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Original Article

ORAOV1 and WWOX are metastatic and prognostic biomarker for infiltrating breast cancer

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Abstract: Background and purpose: Oral cancer overexpressed 1 (ORAOV1, a novel oncogene) and WW domain-containing oxidoreductase (WWOX, a suppressor gene of tumor) are both usefully predictive indicators for metastasis and prognosis in various cancers. However, the metastatic and prognostic value of ORAOV1 and WWOX in infiltrating breast cancer (IBC) are still unclear. The aim of this study to analyze relationship between ORAOV1 and WWOX in IBC, and their respective relationships with clinicopathological features and survival in IBC. Methods: The expression of ORAOV1 and WWOX in 232 whole IBC tissues and control specimens were detected by immunohistochemistry staining. Patients clinical and follow-up data were also collected. Results: Level of ORAOV1 was significantly higher and level of WWOX is significantly lower in IBC tissues than those in control tissues. Level of ORAOV1 is positively related with size of tumors, T stage, grade of tumors, lymph node metastasis (LNM), tumor-node-metastasis (TNM) and expression of HER2, and negatively with expression of ER and PR, as well as patients overall survival (OS) time. Level of WWOX is negatively related with size of tumors, T stage, LNM, TNM, and expression of HER2, and positively with expression of ER and PR, as well as patients with OS time. Multivariate logistic regression analysis and COX regression analysis showed that positive expression of ORAOV1 and WWOX, as well as TNM stages were potential to be metastatic or independent and prognostic indicator for patients with IBC. Conclusions: ORAOV1 and WWOX represent promising metastatic and prognostic indicators, as well as potential therapeutic targets for patients with IBC.

Keywords: IBC, ORAOV1, WWOX, metastasis, prognosis

Introduction

Breast cancer is the second frequently diagnosed cancer which was estimated 1.7 million cases and the most cause of cancer death of females which was estimated 520,000 deaths in 2012 [1]. It was estimated that about 270,000 Chinese new cases and 70,000 deaths in 2015 [2]. In China, cancer incidence and mortality have been increasing which make it the most cause of death. Because breast cancer is generally asymptomatic at its early stages, many females diagnosed in China have advanced stage cancer.

Oral cancer overexpressed 1 (ORAOV1), also named as TAOS1, was identified as a potential oncogen and treatment target for oral squamous cell carcinoma (OSCC) [3-5]. ORAOV1 gene is located on chromosome 11q13 which is one of the most frequently amplified lesions in OSCC [3]. Some studies indicated that the overexrpession of ORAOV1 was significantly related with tumor grades, size, lymph node metastasis (LNM), tumor-node-metastasis (TNM) stages and prognosis in some cancers [6, 7]. ORAOV1 should play pivotal roles in the tumorigenesis by regulating cells proliferation and tumor angiogenesis [4]. Some other studies have also showed that ORAOV1 could be associated with cell cycle and apoptosis [4, 5, 7]. Therefore, it is indicated that ORAOV1 should be considered as a novel useful prognosis and treatment target for cancers [4-9].

The WW domain-containing oxidoreductase (WWOX) which is located on human chromosome 16q23 is considered as a tumor suppressor gene [10]. WWOX, which encompasses the chromosomal fragile site FRA16D, encodes a 46 kDa protein and possesses 2 N-terminal WW domains and a C-terminal high homology

Table 1. Patients characteristics

Patients characteristics	Frequency (n)	Percentage (%)
Ages (years)		
≤50	106	45.7
>50	126	54.3
Location		
Left	115	49.6
Right	106	45.7
Bilateral	11	4.7
Type		
Ductual	159	68.5
Lobular	50	21.6
Other	23	9.9
Size (cm)		
≤2.0	66	28.4
2.0<S≤5.0	139	59.9
>5.0	27	11.6
T stage		
T1	64	27.6
T2	133	57.3
T3	24	10.3
T4a	11	4.7
Grade		
G 1	51	22.0
G 2	121	52.2
G 3	60	25.9
Lymph node metastasis		
No	112	48.3
Yes	120	51.7
TNM stage		
I	34	14.7
II	156	67.2
III	30	12.9
IVa	12	5.2
ER expression		
Negative	110	47.4
Positive	122	52.6
PR expression		
Negative	111	47.8
Positive	121	52.2
HER2 expression		
Negative	138	59.5
Positive	94	40.5

