RESEARCH ARTICLE

Editorial Process: Submission:09/17/2023 Acceptance:05/03/2024

Investigating Radiotherapy Effects on *PD-L1* Expression in Circulating Tumor Cells: An Exploratory Study

Rhandyka Rafli[1], Wirsma Arif Harahap[2]*, Soehartati Gondhowiardjo[3], Andani Ekaputra[2]

Abstract

Introduction: Circulating tumor cells (CTCs) and Programmed death-ligand 1 (*PD-L1*) play pivotal roles in cancer biology and therapy response. This exploratory study aimed to elucidate the influence of neoadjuvant radiotherapy on *PD-L1* expression in tumor tissues and CTCs of patients with inoperable locally advanced breast cancer. **Methods:** We conducted a prospective cohort study at Universitas Andalas Hospital Padang from January to December 2022 with 27 patients. Biopsies and blood draws were executed before and after the tenth fractions of neoadjuvant radiotherapy. Following radiotherapy, CTCs were isolated using magnetic beads enrichment, followed by an RT-PCR analysis for *PD-L1* expression. Correlations between *PD-L1* expression and tumor response, evaluated via local response and RECIST criteria before and after radiotherapy breast CT scan, were examined using Fisher's exact and chi-square tests. **Results:** Our data revealed no significant alterations in *PD-L1* expression in either tumor tissues or CTCs during radiotherapy (p=0.848 for tissue, p=0.548 for CTCs). Notably, *PD-L1* expression in tumor tissue before treatment was significantly associated with RECIST (p=0.021), while other correlations with local response and RECIST were not statistically significant. **Conclusion:** The study implies radiotherapy may not significantly influence *PD-L1* expression in tumor tissue and CTCs. However, pre-treatment *PD-L1* expression in tumor tissue correlates with RECIST criteria. These findings highlight the need for additional, comprehensive studies to elucidate further the interplay between *PD-L1*, CTCs, and radiotherapy response.

Keywords: Circulating Tumor Cells (CTCs)- *PD-L1*- neoadjuvant radiotherapy- locally advanced breast cancer

Asian Pac J Cancer Prev, **25 (5)**, 1559-1566

Introduction

The landscape of cancer treatment has dramatically evolved over recent decades, focusing on leveraging immune checkpoint modulation to enhance the body's natural defense against cancerous cells [1]. Central to this approach is the programmed death-ligand 1 (*PD-L1*), a pivotal modulator that facilitates tumor cells in evading the immune response. *PD-L1*, present in various cell types, including tumor cells, binds to the programmed cell death protein 1 (PD-1) on immune cells, inhibiting T-cell mediated immunity and aiding tumor survival. Grasping the nuances of *PD-L1* expression is vital for tailoring cancer therapy, as it can offer insights into tumor-immune interactions and inform the design of effective treatments.

Recent findings highlight that *PD-L1* expression is not limited to the tumor environment. Circulating tumor cells (CTCs), as cancer cells detach from the main tumor mass and enter the bloodstream, also express *PD-L1* [2]. As precursors to cancer metastasis, CTCs serve as real-time indicators of disease progression, therapeutic resistance, and potential relapse. Notably, in breast cancer, *PD-L1* expression in CTCs correlates with disease severity and prognosis, underscoring *PD-L1*'s significance in cancer development and treatment response.

However, the relationship between *PD-L1* expression, CTCs, and the effectiveness of treatments like radiotherapy, especially concerning locally advanced breast cancer, remains to be fully explored. Radiotherapy is fundamental in treating breast cancer, but its effects on *PD-L1* expression and subsequent tumor behavior are not yet thoroughly understood [3]. Deciphering this relationship may offer pivotal therapeutic insights, facilitating personalized treatment approaches that enhance patient outcomes.

Hence, this study seeks to investigate the impact of neoadjuvant radiotherapy on *PD-L1* expression in tumor tissues and CTCs in patients with inoperable locally advanced breast cancer. We aim to identify links between *PD-L1* expression and therapeutic outcomes, assessed via

*[1]Faculty of Medicine, Universitas Andalas and Universitas Baiturrahmah, Padang, Indonesia. [2]Faculty of Medicine, Universitas Andalas, Padang, Indonesia. [3]Department of Radiation Oncology, Cipto Mangunkusumo National General Hospital, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia. *For Correspondence: Wirsma@unand.med.ac.id*

local response and RECIST criteria, to determine if *PD-L1* is a reliable indicator of treatment efficacy. Through this research, we aim to augment the existing knowledge regarding *PD-L1* and CTCs in cancer treatment and offer insights into the potential of immune checkpoint inhibitors for treating locally advanced breast cancer.

