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ORIGINAL RESEARCH

The Detection of Plasma Soluble Podoplanin of Patients with Breast Cancer and Its Clinical Signification

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Background: Podoplanin (PDPN) is a type-1 membrane sialoglycoprotein that is expressed in many cancer tumors including breast cancer; nonetheless, its roles in tumor occurrence, development, and metastasis are unclear. In this study, we aimed to investigate the clinical significance of plasma soluble PDPN (sPDPN) levels in patients with breast cancer and its significance in the diagnosis and metastasis.

Materials and Methods: Blood samples from healthy controls (CTL), patients with fibroadenomas of breast (FOB), and breast cancer (pathological type: invasive ductal carcinoma, IDC) were collected. sPDPN levels in the plasma of CTL and patients with FOB and IDC were measured by the ELISA.

Results: The plasma sPDPN levels in IDC patients (159 cases, 22.59±3.70 ng/mL) were higher than those in FOB patients (50 cases, 8.29±1.09 ng/mL; $P<0.05$) and CTL (100 cases, 1.21±0.12 ng/mL; $P<0.0001$). The sPDPN levels in patients at stage III and stage IV (30.08 ±4.66 ng/mL) were higher than in patients at stage I and stage II (11.84±1.12 ng/mL; $P=0.005$). The sPDPN levels in patients with high-moderate and moderate differentiation (17.50±3.02 ng/mL) were lower than those in patients with moderately low and low differentiation (35.73±4.26 ng/mL; $P=0.026$). The sPDPN levels in patients with metastasis (30.60±4.27 ng/mL) were much higher than those in patients without metastasis (13.02±1.30 ng/mL; $P=0.017$).

Conclusion: Plasma sPDPN may be used as a new marker for the determination of the clinical stage, differentiation degree, and metastasis status of breast cancer.

Keywords: podoplanin, breast cancer, ELISA, monoclonal antibody

Introduction

Although its mortality has declined, breast cancer is the most common malignant tumor and the second leading cause of cancer-related death among women.[1,2] According to available data, about two million new patients were diagnosed with and 0.6 million patients died of breast cancer in 2018. Breast cancer has early age onset and survival of patients is closely associated with their clinical stages, where earlier stage usually implies better outcome. Thus, early diagnosis and accurate determination of disease status are very critical for breast cancer patients. At present, clinical detection methods of breast cancer include primarily imaging, cytology, serum tumor marker detection and histopathology. Histopathology is currently the gold standard for diagnosis. However, it is highly traumatic and challenging to obtain biopsy tissue and small nodules are difficult to diagnose with. Therefore, the search for a reliable marker of breast cancer is of great clinical significance for patients.

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Podoplanin (PDPN), a mucin-type transmembrane glycoprotein known as the marker of lymphatic endothelial cells,[3] is highly expressed in many types of cancer tissue and cells,[4] such as lung cancer,[5,6] malignant melanoma,[7] osteosarcoma[8] and brain gliomas.[9] PDPN is the only known endogenous ligand of the C-type lectin-like receptor 2 (CLEC-2) expressed on platelets.[10] The binding of tumor cell PDPN to platelet CLEC-2 triggers platelet activation and aggregation.[11,12] To date, many studies have shown that the expression of PDPN is related to the malignancy, invasiveness, and metastasis of tumor.[7,13,14]

Recently, we developed two monoclonal antibodies (mAb) against human PDPN, SZ-163, and SZ-168, and established an enzyme-linked immunosorbent assay (ELISA) to quantitate plasma soluble PDPN utilizing these two mAbs.[15] In this study, we examined plasma sPDPN levels in controls (CTL, 100 cases), patients with fibroadenomas of breast (FOB, 50 cases), and breast cancer patients (pathological type: invasive ductal carcinoma, IDC, 159 cases) were measured with the newly established ELISA method to evaluate the correlation between sPDPN and tumor occurrence and metastasis status of breast cancer.

