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RESEARCH ARTICLE

Optimization of CTLA-4 and PD-1 proteins in EMT6 Mouse Mammary Cancer Cells by Western Blot

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Abstract Targeting the activation of immune checkpoints is recognized as an effective strategy for triggering anti-tumour immune responses in cancer cells. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) were identified as potential crucial targets for cancer treatment. Overexpression of CTLA-4 and PD-1 proteins in primary tumour and human cell lines is well documented. In contrary, lack of data was available using animal cell lines. The presence study aims to optimize the expression of CTLA-4 and PD-1 proteins in EMT6 mouse mammary cancer cells using Western blot, and provide basic understanding of their association with breast cancer cell progression. Proteins extracted from EMT6 parental cells were adjusted to 30ng for gel electrophoresis. Afterwards, the protein was transferred to a nitrocellulose membrane for blotting. The membrane was then subjected to chemiluminescent for band detection. Results obtained using beta-actin as a housekeeping gene show that both CTLA-4 (32 kDa) and PD-1 (34 kDa) proteins were expressed by using a 1:1000 dilution for each antibody from the lysate of EMT6 mouse mammary cancer cells. The relative expression of PD-1 (4.0 ± 0.26) is higher compared to CTLA-4 (1.2 ± 1.8). As a conclusion, both CTLA-4 and PD-1 proteins were indeed expressed in EMT6 mouse mammary cancer cells and this outcome provide the platform for extensive in vivo research on the link of both proteins with breast cancer using animal model.

Keywords: CTLA-4 and PD-1 proteins, EMT6 cells, Western blot.

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Introduction

Chemotherapy, radiation, and surgery are the three main cancer treatments in prior decades. Despite significantly improvement in cancer management, some limitations occurred where normal cells were also affected and leading to unnecessary harm. In general, the immune system was able to attack and destroy tumour cells for instance, T-cells acted against tumour cells with benefits including specificity, memory, and adaptability [1]. In addition, the ability of immunotherapy inhibiting T-cells immune regulatory checkpoints by antibodies has tremendous potential as ground-breaking therapeutic benefits [2]. Antigen receptor signalling and CD28 co-stimulatory signalling were both necessary for T-cells to become completely activated in these two-signal concepts. The suppression of CTLA-4 by blocking the first checkpoint protein blockade through antibody-mediated was proven to be effective in cancer immunotherapy. CTLA-4 translocated to T-cell surface and competes with CD28 for CD80 and CD86 bindings, which results in the inhibition of T-cell proliferation [3]. Unlike CTLA-4, which is expressed only by T cells, PD-1 is expressed by activated T cells, B cells, and monocytes. PD-1 interacts with its two

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ligands, PD-L1 and PD-L2, which are largely expressed by antigen-presenting cells and tumour cells, respectively [4], [5]. PD-1 was also expressed by activated dendritic cells (DCs), regulatory T-cells (Tregs), and natural killer T-cells (NKT) [5]. Generally, these proteins were typically expressed in T-cells of cancer cells. CTLA-4 was also expressed in human melanoma [6], while PD-1 was expressed in breast (MDA-MB-231, MCF-7 and ZR-75), ovarian (OV-17, OVCAR-3, ES-2 and SKOV-3), prostate (LnCap and PC-3), hepatoma (HepG2), lung (A549, H441, H1703 and H460), melanoma (A375) [7], pancreatic (CFPAC-1 and ASPC-1), and colon (Caco-2, SW620, SW480, Colo-205 and HT-29) cancer cells [8]. Based on previous studies, it clearly showed that the involvement of both proteins in cancer progression was extensively performed using *in vivo* model, contrary to *in vitro* study. Thus, the objective of the presence study was to optimize the detection of CTLA-4 and PD-1 proteins in EMT6 mouse mammary cancer cells using Western blot.

Materials and Methods

Antibodies

Anti-PD1 (EPR20665, 1:1000), anti-CTLA4 (CAL49, 1:1000), anti-beta-actin (EPR21241, 1:5000), horseradish peroxidase (HRP)-conjugated with goat anti-rabbit IgG (1:100000) and beta-actin (1:100000; housekeeping gene) were purchased from Abcam (Cambridge, United Kingdom).

