Original Article
Aberrant expression of WWOX and its association with cancer stem cell biomarker expression

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Abstract: Background: Nanog and CD133 are biomarkers of cancer stem cells (CSCs) that regulate cancer progression. The WW domain-containing oxidoreductase (WWOX) is a tumor suppressor protein that can inhibit tumor cell proliferation. The purpose of this study was to investigate the expression and clinical significance of Nanog, CD133, and WWOX in infiltrating breast cancer (IBC). Methods: Expressions of Nanog, CD133, and WWOX in 204 IBC specimens and their corresponding control specimens were detected by immunohistochemistry. Patients' clinicopathologic and follow-up data were also collected. Results: The rates of positive expression of Nanog and CD133 were significantly higher in IBC specimens than in control specimens, and their expression was positively associated with tumor size, grade, and tumor stages, lymph node metastasis (LNM), and tumor-node-metastasis (TNM) stage. The rate of positive expression of WWOX was significantly lower in IBC specimens than in control specimens, and its expression was inversely associated with tumor size, grade, and tumor stages, LNM, and TNM stage. Patients whose specimens expressed Nanog, CD133, or HER2 had a reduced overall survival (OS) when compared with patients not expressing these proteins. However, patients whose specimens expressed WWOX, ER, or PR had an increased OS when compared with patients who did not show expression. Multivariate analysis demonstrated that expression of Nanog, CD133, WWOX, ER, and HER2, and the TNM stage were independent prognostic factors for IBC patients. Conclusions: Therefore, Nanog, CD133, and WWOX should be considered as promising prognostic factors and therapeutic targets in IBC.

Keywords: Infiltrating breast cancer, Nanog, CD133, WWOX, cancer stem cells, prognosis

Introduction

In 2018, there were an estimated 2.1 million new breast cancer cases worldwide, which accounted for approximately 1 in 4 cancer cases among women [1]. In China, there was an estimate of 270,000 new cases in 2015 [2]. Many breast cancer patients are diagnosed at advanced stages in China because the cancer does not show obvious symptoms during the early stages.

Tumor heterogeneity has emerged as a hallmark of the malignant state, leading to persistent growth, therapy resistance, and metastasis [3]. This heterogeneity may arise from a subpopulation of tumor cells called cancer stem cells (CSCs). CSCs are capable of self-renewal, multi-directional differentiation, and progression and are naturally resistant to chemo or radiotherapy.

Nanog is a homeodomain-containing transcription factor that plays a critical role in the self-renewal and maintenance of the embryonic stem cell (ESC) network [4, 5]. The human Nanog gene is located at chromosome 12 and contains 4 exons and 3 introns. The protein consists of 305 amino acids and is divided into an N-terminal, homeobox domain, and C-terminal regions. Nanog is overexpressed in many cancers [6], and its knockdown or knockout can reduce cancer malignancy [7, 8]. Moreover, Nanog overexpression has been often associated with poor prognosis in cancers such as head and neck squamous cell carcinoma, lung carcinoma, breast carcinoma, and colorectal carcinoma [5, 9-12].

CD133, also known as prominin-1, is a common CSC biomarker. The protein is a member of the 5 transmembrane glycoprotein family and a cell surface marker of hematopoietic and progenitor cells. It has 97 kDa and contains an extracellular N-terminal domain, five transmembrane segments, two extracellular loops, and an intracellular C-terminal domain. CD133 overexpression often correlates with unfavorable overall survival (OS) and increased recurrence rates [13, 14]. Cells expressing CD133 are resistant to chemo- and radiotherapy [15].

The inactivation of tumor suppressor genes is a hallmark of cancer [16] that leads to reduced OS. The WW domain-containing oxidoreductase (WWOX) was initially considered as a tumor suppressor gene in breast cancer [17]. Most cancer types inactivate WWOX, mostly by hemizygous but also by homozygous deletions [18]. WWOX is a 46 kDa protein that contains 2 N-terminal WW domains and a C-terminal domain [19]. Recent studies demonstrated that WWOX inactivation could cause tumorigenesis and promote tumor progression and angiogenesis [20], whereas increased WWOX expression inhibited tumor metastasis [21].