domain of short-chain alcohol dehydrogenase/reductase family [11-13]. WWOX can interact with ERK and JNK1 by its N-terminal domain and bind tau and GSK3β by C-terminal domain [13]. Loss of heterozygosity and promoter hypermethylation of WWOX can promote tumorigenesis [14, 15]. Lost expression of WWOX, which can promote tumor progression and angiogenesis [16], is a common event in the most cancer [17]. Furthermore, overexpression of WWOX can suppress the metastasis of human cancer [17].

Overall, evidence of ORAOV1 and WWOX in relation to metastasis and prognosis demonstrated that both biomarkers should be involved in tumor progression and metastasis. However, association between ORAOV1 and WWOX in IBC has not yet been widely studied. In this study, we evaluated the hypothesis that both biomarkers are mutual correlated and related to metastasis and prognosis in IBC.

Materials and methods

Patients and tissue samples

We collected samples from 232 patients (median age: 49.4 years; range: 27-71 years) who were diagnosed for IBC at the Department of Pathology of the First Affiliated Hospital of Bengbu Medical College, from January 2010 to December 2012, along with 232 samples of the corresponding adjacent mammary tissues (removed the same patients, from surrounding mammary tissue at least 5 cm away from the tumor edge). Patients who had received any preoperative anti-cancer therapy were exclude. All tissue samples were obtained with patients writing consent. The study was approved by the ethics committee of Bengbu Medical College and performed in accordance with the guidelines of the Declaration of Helsinki. We collected the completely demographic, clinicopathological and follow-up data (at 6 months intervals by phone, mail, and social application). Overall survival (OS) was counted from patients operation date to her death date or December 2016 (mean OS: 50.7 months, range: 8-82 months). Grades of tumor was according to World Health Organization (WHO) standard. Tumor stage and tumor-node metastasis stage were evaluated according to the 7th edition of American Joint Committee on Cancer (AJCC). Other parameters see **Table 1**.

Immunohistochemistry

Immunohistochemistry was performed by the guideline of Elivision™ Plus detection kit instructions (Lab Vision, USA). All IBC and correspond-

