Original Study

Serum sPD-1 and sPD-L1 as Biomarkers for Evaluating the Efficacy of Neoadjuvant Chemotherapy in Triple-Negative Breast Cancer Patients

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Abstract

Serum biomarkers for monitoring the efficacy of neoadjuvant chemotherapy in triple-negative breast cancer patients have not been established. We found that serum levels of sPD-L1 and sPD-1 were increased with tumor stages in triple-negative breast cancer patients with higher tumor stage and were significantly reduced after neoadjuvant chemotherapy in patients with positive outcomes.

Background: Neoadjuvant chemotherapy (NAC) is widely administered in the primary treatment of triple-negative breast cancer (TNBC). However, serum biomarkers for evaluating or monitoring the curative efficacy of NAC have not been established. Accumulating data have shown that soluble programmed death 1 (sPD-1) and its ligand (sPD-L1) might be potential biomarkers for evaluating the curative efficacy of chemotherapy and patient prognosis in several cancers but not yet in breast cancer. **Patients and Methods:** Blood specimens were obtained from 66 TNBC patients who received NAC and 59 healthy women. The serum concentrations of sPD-1 and sPD-L1 were measured by enzyme-linked immunosorbent assay. **Results:** Compared to healthy women, the serum concentration of sPD-1 was significantly elevated in TNBC patients before NAC (549.3 ± 58.76 pg/mL vs. 379.2 ± 17.30 pg/mL, $P = .007$), but there was only an increase tendency for sPD-L1 (227.7 ± 23.99 pg/mL vs. 195.0 ± 8.49 pg/mL, $P = .22$). The serum levels of sPD-1 and sPD-L1 before NAC in TNBC patients increased with tumor stage ($P = .038$ and .030, respectively). Patients who experienced complete or partial remission after NAC had significantly decreased serum levels of sPD-1 and sPD-L1 compared to patients with a poor response to NAC ($P = .019$ and .021, respectively). **Conclusion:** Serum levels of sPD-1 and sPD-L1 could be used as noninvasive biomarkers for evaluating the malignancy of TNBC before NAC and for predicting the NAC response in TNBC patients.

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Keywords: Biomarker, Neoadjuvant chemotherapy, Soluble programmed death ligand 1, Soluble programmed death 1, Triple negative breast cancer

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Introduction

Triple-negative breast cancer (TNBC) is defined as breast cancer that is negative for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2); therefore, TNBC does not respond to hormone therapy or therapies that target HER2, and usually has a poor prognosis.[1] Currently, neoadjuvant chemotherapy (NAC) followed by definitive surgery has become a standard of care for locally advanced breast cancer, including TNBC. Accumulating evidence has shown that TNBC patients can benefit from NAC, with a higher rate of pathologic

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complete response (pCR) relative to non-TNBC patients.[2,3] In the clinical practice of NAC for TNBC, evaluating the efficacy in real time is an important issue. Traditionally, NAC efficacy is evaluated either by the application of Response Evaluation Criteria in Solid Tumors (RECIST) or by pathologic examination. RECIST is based on tumor shrinkage, which is a later event compared to other changes triggered by treatments,[4] while pathologic examination must be performed after surgery.[5] Therefore, these parameters are unable to monitor the efficacy of chemotherapy in real time and may not be conducive for avoiding the use of ineffective protocols and customizing the optimal strategies.

To evaluate the efficacy of NAC over time, serum biomarkers have the advantages of being noninvasive and easy to use. In recent years, several serum biomarkers have been shown to have potential for assessing NAC responses in breast cancer. Serum concentration of vascular endothelial growth factor (VEGF) was significantly decreased in pCR patients with TNBC relative to non-pCR patients after NAC, and the change in VEGF before the third cycle of NAC had a remarkable predictive value for both pCR and pathologic response with high sensitivity and specificity.[6] It has also been reported that serum activity of IDH-2 was closely related to the NAC response in breast cancer.[7] In addition, variations in serum microRNA levels during NAC treatment may be therapeutically significant for predicting response and survival outcomes of breast cancer patients. For example, the levels of miR-155 showed a significant decrease after chemotherapy.[8] However, none of these serum biomarkers has been shown to be sufficiently reliable to be used in clinical practice, and new serum biomarkers remain to be further explored.