Nanog, CD133, and WWOX are associated with tumor progression and metastasis in different types of cancer. However, an association between these proteins and IBC has not been reported. This study aimed to assess the hypothesis that these proteins correlate with IBC progression and prognosis.

Materials and methods

Patients and specimens

We recruited 204 patients (median age: 50.8 years; range: 26-77 years) who were diagnosed with IBC from January 2011 to December 2012 by the Department of Pathology of our hospital and collected samples of cancer tissue and the corresponding normal mammary epithelial tissue form all patients. Patients who received any anti-cancer therapy were excluded. This study was approved by the ethical committee of Bengbu Medical University and carried out according to the guidelines issued in the Declaration of Helsinki. All patients provided written informed consents. Patients' data included clinicopathologic characteristics, demography, and follow-up data. OS was calcu-

lated from the patients' surgery date to their death date or to December 2017 (range: 11-82 months; mean OS: 56.0 months). The tumor-node-metastasis (TNM) stage was evaluated using the 8th edition of the guidelines issued by the American Joint Committee on Cancer (AJCC). See **Table 1** for specific characteristics.

Immunohistochemistry

The rabbit anti-human polyclonal antibodies against Nanog and WWOX, and the mouse anti-human monoclonal antibody against CD133 were purchased from Abcam, Co., Ltd (USA). The mouse anti-human monoclonal antibodies against HER2, ER, and PR and other reagents were purchased from Fuzhou Maixin Biotechnology Development Co., Ltd (China). All the tissues were fixed in 10% formalin buffer solution, embedded in paraffin, and cut into 4-μm-thick sections. Immunohistochemistry was carried out following the Elivision™ Plus method and the Kit instructions. A citrate buffer solution was used for antigen retrieval, and a solution of methanol containing 3% H_2O_2 was used to block endogenous peroxidase activity. Nanog, CD133, WWOX, ER, PR, and HER2 primary antibodies were added to all sections and incubated at 4°C overnight. Reagents A and B were added by turns. Finally, sections were developed in DAB substrate and re-dyed with hematoxylin.

Immunohistochemistry evaluation

We randomly selected ten high-power-fields (HPF) for each section to reduce potential intratumoral cell heterogeneity in biomarker expression. The intensity and extent of the immunohistochemistry were calculated as previously described [22]. Intensity: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. Extent: 1, < 11%; 2, 11%-50%; 3, 51%-75%; 4, > 75%. The final scores (range 0-12) were calculated by multiplying the intensity score by the extent score. We followed the 2013 ASCO/CAP guidelines to asses positive expression. HER2 expression in at least 10% and ER and PR expression in at least 1% of IBC cells was considered positive. A score above 2 indicated positive staining. For samples that were positive for Nanog, CD133, WWOX, ER, PR, and HER2, an average of the final score of each section was considered.

Table 1. Patient characteristics

Patient characteristics	Frequency (n)	Percentage (%)
Age (years)		
< 50	134	65.7
≥ 50	70	34.3
Location		
Left	103	50.5
Right	94	46.1
Bilateral	7	3.4
Smoking		
No	170	83.3
Yes	34	16.7
Size (cm)		
≤ 2.0	59	28.9
2.0 < S ≤ 5.0	122	59.8
> 5.0	23	11.3
Differentiation		
G 1	50	24.5
G 2	98	48.0
G 3	56	27.5
Tumor stage		
T1	60	29.4
T2	117	57.4
T3	18	8.8
T4a	9	4.4
Lymph node metastasis		
N0	106	52.0
N1	69	33.8
N2	24	11.8
N3	5	2.5
TNM stagesII		
I	33	16.2
II	126	61.8
III	45	22.1
ER expression		
Negative	94	46.1
Positive	110	53.9
PR expression		
Negative	120	58.8
Positive	84	41.2
HER2 expression		
Negative	145	71.1
Positive	59	28.9

Statistical analysis

We used the Chi-square test to evaluate the expression of Nanog, CD133, and WWOX in the IBC and control tissues as well as the associations between their expression and the IBC clinicopathologic characteristics. The correlation analysis was performed using the Spearman correlation test. The univariate OS analysis was carried out using the Kaplan-Meier method with log-rank tests. The multivariate OS analysis was conducted using the Cox regression model test. $P < 0.05$ was considered significant. We acquired all data for statistical analysis using the SPSS 19.0 software (Chicago, IL).

