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Original Article

Sox9 upregulation in breast cancer is correlated with poor prognosis and the $CD44^{+}/CD24^{-/low}$ phenotype

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Abstract: Sox9, an important member of the Sox gene family, plays a key role in regulating sex determination and cartilage formation in mammals. Sox9 is associated with tumorigenesis in several cancers including breast cancer. Here, we investigated the correlation between the expression of Sox9 and breast cancer stem cells (BCSCs) and the clinicopathological characteristics and prognosis of patients with breast cancer. Sox9 expression was detected by immunohistochemistry and double staining was used to detect the expression of CD44 and CD24 in 420 breast cancer specimens. We used statistical methods to evaluate the relationship between Sox9 and clinicopathological features and the impact of BCSCs on overall survival (OS) and disease-free survival (DFS). Our results showed that Sox9 expression was closely associated with estrogen receptor and progesterone receptor levels, Ki-67, p53, lymph node metastasis (LNM), and BCSCs. High expression levels of Sox9 and positive BCSCs status were correlated with poor OS and DFS, and patients with both Sox9 upregulation and BCSCs had the worst prognosis. Multivariate analysis showed that Sox9 expression, LNM, and BCSCs were independent prognostic factors for OS and DFS. Taken together, our results suggest that high Sox9 expression levels are correlated with positive BCSCs status and both are predictors of poor OS and DFS in patients with breast cancer. Sox9 may be used as a biomarker and a therapeutic target for eliminating BCSCs.

Keywords: Sox9, BCSCs, prognosis, breast cancer, biomarker

Introduction

The biggest challenge in cancer research is determining why relapse occurs and why current therapies fail to remove all cancer cells. The cancer stem cell hypothesis is based on the fact that not all cancer cells within a tumor are similar. Moreover, the hypothesis has evolved to explain the complexity, heterogeneity, and recurrence of cancer following surgery and chemotherapy [1]. Breast cancer is not only one of the leading causes of cancer-related death among women, but also a complex and heterogeneous disease [2]. Recent studies have shown that breast canceris driven by a subpopulation of cells that display stem cell properties. These cells mediate metastasis and resistance to radiation and chemotherapy, and contribute to relapse [3]. Experimental evidence supporting the breast cancer stem cell (BCSC) hypothesis was first reported in 2003 by Al-Hajj's group, who demonstrated that human breast cancers contain a cell population with stem cell properties bearing the surface markers $CD44^{+}/CD24^{-}/Lin^{-}$. They found that the $CD44^{+}/CD24^{-/low}$ subpopulation of Lin^{-} cells in tumors was highly tumorigenic. As few as 1000 $CD44^{+}/CD24^{-}$ cells were able to form tumors when xenotransplanted into NOD/SCID mice [4]. Another interesting observation in the same study was the fact that the phenotypic heterogeneity of the initial tumor was conserved in tumors arising from injected $CD44^{+}/CD24^{-/low}$ cells, indicating that BCSCs could generate tumorigenic and non-tumorigenic cells and showed diverse expression patterns of CD44 or CD24. Targeting BCSCs provides new hope for cancer relapse prevention and has become a hot research topic in recent years [5, 6].

Sex determining region Y-box9 (SOX9) belongs to a family of master regulators of sex-determining function in the gonads [7]. Previous studies demonstrated that SOX9 is characterized by a

Table 1. The demographic characteristics of study subjects

Characteristics	No. of cases (%)
Age	
<50	224 (53.3)
≥50	196 (46.7)
Tumor size	
<2 cm	99 (23.6)
≥2 cm	321 (76.4)
LNM	
Negative	211 (50.2)
Positive	209 (49.8)
BCSCs	
Negative	338 (80.5)
Positive	82 (19.5)
ER	
Negative	177 (42.1)
Positive	243 (57.9)
PR	
Negative	137 (32.6)
Positive	283 (67.4)
HER2	
Negative	398 (94.8)
Positive	22 (5.2)
Ki67	
<14%	190 (45.2)
≥14%	230 (54.8)
P53	
Negative	296 (70.5)
Positive	124 (29.5)

high-mobility group box (HMGB) DNA-binding domain, which plays an important role in development, differentiation, and lineage commitment in multiple tissues during embryogenesis [8-10]. In addition, Sox9 is closely related to the occurrence and development of many tumors. Sox9 is amplified in coloncarcinoma, prostate cancer, and gastric carcinoma [11-13]. In addition, Sox9 is associated with breast cancer development and the tumor microenvironment [14-16]. However, the relationship between Sox9 and the $CD44^{+}/CD24^{-/low}$ immunophenotype in breast carcinomas is poorly understood. In the present study, we investigated the expression of the Sox9 protein in breast tumors in relation to various clinicopathological characteristics. The prognostic relevance of $CD44^{+}/CD24^{-/low}$ phenotypes was also examined to understand its relationship with Sox9, clinical outcomes, clinicopathological findings, the expression of human epidermal growth factor receptor-2 (Her-2) and estrogen and progesterone receptors (ER and PR), the proliferation marker Ki67, and p53.

Materials and methods

Patients and clinicopathologic information

Data on 420 cases of invasive ductal carcinoma were retrieved from the Department of Pathology at the Harbin Medical University Cancer Hospital. All patients included in the study had complete case information. All patients had a pathological diagnosis of breast cancer and the corresponding samples were collected before any radiotherapy or chemotherapy. From the medical registers, the following clinical and pathological features were collected: age, tumor size, lymph node metastasis (LNM), recurrence, and disease-free survival (DFS). None of the patients received adjuvant chemotherapy or radiotherapy or anti-estrogen therapy before surgery. The DFS interval was defined as the time from diagnosis to the date of breast-cancer-derived relapse/metastasis, whereas overall survival (OS) was considered as the time from diagnosis to disease-related death. This study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital. All study participants provided written informed consent.