Studies have demonstrated that approximately 20% to 30% of TNBC patients had enriched programmed death ligand 1 (PD-L1) in the membranes of cancer cells, as well as a highly expressed programmed cell death 1 (PD-1) in infiltrated immune cells.[9-11] Moreover, it has been reported that PD-1 and PD-L1 expression may be useful as biomarkers to predict treatment responses to NAC in TNBC.[12] However, PD-1 and PD-L1 are mainly expressed on the cell membrane, ie, membrane PD-1 and membrane PD-L1, and the measurement of these proteins relies on immunohistochemical staining after surgery. Recently, soluble PD-1 and PD-L1 (sPD-1 and sPD-L1) have been found in blood, so we speculate that sPD-1 and sPD-L1 might be new blood-based biomarkers for monitoring the efficacy of NAC in TNBC patients.

This study aimed to investigate the changes in serum sPD-1 and sPD-L1 concentrations at the beginning and end of NAC treatment, and to evaluate their predictive value for NAC response in TNBC.

Patients and Methods

Patient Specimens and NAC Regimen

Blood specimens were obtained from 66 TNBC patients who received NAC at the Breast Disease Center in Southwest Hospital of the Third Military Medical University from 2010 to 2016. The patients were between 24 and 68 years old (median, 47 years) (Table 1). As a control cohort, blood specimens from 59 healthy women who underwent examination at the physical examination center of the same hospital, aged between 22 and 84 years (median, 47 years), were also collected. All blood samples were obtained from each participant by routine venipuncture using a DTA-K2 anticoagulation blood collection tube (WeiGao Group Medical, China). Samples were collected at the start of chemotherapy (before NAC) and the end of chemotherapy (after NAC). Plasma was obtained by centrifuging blood specimens at 1000 × *g* for 15 minutes at 4°C using a centrifuge (Micro 21R; Thermo Fisher Scientific, USA). Subsequently, plasma samples were numbered and stored in aliquots at −80°C until analysis. NAC regimens were docetaxel/anthracycline or docetaxel/anthracycline/cyclophosphamide. Written informed consent for biological studies was obtained from the patients or their guardians, as well as from the healthy volunteers. All experiments were approved by the ethics committee of Southwest Hospital (approval KY201848).

Table 1 Clinicopathologic Features of 66 Triple-Negative Breast Cancer Patients

Characteristic	Value
Age	
Median (range), years	47 (24-68)
≤45 years	31 (47.0)
>45 years	35 (53.0)
Tumor Stage	
1	23 (34.8)
2	28 (42.4)
3	8 (12.2)
4	7 (10.6)
Lymph Node Stage	
0	37 (56.1)
1/2/3	29 (43.9)
Clinical Stage	
1	19 (28.8)
2	32 (48.5)
3	15 (22.7)
Lymph Node Metastasis	
No	42 (63.6)
Yes	24 (36.4)
RECIST	
CR	16 (24.2)
PR	20 (30.3)
SD	28 (42.4)
PD	2 (3.1)

Data are presented as n (%) unless otherwise indicated.
Abbreviations: CR = complete response; PD = progressive disease; PR = partial response; RECIST = Response Evaluation Criteria in Solid Tumors; SD = stable disease.

Enzyme-Linked Immunosorbent Assay

Both sPD-1 and sPD-L1 were measured with a human sPD-1 and human sPD-L1 enzyme-linked immunosorbent assay kit (Jianglai Biological, China). The reagent and materials were kept at room temperature before use. For sPD-1 and sPD-L1 detection, the plasma was diluted 5 times and 2 times with the sample diluent provided in the kit, respectively. The testing samples, blank samples, and standard controls were used to analyze the quality of sPD-1 and