Results

Expression of Nanog, CD133, and WWOX in IBC, and its association with clinicopathologic characteristics

The positive staining of Nanog was confined to the nuclei and cytoplasm of IBC cells, that of CD133 was confined to the cell membrane and cytoplasm, and that of WWOX was confined to the cell cytoplasm. Overall, 59.3% (121/204) of IBC cells and 2.0% (4/204) of normal breast epithelial cells were positive for Nanog expression (**Figure 1A** and **1B**). The difference between the groups was significant ($P < 0.001$). Nanog expression positively correlated with tumor size, differentiation, and tumor stages, LNM, and TNM stages, but not with patient age, smoking status, and tumor location (**Table 2**).

There was a significant difference ($P < 0.001$) in CD133 expression between the IBC (61.8%, 126/204) and control cells (12.3%, 25/204; **Figure 1C** and **1D**). The expression of CD133 positively correlated with tumor size, differentiation, and tumor stages, LNM, and TNM stages, but not with patient age, smoking status, and tumor location (**Table 2**).

There were significantly fewer IBC cells (49.0%, 100/204) expressing WWOX than control cells (85.8%, 175/204; $P < 0.001$; **Figure 1E** and **1F**). The positive expression of WWOX inversely correlated with tumor size, differentiation, tumor T stageLNM, and TNM stage, but not with patient age, smoking status, and tumor location (**Table 2**).

Univariate and multivariate analyzes

As shown in **Figure 2A**, the Kaplan-Meier survival analysis indicated that the OS time of IBC
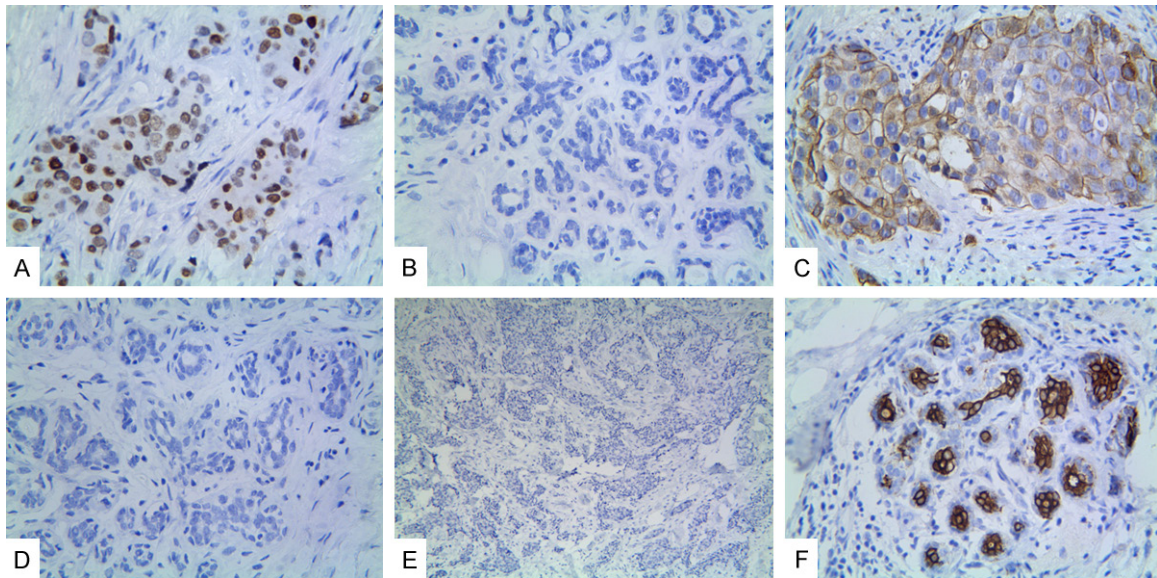
Figure 1. Expression of Nanog, CD133, and WWOX in invasive breast cancer. A. Positive Nanog expression in the cytoplasm and nucleus of cancer cells (×400 magnification). B. Negative Nanog expression in the "normal" breast epithelial cells (×100 magnification). C. Positive CD133 expression in the membrane and cytoplasm of cancer cells (×400 magnification). D. Negative CD133 expression in the "normal" breast epithelial cells (×100 magnification). E. Negative WWOX in the cancer cells (×40 magnification). F. Positive WWOX expression in the cytoplasm of "normal" breast epithelial cells (×100 magnification).

