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Effect of anthracycline and taxane on the expression of programmed cell death ligand-1 and galectin-9 in triple-negative breast cancer

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ABSTRACT

This study identified chemotherapeutic agents that up-regulate programmed cell death ligand-1 (PD-L1) and galectin-9 (Gal-9) in breast cancer cells. Immunohistochemical (IHC) staining was used to evaluate changes in PD-L1 and Gal-9 expression in the tumor tissue of triple-negative breast cancer (TNBC) patients who received anthracycline- and taxane-based neoadjuvant chemotherapy. To determine whether PD-L1 and Gal-9 expression changes were attributable directly to chemotherapeutics, MDA-MB-231 cells and HS578T cells were treated with different concentrations of anthracycline and taxane. Expression levels of PD-L1 and Gal-9 were evaluated and the activation status of NFκB in MDA-MB-231 and HS578T cells was determined to identify the PD-L1 and Gal-9 up-regulation mechanism. Three cases of increased PD-L1 expression and two of increased Gal-9 expression were observed among the TNBC patients. PD-L1 and Gal-9 expression were up-regulated by anthracycline and taxane in MDA-MB-231 cells, but not in HS578T cells. Increased nuclear levels of NFκB were observed in MDA-MB-231 cells treated with 0.5 μM epirubicin. Anthracycline and taxane up-regulated PD-L1 and Gal-9 expression in some subtypes of TNBC. This study provides useful reference data for clinical trials investigating combination treatments with immune checkpoint inhibitors and chemotherapy.

1. Introduction

Immune checkpoints (ICPs) are immune system regulators essential for self-tolerance. ICPs consist of stimulatory and inhibitory checkpoint molecules with immunomodulatory roles [28]. Inhibitory ICPs have been investigated as novel targets for cancer immunotherapy, and in many clinical trials for metastatic cancer treatment. Representative inhibitory surface receptors, which downregulate T cell function, include CTLA-4, PD-1, LAG-3, T cell immunoglobulin mucin 3 (TIM-3), TGFβRs, IL-10R, KIRs, and NKG2 A. These proteins and their ligands are located on the surface of immune cells and can be blocked by monoclonal antibodies. Common examples of monoclonal antibodies are those that interfere with PD-1/PD-L1 and TIM-3/GAL9 interactions [30].

Combination therapies with cytotoxic drugs are preferred for ICP inhibition because inhibition using a single agent has an insufficient effect, and cytotoxic drugs provide an immunogenic benefit [22]. Previous studies have reported on the immunogenic role of chemotherapy, demonstrating that such therapy can promote the anti-tumor immune response by enhancing the immunogenicity of intrinsic tumor cells or modulating immune cells [5,10]. Immunogenic cell death (ICD) of tumors has also been reported. ICD leads to the release of tumor antigen and damage-associated molecular patterns (DAMPs), which activate immune cells and induce adaptive immunity [4,33].

Anthracycline and taxane are essential chemotherapy drugs for breast cancer. Doxorubicin enhances innate immune cells and cytotoxic T-lymphocyte function [9,20]. Doxorubicin can induce immunogenic death of cancer cells *via* the release of high-mobility group box 1 (HMGB1) protein from dying cancer cells [3]. Paclitaxel enhances the anti-tumor activity of lymphocytes by the induction of various cytokines [16]. Combination chemotherapy with doxorubicin and paclitaxel has been shown to activate T-cells, and increase their number in the peripheral blood of breast cancer patients [24].

Breast cancer is considered a less immunogenic tumor compared to

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tumors such as melanoma and lung cancer [7]. Specific molecular subtypes of breast cancer have exhibited immunogenicity. Cancers that are more heterogeneous and show high levels of tumor infiltrating lymphocytes (TILs) are considered more immunogenic. Triple-negative breast cancer (TNBC) is characterized by extremely high TIL levels and is more heterogeneous than other subtypes [1,14]. Potential mechanisms of immunogenicity in TNBC include genetic instability and increased mutations [8]. The expression of PD-1 in TILs, and of PD-L1 in tumor cells, is subject to markedly greater upregulation in TNBC [2]. The expression of PD-L1 is associated with better results of PD-L1 monoclonal antibody treatment in various cancers [13]. Additionally, ICD due to chemotherapy induces interferon (IFN)-γ in the tumor microenvironment and promotes PD-L1 expression in tumor cells [18].

Cytotoxic drugs, which increase the expression of immune checkpoints in tumor cells, may increase the efficacy of immune checkpoint inhibitor treatment. This study evaluates outcomes of TNBC patients who received neoadjuvant chemotherapy (NAC) and TNBC cell lines which were treated with cytotoxic drugs.

2. Materials and methods

2.1. Patients and tissue samples

A total of 13 patients with TNBC (clinical stage II–III) received NAC and underwent curative surgery. All patients were pathologically diagnosed with breast cancer by core needle biopsy prior to NAC; the same specimen was used to evaluate PD-L1 and Gal-9 expression. Patients were treated with three to six cycles of anthracycline and/or taxane-based chemotherapy (three to six cycles of doxorubicin 60 mg/m^2 and docetaxel 75 mg/m^2 with a 3-week interval, three to six cycles of epirubicin 90 mg/m^2 and docetaxel 75 mg/m^2 with a 3-week interval, four cycles of doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 with a 3-week interval, and four cycles of docetaxel 75 mg/m^2 with a 3-week interval). Patients then underwent curative breast surgery. Tumor tissue was collected from the surgical specimen to evaluate PD-L1 and Gal-9 expression after NAC. This study was approved by our Institutional Review Board (IRB number: 16-0179).