sPD-L1. Then, 50 μL of standard controls or samples were added to the appropriate wells in triplicate. Subsequently, 100 μL of horseradish peroxidase–conjugated antibody was added to each well except the blank wells, covered with an adhesive strip, and incubated for 60 minutes at 37°C. After washing the microtiter plate 5 times, 50 μL of substrate A and 50 μL of substrate B were added to each well. The samples were gently mixed and incubated in the dark for 15 minutes at 37°C. Finally, 50 μL of stop solution was added to each well, and the optical density at 450 nm was measured using a microtiter plate reader (Thermo Multiskan, USA) within 15 minutes. A standard curve was generated by plotting the average optical density (450 nm) on the vertical axis versus the corresponding concentration on the horizontal axis; this was used to determine the concentration of unknown samples (Supplemental Figure 1 in the online version). All the concentrations are expressed as mean ± standard error.

Response Evaluation Criteria in TNBC

The definition of complete response (CR) of TNBC is that all of the lesions disappeared in the patient after NAC. Patients with tumors reduced by more than 30% are defined as experiencing partial remission (PR). Progressive disease (PD) means that patient tumors increased by more than 20% or that new lesions appeared. Patients with a changed tumor size that did not reach to PR and PD were defined as having stable disease.

Statistical Analyses

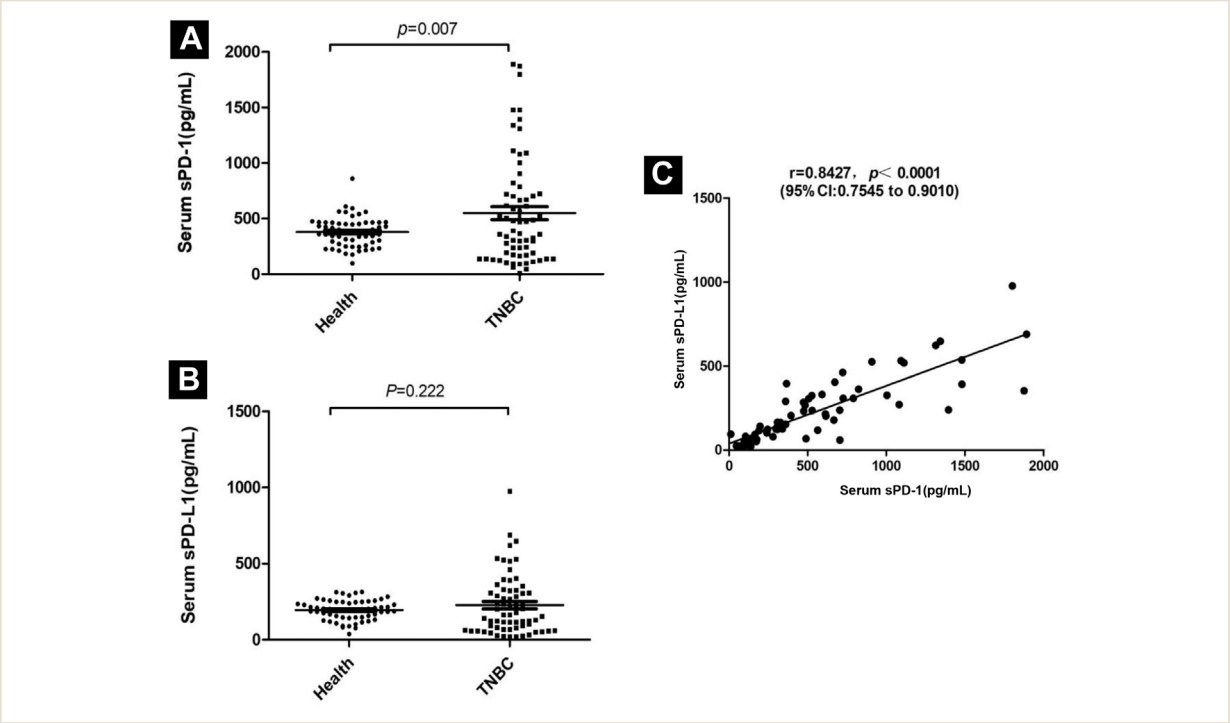
All experiments were performed at least in triplicate and results presented as mean ± standard error. The homogeneity test and normal test of the measurement data were performed by SPSS PASW 18.0 software (IBM, USA), and the nonnormally distributed data were tested using an unpaired Student *t* test. Ranked data were tested using the rank-sum test. Pairing data were tested by a paired Student *t* test. Differences between rates were analyzed by the chi-square test; correlation analyses were performed using the Pearson coefficient analysis. GraphPad Prism 5 (GraphPad Software, USA) was used to create all charts. $P < .05$ was considered statistically significant.