Table 2. Associations between expression of Nanog, CD133, and WWOX and clinicopathologic characteristics of invasive breast carcinoma (IBC)

Variable	Nanog		P	CD133		P	WWOX		P
	-	+		-	+		-	+	
Age (years)			0.876			0.592			0.619
< 50	54	80		53	81		70	64	
≥ 50	29	41		25	45		34	36	
Location			0.477			0.537			0.885
Left	46	57		40	63		52	51	
Right	34	60		34	60		49	45	
Bilateral	3	4		4	3		3	4	
Smoking			0.483			0.122			0.168
No	71	99		69	101		83	87	
Yes	12	22		9	25		21	13	
Size (cm)			< 0.001			0.014			0.002
≤ 2.0	38	21		31	28		23	36	
2.0 < S ≤ 5.0	43	79		42	80		62	60	
> 5.0	2	21		5	18		19	4	
Differentiation			< 0.001			< 0.001			< 0.001
G 1	39	11		34	16		13	37	
G 2	37	61		38	60		52	46	
G 3	7	49		6	50		39	17	
Tumor stage			< 0.001			0.046			0.005
T1	39	21		31	29		22	38	
T2	40	77		41	76		61	56	
T3	2	16		4	14		14	4	
T4a	2	7		2	7		7	2	

LNM			0.001			< 0.001		< 0.001
N0	57	49		55	51		34	72
N1	20	49		21	48		45	24
N2	6	18		1	23		20	4
N3	0	5		1	4		5	0
TNM stages			< 0.001			< 0.001		< 0.001
I	26	7		24	9		7	26
II	48	78		49	77		60	66
III	9	36		5	40		37	8
ER expression			0.074			0.255		0.011
Negative	32	62		32	62		57	37
Positive	51	59		46	64		47	63
PR expression			0.011			0.085		0.026
Negative	40	80		40	80		69	51
Positive	43	41		38	46		35	49
HER2 expression			0.002			0.002		< 0.001
Negative	69	76		65	80		57	88
Positive	14	45		13	46		47	12
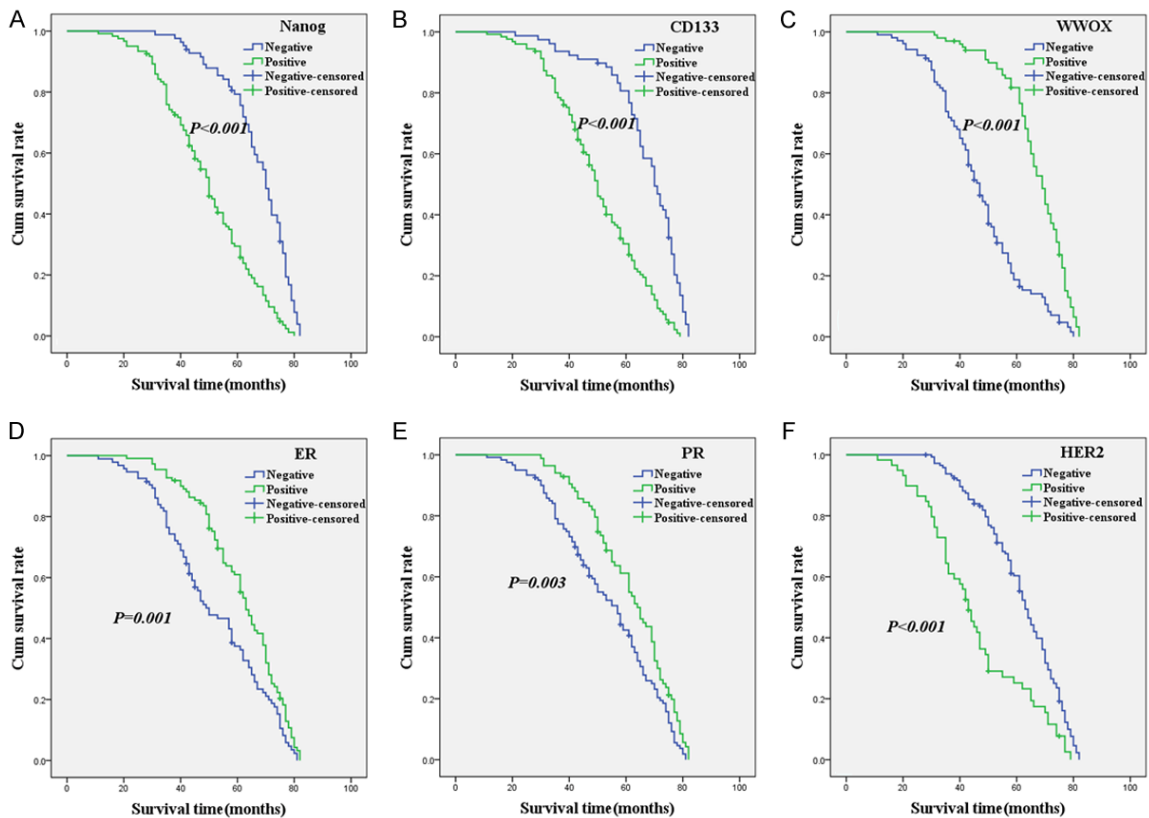
Figure 2. Kaplan-Meier analysis of the survival rate of patients with IBC. The y-axis represents the percentage of patients; the x-axis represents their survival in months. (A) Overall survival of all patients in relation to Nanog (log-rank = 54.217, P < 0.001); (B) Overall survival of all patients in relation to CD133 expression (log-rank = 53.793, P < 0.001); (C) Overall survival of all patients in relation to WWOX expression (log-rank = 63.399, P < 0.001); (D) Overall survival of all patients in relation to ER expression (log-rank = 10.061, P = 0.001); (E) Overall survival of all patients in relation to PR expression (log-rank = 8.661, P = 0.001); Overall survival of all patients in relation to HER2 expression (log-rank = 27.043, P < 0.001). In (A-F) analyses, the green line represents patients with positive expression of biomarkers and the blue line represents the negative expression of biomarkers.

