The Effects of Chemotherapeutic Drugs on PD-L1 Gene Expression in Breast Cancer Cell Lines

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Abstract

Breast cancer is the most common cancer among women in terms of prevalence and mortality, and chemotherapy is one of the most effective treatments at higher stages. However, resistance to chemotherapy is the main obstacle in the treatment of this cancer. Accumulated evidence identified the PD-L1 protein as an essential protein in the development of different cancers. Abnormal expression of this protein in various tumor cells is linked to cancer development and inhibiting the function of immune cells, which correlated with reduced beneficial effects of chemotherapy drugs. In the present study, the effects of common chemotherapy drugs including doxorubicin, paclitaxel, and docetaxel on the expression of the PD-L1 gene were investigated by qRT-PCR before and after the treatment with these drugs in MD231, MD468, SKBR3 breast cancer cell lines. Also, the MTT test was applied to examine the effects of drugs on the growth and proliferation of cancer cells considering PD-L1 expression. The expression of the PD-L1 gene increased after 24 and 48 hours of treatment with chemotherapy drugs. The obtained results indicate the enhancing effects of chemotherapy drugs on PD-L1 gene expression, which have a suppressive effect on the immune system against breast cancer. The use of these drugs as the first line of chemotherapy in triple-negative breast cancer is not recommended. However, there is still a need for further experimental and clinical research on the exact effects of these drugs on undesired immune cells exhaustion in breast cancer therapy.

Introduction

Breast cancer is the most common cancer in women worldwide with two million new cases and with a mortality rate of 600,000 deaths in 2018 [1]. This condition of the disease needs special attention in terms of prevention and treatment. However, due to the significant progress in screening methods, early detection and advances in the treatment of breast cancer have reached in recent years. At the molecular level, breast cancer is classified into one of the major subtypes, using three standard immunohistochemical markers to facilitate targeted treatment, including; a) Luminal A [Estrogen receptor positive (ER+) and/or progesterone receptor positive (PR+), human epidermal growth factor receptor negative (HER2-)], b) Luminal B [ER + and/or PR+, HER2+], c) HER2 overexpression [ER-, PR-, HER2+], d) triple-negative breast cancers (TNBC) [ER-, PR-, HER2-], and e) Basal-like [ER-, PR-, HER2-, with an expression of proteins that TNBC usually doesn't have] [2–6]. Basal-like and TNBC subsets account for about 15% of invasive breast cancers. TNBCs have the most recurrences after treatment and are more aggressive than other subtypes, which have a very high risk of metastasis [7]. The 1-year and 3-year survival rates of patients with TNBC are slightly lower than other cases of breast cancer. While 5-year and 10-year survival rates, without association with other diseases, are very low in these patients [8, 9].

Programmed cell death protein 1 (PD-1) receptor is a co-stimulatory helper molecule that negatively regulates T cell immune responses. PD-1 is expressed on T cells and pro-B cells and causes T cells to be unresponsive to the target cell by binding to its ligand PD-L1, which is normally expressed on both hematopoietic and non-hematopoietic cells [10]. This mechanism prevents autoimmune reactions and damage to the organ cells. However, the expression of these ligands in cancer cells causes the T cells to

react to the cancer cells and consider them as native cells. It is now widely accepted that the expression of PD-L1 in cancer cells contributes to the resistance of cancer cells to the immune system. PD-L1 expression causes weak immune responses and makes the tumor cells escape from the activity of T cells. The binding of PD-L1 expressed in cancer cells to PD-1 expressed on T cells suppresses T cell activation and proliferation and induces T cell apoptosis. Also, continuous expression of high levels of PD-1 on antigen-exposed CD8 + T cells leads to a phenotype of CD8 + T cells that is characterized by dysfunction and persistent expression of inhibitory receptors, known as the T cell exhaustion. As a result, it causes to weakens of T cells functions against cancer cells [11]. Therefore, the application of PD-1 inhibitors can prevent T cells from exhaustion and become more susceptible to cancer cells and prevent the immune system from collapsing against cancer cells. In general, dysfunction of both PD-1 protein and its ligand, PD-L1, impairs the function of T cells against cancer cells, and this weakness of the immune system provides an opportunity for cancer cells to grow [12].