Materials and Methods

Patient Selection and Sample Collection

Patients (159 cases of IDC, 50 cases of FOB and 100 cases of CTL) were selected from the First Affiliated Hospital of Soochow University or Luoyang Central Hospital Affiliated to Zhengzhou University in China.

All the 159 IDC patients were female, aged 25–78 years, with a median age of 58.5 years. Inclusion criteria of IDC patients: all the patients were diagnosed as IDC of the breast by histopathology and had not received anti-tumor treatment before blood collection such as surgery, radiotherapy and chemotherapy. Breast cancer staging was determined according to the TNM staging standard published by the Union for International Cancer Control (UICC).[16]

All FOB patients were female, aged 23–68 with a median of 52.5. The control group were female with various ABO blood groups, aged 24–72, with a median of 57.

Exclusion criteria: Patients with viral infectious diseases such as hepatitis B, hepatitis C, acquired immune deficiency syndrome (AIDS) and syphilis, immune diseases such as rheumatoid and systemic lupus erythematosus, burn wounds and dysfunction of major organs such as liver and kidney were excluded.

Two milliliter of blood was collected from above patients during 2017–2019 by vein puncture and the blood was stored in tubes containing 3.6 mg of ethylene diaminetetraacetic acid. Plasma was prepared by centrifugation at 3000 rpm for 5 min and the supernatant was stored at −80°C.

Forty tissue samples of invasive breast cancer patients embedded in paraffin were collected in 2018 from the Department of Pathology of the First Affiliated Hospital of Soochow University, China. These patients were diagnosed according to UICC recommendations.[16]

Mice

Female Babl/c mice (4–6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All the animals were housed in an environment with a temperature of 22±1°C, relative humidity of 50±1%, and a light/dark cycle of 12/12 hr. The CO_2 anesthesia was used when hybridoma cells were injected into the mice and ascites were collected through abdominal puncture. Compressed CO_2 asphyxiation was used to sacrifice mice.

Antibodies

Hybridoma of SZ-163 and SZ-168, two mouse anti-human PDPN mAbs developed as described previously, were injected into mice sensitized by pristane.[15] Ascites were collected 10 days later and were applied to Protein G affinity chromatography. IgG from the ascites was eluted with glycine hydrochloride, pH 2.8–3.0.

Detection of Plasma sPDPN by ELISA

A total of 100 μL of 5μg/mL SZ-163 IgG was coated into each well of a 96-well microtiter plate overnight at 4°C. After washing with PBS-0.05% Tween-20 (PBST) washing buffer, the wells were blocked with PBS containing 2% BSA (w/v) at 37°C for 2–3 hours. Then, 100μL of plasma samples or recombinant human PDPN-Fc (R&D Systems, Minneapolis, MN, USA) were added to the wells and incubated at 37°C for 2 hours. After washing, 100 μL of SZ-168-HRP was added and incubated at 37°C for 1 hour. To detecting the binding of SZ-168-HRP to PDPN, OPD substrate was used according to instruction and the signal was measured with a plate reader.

Any plasma sample was tested 20 times under the same conditions and at the same time (intra-assay), and was tested another 20 times at different times under the same conditions (inter-assay).

Detection of PDPN Expression in Breast Cancer Tissue by Immunohistochemistry (IHC) Staining

All paraffin-embedded tissue samples of 40 invasive ductal cancer patients were cut into 6-μm sections and stained with hematoxylin-eosin (HE) staining according to the kit's procedure instructions (G1122, Solarbio, Beijing, China). 100μL of mAb against human PDPN, D2-40 (ab77854, Abcam, Cambridge, UK) was used to label podoplanin after dewaxing, hydration, and antigen retrieval. Then the sections were treated with Envision kit and 3,3-diaminobenzidine tetrahydrochloride, and finally, counterstained with hematoxylin. The results were determined by a professional pathologist.

Statistical Analysis

sPDPN levels are described as the mean ± SD. Mann–Whitney *U*-test and the nonparametric test were used to determine the statistical significance of the results in the levels of sPDPN. **P*<0.05 was considered to be statistically significant. All statistical tests were two-sided.