Table 3. Results of multivariate analyses of overall survival (OS) time

Covariate	B	SE	P	HR	95% CI
Nanog	0.822	0.211	< 0.001	2.276	1.505-3.443
CD133	0.598	0.200	0.003	1.818	1.227-2.691
WWOX	-0.598	0.182	0.001	0.550	0.385-0.786
ER	-0.621	0.259	0.016	0.538	0.324-0.892
HER2	0.462	0.189	0.014	1.588	1.096-2.299
TNM stage	0.534	0.268	0.046	1.706	1.008-2.886

Table 4. Correlation among expression of Nanog, CD133, and WWOX in IBC

Variable	Nanog -	Nanog +	r	P	CD133 -	CD133 +	r	P
Nanog							0.416	< 0.001*
-					52	31		
+					26	95		
WWOX			-0.485	< 0.001@			-0.480	< 0.001@
-	18	86			16	88		
+	65	35			62	38		

*: positive association; @: negative association.

patients who expressed Nanog was significantly lower than that of patients who did not express the protein (log-rank = 54.217, P < 0.001). The OS time for CD133-positive patients was significantly lower than that of CD133-negative patients (log-rank = 53.793, P < 0.001; **Figure 2B**), showing similar results to those of Nanog expression. The relationship between WWOX expression and OS time was the opposite to that of Nanog and CD133, with patients who expressed WWOX surviving longer than those who did not express the protein (log-rank = 63.399, P < 0.001; **Figure 2C**). Moreover, OS was significantly associated with ER (log-rank = 10.061, P = 0.001; **Figure 2D**), PR (log-rank = 8.661, P = 0.003; **Figure 2E**), and HER2 (log-rank = 27.043, P < 0.001; **Figure 2F**) expression.

The multivariate analysis suggested that the expression of Nanog, CD133, WWOX, ER, and HER2, as well as the TNM stage, were independent prognostic factors for IBC (**Table 3**).