The National Comprehensive Cancer Network (NCCN) treatment recommendations for breast cancer include local treatment with surgery and radiotherapy, as well as systemic treatment with chemotherapy, hormonal and biological therapies [13]. Chemotherapy is recommended for the vast majority of TNBCs, HER2-positive breast cancers, and high-risk luminal tumors [14]. Chemotherapy involves the administration of drugs that kill the individual and the individual. The drug can be used alone or in combination with each other to give better results. Each of these regimens has advantages and disadvantages that are selected based on genetics, age, tumor type, tumor size, metastasis, and other factors. In the present study, we examined the potential effects of common regimens applied in breast cancer therapy, including doxorubicin and taxanes (i.e. paclitaxel and docetaxel) on PD-L1 expression in breast cancer cell lines.

Materials And Methods

Cell culture

First, SK-BR-3 (isolated by Trempe and Old; ATCC number HTB-30), MDA-MB-231 (isolated by Cailleau; ATCC number HTB-26), and MDA-MB-469 (isolated by Cailleau; ATCC number HTB-132) were purchased from Pasteur Institute Cell Bank and cultured in RPMI-1640 (Roswell Park Memorial Institute) medium supplemented with 10% FCS then they were grown in an incubator containing 5% CO₂ with a temperature of 37°C and 95% humid air. Cells with an initial concentration of 5x10⁴ per ml were passaged and used in all experiments.

MTT Assay

For this purpose, the cells were cultured in small flasks. Then, 200 µl of suspension containing 15,000 cells from MDB-468, MD-231, SK-BR-3 cell lines separately with RPMI-1640 culture medium and 10% bovine fetal serum were distributed in each well from a 96-bottom plate (U bottom) and kept in an incubator at 37°C, 95% humidity and 5% carbon dioxide for 24 hours (h) until the cells reached the bottom of the plate to adhere. The mentioned drugs in different doses (0, 1, 10, 20, 30, 40 and 50 µg/ml) were

added to the culture and incubated for 24 and 48 h. To perform the test, first, the supernatant culture medium was removed, then 50 µl of the MTT solution (2 mg/ml MTT in PBS) along with 100 µl of complete culture medium was added to each well and the plate was incubated every 4 h. After the incubation time, the supernatant was removed again and 100–200 µl of dimethyl sulfoxide (DMSO) was added to each well. After 30 minutes, it was placed in an ELISA reader, and the color change of the wells was measured at 570–630 nm wavelength.

RNA extraction and PD-L1 gene expression measured by qRT-PCR

To determine PD-L1 gene expression, total RNA was isolated from cultured cell lines using RiboEx reagent (Geneall, Korea) according to the manufacturer's instructions. Then the purity and amount of RNA extracted were determined by Nanodrop. Changes in gene mRNA expression were measured by SYBR Premix Ex Taq II (TAKARA, Japan) and the Applied Biosystems StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, USA). GAPDH was used as an internal control to normalize the expression of the main gene. The synthesized cDNA was examined by qRT-PCR using specific primers. PD-L1 primer sequences were blasted using NCBI site software (<http://www.ncbi.nlm.nih.gov>). The primers were synthesized by Takapouzist Company (Takapouzist, Tehran) and presented in Table 1.

Table 1
Primer sequences.

Gene	Strand	Sequence 5' → 3'
GAPDH	Forward	CAAGATCATCACCAATGCCT CCCATCACGCCACAGTTTCC
	Reverse	
PD-L1	Forward	TGCCGACTACAAGCGAATTACTG CTGCTTGTCCAGATGACTTCGG
	Reverse	

Statistical analysis

The results obtained from the experiment were analyzed with GraphPad prism 6. To determine the variable changes between control and treated groups, Student's t-test and one-way ANOVA were employed to measure the statistical differences between the two and multiple groups, respectively. P-value < 0.05 was considered statistically significant.

Results

Measurement of PD-L1 gene expression in breast cancer cell lines

In this study, first the MDA-MB-231, MDA-MB-468, SK-BR-3 cell lines were cultured, then the RNA of all cultured cells was extracted and PD-L1 gene expression in the cell lines was measured by Quantitative

Real-Time PCR based on comparison with internal control. In this technique, GAPDH was used as an internal control for the gene.