Cell Culture

EMT6 mouse mammary cancer cells (EMT6 parental cells, American Type Culture Collection, Virginia, USA) were cultured in T25 flask (BioMedia Scientific, Singapore) with complete culture media consisting of Dulbecco's modified eagle's medium (DMEM) and supplemented with 10% foetal bovine serum (FBS) and 1% streptomycin and penicillin (CELLGRO Mediatech, Virginia, USA). Cells were incubated in a CO_2 incubator (Binder, GmbH, Germany) at 37˚C with 5% CO_2 and were allowed to grow until 70% to 80%confluence. All the culture media components were tissue culture grade and purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA) except mentioned.

Cells were rinsed thrice with cold phosphate buffer saline (PBS) (Sigma-Aldrich, MA, USA) and then resuspended with 5 mL PBS in a 15 mL centrifuge tube. After 3 minutes of centrifugation at 124 x g (Eppendorf, Hamburg, Germany), the supernatant was discarded, and cell pellet was resuspended in 1 mL cold PBS and placed in a cold microfuge tube. At 4°C, the cell suspension was centrifuged again at 3000 rpm for another 3 minutes. The supernatant was discarded once more. This procedure was repeated thrice. A total of 1 x 10^6 cells were resuspended with a mixture of 300 µL radioimmunoprecipitation assay (RIPA) lysis buffer and protease inhibitor (ThermoFisher Scientific, Waltham, Massachusetts, USA).

Determination of Protein Concentration

Protein concentration was determined using a standard curve prepared from bovine serum albumin (BSA) protein assay kit (ThermoFisher, Waltham, Massachusetts, USA). A standard curve with nine concentrations was prepared using 2 mg/mL BSA and diluted in distilled water to different final concentrations ranging from 0 to 2000 µg/mL. A 25 µL from each standard concentration and each protein sample were added into a microplate well. The microplate was covered with aluminium foil and incubated at 37°C for 30 minutes, followed by absorbance measurement using an ELISA plate reader (Victor X5) (Perkin Elmer, MA, USA) at 562 nm wavelength. The standard curve was plotted using the absorbances of each concentration against the concentration of the standards, and the protein concentrations of the EMT6 samples were determined accordingly.

Gel loading, Electrophoresis, and Western Blot

A 10 µL from each of the different protein samples was added with 10 µL Laemilli sample buffer (Sigma Aldrich, MA, USA) with the final for each well is 30 ng. The protein and buffer samples were thoroughly mixed using a vortex (Eppendorf, Hamburg, Germany). The tubes were heated in a thermal cycler (Eppendorf, Hamburg, Germany) for 5 minutes at 95°C to denature the proteins. Denaturation reversal was prevented by immediately placing the protein and buffer samples onto ice before mixing and centrifugating at 15,000xg for 30 seconds. A 12% SDS-polyacrylamide gel (Sigma Aldrich, MA, USA) with pH 8.9 was prepared according to the size of protein samples (kDa) as optimised earlier and summarized in Table 1.

Gel electrophoresis was performed at different voltages and durations of 100V for 90 minutes, 120V for 60 minutes, 140V for 60 minutes and 140V for 90 minutes. The migrating of the blue dye front, and the

colourful bands of the standard protein markers indicated successful electrophoresis. After completion, the proteins were harvested from the gel and transferred onto a nitrocellulose membrane for blotting.

The maximum protein transferred was ensured by stacking in the order of foam pad, filter papers, gel, nitrocellulose membrane and filter papers on a translucent cassette's cover and immersing the stacked arrangement into a cold transfer buffer band; before pressing across them with a cylindrical rod to remove air bubbles. After closing the cassette's cover, an ice block was placed nearby, before the electrophoretic transfer was carried out at different voltages and durations of 400mA for 60 minutes, 350mA for 90 minutes and 120mA for 240 minutes at 4°C.

The blotted membrane was blocked with blocking buffer, Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween 20 (TBS-T) to prevent nonspecific binding for 40 minutes with constant shaking. The membrane blot was rinsed with distilled water before the addition of primary antibodies of anti-PD-1, anti-CTLA-4, and anti-beta-actin. Prior to this, each primary antibody was diluted in milk solution at the indicated concentrations after optimization as summarized in Table 2. Beta-actin served as a housekeeping gene. A 0.1% Tween 20 was included in all the incubation steps. After constant shaking for 24 hours of incubation at 4°C, the membrane was washed thrice with TBS-T for 1 to 3 minutes each time before incubating with the secondary antibody for 1 hour at room temperature. After a secondary incubation, the membrane blot was washed four times with TBS-T for 5 to 10 minutes. The chemicals and disposable items used were from Abcam (Cambridge, United Kingdom) except those mentioned specifically.