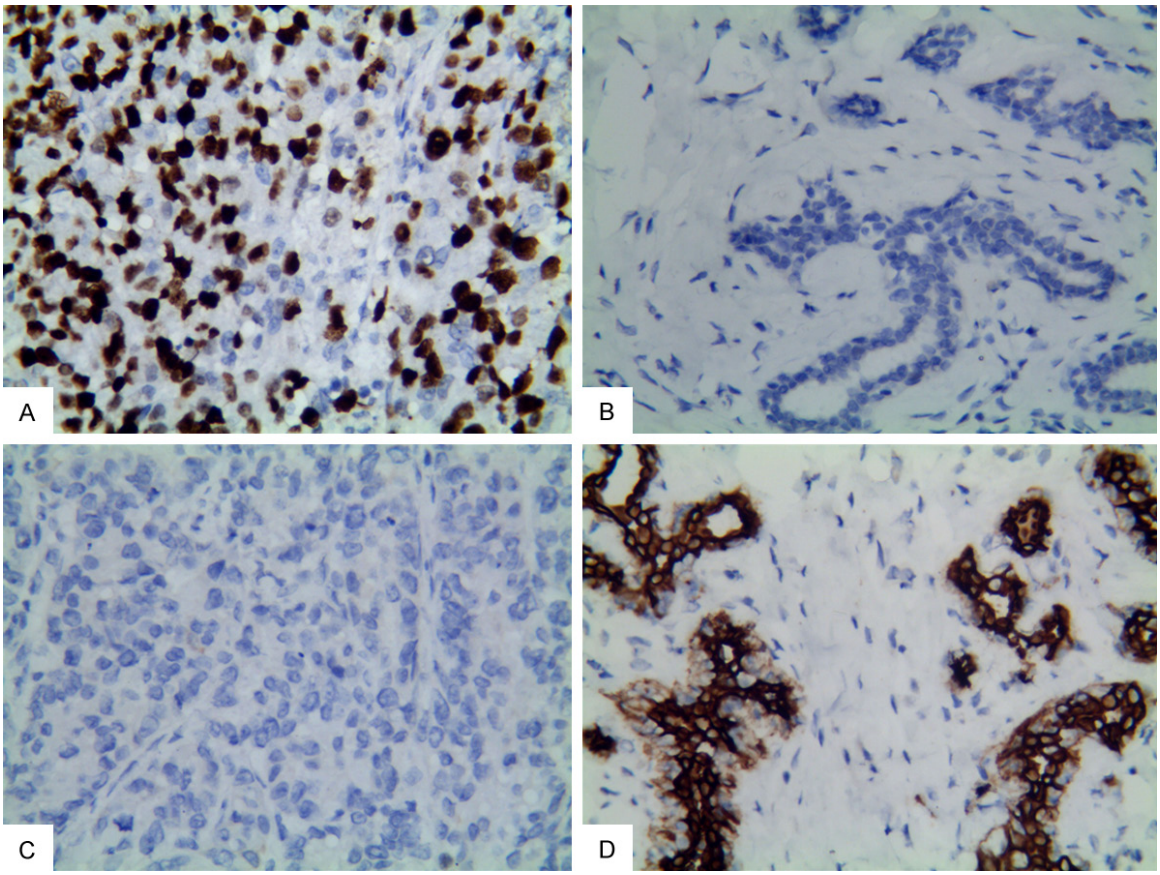

Figure 1. Immunostaining of ORAOV1 or WWOX in IBC or the control tissues (400×). A: Positive staining of ORAOV1 in nuclei of the IBC cells; B: Negative staining of ORAOV1 in the control tissues; C: Negative staining of WWOX in the IBC cells; D: Positive staining of WWOX in the cytoplasm of the IBC cells.

ing normal mammary tissues were fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections (4 μm thick) of both IBC and control tissues were cut and deparaffinized in xylene and dehydrated in a series graded alcohol. Then washed for min with phosphate buffer saline (PBS, pH 7.2). The endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 10 min at room temperature. Consequently placed in citrate buffer (pH 6.0) and heated to 95°C for antigen repair for 30 min. After several washes with PBS, all specimens were blocked by goat serum for 30 min, then incubated with rabbit polyclonal antibody against human ORAOV1 (Abcam, USA), rabbit polyclonal antibody against human WWOX (Abcam, USA) for 1 h at 37°C. All specimens were counterstained with hematoxylin, dehydrated, air-dried, and mounted. Negative controls were prepared by omitting primary antibody from the staining procedure.

Assessment of immunostaining

To assess the immunostaining of ORAOV1 and WWOX, the number of positive immunostaining cancer cells at least 10 representative high-power field from each IBC section. Positive immunostaining was scored according to extent (extent score was grades as follows: 1: <11% positive cells; 2: 10% <positive cells ≤50%; 3: 50% <positive cells ≤75%; 4: positive cells >75%) and intensity (intensity score was calculated as follows: 0: no positive staining; 1: weak positive staining; 2: moderate positive staining; 3: strong positive staining). Then the extent and intensity scores were multiplied to yield final scores that ranged 0-12. Result was considered positive when the final score was >2. Immunostaining results were evaluated by two independent pathological doctors who were blind to patients' demographic, clinicopathological and follow-up data.