Materials and Methods

Our study employed a meticulous approach to evaluate the effects of neoadjuvant radiotherapy on *PD-L1* expression in patients with inoperable locally advanced breast cancer.

Patient Selection

We conducted an observational cohort study within the radiotherapy department of Universitas Andalas Hospital from January through December 2022. Our patient pool consisted of breast cancer patients and candidates for neoadjuvant radiotherapy. These patients included cases deemed inoperable post-neoadjuvant chemotherapy, non-operable cases, and patients with bilateral breast cancer without other metastatic lesions.

Sample Collection

Core biopsies and peripheral blood were collected from patients before and after the tenth fraction of their radiotherapy treatment. The rationale behind choosing the tenth fraction was the hypothesis that a sufficient radiation-induced immune response would have occurred at this stage and secondary core biopsies would still be feasible.

CTC Isolation

CTCs were isolated using the ADNA-breast cancer select/detect method. We drew peripheral blood both before the first and after the tenth fraction of radiotherapy. The initial half cc of blood was discarded to avoid skin cell contamination. Once drawn, we processed 10 cc of the blood in an EDTA vial for enrichment within a 4-hour window to avoid cell lysis. Using magnetic beads coated with a mix of Ep-Cam, Her2, and EGFR antibodies, we successfully bound the CTCs. A magnetic rack and a sequence of leucocyte washings helped us separate the CTCs from other blood cells.

RNA Extraction and RT-PCR

Following CTC isolation, we lysed the captured CTCs and proceeded with nucleic acid concentration measurement using a nanodrop spectrophotometer and the ADNA breast cancer detection method. In cases where none of the markers were detected or nucleic acid concentrations were below five ng/ul, we considered the CTCs negative.

Our RNA extraction from tissue involved a five-step process

homogenization of core biopsy tissues and separation phases involving chloroform and Genezol reagent. We then precipitated the RNA using isopropanol and centrifugation, washed it with 70% ethanol, and resuspended it in RNAase-free water. The extracted tissue nucleic acid concentration was then measured.

We converted 100ng of RNA from each tissue and CTC sample into complementary DNA (cDNA). The qPCR was performed using the Bio-Rad CFX96 machines and SYBR-Green reagent. We carried out annealing optimization tests for the *PD-L1* primer and performed melt-profile analyses after the three-step cycling of q-PCR.

Radiotherapy Regimen

A radiation oncologist planned and approved the radiotherapy regimen for our patients. Our process involved utilizing virtual simulations derived from 3mm-thick computed tomography slices. Target volumes were delineated following the RTOG breast cancer delineation guidelines. Our patients received 40Gy in 15 fractions of locoregional radiotherapy targeted at the breast and regional lymph nodes using Intensity-Modulated Radiation Therapy (IMRT). This dose was followed by a booster dose of 20Gy in 10 fractions, delivered by a linear accelerator machine.

Clinical Response Examination

The evaluation of clinical responses involved a two-fold approach: assessing the local response and employing the RECIST 1.1 criteria.

The local response was assessed by comparing the Gross Tumor Volume (GTV) in the irradiated area before and after radiotherapy. GTV is a measure of the volume of known disease and is defined as the gross demonstrable extent and location of the tumor. A CT scan was performed before the initiation of radiotherapy and three months after its completion. The comparison involved calculating the shrinkage of the GTV by analyzing the change in size and volume of the tumor within the irradiated area.

The RECIST (Response Evaluation Criteria In Solid Tumors) 1.1 criteria was used for a more holistic evaluation of the patient's response to treatment. This criteria included consideration of lesions outside the irradiated area. The assessment involved combining data from the CT scan performed before and after radiotherapy and other clinical data obtained from the physical examination. Physical examinations were conducted to check for any palpable lymph nodes or masses and to monitor the patient's general condition. Any changes in the size of the lymph nodes or masses were documented. The RECIST criteria consider the sum of the diameters of target lesions and the presence of new lesions. The criteria define four categories of response: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD).