Results

Plasma Levels of sPDPN in IDC, FOB Patients and CTL

As shown in Table 1 and Figure 1, there were significant differences in the sPDPN levels among CTL, FOB, and IDC (*P*<0.0001). The sPDPN levels in IDC patients were much higher than in FOB patients (22.59 ±3.70 ng/mL vs 8.29±1.09 ng/mL, *P*<0.05) and CTL (22.59±3.70 ng/mL vs 1.21±0.12 ng/mL, *P*<0.0001), and the sPDPN levels in FOB patients were much higher than in CTL (8.29±1.09 ng/mL vs 1.21±0.12 ng/mL, *P*<0.0001).

In addition, in our ELISA method, we found that the high sPDPN level's intra-assay coefficient of variation was 4.66% (0.5566/1.1935), and the inter-assay coefficient of variation was 6.50% (0.07788/1.1985); the low sPDPN level's intra-assay coefficient of variation was 6.26% (1.37927/22.0475), and the inter-assay coefficient of variation was 9.68% (2.17894/22.513).

Table 1 Plasma sPDPN Levels in FOB, IDC Patients and CTL

Groups	Cases	sPDPN (ng/mL)
FOB	50	8.29±1.09[&]
IDC	159	22.59±3.70[&]*
CTL	100	1.21±0.12

Notes: [&]Compared with CTL, *P*<0.0001; *Compared with FOB, *P*<0.05.
Abbreviations: sPDPN, soluble podoplanin; FOB, fibroadenomas of breast; IDC, invasive ductal carcinoma; CTL, control.

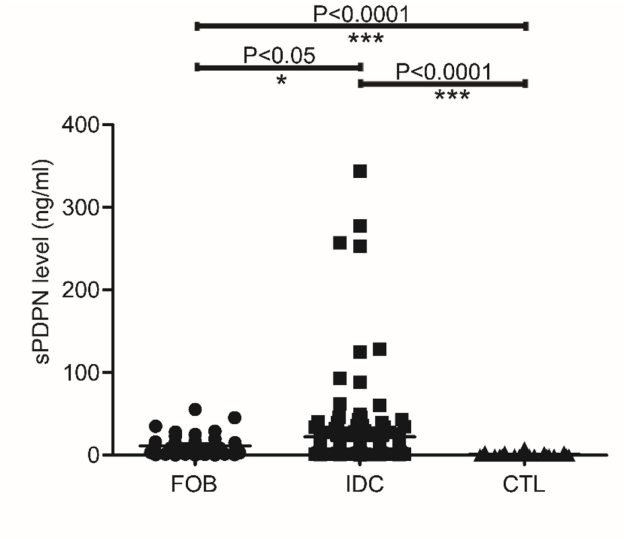

Figure 1 Comparison of plasma sPDPN between FOB, IDC patients and CTL. **P* <0.05; ****P* <0.0001.
Abbreviations: sPDPN, soluble podoplanin; FOB, fibroadenomas of breast; IDC, invasive ductal carcinoma; CTL, control.

Relationship Between Plasma sPDPN Levels and Clinicopathological Parameters in IDC

One hundred fifty-nine cases of IDC were classified according to age, clinical stage, degree of differentiation, tumor size, and other parameters, and then sPDPN levels were statistically analyzed (Table 2).

The results showed that the levels of plasma sPDPN in stage III and IV IDC patients (according to Tumor-Node-Metastasis classifications) were higher than those in stage I and II patients (30.08±4.66 ng/mL vs 11.84 ±1.12 ng/mL, *P*=0.005, Figure 2A). The levels of plasma sPDPN in IDC patients with high-moderately or moderately differentiated carcinoma were lower than those with moderately poorly or poorly differentiated (17.50±3.02 ng/mL vs 35.73±4.26 ng/mL, *P*=0.026, Figure 2B). The levels of plasma sPDPN in IDC patients with metastasis were significantly higher than those with non-metastasis (30.60±4.27 ng/mL vs 13.02 ±1.30 ng/mL, *P*=0.017, Figure 2C). The levels of plasma sPDPN in IDC patients with human epidermal growth factor receptor 2 (HER2) protein were lower than in those without HER2 protein (12.59±2.56 ng/mL vs 29.40±4.32 ng/mL, *P*=0.012).