Table 1. The gel percentage requirement for optimum separation according to the size of target protein

Gel percentage	Protein size
20%	4 - 40 kDa
15%	12 - 45 kDa
12%	10 - 70 kDa
10%	15 -100 kDa
8%	25 -100 kDa

Table 2. The differential dilutions attempted for the primary and secondary antibodies in Western blot with specific incubation time

Antibody	Dilution	Incubation time
Primary		
Anti-PD-1	1:500	24 hours
	1:1000	24 hours
	1:5000	24 hours
Anti-CTLA-4	1:500	24 hours
	1:1000	24 hours
	1:5000	24 hours
Anti- beta-actin	1:500	24 hours
	1:1000	24 hours
	1:5000	24 hours
Secondary		
HRP-conjugated goat anti-rabbit IgG	1:5000	1 hour
	1:50000	1 hour
	1:100000	1 hour

Chemiluminescent Detection and Image Processing

The enhanced luminol-based chemiluminescent (ECL) substrate kit was used as a substrate for detecting HRP and consisted of two components: luminol/enhancer solution, and stable peroxide solution. These components were mixed in a 1:1 ratio to create a working solution. Four mL of each component produced enough solution to completely covered the nitrocellulose membrane. The membrane was incubated in the working solution with mild agitation for 5 minutes devoid of light. The membrane was exposed to C-Digits (Biomedia Scientific, Singapore) for 12 minutes. The image was captured by Image Studio software version 5.2.5 (LI-COR Biosciences, LI-COR Biosciences, USA). ImageJ software was used to quantitate the relative expression of proteins, The chemicals and

disposable items used were from Abcam (Cambridge, United Kingdom) except those mentioned specifically.

Statistical Analysis

The data was obtained from three sets of experiments and presented as mean ± standard deviation. The graphs were generated with GraphPad Prism version 8.0 for Windows (San Diego, California USA).

Results and Discussion

Protein Quantification

The protein concentrations harvested from the samples were determined using BSA standard curve following the equation y=0.0012x+0.165 as summarized in Figure 1. The final concentration of protein lysates yielded a total of 1859.585 ± 22.60 µg/mL of protein (Table 3). This concentration is adequate and adjusted to 20 µg in 20 µL Laemmili buffer for the following experiment.