2.2. Assessment of molecular subtypes

Immunohistochemical (IHC) stains were performed using the VENTANA BenchMark ULTRA instrument and the ultraView Detection Kit (Roche Diagnostics Corp., Tucson, AZ, USA). Paraffin-embedded tissue sections were de-waxed in xylene and rehydrated in a graded alcohol series. Antigen retrieval was carried out using Tris-EDTA buffer (pH 8.0), except for the retrieval of epidermal growth factor receptor (EGFR), which was done using protease 1, for 36–52 min. The primary antibodies used were ER (1:90; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), PR (1:170; Novocastra), HER2 (ready to use; Ventana Medical Systems, Inc., Tucson, AZ, USA), Ki-67 (1:280; Dako, Glostrup, Denmark), CK5/6 (1:100; Dako), and EGFR (1:90; Invitrogen, Camarillo, CA, USA). Visualization was conducted using DAB (Dako) and a counterstain with hematoxylin.

The expression levels of ER and PR were assigned an IHC score, based on the Allred scoring system [12]. IHC and SISH were performed for evaluation of HER-2/ErbB2 status. An IHC score of 3+ or a HER-2 loci/chromosome 17 centromere ratio > 2.2 was defined as HER-2/ErbB2 positive. Expression of CK5/6 and EGFR was defined as positive if > 10% of the tumor cells showed cytoplasmic or membranous expression.

2.3. Tissue microarray construction and immunohistochemistry

Needle biopsy slides from 13 TNBC patients, obtained prior to neoadjuvant therapy, and surgical tissue sections obtained after neoadjuvant therapy, were stained with hematoxylin-and-eosin (H&E) and reviewed. To obtain sufficient tumor tissue from the needle biopsy samples, 3 cores including tumor tissues were obtained. To make tissue microarray (TMA) blocks, three representative foci of invasive carcinoma were selected from each H&E slide. The TMAs were assembled using a commercially available manual tissue microarrayer (Quick-Ray; Unitma Co., Ltd, Seoul, Korea). Three representative 2.0-mm diameter tumor cores were punched from each block and arrayed onto recipient blocks. The three cores from each case were arrayed to increase the concordance rate between the IHC results of the TMAs and the whole tissue sections. In the remaining three cases, whole tissue biopsy sections and surgical tissue sections were subjected to IHC staining. Serial-sectioned 4-μm tissue sections of each TMA block were produced, in addition to whole tissue sections; H&E staining was performed to identify tumor tissues. Cases with only stromal tissue or insufficient amounts of carcinoma tissue in the cores were excluded from the analysis. IHC was performed using the Benchmark XT/Ultra instrument (Ventana Medical Systems) according to the manufacturer's instructions, using primary monoclonal antibody to PD-L1 (clone: SP142, 1:50 dilution; Ventana) and Gal-9 (clone: D9R4A, 1:200 dilution; Cell Signaling Technology, Danvers, MA, USA). Hematoxylin was used to counterstain the tissues. The negative control was treated identically, substituting primary antibody with mouse IgG. As positive control for PD-L1, tonsil and placental tissues were used.

2.4. Interpretation of IHC staining results and assessment of the NAC response

Slides were reviewed independently by two pathologists blind to patient information.

A positive reaction for PD-L1 and Gal-9 was defined as membranous and/or cytoplasmic expression of more than 5% and 10% of the tumor cells respectively, both before NAC and after NAC.

Pathological responses to NAC were assessed using the Miller and Payne system as follows: Grade 1: no change or some minor alteration in individual malignant cells, but no reduction in overall cellularity; grade 2: minor loss of tumor cells, but overall high cellularity; up to 30% reduction in cellularity; grade 3: 30–90% reduction in tumor cellularity; grade 4: marked disappearance of > 90% of tumor cells, such that only small clusters or widely dispersed individual cells remained; and Grade 5: no invasive malignant cells identifiable in sections from the tumor site [27].

2.5. Reagents and cell lines

Epirubicin, doxorubicin, docetaxel, and paclitaxel were purchased from Sigma (St. Louis, MO, USA). Recombinant human IFN-γ was purchased from R&D Systems (Minneapolis, MN, USA). The human breast cancer cell line MDA-MB-231 and HS578 T cells were purchased from the American Type Culture Collection (ATCC, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) of RPMI with 10% fetal bovine serum and 1% penicillin, and cultured at 37 °C with 5% CO_2.

2.6. Cell viability assay

Viability of the breast cancer cells was measured with a TOX6 *in vitro* toxicology assay kit (Sigma). Cells were seeded at a rate of 0.5–1 × 10^4 cells/well in 96-well culture plates. Chemotherapy drugs were administered for 72 h at the indicated doses. Cells were fixed in 10% trichloroacetic acid for 1 h at 4 °C, stained with sulforhodamine B (SRB) for 15 min, and washed three times with 1% acetic acid. The incorporated dye was solubilized with 10 mM Tris Base, pH 8.8. Absorbance was measured spectrophotometrically at 565 nm using an EL800 micro plate reader (BioTek Instruments, Winooski, VT, USA).