Table 2 Relationship Between Plasma sPDPN Levels and Clinicopathological Parameters in IDC Patients

Clinicopathological Parameters	Cases	sPDPN (ng/mL)	P-value
Age(Y)			0.242
≤50	56	18.17±2.74	
50~60	55	16.54±1.54	
>60	48	25.03±3.81	
Clinical stage*			0.005
I+II	84	11.84±1.12	
III+IV	75	30.08±4.66	
Differentiation degree*			0.026
High-moderately+ moderately	74	17.50±3.02	
Moderately low+ low	85	35.73±4.26	
Tumor diameter(mm)			0.480
≤20	102	18.12±2.83	
>20	57	24.56±3.61	
Metastasis condition*			0.017
Metastasis	86	30.60±4.27	
Non-metastasis	73	13.02±1.30	
Number of lesions			0.149
Single	117	20.82±3.62	
Multiple	42	23.66±4.25	
Estrogen receptor			0.114
Negative	62	25.39±3.12	
Positive	97	20.17±1.97	
Progesterone receptor			0.537
Negative	78	23.92±2.21	
Positive	81	20.12±1.92	
HER2 protein*			0.012
Negative	73	29.40±4.32	
Positive	86	12.59±2.56	

Note: *$P<0.05$.
Abbreviations: sPDPN, soluble podoplanin; IDC, invasive ductal carcinoma; HER2 protein, human epidermal growth factor receptor 2 protein.

The Diagnostic Value of Plasma sPDPN

The sPDPN level, as a potential tumor marker, had the largest area under the curve (AUC) in Figure 3 compared with other tumor markers such as cancer antigen 125 (CA125), cancer antigen 153 (CA153), and carcinoembryonic antigen (CEA) in the diagnosis of breast cancer (0.961 vs 0.741, 0.860 and 0.716, respectively). Moreover, sPDPN had the highest sensitivity (88.98%) and specificity (96.00%) among all the markers evaluated (Table 3).

On the other hand, the sPDPN level in the AUC provided no assistance for differentiating benign and malignant tumors nor for identifying benign FOB from the normal population.

The Value of Plasma sPDPN for Cancer Prediction

First, the single factor analysis was carried out using the values of the diagnosed breast cancer as the dependent variable, and age, sPDPN, CA125, CA153, and CEA as the independent variables. Next, multivariate analysis was performed to identify indicators with statistical significance (Table 4). The results of multivariate analysis showed that sPDPN and CA153 were independent predictors of breast cancer, especially if adjusted for other confounding factors, with sPDPN having the highest odds ratio (OR) value of 4.024 ($P<0.001$).

HE Staining and IHC Analysis by D2-40 of Breast Cancer Tissues

The tumor tissue was confirmed by HE staining (Figure 4A), indicated by large and deformed cell nucleus, proliferated interstitial fibrous tissue, and visible infiltration of inflammatory cells. PDPN expression in tumor tissues was confirmed by D2-40 staining (Figure 4B and C). Based on cell morphology, we suggested the positive tumor stroma was cancer-associated fibroblasts (CAFs).

Relationship Between D2-40 Staining in Breast Tumor Tissue and Clinicopathological Parameters

A total of 40 IDC patients were classified according to age, clinical stage, degree of differentiation, lymph node metastasis status and other parameters. Statistical analysis was then performed for the positive rate of D2-40 staining in cancer tissues. The results showed that the positive rate was much higher in groups of stage III and IV, lymph node metastasis, negative estrogen receptor and negative progesterone receptor than that of the counter group. The P values are: clinical stage, $P=0.022$; lymph node metastasis, $P=0.042$; estrogen receptor, $P=0.010$, progesterone receptor, $P=0.037$ (Table 5). The sPDPN levels in the positive D2-40 staining group were significantly higher than those in the negative staining group (23.74±2.71 ng/mL vs 21.09±1.89 ng/mL, $P=0.003$). The Ki-67 labeling rate in the positive D2-40 staining group was also higher than that in the counter group (57.14±12.54% vs 21.54 ±8.86%, $P<0.001$).