Association between the expression of Nanog, CD133, WWOX, ER, PR, and HER2 and IBC

The Spearman correlation coefficient analysis showed a negative association between WWOX expression and Nanog (r = -0.485, P < 0.001), CD133 (r = -0.480, P < 0.001), or HER2 (r = -0.366, P < 0.001) expression and a positive relationship between WWOX expression and ER (r = 0.179, P = 0.011) or PR (r = 0.156, P = 0.026) expression. There was also a positive relationship between Nanog expression and CD133 (r = 0.416, P < 0.001) or HER2 (r = 0.220, P = 0.002) expression. The expression of Nanog negatively correlated with PR expression (r = -0.179, P = 0.010) and the expression of CD133 was positively associated with HER2 expression (r = 0.213, P = 0.002) (**Table 4**).

Discussion

IBC is the most common malignant tumor among women. Its heterogeneity makes it a threat to life and health. Therefore, it is urgent to find effective biomarkers that comprehensively predict the biologic behavior of this type of cancer.

Nanog is a biomarker of CSCs, whose overexpression can promote tumor cell proliferation, invasion, and resistance to therapy [23]. Inhibiting Nanog expression can induce apoptosis [23]. In this study, we investigated Nanog expression in IBC and the corresponding normal mammary (control) tissues and found that the IBC tissue expressed higher levels of the protein than control tissue. Moreover, Nanog expression positively correlated with tumor size, tumor stage, differentiation, and LNM and TNM stages. The OS analysis showed that IBC patients expressing Nanog survived for less time than patients who did not express Nanog. Our results suggested that Nanog overexpression is involved in the progression and metastasis of IBC and that Nanog should be considered as a biomarker to predict IBC prognosis.

CD133 is a CSC marker in IBC. Its overexpression can promote tumorigenesis and tumor cell proliferation, invasion, and metastasis [24, 25]. In this study, CD133 overexpression was significantly associated with tumor size, tumor stage, differentiation, and LNM and TNM stages, which agrees with the results of previous stud-

ies [22, 24-26]. The OS analysis indicated that patients whose tumor expressed CD133 survived for less time than those who did not express the protein. These results indicated that CD133 expression plays an important role in IBC progression, invasion, and metastasis and that the protein is associated with poor prognosis, which is consistent with previous studies [22, 24-27].

WWOX acts as a tumor suppressor in human cancer, it suppresses tumorigenesis by inducing apoptosis and inhibiting tumor cell proliferation, invasion, metastasis, and angiogenesis [28, 29]. Our data demonstrated that WWOX expression was inversely associated with tumor size, tumor stage, differentiation, and LNM and TNM stages. Furthermore, the OS analysis indicated that patients expressing WWOX lived longer than those who did not express the protein. These results suggested that the reduction or loss of WWOX expression promoted IBC progression and metastasis and should be associated with a poor prognosis, which is in accordance with other studies [27, 30-32].

The results of our study showed that the expression of Nanog, CD133, WWOX, ER, and HER2 and the TNM stage were independent prognostic factors of OS for patients with IBC. We also demonstrated that WWOX expression was negatively associated with Nanog, CD133, and HER2 expression and positively associated with ER and PR expression and that Nanog expression was positively associated with CD133 expression.

Previous studies have suggested that CSCs promote tumorigenesis [33]. CSCs can induce malignant transformation partly by activating the Wnt/β-catenin signaling pathway [34]. Overexpression of Nanog and CD133 is considered to cause IBC progression and metastasis. WWOX is considered to be associated with hormonal status and breast carcinoma [35]. Normal WWOX can suppress cells' tumorigenicity and decrease cells invasive ability [36]. Aberrant expression of WWOX can cause cell proliferation, mobility, migration, and metastasis. Therefore, we speculate that the overexpression of Nanog and CD133 and the underexpression of WWOX synergistically promote IBC cell proliferation, progression, and metastasis.

Conclusions

The results of this study suggested that the overexpression of Nanog and CD133 and that underexpression of WWOX could affect the initiation, progression, and metastasis of IBC. Therefore, Nanog, CD133, and WWOX should be considered as valuable biomarkers to predict metastasis and prognosis in IBC patients.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

IBC, infiltrating breast carcinoma; WWOX, WW domain-containing oxidoreductase; LNM, lymph node metastasis; TNM, tumor-node-metastasis; OS, overall survival; CSCs, cancer stem cells; ESC, embryonic stem cell; AJCC, American Joint Committee on Cancer; HPF, high-power-field.

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