Then, to determine the effective dose (IC50) of the drugs, including Doxorubicin, Paclitaxel, and Docetaxel in the MDA-231 (Fig. 1), MDA-468 (Fig. 2), SKBR3 cell lines (Fig. 3), the values of which were interpreted according to Table 2. The half-life of paclitaxel is 52 h, docetaxel is 86 h, and doxorubicin is 48 h.

Table 2
IC50 values of investigated drugs after incubation for 24 and 48 hours.

	MDA231		MDA468		SKBR3	
	24 h (µg/ml)	48 h (µg/ml)	24 h (µg/ml)	48 h (µg/ml)	24 h (µg/ml)	48 h (µg/ml)
Paclitaxel	10.79	1.78	9.4	0.97	19.32	6.03
Docetaxel	28.45	4.3	21.39	2.9	57.50	20.58
Doxorubicin	27.19	0.21	0.99	0.2	24.41	5.76

Measurement of PD-L1 gene expression after treatment with chemotherapy agents

After measuring the IC50 of the drugs, the expression level of the PD-L1 gene in the presence of chemotherapy agents was measured by the qRT-PCR method as mentioned above. In the MDA-MB-231 cell line, according to Fig. 4, the expression of the PD-L1 gene under the influence of all three drugs paclitaxel, docetaxel, and doxorubicin increased significantly at 24 and 48 h after treatment compared to the control. ($P \leq 0.0001$). Also, the expression of the PD-L1 gene in the MDA-MB-468 cell line did not change significantly in the first 24 h according to Fig. 5 with treatment with paclitaxel ($P \leq 0.05$) and docetaxel ($P > 0.05$). However, paclitaxel was significantly increased in the next 48 h ($P \leq 0.001$). Doxorubicin significantly increased gene expression in 24 h and 48 h after treatment ($P \leq 0.0001$). Similarly, the expression of the PD-L1 gene in the SK-BR-3 cell line, according to Fig. 6, was significantly increased under the influence of all three drugs paclitaxel, docetaxel, and doxorubicin ($P \leq 0.0001$).

Discussion

PD-L1 expression is found in a variety of solid tumors, such as melanoma, lung cancer, thymoma, ovarian cancer, renal cell carcinoma, esophageal squamous cell carcinoma, colorectal, prostate, and bladder cancer, and the expression of the PD-L1 gene is directly related to the growth of cancer cells [11, 15, 16]. The expression of PD-L1 in breast cancer varies according to its subtypes. According to studies by Gebbeh et al., the PD-L1 gene is expressed in about 50% of breast cancers [17]. TNBCs have the highest expression among breast cancer subtypes, followed by HER2 overexpression subtypes [18]. Finally, luminal A and B subgroups are associated with the lowest expression that has little effect on the prognosis of patients and the reduction in survival [10, 19]. The present study measured the expression of

PD-L1 gene expression in MDA-231, MDA-468, SK-BR-3 cell lines, which represents TNBC subtypes for MDA-231,468 and HER2 overexpression for SK -BR-3 and comparing its results with PD-L1 gene expression in these categories after the addition of common chemotherapy drugs.

In previous studies, it is proven that the expression of PD-L1 increases in different breast cancer cell lines and other cancers such as lung cancer, prostate cancer, and esophageal cancer. In the present study, the increase in PD-L1 gene expression in the studied cell lines related to triple-negative and basal-like breast cancer was calculated, which is consistent with these studies. A study by Bailey C, et al. shows that chemotherapy drugs such as Doxorubicin, Etoposide, Busulfan, and others can increase PD-L1 gene expression, which is consistent with the results of our study [20]. Another study by Yang et al. on stromal tuberculosis cancer cell lines of the bone marrow showed that the expression level of PD-L1 cells in stromal tuberculosis cells was low before the addition of drugs [21]. Common chemotherapy drugs used in these cancers, including Doxorubin, Cytarabine, Oxaliplatin, Vincristine, and Etoposide, increase the expression of PD-L1 in these cells, which is consistent with the effect of Doxorubicin in the present study. In another study by Yan Hoy et al. PD-L1 gene expression was measured in esophageal squamous cell carcinoma cell lines under the influence of Paclitaxel and Carboplatin and 5-Fluorouracil plus Cisplatin which was found to be associated with an increase in PD-L1 gene expression [16]. This finding is consistent with the results of the present study on the increased expression of the PD-L1 gene under the influence of Paclitaxel. In a study by Zhang P. et al., the expression of PD-L1 protein expression in cell lines MDA-MB-468, MDA-MB-435, MCF-7 with Paclitaxel, and Etoposide and 5-Fluorouracil was performed by flow cytometry of cell surface proteins, which resulted in increased gene expression in all three cell lines [22]. The results of the effect of paclitaxel on the MDA-MB-468 cell line are similar to the present study. In a study by Funaki et al., the expression of genes in lung cancer cell lines and human living tissue samples under the influence of Paclitaxel and Cisplatin were examined, which resulted in increased expression of the PD-L1 gene which was in line with the results of our study [23]. In a study by Zili et al., the effect of monoclonal antibodies against PD-L1 along with Docetaxel on melanoma cell lines was studied, which resulted in a positive and enhanced effect of these drugs on their use [24]. It alone can induce the inhibition of PD-L1 protein by antibodies and enhance the effect of Docetaxel. However, no separate study was found on the effect of Docetaxel on different cell lines, which remains to be another study to evaluate the effects of this drug on PD-L1 expression.