Results

Serum Levels of sPD-1 and sPD-L1 in Healthy Women and TNBC Patients Before NAC

Initially, we measured the levels of serum sPD-1 and sPD-L1 in healthy women and TNBC patients before NAC. Serum sPD-1 levels in TNBC patients were significantly higher than those in healthy individuals (549.3 ± 58.76 pg/mL vs. 379.2 ± 17.30 pg/mL, $P = .007$) (Figure 1A). However, there was an increased tendency for in-serum sPD-L1 only levels in TNBC patients compared to healthy women (227.7 ± 23.99 pg/mL vs. 195.0 ± 8.49 pg/mL, $P = .22$) (Figure 1B). We observed a close correlation between the levels of serum sPD-1 and sPD-L1 in TNBC patients before NAC ($r = 0.8427$; 95% confidence interval, 0.7545-0.9010; $P < .0001$) (Figure 1C).

Figure 1 Concentrations of Serum sPD-1 and sPD-L1 in Healthy Women and TNBC Patients Before NAC. (A) Level of Serum sPD-1 Was Significantly Higher in TNBC Patients Before NAC than in Healthy Women. (B) Level of Serum sPD-L1 in TNBC Patients Before NAC Compared to Healthy Women. (C) Concentration of sPD-1 Correlated With sPD-L1 in TNBC Patients Before NAC



Abbreviations: NAC = neoadjuvant chemotherapy; sPD-1 = soluble programmed death 1; sPD-L1 = soluble programmed death 1 ligand; TNBC = triple-negative breast cancer.

Correlation Between Serum Levels of sPD-1 and sPD-L1 and Clinicopathologic Parameters in TNBC Patients Before NAC

The relationship between the serum sPD-1 or serum sPD-L1 levels in pre-NAC patients and the clinicopathologic features of TNBC patients was analyzed. Results indicated that the levels of serum sPD-1 and sPD-L1 were significantly associated with different tumor stages of TNBC patients ($P = .038$ and .030, respectively) (Table 2). The higher the tumor stage, the higher the sPD-L1 and PD-L1 levels (Figure 2). Both serum sPD-1 and PD-L1 levels in pre-NAC patients were not significantly associated with other clinicopathologic features investigated, including age, clinical stage, lymph node stage, and lymph node metastasis ($P \geq .05$ for all) (Table 2).

Reduced Serum sPD-L1 and sPD-1 Levels in NAC Response in TNBC Patients

We next compared the levels of serum sPD-1 and sPD-L1 before and after NAC, and we analyzed the correlation between changes in serum sPD-1 and sPD-L1 levels and the NAC response in TNBC patients. Both serum sPD-1 and sPD-L1 levels were reduced at the end of NAC compared to pre-NAC values in the whole cohort (494.2 ± 79.64 vs. 549.3 ± 58.76 for serum sPD-1 and 190.8 ± 26.07 vs. 227.7 ± 23.99 for serum sPD-L1), but this finding was not statistically significant ($P = .396$ and .065, respectively) (Figure 3A, B). Among the 66 patients, 16 patients experienced CR, 28 patients partial remission, 20 patients stable disease, and 2 patients PD after NAC.