Table 2. Correlation between the expression of ORAOV1 and WWOX and clinicopathololgical characteristics in IBC

Variable	ORAOV1		*P* value	WWOX		*P* value
	Negative	Positive		Negative	Positive	
Ages (years)			0.175			0.005
≤50	37	69		66	40	
>50	55	71		55	71	
Location			0.393			0.897
Left	48	67		60	55	
Right	38	68		56	50	
Bilateral	6	5		5	6	
Type			0.433			0.886
Ductual	61	98		83	76	
Lobular	19	31		27	23	
Other	12	11		11	12	
Size (cm)			<0.001			0.028
≤2.0	36	30		26	40	
2.0<S≤5.0	54	85		77	62	
>5.0	2	25		18	9	
T stage			<0.001			0.036
T1	36	28		25	39	
T2	54	79		72	61	
T3	2	22		17	7	
T4a	0	11		7	4	
Grade			<0.001			0.531
G 1	32	19		25	26	
G 2	55	66		61	60	
G 3	5	55		35	25	
Lymph node metastasis			<0.001			<0.001
No	66	46		38	74	
Yes	26	94		83	37	
TNM stage			<0.001			0.002
I	27	7		8	26	
II	63	93		86	70	
III	2	28		20	10	
IVa	0	12		7	5	
ER expression			0.020			<0.001
Negative	35	75		104	6	
Positive	57	65		17	105	
PR expression			0.031			<0.001
Negative	36	75		90	21	
Positive	56	65		31	90	
HER2 expression			<0.001			0.033
Negative	80	58		64	74	
Positive	12	82		57	37	
WWOX expression			<0.001*			
Negative	31	90				
Positive	61	50				

*negative correlation.

Table 3. Univariate analysis and multivariate analysis of factors affecting lymph node metastasis

Variables	Categories	Univariate analysis	Multivariate analysis		
		P	HR	95% CI	P
Ages	≤50/>50	0.008	0.608	0.332-1.114	0.107
Size	≤2.0/2.0<S≤5.0/>5.0	0.050	0.636	0.321-1.260	0.195
Grade	G 1/G 2/G 3	0.032	1.014	0.629-1.636	0.954
TNM	I/II/III/IVa	<0.001	2.135	1.084-4.206	0.028
ER	Negative/Positive	0.004	3.839	0.842-17.505	0.082
ORAOV1	Negative/Positive	<0.001	2.858	1.416-5.767	0.003
WWOX	Negative/Positive	<0.001	0.098	0.021-0.451	0.003

Statistical analysis

Associations between either ORAOV1- or WWOX expression and clinicopathological parameters were compared using Chi-square test or Fisher's exact test. The association between ORAOV1 and WWOX expression was compared using Spearman's coefficient test. The effects of ORAOV1 and WWOX expression on metastasis were determined using logistic regression analysis by univariate and multivariate analyzes. The effects of ORAOV1 and WWOX expression on survival were determined using COX regression analysis for multivariate analysis. The Kaplan-Meier method with log-rank test for OS analysis was used to evaluate the association between the expression of ORAOV1 or WWOX and clinicopathological and survival time for using SPSS 19.0 software for Windows (Chicago, IL). A value of $P<0.05$ was considered as statistically significant.

Results

Expression of ORAOV1 and WWOX in IBC, and their associations to clinicopathological parameters

ORAOV1 staining was mainly located on the cancer cell nuclei; WWOX staining was mainly located on the cancer cell cytoplasm. The positive rate of ORAOV1 expression was 60.3% (140/232) in IBC tissues and 6.5% (15/232) in normal mammary tissues (**Figure 1A** and **1B**). There was a significant difference between two groups (P<0.001). The positive expression rate of ORAOV1 in IBC was positively associated with tumor size, T stages, grades, LNM, and TNM stages, but not patients ages, tumor location, and tumor type (**Table 2**).

The positive expression rate of WWOX was significantly lower in IBC tissues (47.8%, 111/232) than that in the normal mammary tissues (95.3%, 221/232; P<0.001; **Figure 1B** and **1C**). The positive expression rate of WWOX in IBC was negatively associated with tumor size, T stages, LNM, and TNM stages, but not with tumor location, tumor type, and tumor grades (**Table 2**).

Univariate and multivariate analyzes of metastasis

Univariate analysis demonstrated that ages of patients, grades of tumor, TNM stages, and expression of ORAOV1, WWOX, and ER were significantly associated with lymph node metastasis of patient with IBC (P<0.05). In multivariate logistic regression analysis, the expression of ORAOV1 and WWOX, as well as TNM stages was significantly associated with lymph node metastasis of patients with IBC (**Table 3**).