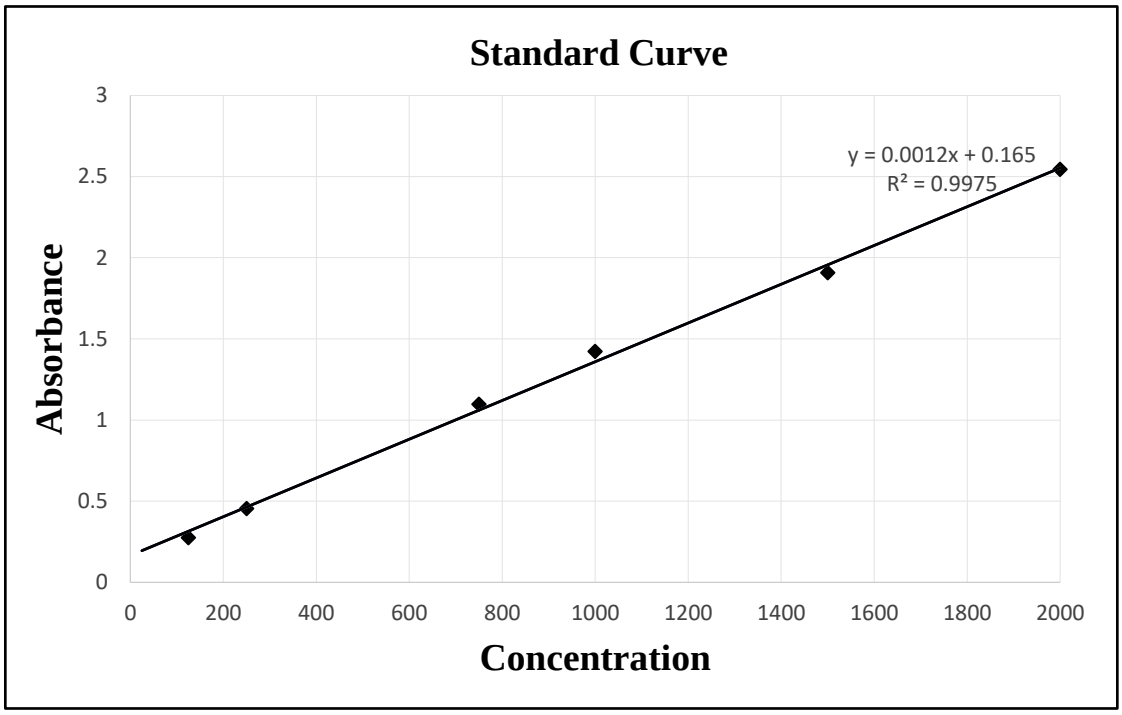

Figure 1. Protein concentration (x) was obtained using the standard curve following the equation y= 0.0012x +0.165. R^2 is the Pearson coefficient, and a value of > 0.980 implies both the assay and data as efficient

Table 3. The absorbance of sample and protein concentration of each sample

Sample	Absorbance of unknown (y)	Concentration of unknown (x) (y-0.165)/0.0012	Average concentration (µg/mL)
Parental EMT6 (1)	2.410	1870.575	1859.58
Parental EMT6 (2)	2.362	1830.682	
Parental EMT6 (3)	2.418	1877.498	

CTLA-4 and PD-1 Expressions were Detected at the Optimum Antibody Dilution

The concentrations of beta-actin and both antibodies used on protein lysates of parental EMT6 cell lines were optimized. The optimum dilution for beta-actin used as a housekeeping gene attempted at 1:500 and 1:1000 failed to produce any visible bands (Figures 2a-b). The trial was successful and a band of 42 kDa was detected using a dilution of 1: 5000 (Figure 2c). Similarly, after several attempts, both CTLA-4 and PD-1 proteins were successfully detected at respective 34 kDa (Figure 3c) and 32 kDa (Figure 4c) using a dilution of 1:1000 after an overnight incubation. The relative expressions of beta-actin, PD-1 and CTLA-4 proteins were summarized in Figures 5 (a - c), and figure 6 respectively. The relative expression of PD-l is higher (4.0 ± 0.26) compare to CTLA-4 (1.2 ± 1.83)

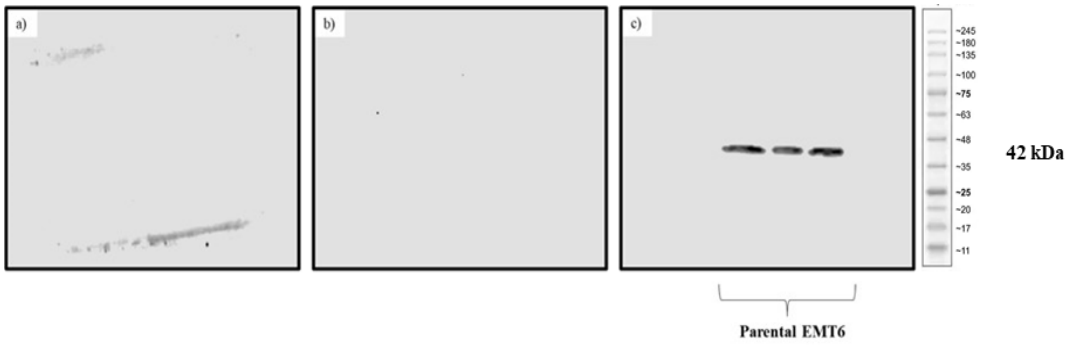

Figure 2 (a-c). Optimization of beta-actin antibody dilutions at a) 1:500 b) 1:1000 and c) 1:5000 after an overnight membrane incubation. Beta-actin protein band appears at a dilution of 1:5000 with a molecular weight of 42 kDa