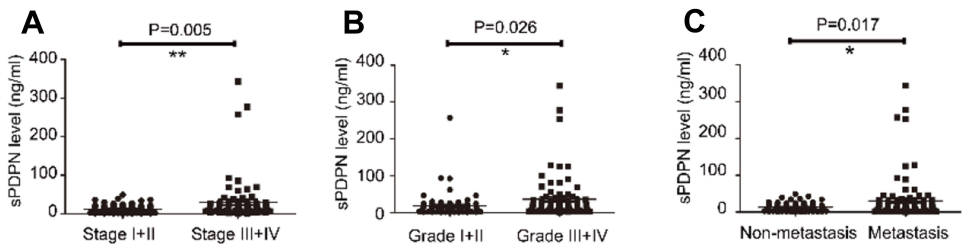

Figure 2 Analysis of the plasma sPDPN levels in IDC patients with different clinical-stage, degree of differentiation, and metastasis status. (**A**) The sPDPN levels in stage I and II patients compared with stage III and IV patients. (**B**) The sPDPN levels in grade I and II patients compared with grade III and IV patients. (**C**) The sPDPN levels in patients with metastasis compared with patients without metastasis. *P<0.05; **P <0.01.
Abbreviations: sPDPN, soluble podoplanin; IDC, invasive ductal carcinoma.

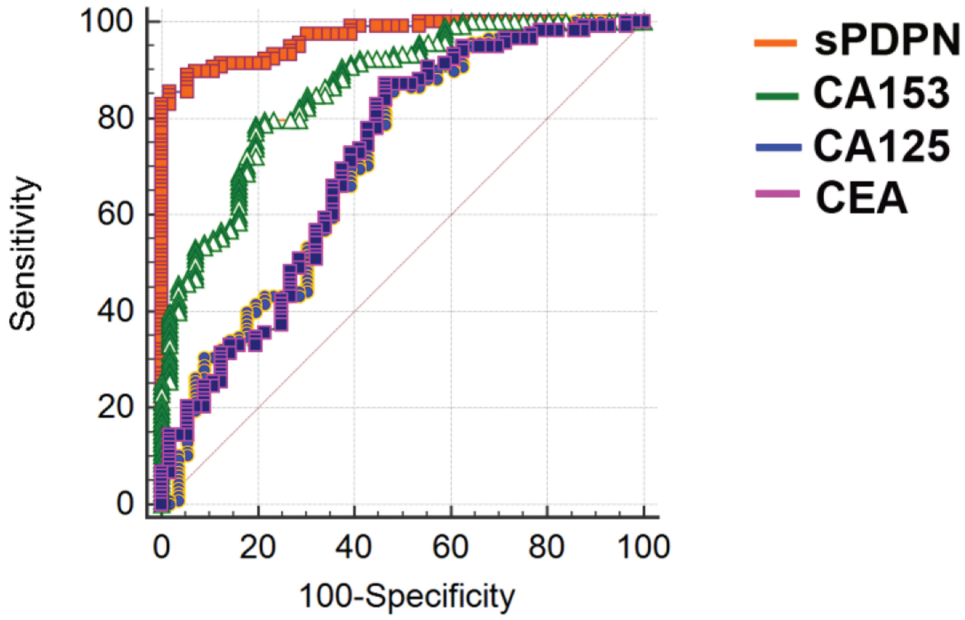

Figure 3 ROC curve of sPDPN, CA125, CA153, and CEA in diagnosis of breast cancer.
Abbreviations: AUC, area under the curve; sPDPN, soluble podoplanin; CA125, cancer antigen 125; CA153, cancer antigen 153; CEA, carcinoembryonic antigen.