Survival analysis and COX regression analysis

Follow-up data demonstrated that OS was significantly lower in IBC patients with ORAOV1-positive samples (43.2±16.9 months) compared with those with ORAOV1-negative patients (62.2±20.0 months; log-rank=33.103, P< 0.001; **Figure 2A**). Inversely, OS of WWOX –positive patients (58.9±18.1 months) was significantly higher than those of WWOX-negative patients (43.2±20.8 months; log-rank=26.084, P<0.001; **Figure 2B**). Furthermore, OS was significantly associated with clinicopathological parameters, including ER expression (log-rank=6.903, P=0.009, **Figure 2C**), PR expression (log-rank=11.076, P=0.001, **Figure 2D**); HER2 expression (log-rank=10.330, P=0.001, **Figure 2E**); T stages (log-rank=30.889, P< 0.001, **Figure 2F**); tumor size (log-rank=20.044, P<0.001, **Figure 2G**); LNM (log-rank=23.395, P<0.001, **Figure 2H**), and TNM stages (log-rank=47.092, P<0.001, **Figure 2I**). COX regression analysis suggested that ORAOV1-positive and WWOX-positive patients, as well as TNM stages were independent prognostic factors for IBC (**Table 4**).

Association between the expression of ORAOV1 and WWOX in IBC

Spearman association coefficient analysis indicated that there was a negative association

