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Research Protocol

Pathology Section

Expression of PD-1 and PD-L1 in Breast Carcinoma: Research Protocol for a Cohort Study

SHREYA GHOSH[1], JAYANT S MAKARANDE[2], SUNITA VAGHA[3], ANIL K AGRAWAL[4], SAHITYA VODITHALA[5]

ABSTRACT

Introduction: Breast cancer is the most common malignant tumour and the leading cause of cancer-related death in women is breast cancer. The Immunohistochemistry (IHC) method utilised antigens and antibodies to interact to identify cellular or tissue constituents (antigens). This research has been employed as a diagnostic tool for specific cancers. When Programmed Cell Death Ligand 1 (PD-L1) binds to Programmed Cell Death 1 (PD-1), it suppresses the cellular immune response by killing and depleting T-cells. Monoclonal antibodies that block the PD-1/PD-L1 pathway have shown promise as a treatment strategy currently being tested in human cancer trials.

Need for the study: Breast cancer is a global issue, and PD-L1 expression is emerging as a promising biomarker for breast cancer prognosis. It can provide valuable information treatment planning.

Aim: The current study aims to examine the expression of PD-1 and PD-L1 in breast cancer using IHC in all subgroups of breast cancer patients. Both tests can serve as a biomarkers to guide immunotherapeutic interventions, improving prognosis, and correlating with other clinicopathological individual parameters such as age, tumour size, distant metastasis, lymph node involvement, Estrogen Receptor (ER) and Progesterone Receptor (PR) status, Her2neu expression, histological type, and TNM stage.

Materials and Methods: This will be a two-year cohort study conducted in the Department of Pathology, Jawaharlal Nehru Medical College (JNMC), Maharashtra, India. The study will include 70 specimens from all cases with a histopathological diagnosis of breast cancer. The Nottingham variant of the Bloom-Richardson Grading System will be used to determine the histological grade of the tumour, and immunostaining for PD-1 and PD-L1 will be performed to evaluate their protein expression.

Keywords: Breast cancer, Immunity, Programmed cell death 1, Programmed cell death ligand 1

INTRODUCTION

The most prevalent malignant tumour in women and the leading cause of cancer-related death is breast cancer. It is more commonly seen in developed countries [1]. Breast cancer has now surpassed cervical and oral cavity malignancies to become the most prevalent cancer and the main cause of cancer death in India [2].

The likelihood of breast cancer in women is higher from their early thirties to their fifties, and the risk continues to rise until it reaches a peak between the ages of 50 and 64 years. Indian women have a one in 28-lifetime likelihood developing breast cancer. It is more common in urban women (1 in 22) compared to rural women (1 in 60) [3]. Female breast cancer accounts for 11.7% of all cancer cases, making it the most prevalent type. It ranks as the fifth most common cause of cancer mortality worldwide, with 685,000 fatalities. One in four women is affected by breast cancer, which also causes one in every six cancer deaths [4]. Breast cancer can occur due to certain genetic mutations and Deoxyribonucliec Acid (DNA) damage. It has been associated with oestrogen exposure. Some people inherit DNA and gene defects, including those caused by the BRCA1, BRCA2, and P53 genes, among others. Individuals with a family history of ovarian and breast cancer have an increased risk of developing breast cancer. The immune system detects DNA-damaged cells and cancerous cells and eliminates them. Breast cancer occurs when there is inadequate immune defense and surveillance. Disruption of the numerous growth factors and other mediator signaling systems that interact between stromal and epithelial cells can contribute to the development of breast cancer [5].

The two biggest risk factors are gender (99% of those affected are female) and advancing age. Other significant risk factors include early menarche (before the age of 12), late menopause (beyond the age of 55), a late first pregnancy (after the age of 35), nulliparity, the absence of breastfeeding, exogenous hormone therapy, postmenopausal obesity, and inactivity [6].

The most common breast signs and symptoms reported by women with breast cancer are pain, inflammatory changes, nipple discharge, lumpiness, or a palpable mass [6]. Although there are many different forms of breast carcinomas, infiltrating ductal carcinoma is the most prevalent histological type [6].