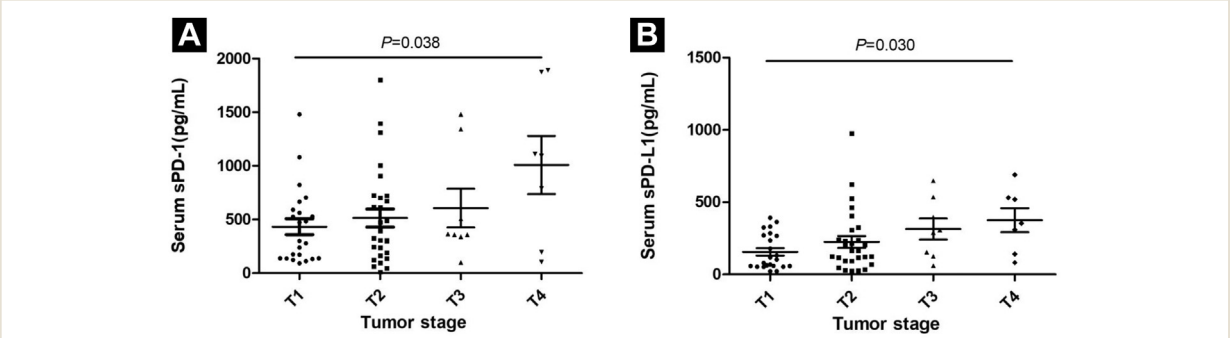
We analyzed changes in serum sPD-1 and sPD-L1 levels in patients with different NAC responses. Because there were only 2 patients with PD status who did not meet the criteria for the Student *t* test, the patients were divided into 2 groups as follows: a good NAC response group, including CR and PR patients, and a poor NAC response group, including stable disease and PD patients. Compared to pre-NAC values, both serum sPD-L1 and sPD-1 levels after NAC were significantly reduced in the good NAC response group ($P = .021$ and .019, respectively) (Table 3, Figure 3C, D). However, there were no significant changes in serum sPD-L1 and sPD-1 levels between pre- and post-NAC values in the poor NAC response group ($P = .276$ and .681, respectively) (Table 3).

Table 2 Correlation Between Serum sPD-L1 and sPD-1 Levels Before NAC and Clinicopathologic Features in Triple-Negative Breast Cancer Patients

	Pre-NAC Serum sPD-L1		Pre-NAC Serum sPD-1	
Characteristic	**sPD-L1 (pg/mL)**	***P***	**sPD-1 (pg/mL)**	***P***
Age				
≤45 years	234.9 ± 31.10	.389	559.2 ± 77.31	.398
>45 years	221.4 ± 36.29		540.6 ± 88.15	
Tumor Stage				
1	156.0 ± 25.51	.03[a]	433.2 ± 73.53	.038[a]
2	225.1 ± 40.31		513.6 ± 84.00	
3	314.4 ± 72.35		606.5 ± 180.3	
4	374.8 ± 82.95		1009.0 ± 270.4	
Lymph Node Stage				
0	220.8 ± 31.23	.907	491.9 ± 67.69	.462
1/2/3	236.5 ± 37.90		622.7 ± 101.9	
Clinical Stage				
1	214.5 ± 37.70	.987	488.6 ± 79.42	.881
2	229.3 ± 36.11		525.3 ± 80.73	
3	241.0 ± 57.07		677.6 ± 167.0	
Lymph Node Metastasis				
No	211.8 ± 27.06	.509	506.7 ± 67.17	.509
Yes	255.6 ± 46.22		624.0 ± 111.3	
RECIST				
CR	207.9 ± 32.10	.979	521.5 ± 97.95	.867
PR	217.6 ± 34.64		596.2 ± 113.5	
SD	252.4 ± 47.77			
PD	142.0 ± 23.63		435.2 ± 127.7	

Values are presented as mean ± SE.
Abbreviations: CR = complete response; NAC = neoadjuvant chemotherapy; PD = progressive disease; PR = partial response; RECIST = Response Evaluation Criteria in Solid Tumors; SD = stable disease; sPD-1 = soluble programmed death 1; sPD-L1 = soluble programmed death 1 ligand.
[a]Statistically significant ($P < .05$). Unpaired Student *t* test was used.

Figure 2 Correlation Between Levels of Serum sPD-1 and sPD-L1 and Tumor Stage in TNBC Patients Before NAC. (A) Levels of Serum sPD-1 Correlated with Tumor Stages in TNBC Patients Before NAC. (B) Serum sPD-L1 Levels Correlated With Tumor Stage in TNBC Patients Before NAC



Abbreviations: NAC = neoadjuvant chemotherapy; sPD-1 = soluble programmed death 1; sPD-L1 = soluble programmed death 1 ligand; TNBC = triple-negative breast cancer.