Discussion

Podoplanin, a mucin-type membrane glycoprotein rich in O-glycoside chains,[17] is mainly expressed on the lymphatic endothelium,[18] pneumocytes,[19] and glomerular podocytes[20] and plays essential roles in the development of lymphatic vessels,[21,22] cerebrovascular system formation and maintenance of its integrity,[23] as well as in the promotion of natural regulatory T cells.[24] Besides normal tissues and cells, PDPN is also found in human inflammatory diseases[25–27] and various cancer tissues[28] indicted by D2-40 staining technique or flow cytometry analysis. Previous studies have suggested that PDPN is not only expressed in cancer cells,[8] but also in cancer stroma,[9] especially in fibroblasts.[4] After the surface plasmon resonance imaging biosensor was applied for the detection of sPDPN in the serum sample,[29] our lab established an efficient ELISA method with that we successfully and conveniently detected sPDPN in human plasma.[15] In this study, we found that the sPDPN levels in IDC patients (22.59±3.70 ng/mL) were significantly higher than those in FOB patients (8.29±1.09 ng/mL; P<0.05) and CTL (1.21±0.12 ng/mL; P<0.0001). In addition, expression of PDPN may be correlated to metastasis,[30,31] as we observed that patients of later clinical-stage, poor differentiation, lymph node metastasis, and negative HER2 expression had higher level of sPDPN. sPDPN may be proteolytically cleaved from the extracellular domain and enter the blood circulation. Alternatively, it can be secreted by tumor cells and stromal cells as a full-length protein attached to extracellular vesicles.[32]

Table 3 Comparison of the Diagnostic Value of sPDPN, CA125, CA153, and CEA for Breast Cancer

Index	AUC	95% CI	Cut-Off Value	Sensitivity	Specificity
sPDPN	0.961	0.926 ~ 0.983	3.03ng/mL	88.98%	96.00%
CA125	0.741	0.667 ~ 0.798	8.20ng/mL	85.59%	56.00%
CA153	0.860	0.800 ~ 0.908	8.74ng/mL	78.81%	80.36%
CEA	0.716	0.652 ~ 0.775	1.14ng/mL	87.29%	48.00%

Abbreviations: AUC, area under the curve; CI, confidence interval; sPDPN, soluble podoplanin; CA125, cancer antigen 125; CA153, cancer antigen 153; CEA, carcinoembryonic antigen.

Table 4 Logistic Regression Analysis of Independent/Related Indexes of Breast Cancer

Index	Single Analysis OR (95%)	P-value	Multivariate Analysis OR(95%)	P-value
Age	0.998 (0.977 ~ 1.020)	0.880		
sPDPN	2.568 (1.897 ~ 3.476)	<0.001	4.024 (2.035~7.957)	<0.001
CA125	1.135 (1.073 ~ 1.201)	<0.001	1.051 (0.915~1.207)	0.480
CA153	1.489 (1.301 ~ 1.704)	<0.001	1.522 (1.178~1.967)	0.010
CEA	1.806 (1.375 ~ 2.373)	<0.001	1.131 (0.608~2.103)	0.698

Abbreviations: OR, odd radio; sPDPN, soluble podoplanin; CA125, cancer antigen 125; CA153, cancer antigen 153; CEA, carcinoembryonic antigen.

Furthermore, compared with CA125, CA153, and CEA, sPDPN had the best diagnostic value in IDC patients (AUC=0.961, sensitivity=88.98%, specificity=96.00%) as well as was an independent predictor of breast cancer with OR = 4.024. CAF has been reported as a sign of breast cancer recurrence.[33] Our IHC results are consistent with that finding suggesting that infiltration of positive PDPN CAFs can predict poor prognosis.[34,35] The expression of PDPN (probably in tumor stroma) was detected in the surgical specimens of 15 out of 40 IDC patients, especially in the patients of late clinical stage who had lymph node metastasis, high expression of Ki67 and sPDPN, and were estrogen receptor- and progesterone receptor-negative. Recently, many studies have shown that the PDPN expressed on macrophages[36] and CAFs[37] could promote the metastasis of breast cancer in vivo and in vitro. We detected the elevated sPDPN in the plasma of breast cancer patients for the first time. We believe that sPDPN is a reliable indicator of breast cancer with relatively poor prognosis.