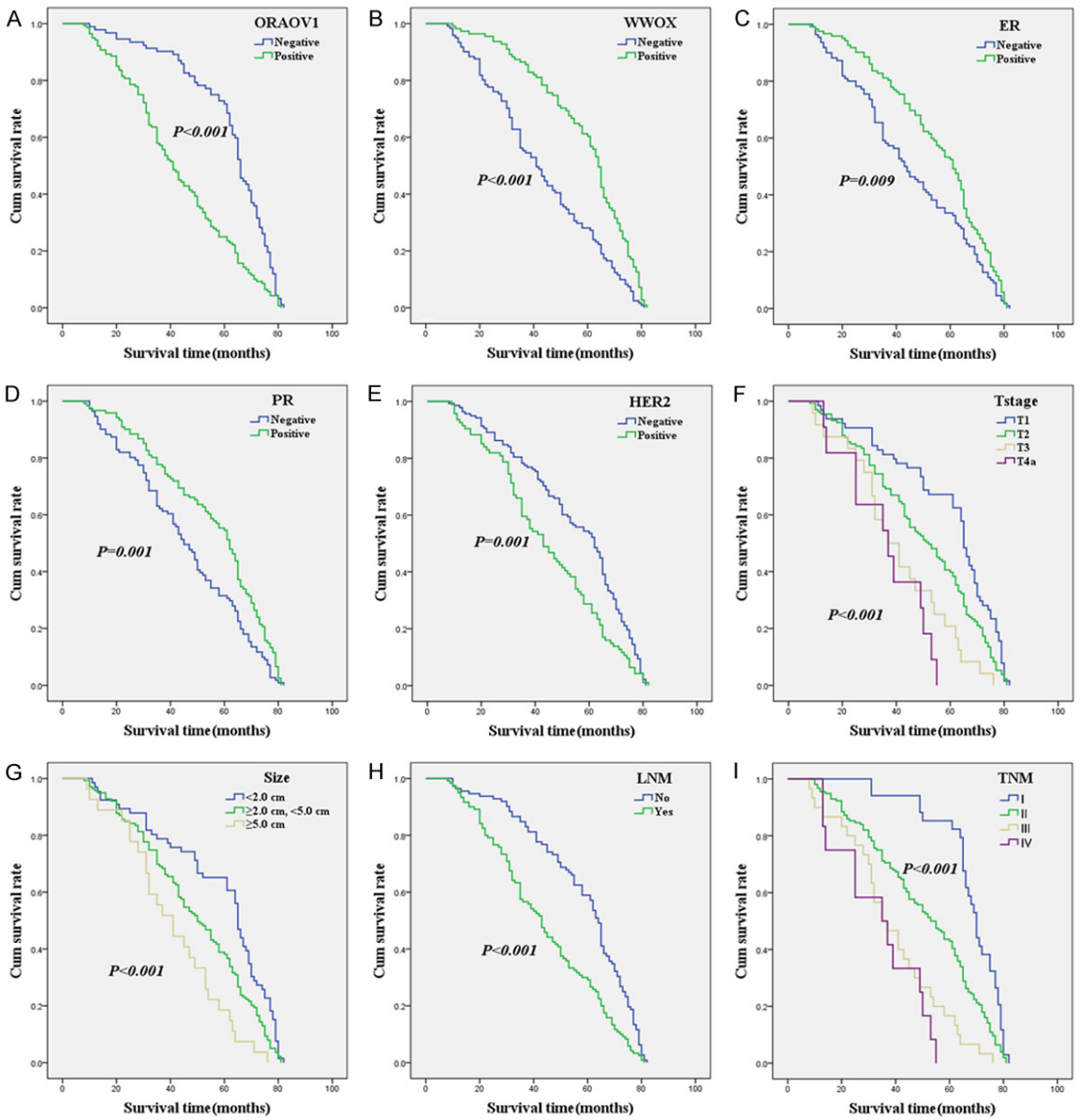

Figure 2. Kaplan-Meier analysis of the survival rate of patients with IBC. The y-axis represents the percentage of patients; the x-axis represents their survival in months. (A) Overall survival of all patients in relation to ORAOV1 (log-rank=33.103, P<0.001); (B) Overall survival of all patients in relation to WWOX expression (log-rank=26.084, P <0.001); (C) Overall survival of all patients in relation to ER expression (log-rank=6.903, P=0.009); (D) Overall survival of all patients in relation to PR expression (log-rank=11.076, P=0.001); (E) Overall survival of all patients in relation to HER2 expression (log-rank=10.330, P=0.001); In (A-E) analyses, the green line represents patients with positive expression of biomarkers and the blue line representing the negative expression of biomarkers. (F) Overall survival of all patients in relation to T stages (log-rank=30.889, P<0.001; the blue line represents patients with T1 stages, the green line represents patients with T2 stages; the brown line represents patients with T3 stages; the purple line represents patients with T4 stages; (G) Overall survival of all patients in relation to tumor size (log-rank=20.044, P<0.001; the blue line represents patients with tumor size <3.0 cm group, the green line represents patients with 3.0 cm≤ tumor size <7.0 cm group, the brown line represents patients with tumor size ≥7.0 cm group); (H) Overall survival of all patients in relation to LNM (log-rank=23.395, P<0.001; the blue line represents patients with no LNM group, the green line represents patients with yes LNM group); (I) Overall survival of all patients in relation to TNM stages (log-rank=47.092, P<0.001; the blue line represents patients with I stages; the green line represents patients with II stages; the brown line represents patients with III stages; the purple line represents patients with IVA stages).

between expression of ORAOV1 and WWOX (r=-0.300, P<0.001). The positive expression of ORAOV1 was negatively associated with ER (r=-0.152, P=0.020) or PR (r=-0.141, P=0.031)

Table 4. Multivariate survival analysis of 232 patients with IBC

Covariate	B	SE	*P* value	Exp (B)	95% CI
ORAOV1	0.422	0.187	0.024	1.526	1.058-2.199
WWOX	-0.777	0.263	0.003	0.460	0.275-0.769
TNM stage	0.598	0.239	0.012	1.818	1.139-2.903

expression, and positively with HER2 expression (r=0.454, P<0.001). The positive expression of WWOX was positively associated with ER (r=0.806, P<0.001) or PR (r=0.555, P< 0.001) expression, and inversely with HER2 expression (r=-0.140, P=0.033; **Table 2**).