Statistical Analysis

The analysis involved both univariate and bivariate exploratory methods. Descriptive statistics summarized the demographic and clinical characteristics. The medians of *PD-L1* expression in tissue and CTCs pre- and post-radiotherapy were calculated. The association between *PD-L1* expression and radiotherapy response was assessed employing RECIST 1.1 criteria and local tumour response categorizations. Fisher's exact test evaluated the correlations between *PD-L1* expressions (pre- and

post-radiotherapy) and both RECIST and local tumour responses.

In summary, this study employed a meticulous and comprehensive approach, integrating multiple key stages of research, to evaluate the effects of neoadjuvant radiotherapy on *PD-L1* expression in patients with inoperable locally advanced breast cancer, as illustrated in Figure 1.

Results

Our patient cohort comprised 27 individuals with a higher proportion above 50 years (55.6%), as depicted in Table 1. Notably, the incidence of breast cancer was higher on the left side (44.4%), and 22.2% of the patients presented with bilateral affliction. The majority of patients were grappling with stage 3B breast cancer (65.6%), with the Non-Special Type (NST) being the dominant histopathological type (77.8%). The most prevalent immunohistochemical type was Luminal B Her2 (+), seen in 37% of patients. Many patients (74.1%) had undergone neoadjuvant chemotherapy in their treatment journey.

An interesting observation from Figure 2 indicated a median increase in *PD-L1* expression in both tissue and circulating tumor cells (CTCs) during radiotherapy. Specifically, the median *PD-L1* expression in tissue increased from a log10 value of 4.50 before radiotherapy to 4.78 during radiotherapy. Similarly, the median *PD-L1*