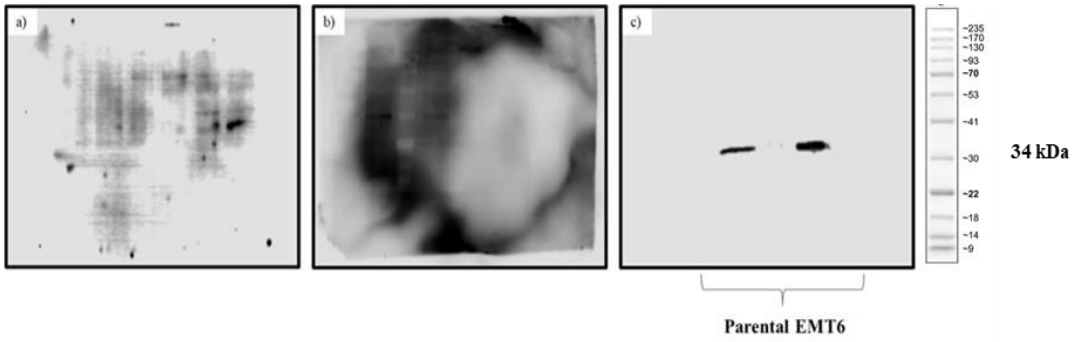

Figure 3 (a-c). Optimization of CTLA-4 antibody dilutions at a) 1:500 b) 1:5000 and c) 1:1000 after an overnight membrane incubation. CTLA-4 protein band appears at a dilution of 1:1000 with a molecular weight of 42 kDa

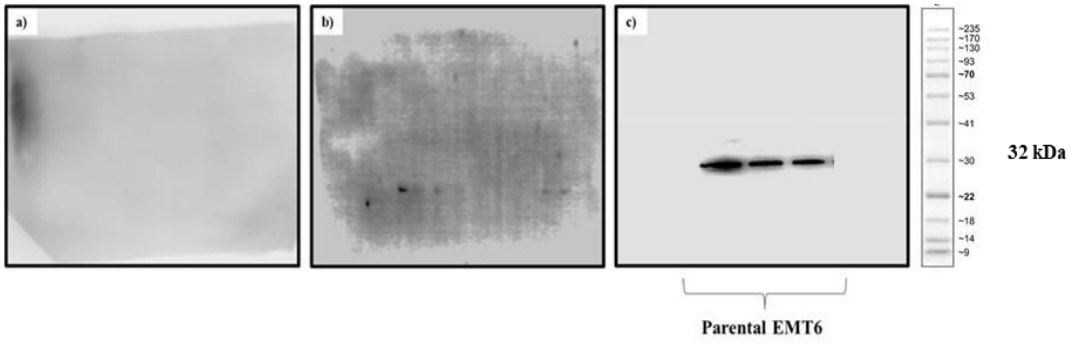

Figure 4 (a-c). Optimization of PD-1 antibody dilutions at a) 1:500 b) 1:5000 and c) 1:1000 after an overnight membrane incubation. PD-1 protein band appears at a dilution of 1:1000 with a molecular weight of 32 kDa

(The data in Figure 2c, 3c and 4c are partially been published in Scientific Report (Sham *et al*. 2023)

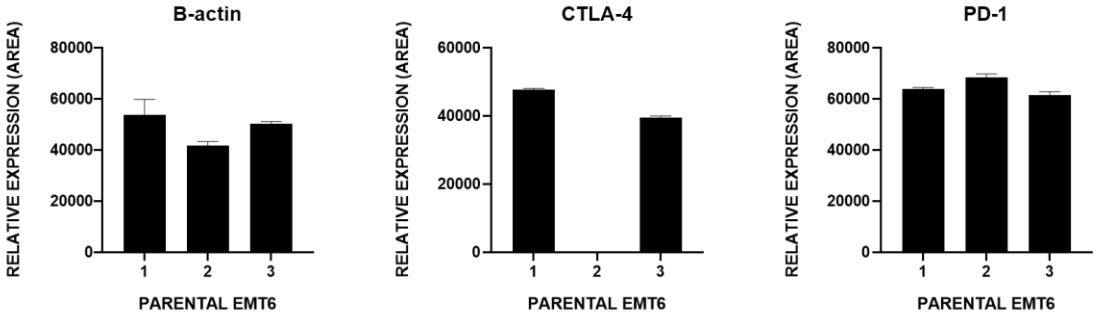

Figure 5. Band quantification for a) beta-actin with a 1:5000 dilution of primary antibodies b) CTLA4 and c) PD-1 both at 1:1000 dilutions after an overnight membrane incubation