Breast cancer can be detected through various procedures, including breast examination, mammograms, breast ultrasounds, breast Magnetic Resonance Imaging (MRIs), and biopsies. A biopsy is the only reliable method that allows us to make a conclusive diagnosis of breast cancer. The samples are evaluated to determine the cell types that contribute to breast cancer, the degree (grade) of the illness, and whether the cancer cells have hormone receptors or other receptors that could affect the available treatments that are available. Sentinel lymph node biopsy, fine needle aspiration biopsy, core needle biopsy, surgical biopsy, and imaging-guided biopsy are among the numerous forms of biopsy that can be employed [6].

If the tumour is localised, the mainstay treatment for breast carcinoma is surgery, followed by chemotherapy, and radiotherapy. For ER- and PR-positive tumours, adjuvant hormonal therapy, and immunotherapy are also used [7].

The transmembrane glycoprotein death ligand encoded as PD-L1 is found on the membranes of various tumour cells, epithelial cells, and immune cells, including B cells, macrophages, dendritic cells, and T-cells. PD-L1 binds to PD-1, an immuno-inhibitory receptor

belonging to the B7-CD28 gene superfamily, which regulates the immune response to tumour cells. When PD-1 on immune cells binds to PD-L1 on tumour cells, T-cell activation is inhibited, leading to reduced T-cell-mediated anti-cancer immunity. Overexpression of PD-L1 has been linked to increased neoplastic development, treatment resistance, and cancer recurrence. Consequently, several immunotherapeutic approaches have been developed. Notably, PD-L1 inhibitor atezolizumab and PD-1 inhibitor pembrolizumab have received Food and Drug Administration (FDA) approval for use in patients with locally advanced or metastatic PD-L1 positive Triple Negative Breast Carcinoma (TNBC) in combination with chemotherapy, indicating the increasing use of immunotherapy in breast cancer treatment [8].

The present study aims to investigate PD-1 and PD-L1 expression through Immunohistochemistry (IHC) in relation to clinicopathological parameters such as age, tumour size, distant metastasis, lymph node involvement, ER/PR/Her2neu status, histological type, and TNM stage. The study objectives include determining the histological grade using the Nottingham histological grading system, examining PD-1 and PD-L1 expression in breast cancer tumour tissue using IHC, analysing the distribution of positive PD-1 and PD-L1 expression in all subtypes of breast cancer patients, and evaluating the potential utility of PD-1 and PD-L1 expression in guiding immunotherapeutic interventions.

REVIEW OF LITERATURE

Breast cancer is a global issue, with invasive breast cancer being the most common type of cancer in women worldwide [9]. Women's cancers account for 23% of all cancers globally. There are several genetic markers, such as CD9, CD63, CD81, CD53, and CD37, that may have significant value as prognostic indicators in breast cancer patients. PD-L1 expression is emerging as a promising biomarker for breast cancer prognosis, and it may be useful for doctors in selecting the best treatment for breast cancer. Various grading techniques can be used to determine the grade of breast cancer, with the Nottingham composite histologic grade (Elston-Ellis version of the Scarff-Bloom-Richardson grading system) being one of them, commonly used for reporting. There is a correlation between histologic grade and outcome within each stage grouping. The Nottingham composite histologic grade evaluates factors such as the amount of tubule formation, the degree of nuclear pleomorphism, and the mitotic count.

PD-L1 binds to PD-1 and leads to T-cell death or exhaustion, suppressing the cellular immune response. Monoclonal antibodies that block the PD-1/PD-L1 pathway are a promising treatment strategy currently being tested in human cancer trials [10]. These findings suggest that PD-L1 promotes the activation of the PD-1/PD-L1 pathway, facilitating immune escape by tumour cells. PD-L1 expression has been observed in solid tumours such as hepatocellular carcinoma, renal cell carcinoma, testicular cancer, papillary thyroid cancer, breast cancer, lung cancer, gastric cancer, and colorectal cancer. Li CJ et al., reported that breast cancer is a common malignant tumour in women, with triple-negative breast cancer being a particularly aggressive form of the disease with a poor prognosis and a high risk of metastasis. PD-L1 is involved in the evasion of tumour cells from immune surveillance. Significant progress has been made in understanding the biology of triple-negative breast cancer [11]. Sun WY et al., found that the detection of PD-L1 in both cancerous and immune cells varied depending on the antibody clone used. PD-L1 (E1L3N) showed the highest staining, followed by PD-L1 (28-8), and then PD-L1 (SP142). Further investigation of the PD-L1 inhibitor clinical trial group is needed to determine the best cut-off value and the gold standard antibody [12].