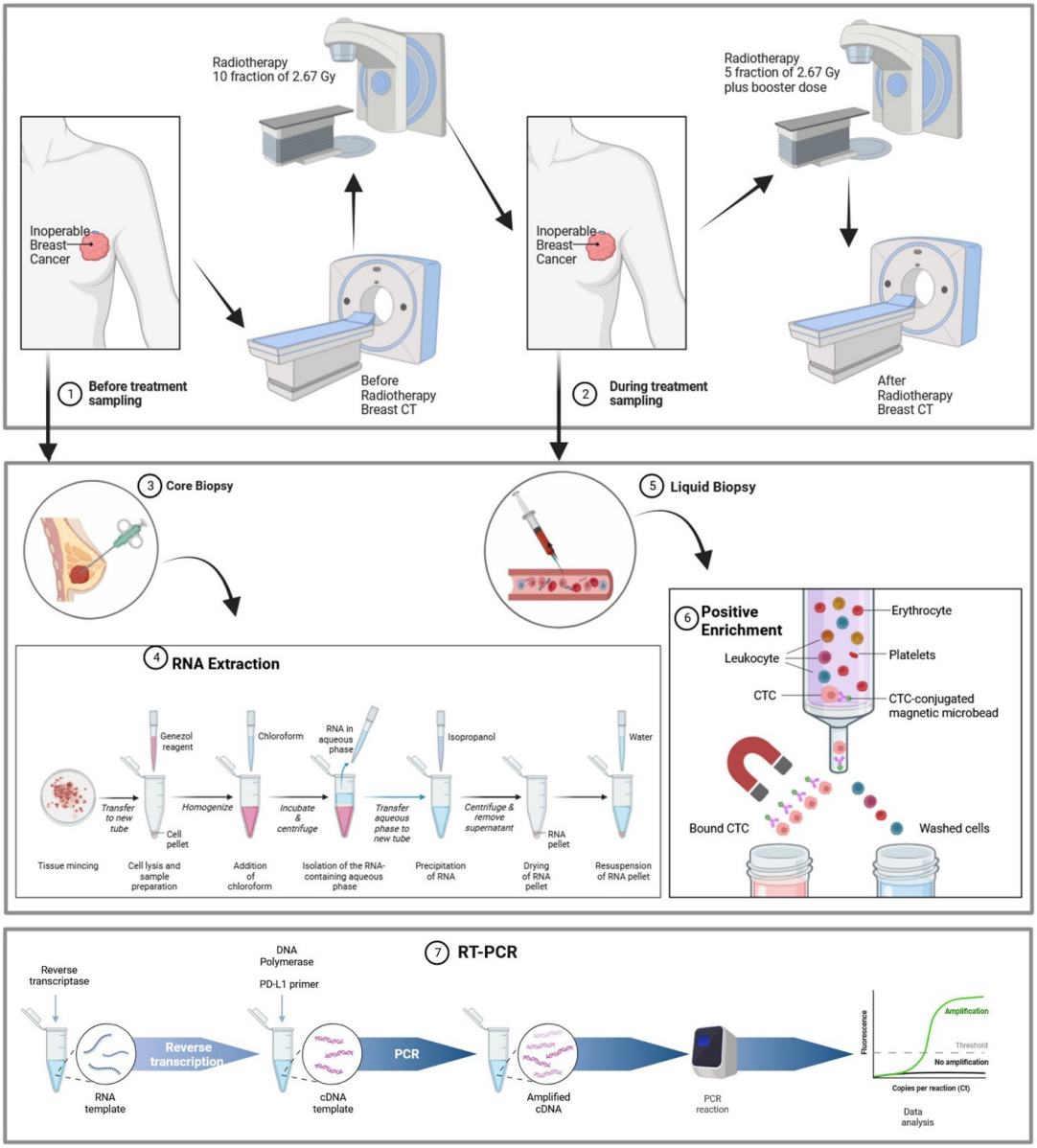

Figure 1. Workflow of the Study. This illustration depicts the steps undertaken in this study: (1) Before treatment, sampling involves collecting core biopsies and peripheral blood from patients before the start of their radiotherapy treatment. (2) During treatment, sampling involves collecting peripheral blood during the tenth fraction of their radiotherapy treatment. (3) Core biopsy involves obtaining tissue samples from the breast tumour. (4) RNA extraction involving homogenization, separation, precipitation, washing, and resuspension of the core biopsy tissues. (5) Liquid biopsy involves the isolation of CTCs from the peripheral blood using the ADNA-breast cancer select/detect method. (6) Positive enrichment involves using magnetic beads coated with a mix of Ep-Cam, Her2, and EGFR antibodies to bind the CTCs and separate them from other blood cells. (7) RT-PCR involves converting the RNA extracted from tissue and CTCs into cDNA and performing qPCR using Bio-Rad CFX96 machines and SYBR-Green reagent.

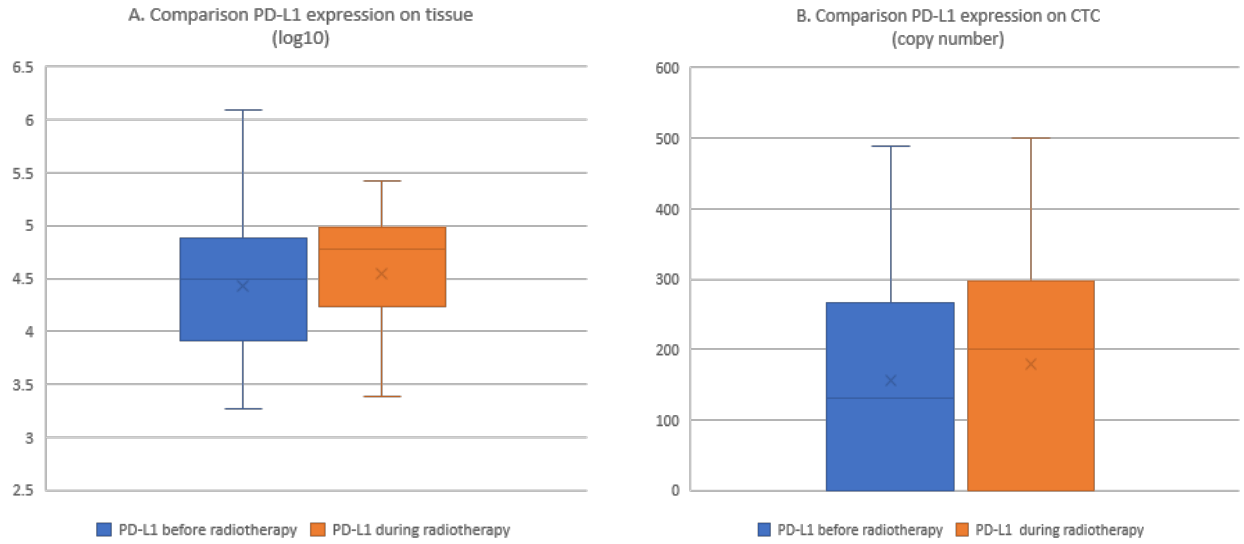

Figure 2. *PD-L1* Expression in Tissue and CTCs Before and During Radiotherapy. (A) Illustrates the increase in median *PD-L1* tissue expression from a log10 value of 4.50 (min: 3.27, max: 6.09) before radiotherapy to 4.78 (min: 3.39, max: 5.42) during radiotherapy. (B) Highlights the rise in median *PD-L1* expression in CTCs from 131 copies (min: 0, max: 489) before radiotherapy to 201 copies (min: 0, max: 500) during radiotherapy.

expression in CTCs increased from a copy number of 131 before radiotherapy to 201 during radiotherapy.