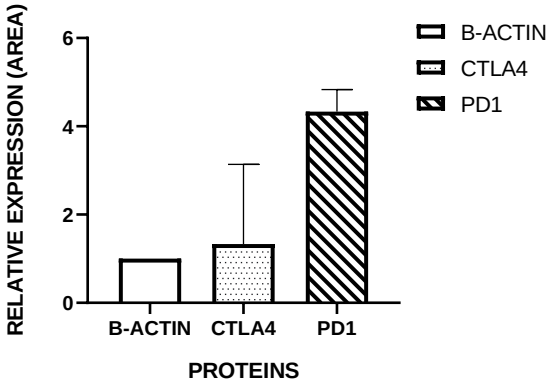

Figure 6. Figure showed comparison of band quantification between three markers (B-actin, CTLA4, PD1). PD1 protein has the higher expression compared to CTLA4

The prospect of immune cell's involvement in the prevention and defence against cancer development was enhanced tremendously. This was due to an enhancement in our understanding of the immune system and immune surveillance as numerous methods for triggering immunological responses were available. One of the approaches in cancer immunotherapy was to challenge the T-cell by using interleukin-2 (IL-2), which promotes T-cell proliferation. IL-2 is also one of the oldest immune-based medications approved for the treatment of metastatic renal carcinoma and melanoma [9], [10]. Checkpoints of T-cell activation were identified as reliable targets for the regulation of immunological responses [11]–[14]. CTLA-4 and PD-1 were identified as the most reliable targets among checkpoints to significantly altered the course of treatment for advanced malignancies.

There are various types of methods to determine CTLA-4 and PD-1 protein expressions such as western blot [15] and immunohistochemistry [16], [17]. Both western blot and immunohistochemistry principles are based on the binding of the antibody to the antigen in the extracted protein sample. With advances in molecular techniques in detecting protein expression, the western blot technique still significant despite several limitations such as the requirement for optimum sample protein is needed for loading and the volume of antibodies used. Despite these measures, results with no bands appearing, faint bands, or high background on the blot will occur [18]. So, it is very important to determine the optimum concentration of sample protein used before loading and the dilution of the antibodies as shown in our optimization. Furthermore, the appearance of the internal control band, which is beta-actin in our study is crucial to confirm the validity of the results.

Our findings proved CTLA-4 and PD-1 proteins were expressed in EMT6 mouse mammary cancer cells. However, the physiological roles of these proteins on EMT6 mouse mammary cancer cells remained unclear. CTLA-4 expression in EMT6 cells was in line with another study using melanoma cell lines. However, no identifiable tumour-infiltrating lymphocytes of CTLA-4 were transcripted [6]. CTLA-4 was also highly expressed in breast cancers [19]. CTLA-4 was expressed in various degrees of intensity in

human tumour cell such as in carcinoma, melanoma, neuroblastoma, osteosarcoma and rhabdomyosarcoma and was able to induce apoptosis in these cells [20]. The increment of CTRLA-4 in cats with mammary carcinoma could be related to a continuous inflammatory response in tumour microenvironment, where post-activated T-lymphocytes expressed sCTLA-4 [21]. The prevention of cancer cell death in an immunosuppressive environment was caused by the tolerance of tumour-specific T-lymphocytes that overexpressed inhibitor receptors, which are CTLA-4 and PD-1 [22].

The mRNA levels of PD-1, PD-L1, and PD-L2 were increased in gastric cancer tissues [23]. Other than that, the prostate of Il-17rc wild-type mice also expressed PD-1, PD-L1, and PD-L2 at higher levels compared to Il-17rc [24]. In a PTEN-null background, it was discovered that Il-17rc wild-type mice had more aggressive prostate cancer compared to Il-17rc knockout animals. According to this research, raising PD-1/PD-L1/2 expressions was able to boost immune suppression in the tumour microenvironment, which would therefore encourage the development of prostate cancer [25]. PD-1 expression was upregulated in breast cancer patients with ER-, PR- and HER- groups [26]. Overexpression of PD-1 also was observed in the molecular subtypes such as basal-like and HER-2 [26].

Conclusions

Our protocol can determine the expressions of CTLA-4 and PD-1 in mouse mammary cancer cell lines. The presence findings, together with previous supporting evidence, provide the opportunity to elucidate the involvement of these two proteins in cancer progression in *in vitro* and *in vivo* models.

Conflicts of Interest

The authors have declared that there is no conflict of interest regarding the publication of this paper.

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