Discussion

Infiltrating breast cancer (IBC) is a highly heterogeneous cancer, which can affect the effectiveness of biomarkers assessment. Therefore, it is critical to ensure the metastatic and prognostic value of candidate biomarkers by thoroughly detected. ORAOV1, an oncogene is related to tumorigenesis, cell cycle, and apoptosis, could be considered as a novel metastasis and prognosis in various cancers [3-9]. In this study, we detected ORAOV1 expression in IBC and matched normal mammary tissues from 232 patients and compared it to clinicopathological parameters. We found that overexpression of ORAOV1 was significant higher in IBC tissues than that in the control tissues. Furthermore, ORAOV1 expression was positively associated with tumor size, tumor stage, grades, LNM, and TNM stages. Kaplan-Meier survival analysis indicated that IBC patients with positive ORAOV1 expression had significantly lower OS than did ORAOV-negative patients. These results suggested that ORAOV1 should play an important role in the process of progression of IBC, which are consistent with the previous studies [3-9].

WWOX has been widely considered as a biomarker of tumor suppressor in various cancers [11-17]. WWOX can inhibit tumor cell proliferation, invasion, and angiogenesis and promote apoptosis [11-20], also be considered as a valuable biomarker of metastasis and prognosis of cancers. In this study, we found that the positive expression of WWOX was significantly lower in IBC tissues than that in the control tissues, and its positive rate was negatively correlated with tumor size, tumor stages, LNM, and TNM stages. In addition, Kaplan-Meier survival analysis suggested that WWOX-positive patients had significantly higher OS than did WWOX-negative patients. These findings suggested that down- or lost-expression of WWOX should promote tumor cell invasiveness and progression of IBC, which are similar to other studies [16-21].

Breast cancer incidence and mortality rates have been rising in China, most likely due to lifestyle changes [2]. At the same time, tumorigenesis is closely related to the activation of oncogenes and the inactivation of tumor suppressor genes. ORAOV1 is an oncogene which was originally found in oral squamous cell carcinoma. Overexpression of ORAOV1 can promote tumorigenesis [4], and also promote tumor cell proliferation. Furthermore, overexpression of ORAOV1 can also induce cancer cell invasion through the activation of Cyclins and tumor angiogenesis by regulating VEGF expression [4-7], thus induce metastasis. WWOX gene can inhibit tumorigenicity and lower attachment to fibronectin by integrin [22]. Therefore, down- or loss-regulation of WWOX can promote tumorigenesis, proliferation, progression, invasion, and metastasis. Aberrant expression of WWOX also induces angiogenesis by regulating VEGF expression [16]. In this study, Spearman coefficient analysis demonstrated that there was negative association between the expression of ORAOV1 and WWOX. We speculate that overexpression of ORAOV1 and aberrant (down- or lost-) expression of WWOX synergistically promote development, invasion, and metastasis of IBC. Combined with the results of the univariate and multivariate logistic analyzes, we have reason to believe that the interaction of these two biomarkers is correlated with metastasis in IBC.

From our study, we found that lymph node metastasis is significantly associated with the prognosis (**Figure 2H**). Kaplan-Meier survival analysis indicated that reduction in WWOX, ER, and PR expression and increasing ORAOV1 and HER2 expression are indicators of a poor prognosis in IBC patients (**Figure 2**). Furthermore, large size of tumor, high tumor stages, and high TNM stages are also indicators of a poor prognosis in IBC patients (**Figure 2**). In COX multivariate analysis, ORAOV1 expression and WWOX expression, as well as TNM stages were considered as independent factors for IBC,

which is similar to the previous studies [6, 9, 12, 17, 20] (**Table 4**), suggesting that these two biomarkers play an important role in IBC prognosis.

Although, we used IHC to evaluate the association between these parameters, and the number of samples was relatively small, our findings could still be indentified to reflect the biological behavior of IBC metastasis and prognosis. Moreover, this study also provides a potential target for future molecular studies of IBC.

Conclusion

In summary, our results imply that ORAOV1 and WWOX expression affect the development, invasiveness, and metastasis of IBC; and that combined investigation of ORAOV1 and WWOX are valuable factors of metastasis and prognosis in IBC.

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Disclosure of conflict of interest

None.

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