Evaluation of patient responses to radiotherapy presented a spectrum of outcomes. As shown in Table 2, complete response per RECIST criteria was achieved in a modest 7.4% of patients, while partial response was more common, seen in 40.7% of the cohort. A significant proportion of patients (33.3%) remained with stable disease, and unfortunately, 18.6% showed progressive disease. A more granular analysis of local tumor response painted a positive picture; 70.3% of patients exhibited good to an excellent response, characterized by 51-75% and over 75% tumor shrinkage, respectively.

The bivariate analysis indicated no significant differences in *PD-L1* expression in both tissue and circulating tumor cells (CTCs) after radiotherapy. Specifically, the Wilcoxon Signed Rank Test revealed no significant median difference between *PD-L1* tissue expression before and after radiotherapy (p=0.848) and, similarly, no significant median difference in *PD-L1 CTC* expression before and after radiotherapy (p=0.548). Additionally, Spearman's rho test showed no significant correlation between *PD-L1* tissue and *PD-L1 CTC* expression before (p=0.538) and after (p=0.892) radiotherapy.

Furthermore, Fisher's exact test was used to analyze

Table 1. Patient Characteristics

Characteristics	No. of patients	Percentage (%)
Age (years)		
<50	12	44.4
≥50	15	55.6
Side of Breast Cancer		
Right	9	33.4
Left	12	44.4
Bilateral	6	22.2
Breast Cancer Stage		
3B	18	65.6
3C	9	34.4
Histopathological Type		
Non-Special Type (NST)	21	77.8
Invasive Lobular	4	14.8
Mixed NST and Lobular	1	3.7
Mixed NST and Medullary	1	3.7
Immunohistochemical Type		
Luminal A	2	7.4
Luminal B Her2 (-)	4	14.8
Luminal B Her2 (+)	10	37.0
Her2 Overexpression	8	29.6
Triple Negative	3	11.2
Chemotherapy History		
Neoadjuvant Chemotherapy	20	74.1
Without NAC	7	25.9
Operative Status		
Inoperable	21	77.8
Residual post-MRM	6	22.2

Table 2. Tumor Responses Following Radiotherapy

Response Type	Response Category	Frequency	Percent (%)
RECIST Response	Complete response	2	7.4
	Partial Response	11	40.7
	Stable Disease	9	33.3
	Progressive Disease	5	18.6
Local Response	Poor (<0%)	2	7.4
	Fair (0-25%)	4	14.9
	Moderate (26-50%)	2	7.4
	Good (51-75%)	7	25.9
	Excellent (>75%)	12	44.4

