agents induce programmed death-ligand 1 (PD-L1) surface expression in breast cancer cells and promote PD-L1-mediated T cell apoptosis. *Mol Immunol*, 45, 1470–6.

Zhong J, Chen S, Xu L, et al (2016). Lower expression of PD-1 and PD-L1 in peripheral blood from patients with chronic ITP Lower expression of PD-1 and PD-L1 in peripheral blood from patients with chronic ITP. *Hematology*, 21, 552-7.

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