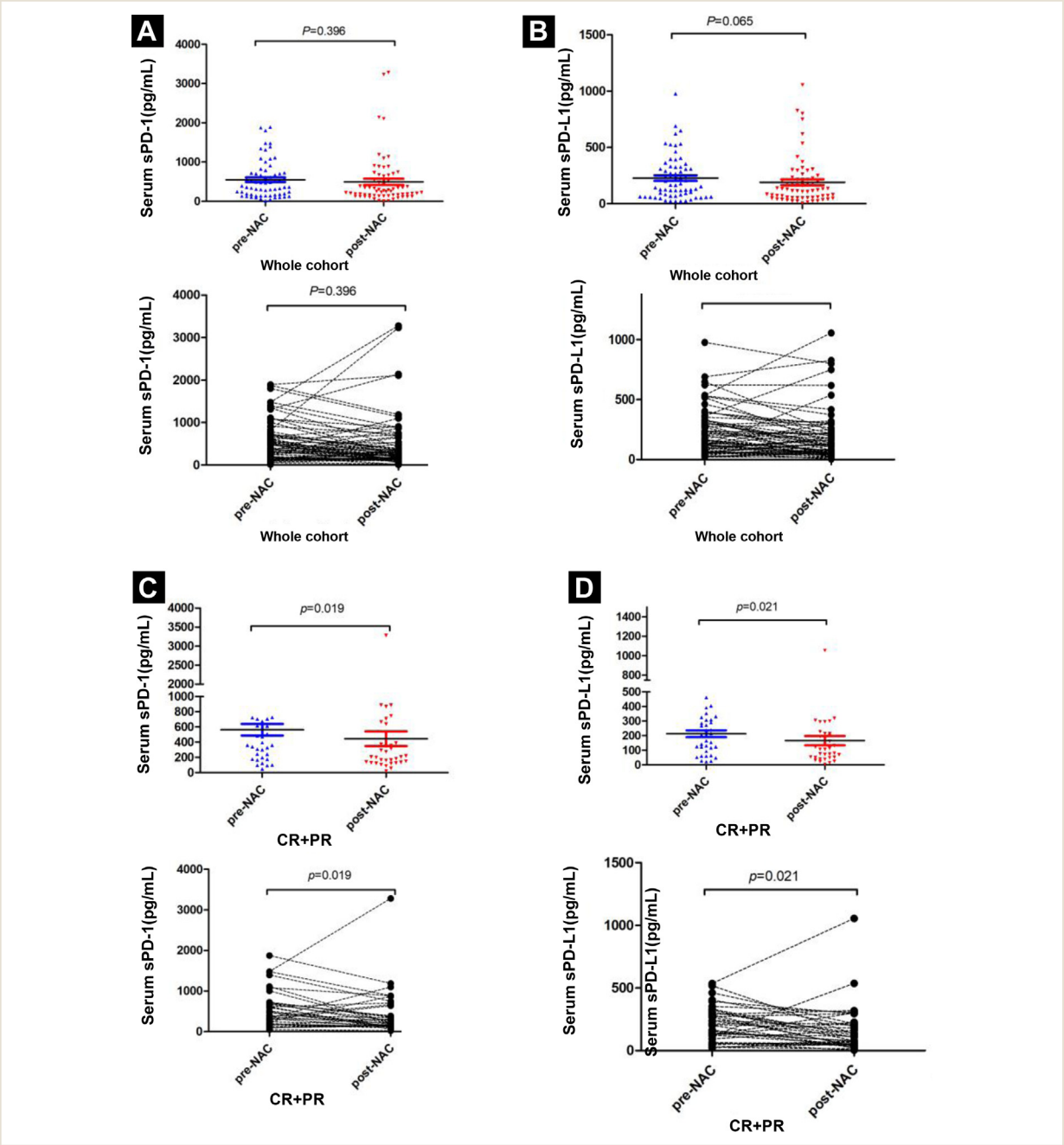
Discussion

Currently, multiple serum tumor markers have been used for aiding early diagnosis, predicting prognosis, and monitoring therapeutic efficacy in breast cancer, such as carbohydrate antigen (CA15-3), BR 27.29 (also known as CA27.29), carcinoembryonic antigen, tissue polypeptide antigen, tissue polypeptide specific antigen, and HER2 (the extracellular domain).[13,14] However, these biomarkers lack sufficient specificity and sensitivity for evaluating NAC responses over time.[15] To our knowledge, our work is the first to reveal the potential of serum sPD-1 and sPD-L1 as blood-based biomarkers for evaluating the NAC response in TNBC.

One of the key interactions between cancer and the immune system involves the signaling of PD-L1 binding to PD-1. PD-L1 is present on the surface of tumor cells, and PD-1 is present on the surface of activated T and B cells.[16,17] The binding of PD-L1 to PD-1 generates a net immunosuppressive effect and allows the tumor to evade immune destruction.[18,19] The clinical impact of PD-1 and PD-L1 has predominantly been addressed with immunotherapy for the treatment of cancer. In recent years, the soluble forms of PD-1 and PD-L1 in the blood of patients with tumors have attracted the attention of researchers. The soluble forms of PD-1 and PD-L1 were initially described in autoimmune disorders where both sPD-1 and sPD-L1 are thought to be produced by immune cells upon stimulation with proinflammatory cytokines.[20] Currently, sPD-1 is considered encoded by the PD-1 Deltaex3 variant,[21] and sPD-L1 is mainly released through proteolytic cleavage of mPD-L1 by matrix metalloproteinases, although other sources, such as immature dendritic cells, macrophages, monocytes and T cells, cannot be excluded.