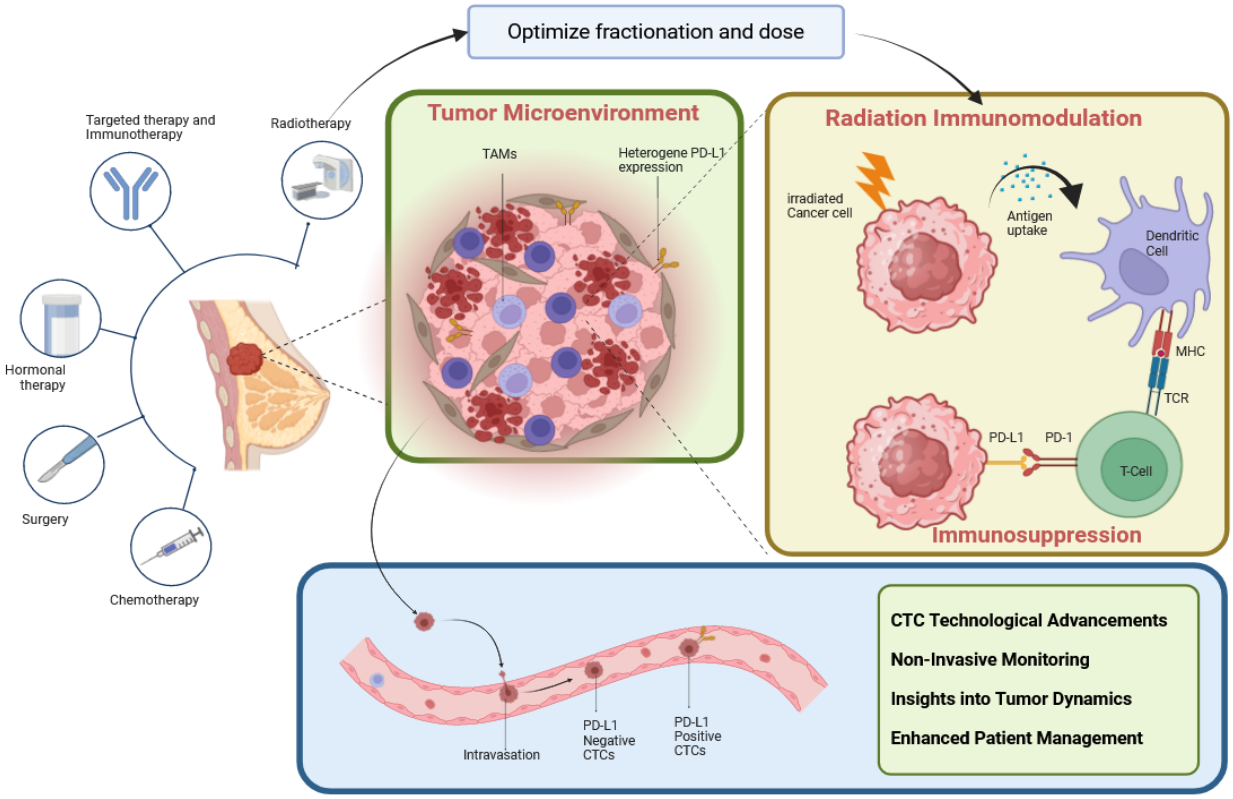

Figure 3. Insights into Tumor Microenvironment and Treatment Implications: This figure delineates the complex interactions within the tumor microenvironment, illustrating the roles and implications of various components such as *PD-L1* expression and immune cells. Additionally, it sheds light on radiation immunomodulation mechanisms and the interplay between dendritic cells and T-cells, highlighting the critical influence of optimized radiation fractionation and dose. Furthermore, the potential of Circulating Tumor Cells (CTCs) technology in enhancing patient management through non-invasive monitoring and providing insights into tumor dynamics.

the relationship between *PD-L1* expression and tumor response. A significant correlation was found between *PD-L1* tissue expression before radiotherapy and the RECIST response (p=0.021), indicating a noteworthy relationship. However, no significant correlations were found in other configurations tested: *PD-L1* tissue expression before radiotherapy and local response (p=0.420), *PD-L1* tissue expression after radiotherapy and RECIST response (p=0.120), *PD-L1* tissue expression after radiotherapy and local response (p=0.206), *PD-L1 CTC* expression before radiotherapy and RECIST response (p=1.000), *PD-L1 CTC* expression before radiotherapy and local response (p=0.696), *PD-L1 CTC* expression after radiotherapy and RECIST response (p=0.420), and *PD-L1 CTC* expression after radiotherapy and local response (p=1.000). These findings hint at the complex interplays between *PD-L1* expression and tumor response to radiotherapy.

Discussion

In our endeavor to decode cancer biology and therapy intricacies, we examined the effects of neoadjuvant radiotherapy on *PD-L1* expression in tumor tissues and circulating tumor cells (CTCs) of patients diagnosed with inoperable locally advanced breast cancer. *PD-L1* is pivotal as a biological marker and a beacon for therapeutic strategies and prognostic implications.

Derived from the cohort at Universitas Andalas Hospital Padang, our findings suggest a subtle, albeit not statistically significant, increase in *PD-L1* expression following radiotherapy. This result contrasts prior research, which pointed to radiotherapy amplifying *PD-L1* expression in tumor cells, potentially enhancing the efficacy of PD-1/*PD-L1* checkpoint blockade therapies [4,5]. However, it's vital to note that some of these observations, like ours, show trends rather than statistically significant results.