In a study conducted by Yuan C et al., immunohistochemical techniques were used to determine the positivity or negativity of PD-1/PD-L1. Positive PD-1/PD-L1 cells in metastatic lymph nodes were found to be associated with unfavourable prognostic factors, including a significant number of metastatic lymph nodes (p=0.002), high TNM stage (p=0.012), high Ki-67 index (p=0.048), and high histological grade (p=0.029). It is important to confirm the presence of PD-L1 in both the primary tumour and metastatic lymph nodes due to heterogeneity [13]. Han Y et al., reported that cancer immunotherapy has shown promising results in recent years. PD-1 regulates T-cell activity, suppressing immune responses and promoting self-tolerance by preventing the death of regulatory T-cells while inducing it in antigen-specific T-cells [14].

Schütz F et al., found that immunomodulation appears to be a feasible approach in solid tumours. Breast cancer subtypes with high proliferation index, such as TNBC and HER2-positive, have been characterised as highly immunogenic. Immune checkpoints play a role in immune escape, with PD-L1 expression on tumour cells leading to lower CD8+ T-cell activity. Clinical studies are investigating antibodies against PD-1 or PD-L1, and predictive markers are needed to identify patients who would benefit from effective treatment [15]. According to Jiang C et al., in addition to T-cell activation, lymphocyte activation, leukocyte migration, T-cell apoptosis, tolerance induction, and cytolysis, genes correlated with PD-1/PD-L1 are also involved in these biological processes. PD-1 expression was associated with immune infiltration, immune regulators, and higher survival rates in patients with breast cancer [16]. Bharadwaj KR et al., found that approximately 52% (68/132) of TNBC cases express PD-L1, suggesting that anti-PD-1/PD-L1 therapy alone or in combination with chemotherapy may be a promising treatment for TNBC in Indian patients [17]. Alsaab HO et al., noted that not all PD-L1-expressing tumours respond to PD-1/PD-L1 inhibitors, and conversely, PD-L1-negative tumours can respond to these agents. Further studies on this subject are still ongoing [18].

According to Costa R et al., PD-1 is an immune checkpoint inhibitor expressed on immune effector cells. The first PD-1 antibody tested in humans was Nivolumab. It was found that only a small number of patients responded to the treatment, but those who did showed impressive long-lasting tumour remission. However, there were reports of serious immune-related toxicity. Currently, the anti-PD-1 antibody Nivolumab and the anti-PD-L1 antibody Durvalumab are being investigated in breast cancer [19]. In a study by Ma W et al., antibodies targeting PD-1 and PD-L1 are showing promising results, but only a subset of patients (20%) respond to immune checkpoint inhibitory treatment. Predictive markers are needed to identify those patients who have the best chance of benefiting from effective treatment [20].

MATERIALS AND METHODS

A cohort study will be conducted for a duration of two years (June 2022 to June 2024) in collaboration between the Department of Pathology and the Department of Surgery at JNMC Maharashtra, India. The study will enroll patients who have been diagnosed with breast carcinoma (IEC No.- DMIMS(DU)/IEC/2022/1070, Date-27/06/2022).

Inclusion criteria: The study will include all cases with a histopathological diagnosis of breast carcinoma, all specimens of modified radical mastectomy, and all female patients diagnosed with breast carcinoma.

Exclusion criteria: The study will exclude cases of adenomas, malignant lymphomas, myoepithelial lesions, nipple tumours, fibroepithelial tumours, and mesenchymal tumours. Additionally,

examples of tumours that can occur in the body such as lactating adenoma, tubular adenoma, pleomorphic adenoma, apocrine adenoma, and ductal adenoma will be excluded. Male patients with breast carcinoma will also be excluded.