Chemotherapy for cancer is divided into three types. After surgery or adjuvant treatment for breast cancer, which is used to treat micrometastatic diseases (i.e., breast cancer cells that have escaped from the breast and lymph nodes in the area but have not yet been identified as a metastasis). Despite surgical treatment, there is an increased risk of recurrence of cancer, so adjuvant therapy reduces the risk of recurrence and death from breast cancer. The next type is neoadjuvant therapy, which is used before surgery for newly diagnosed cancers whose tumor size is too large to be surgically removed. In these cases, the tumor is first reduced in size by chemotherapy and then surgery is performed. The tumor response to chemotherapy drugs can also be measured and used after surgery if needed. And like adjuvant therapy, it reduces the chance of recurrence [25]. Depending on the risk reduction model, breast

cancer adjuvant and neoadjuvant therapy including PD-L1-based therapies may provide an efficient therapeutic strategy for breast cancer therapy [26].

Conclusion

In the current study, the chemotherapy drugs increased the expression of the PD-L1 gene in cell lines MDA-231, MDA-468, and SK-BR-3, which may further weaken the immune system in the presence of drugs. Except for the effect of Docetaxel in the MDA-MB-468 cells, which indicates a low effect of the drug on gene expression, other drugs have considerable effects. These drugs increase the expression of PD-L1 and the use of these drugs is not recommended for breast cancer such as HER2 overexpression and triple-negative cancer, and if they are used, PD-L1 inhibitors are needed.

Declarations

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Conflicts of interest/Competing interests

The authors have no conflicts of interest to declare.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

MM, and BB devised the main conceptual ideas and participated in the design of the work. BB provided biological materials and reagents. MM, SS, MA, and AB performed the experiments. MM, and KH wrote the initial draft of the manuscript. SH, SSS, and AM participated in the analysis of the work and reviewed and edited the manuscript. BB supervised the study.

Ethics approval

All experiments and procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical

research.

Consent to participate

This article does not contain any studies with human or animal subjects performed by any of the authors.

Consent for publication

All authors agree with publication.

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Figures

MDA-231 cell line

Figure 1

Determination of IC₅₀ values of Docetaxel, Doxorubicin and Paclitaxel in MDA-231 cell line after 24 and 48 hours incubation.

MDA-468 cell line

Figure 2

Determination of IC₅₀ values of Docetaxel, Doxorubicin and Paclitaxel in MDA-468 cell line after 24 and 48 hours incubation.

SKBR3 cell line

Figure 3

Determination of IC50 values of Docetaxel, Doxorubicin and Paclitaxel in SKBR3 cell line after 24 and 48 hours incubation.

MDA-231 cell line

Figure 4

Expression of PD-L1 gene after treatment with chemotherapeutic agents in MDA-MB-231 cell line at 24 and 48 hours. **** P < 0.0001.

MDA-468 cell line

Figure 5

Expression of PD-L1 gene in 468MDA-MB cell line after treatment with chemotherapeutic agents at 24 and 48 hours. **** P < 0.0001; ***P < 0.001; *P < 0.05.

Figure 6

PD-L1 gene expression after treatment with mentioned chemotherapeutic agents in SK-BR-3 cell line at 24 and 48 hours. **** P < 0.0001.