A compelling argument in the broader oncology community is that CTCs hold considerable promise in deepening our understanding of tumor dynamics [6]. As liquid biopsy, CTC offers a promising approach to discovering predictive biomarkers for patient selection in immunotherapy [7]. This method overcomes challenges linked to repeated tissue sampling, often complicated by limited tissue samples and tumor evolution [8].

With rapidly evolving CTC detection technology, including advancements like the CellSearch System, RT-PCR, Microfluidic Devices, Filtration Methods, Density Gradient Centrifugation, Magnetic-activated Cell Sorting (MACS), Fluorescence In Situ Hybridization (FISH), Dielectrophoresis, BEAMing (Beads, Emulsions, Amplification, and Magnetics) and CAPP-Seq (Cancer Personalized Profiling by deep Sequencing) [9,10], the

potential of utilizing CTCs for *PD-L1* measurement in breast cancer is immense. This potential is particularly promising for identifying patients most likely to benefit from Immune checkpoint inhibitors [11]. Moreover, *PD-L1* expression's role in breast cancer, especially its association with tumor size, grade, and receptor status, necessitates understanding its dynamics in CTCs post-radiotherapy [12].

Additionally, considering the discrepancies in *PD-L1* expression between primary cancer tissue and metastatic lesions [13], it underscores the necessity to grasp the diverse *PD-L1* expressions across various cells in a patient's body and the potential influences of treatments like radiotherapy. Our patient cohort, composed of those with locally advanced breast cancer, offers a unique perspective. Their specific cancer stage may present biological nuances affecting the interplay between radiotherapy and *PD-L1* expression.

Our protocol, specifically hypo fractionated radiotherapy delivered at 40.05 Gy across 15 fractions, adheres to recognized treatment guidelines. The literature underscores the intricate relationship between radiation therapy and its subsequent effects on the immune system, suggesting a potential boost in anti-tumor activities. The importance of optimally timing the introduction of immune checkpoint inhibitors (ICIs) with radiotherapy fractionations has been highlighted [14]. Similarly, there is an emphasis on the need to calibrate radiation dosages to achieve the best anti-tumor immune responses [15]. The complex implication of CTC technology and effect of radiotherapy to *PD-L1* expression were shown in Figure 3.

PD-L1's regulation in cancer cells is multifaceted. Beyond transcriptional control, *PD-L1* expression undergoes post-transcriptional, post-translational, and epigenetic regulation. These intricate regulatory layers influence how tumors interact with the immune system and also in response to the treatment of radiation or chemotherapy agents, potentially pointing toward avenues to enhance the effectiveness of therapies targeting the *PD-L1*/PD-1 axis [16–18]. Such revelations from the broader scientific community enable us to contextualize our findings better.

Detailed Reflections on Our Observations

Correlation Between Pre-treatment PD-L1 Expression and RECIST Criteria

A nuanced dive into our data reveals a conspicuous relationship here, emphasizing the pivotal role of *PD-L1* status as a predictive biomarker. This resonance with a cohort that demonstrated a sizable proportion of patients with metastatic breast cancer, who exhibited a higher prevalence of PD-L1-rich CTCs, often charted enhanced clinical trajectories when subjected to anti-PD-1 immunotherapy [19].

Zero PD-L1 Expression in Certain CTC Samples and RT-PCR Nuances

The observation of zero PD-L1 expression in some CTC samples is intriguing. While it could be interpreted as a lack of target gene expression, the intrinsic rarity and heterogeneity of CTCs may offer another explanation. Moreover, a study hypothesized that the persistent presence of PD-L1(+) CTCs might indicate latent resistance mechanisms to therapeutic interventions [20]. On the other hand, real-time polymerase chain reaction (RT-PCR) is a sensitive and reliable technique to quantify gene expression, but it is not without challenges. A result of zero copies, as found in some of our CTC samples, might not only indicate a potential lack of PD-L1 expression due to biological variability or radiotherapy influence but could also be influenced by other factors such as the integrity of RNA and the efficiency of reverse transcription and amplification stages. The balance between detection sensitivity and specificity, especially for low copy number targets, demands rigorous optimization.

Influence of Neoadjuvant Radiotherapy

Radiotherapy is a well-known method used in cancer treatment, primarily working by damaging the DNA of cancer cells and causing them to die. Our study looked at how this treatment might also change levels of PD-L1 expression in tumor tissue and CTCs. We found that radiotherapy could increase PD-L1 expression, but it's unclear whether this increase is significant enough to affect the treatment outcomes. We are considering if changing the radiation dose or how often it's given might lead to more noticeable changes. We also acknowledge that other factors could have affected our results.