In the further, we will expand the number of specimens to explore the value of PDPN in differentiating benign and malignant tumors. Meanwhile, we will intend to establish a suitable animal breast cancer model to investigate the role of podoplanin in the occurrence, development and metastasis of breast cancer, so as to promotes PDPN as a breast cancer screening index. Furthermore, we will investigate whether SZ-168, the antibody against PDPN, could inhibit breast cancer progress and thus a potential therapeutic candidate for breast cancer treatment.

Conclusion

Plasma sPDPN levels in patients with breast cancer and PDPN expression in the tumor stroma have a certain

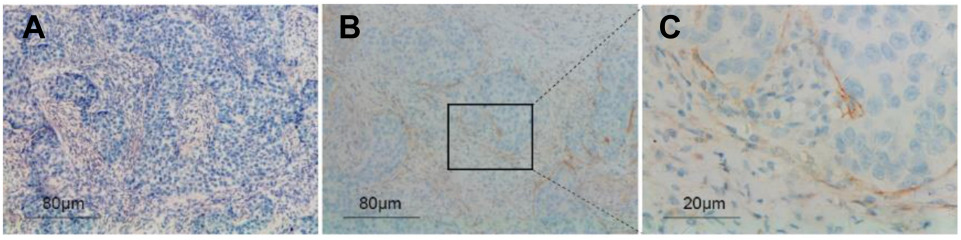

Figure 4 HE staining and IHC staining analysis by D2-40 of breast cancer tissues. Breast cancer sections were stained by HE (**A**), Scale bar, 20μm. Sections were marked with antibody D2-40, Scale bar, 20 μm (**B**) and 80μm (**C**).
Abbreviations: HE, hematoxylin-eosin; IHC, immunohistochemistry.

Table 5 Comparison of D2-40 Staining in Various IDC Patient Groups Classified with Clinical Parameters

Clinicopathological Parameters	D2-40 Staining		P-value
	-	+	
Age (Y)	54.02 ±13.10	58.14 ±15.01	0.529
ki67 (%)***	21.54±8.86	57.14 ±12.54	<0.001
sPDPN (ng/mL)**	21.09±1.89	23.74±2.71	0.003
Clinical stage (cases)			0.022
I+II	16	4	
III+IV	9	11	
Differentiation degree(cases)			0.165
High-moderately+ moderately	14	5	
Moderately low+ low	11	10	
Metastasis status (cases)*			0.042
Non-metastasis	19	4	
Metastasis	8	9	
Estrogen receptor (cases)*			0.010
Negative	8	11	
Positive	17	4	
Progesterone receptor (cases)*			0.037
Negative	12	12	
Positive	13	3	
HER2 protein (cases)			0.810
Negative	11	6	
Positive	14	9	
63 gene (cases)			0.730
Negative	18	10	
Positive	7	5	

Notes: *P<0.05; **P<0.01; ***P<0.001.
Abbreviations: sPDPN, soluble podoplanin; IDC, invasive ductal carcinoma; HER2 protein, human epidermal growth factor receptor 2 protein.

evaluation predictive value for breast cancer patients' general clinical status including clinical stage, degree of differentiation, and metastasis. Plasma sPDPN could be used as a new tumor marker for the diagnosis, clinical stage, degree of differentiation, and metastasis of breast cancer.

Ethics Approval and Consent to Participate

This study conformed to the ethical guidelines of the 2004 Declaration of Helsinki and was approved by the Institutional Ethics Committee at the First Affiliated Hospital of Soochow University, China (2017.11.01), and by the Institutional Ethics Committee at the Luoyang Central Hospital Affiliated to Zhengzhou University, China (2017.11.01). Informed consent was obtained from all healthy control individuals and patients.

The animal studies were also approved by the Animal Use and Ethics Committee of Soochow University (Suzhou, China) (2017.11.06). All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Soochow University (Suzhou, China) institutional animal care and conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Institutional Animal Care and Use Committee (IACUC) guidelines.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no conflicts of interest to declare.

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