The study will involve 70 specimens of breast carcinoma obtained from the Department of General Pathology at JNMC. Formalin-fixed paraffin-embedded blocks will be obtained for analysis. The materials required for the study include 10% formalin, grossing instruments (grossing tray, knife, scalpel, measuring tape, plain forceps, toothed forceps), an automated tissue processing assembly, haematoxylin and eosin stain, PD-1 and PD-L1 markers (PathnSitu), glass slides with dimensions of 7.5×2.5 cm, and a binocular research microscope.

Study Procedure

Prior informed consent will be obtained from the patients who will participate in the study. For new patients, a clinical history and physical examination will be conducted, while the clinical details of previously diagnosed cases will be collected, considering the inclusion and exclusion criteria. Biopsies will be taken from clinically suspected cases and sent for histopathological examination at the Department of Pathology, Jawaharlal Nehru Medical College. Routine laboratory tests will be conducted for every patient. The received specimens will be carefully examined and dissected, and appropriate sections will be collected. Normal tissue preparation will be performed before conducting routine Haematoxylin and Eosin (H&E) staining. The histological grade of the tumour will be determined using the Nottingham variant of the Bloom-Richardson Grading System. Immunostaining for PD-1 and PD-L1 will be performed to evaluate the expression of these proteins [21].

Staining Protocol: Haematoxylin and Eosin staining [22,23]: The paraffin-cut sections of breast cancer tumour tissue will be processed using the standard protocol for Haematoxylin and Eosin staining to evaluate histological features.

Immunostaining [22,23]: Formalin-fixed paraffin-embedded tissue sections, with a thickness of four microns, will be placed on slides coated with L-lysine polymer. The slides will be deparaffinized and rehydrated. Antigen retrieval will be performed using a pressure cooker for heat-induced epitope retrieval, followed by three washes with Phosphate Buffer Saline (PBS). Endogenous peroxidase activity will be blocked by treating the slides with 3% hydrogen peroxide for 10 minutes. The PD-1/PD-L1 primary antibody will be applied and allowed to react at room temperature for 30-40 minutes, followed by three washes with PBS. The secondary antibody (streptavidin-biotin) will be applied and allowed to react at room temperature for 30 minutes, followed by another round of PBS washes. The slides will then be soaked in a 3,3-Diaminobenzidine (DAB) solution for 10-20 minutes. After washing with tap water, the slides will be incubated with haematoxylin for 2 minutes at room temperature. The slides will be dehydrated, cleared, and mounted.

Methodology of interpretation: The Nottingham variation of the Bloom-Richardson system will be used to histologically grade the tumour mass [24].

Evaluation of PD-1 and PDL-1 by IHC [25,26]

- **PD-1:** Positive PD-1 expression will show cytoplasmic and membranous staining in lymphocytes with a cutoff of at least 1%.
- **PD-L1:**
  a) In tumour cells: Positive PD-L1 expression will be defined as partial or full membranous staining in more than 1% of tumour cells.
  b) In immune cells: Positive PD-L1 expression will be defined as cytoplasmic and membranous staining in at least 1% of immune cells.
- A cutoff of >=1% will be considered positive for both PD-1 and PD-L1.

Primary outcome: PD-1 and PD-L1 expression will be studied in all categories of breast cancer patients, and the distribution of positivity will be reported.

STATISTICAL ANALYSIS

A Chi-square test will be conducted to analyse the relationship between PD-1 and PD-L1 protein expression and clinicopathological parameters, including age in years, size in cm, distant metastasis, lymph node involvement, ER status, PR status, Her2neu status, histological type, and TNM stage.

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PARTICULARS OF CONTRIBUTORS:
1. Junior Resident, Department of Pathology, JNMC, Sawangi, Wardha, Maharashtra, India.
2. Associate Professor, Department of Pathology, JNMC, Sawangi, Wardha, Maharashtra, India.
3. Professor and Head, Department of Pathology, JNMC, Sawangi, Wardha, Maharashtra, India.
4. Professor, Department of Pathology, JNMC, Sawangi, Wardha, Maharashtra, India.
5. Assistant Professor, Department of Pathology, JNMC, Sawangi, Wardha, Maharashtra, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Jayant S Makarande,
Associate Professor, Department of Pathology, JNMC, Sawangi,
Wardha-442005, Maharashtra, India.
E-mail: drjayant_mak@rediffmail.com

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