Clinical Implications and Future Directions

Our study underlines the substantial role of PD-L1 expression in sculpting the roadmap for breast cancer diagnostics and treatment, potentially aligning with the RECIST criteria to forge refined trajectories in patient management. The nuances of PD-L1 expression, especially in relation to neoadjuvant radiotherapy, beckon a deeper exploration to ascertain its efficacy as a reliable biomarker. Furthermore, the dynamics between radiotherapy and PD-L1 expression hint at an intricate narrative that might be pivotal in crafting more nuanced treatment regimens. Could a modified radiotherapy dose or a revised fractionation protocol potentially influence PD-L1 expression more substantially? Moreover, the prospect of integrating targeted therapies or immunomodulators poses an interesting avenue for research, possibly unlocking synergistic potentials in treatment strategies.

Additionally, the observed heterogeneity in PD-L1 expression within CTCs raises a pertinent question: does this variation paint a faithful representation of the primary tumor's behavior? Alongside, the advancements in CTC technology beckon further exploration to facilitate non-invasive monitoring and furnish insights into tumor dynamics, possibly offering a window into predicting treatment responses and managing patients more effectively.

Moving forward, a more extensive data repository, encompassing various settings and a wider array of patient backgrounds, would be instrumental in providing a more comprehensive understanding. It seems prudent to ponder on the opportune moments to reassess PD-L1 expressions,

potentially guiding the fine-tuning of treatment approaches in the future. These lingering questions pave the way for future studies, holding the promise of ushering in a new era of personalized and effective cancer treatment strategies.

Limitations and Lessons

Our cohort, sourced from Universitas Andalas Hospital Padang, provided a rich dataset to work on. However, every study comes with inherent limitations. The sample size, though adequate, could benefit from expansion, lending further statistical power. The singular focus on neoadjuvant radiotherapy could complement investigations into other therapeutic interventions and their influence on PD-L1. Additionally, a more prolonged post-treatment monitoring period might yield richer insights into the longer-term effects of radiotherapy on PD-L1 dynamics.

In conclusion, our investigation revealed a significant correlation between pre-radiotherapy PD-L1 tissue expression and RECIST response, despite no notable alterations in PD-L1 expression in both tissue and circulating tumor cells (CTCs) post-radiotherapy. This critical observation underscores the predictive value of PD-L1 tissue expression prior to radiotherapy and points towards a complicated interplay between PD-L1 expression and the tumor's response to radiotherapy.

The implications of these observations are manifold. Primarily, it accentuates the necessity of assessing PD-L1 expression before commencing radiotherapy, as it may be a pivotal predictive marker for treatment response. Moreover, it underscores the imperative need for further investigations to unravel the intricate relationship between PD-L1 expression, CTCs, and tumor response to radiotherapy, as well as the potential involvement of other immune checkpoints.

Moving forward, it is crucial to validate these findings in a more extensive cohort of patients and delve deeper into the potential mechanisms underlying the observed correlations. Additionally, exploring the relationships between other immune checkpoints and radiotherapy response could yield a more comprehensive understanding of the immune landscape in breast cancer patients undergoing neoadjuvant radiotherapy.

Author Contribution Statement

The roles in this study were divided as follows: Conceptualization was done by RR, WAH, and SG; RR and AEP developed the methodology; SG and AEP carried out the validation; RR, WAH, SG, and AEP participated in formal analysis; RR spearheaded the investigation and along with WAH procured the resources; RR took the lead in writing the original draft, while the review and editing were overseen by RR, WAH, SG, and AEP. All authors have reviewed and approved the final version of the manuscript set to be published.

Acknowledgements

This study received generous support from the Universitas Andalas Hospital.

Ethics Approval and Consent to Participate

The current study was conducted in accordance with the Declaration of Helsinki and has been approved by the Ethics Committee of the Faculty of Medicine, Universitas Andalas (approval no. 535/UN.16.2/Kep-FK/2021). Written informed consent was secured from all individuals participating in the research. Additionally, all subjects have given their written consent for the publication of this paper.

Availability of Data and Materials

The datasets generated and analyzed during the current study are not publicly available owing to institutional policies but can be availed upon reasonable request from the corresponding author.

Conflict of Interest

The authors declare that there are no relevant financial or non-financial interests to disclose in relation to this study.

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