[22,23] The soluble forms of PD-1 and PD-L1, the functions of which remain unclear, increase the complexity and diversity of the composition and function of the PD-1/PD-L1 signaling pathway. Shin et al[24] reported for the first time a positive correlation of sPD-1 and sPD-L1 levels in patients with cancer. Immune suppression is one of the major pathways for malignant tumor cells to resist and to escape from antitumor immunity. The PD-1/PD-L1 pathway down-regulates the effect of T cells in the immune response, thereby causing immune suppression. Recently, the levels of serum sPD-1 and sPD-L1 have been reported to be independent prognostic factors in different solid tumors, such as aggressive renal-cell carcinoma,[25] hepatocellular carcinoma,[26] and non–small-cell lung cancer.[27] We elucidated that the level of serum sPD-1 in TNBC was significantly higher than that in healthy women, implying a strong immune suppression in TNBC patients. A similar pattern of serum sPD-1 and sPD-L1 levels was also observed in hepatocellular carcinoma,[28] oral cancer,[29] and lung cancer,[30] indicating that sPD-1 and sPD-L1 may play an important role in the occurrence and development of malignant tumors. In this study, we found that the levels of serum sPD-1/sPD-L1 did not correlate with patient age, lymph node stage, clinical stage, or lymph node metastasis. These results indicate that all pretherapeutic TNBC patients have similar high serum sPD-1/sPD-L1 levels. However, we found that high serum sPD-1/sPD-L1 levels in TNBC patients before NAC were closely correlated to the tumor stage of patients, suggesting that base serum sPD-1 and sPD-L1 levels might be biomarkers for malignant features of TNBC. These results were consistent with the results from the CCTG MA.31 trial,[31] in which, in $HER2^{+}$ metastatic breast cancer patients, higher sPD-L1 levels before trastuzumab treatment were associated with shorter overall survival.

In this study, we compared the serum sPD-1/sPD-L1 levels before and after NAC in TNBC patients. Although we did not find a significant difference in sPD-1/sPD-L1 in the entire test cohort before and after NAC therapy, we showed that the TNBC patients who had a positive response to NAC (CR + PR) showed significantly reduced serum sPD-1 and sPD-L1 levels after NAC compared to serum sPD-1 and sPD-L1 levels before NAC. These results strongly suggest that the reduction of serum sPD-1 and sPD-L1 levels in TNBC patients receiving NAC therapy indicates a good response to NAC therapy in TNBC patients. Despite ongoing arguments about the function and underlying mechanisms of serum sPD-1 and sPD-L1 levels in malignant tumors, our results indicate that decreased serum sPD-1 and sPD-L1 levels after NAC could be a good indicator of the efficacy of NAC in TNBC patients. Therefore, these methods can provide guidance for choosing or

Figure 3 Correlation Between Levels of Serum sPD-1 and sPD-L1 and NAC Response in TNBC Patients. (Top) (A, B) In Whole TNBC Cohort, Levels of Serum sPD-1 and PD-L1 Decreased After NAC Compared to Before NAC, But Statistical Significance Was Not Obtained. (C, D) In CR + PR Patients, Levels of Serum sPD-1 and PD-L1 Markedly Decreased After Compared to Before NAC. (Bottom) Case-by-Case Details in Correlation Graph, With Lines Connecting Pre- Versus Post-NAC sPD-1 or sPD-L1 Measurements



Abbreviations: CR = complete response; NAC = neoadjuvant chemotherapy; PR = partial response; sPD-1 = soluble programmed death 1; sPD-L1 = soluble programmed death 1 ligand; TNBC = triple-negative breast cancer.

adjusting the therapeutic protocol. In addition, because measuring serum sPD-1/sPD-L1 levels is less invasive and more efficient, and because it can be done easily and in real time in clinical practice, we believe that it can be used as new biomarkers for predicting the prognosis of NAC-treated TNBC patients.

However, several limitations of our study should be considered. First, our sample sizes of TNBC patients and healthy women were small, which might explain why serum concentrations of sPD-L1 did not show significant differences between TNBC patients and healthy women ($P = .22$). Second, to better monitor the efficacy of

Table 3 Changes in Serum sPD-L1 and sPD-1 Before and After NAC in Triple-Negative Breast Cancer Patients With Different Outcomes

Efficacy	No. of Patients	sPD-L1 (pg/mL)			sPD-1 (pg/mL)		
		Before NAC	After NAC	*P*	Before NAC	After NAC	*P*
CR + PR	36	213.3 ± 23.64	166.1 ± 31.89	.021[a]	563.0 ± 75.84	444.5 ± 96.50	.019[a]
SD + PD	30	245.0 ± 44.84	220.5 ± 42.70	.276	532.9 ± 93.15	553.8 ± 132.5	.681

Values are presented as mean ± SE.
Abbreviations: CR = complete response; NAC = neoadjuvant chemotherapy; PD = progressive disease; PR = partial response; SD = stable disease; sPD-1 = soluble programmed death 1; sPD-L1 = soluble programmed death 1 ligand.
[a]Statistically significant ($P < .05$). Paired Student *t* test was used.

NAC and adjust the therapeutic protocols over time, dynamic detection of serum sPD-1 and sPD-L1 levels should be performed during NAC, rather than only at the beginning and end of NAC. Third, there is no standard NAC regimen for TNBC to date. However, sequential regimens of anthracyclines and taxanes are recommended.[32] As a result of the retrospective nature of the study, concomitant regimens of anthracyclines and taxanes had been provided. Finally, we lacked information about pathologic residual tumor burden and lymphocytic infiltration, so we thus cannot be absolutely sure that the change in serum PD-1 and PD-L1 follows tumor response rather than an effect of chemotherapy in lymphocyte and/or macrophage depletion elsewhere in the body, such as in lymph nodes, thymus, or bone marrow.

Conclusion

Our results indicate that the levels of serum sPD-1 and sPD-L1 could be used as noninvasive biomarkers for predicting the NAC response in TNBC patients.

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Disclosure

The authors have stated that they have no conflict of interest.

Supplemental Data

Supplemental figure accompanying this article can be found in the online version at https://doi.org/10.1016/j.clbc.2019.03.008.

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Supplemental Figure 1 Standard curve for sPD-1(A) and for sPD-L1(B).