Tissue microarray (TMA) construction and immunohistochemistry

The most representative tumor areas were selected on hematoxylin and eosin (H&E) stained sections and marked on paraffin blocks. For TMA block construction, at least two 3-mm tissue cores from each selected areas of individual paraffin-embedded tissue blocks were punched out using a TMA workstation and deposited into a recipient block. All breast cancer tissue blocks were cut to 4 μm with a microtome and arrayed in triplicate. The result of each tissue core was recorded with the corresponding clinical data. One section was stained with H&E to confirm the presence of the tumor by light microscopy. Non-neoplastic tissue cores were included as controls. Anti-Sox9 antibodies (Abcam, Cambridge, USA), anti-CD44 antibodies (BD Biosciences, NY, USA) and anti-CD24 antibodies (BD Biosciences, NY, USA) were used at a dilution ratio of 1:200.

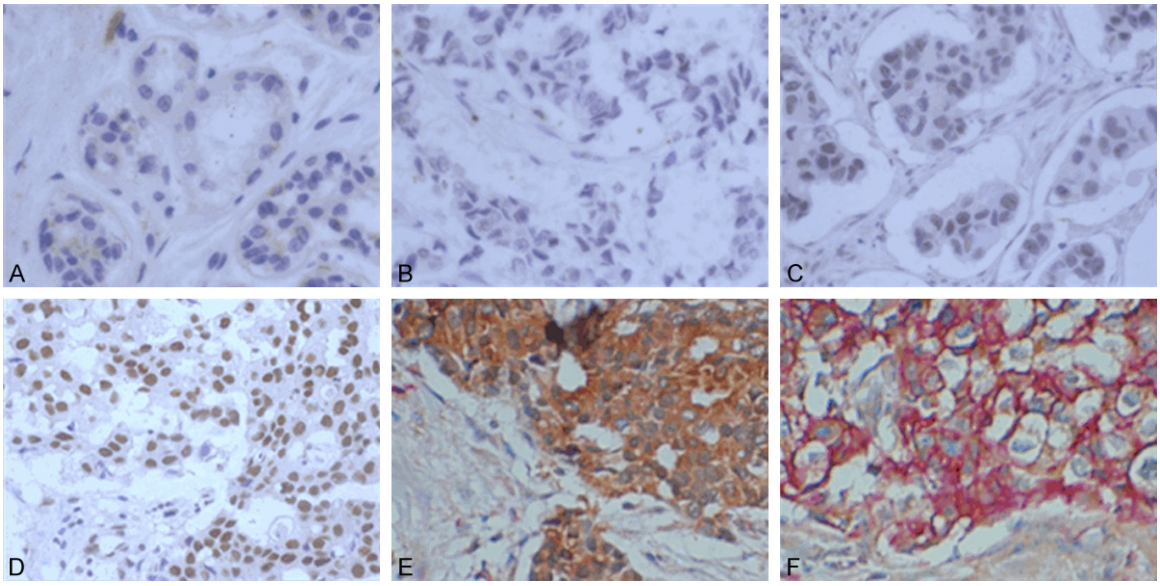

Figure 1. Immunohistochemistry results of Sox9 and BCSC expression in breast tissue microarrays. Representative images shown (magnification × 400) were from 420 paired breast cancer tissues. A: Staining of Sox9 in normal breast tissues. B: Negative staining in breast cancer tissues. C: Low expression levels of Sox9 in breast cancer tissues. D: High expression levels of Sox9 in breast cancer tissues. E: CD24 positive and CD44 negative staining in breast cancer tissues. F: CD44 positive and CD24 negative staining in breast cancer tissues.

Table 2. Correlation between Sox9 expression and clinicopathological features

Characteristics	No.of cases	Negative	Positive	*P*
Age				
<50	224	146	78	0.293
≥50	196	118	78	
Tumor size				
≤2 cm	99	56	43	0.138
>2 cm	321	208	113	
LNM				
Negative	211	145	66	0.012
Positive	209	119	90	
BCSCs				
Negative	338	239	99	<0.001
Positive	82	25	57	
ER				
Negative	177	91	86	<0.001
Positive	243	173	70	
PR				
Negative	137	70	67	0.001
Positive	283	194	89	
HER2				
Negative	398	249	149	0.595
Positive	22	15	7	
Ki67				
<14%	190	135	55	0.002
≥14%	230	129	101	
P53				
Negative	296	201	95	0.001
Positive	124	63	61	

Sox9 single staining was performed according to the manufacturer's instruction. CD44 and CD24 double straining was performed as followed: considering the influence of the space steric hindrance, we started with CD24 using the SAP method and DAB chromogenic detection, followed by immunohistochemical detection of CD44 and AP-red coloration.

A semi-quantitative integral method was used to assess the results of Sox9 staining. The staining intensity and the positive cell percentage points of Sox9 were evaluated in a blinded manner. Grading standards were as follows: positive cell percentage points were scored as 0: nostaining; 1: <10%; 2: 10%-50%; and 3: >5%. The staining intensity was scored as follows: 0: no staining; 1: mild staining; 2: moderate staining; and 3: strong staining. The results were calculated as the product of the staining intensity score and the positive cell percentage points. A product score of 3 was used as the cut-off value. A score >3 was considered positive for Sox9 expression, while ≤3 was considered negative. The status of breast cancer stem cells (BCSCs) was considered positive according to a percentage of $CD44^{+}/CD24^{-/low}$ cells >1%. Results were determined by two pathologists working together.

Table 3. Prognostic factors in the Cox model of proportional hazards for OS

Variable	Univariate			Multivariate		
	RR	95% CI	*P*	RR	95% CI	*P*
Age (years)	0.810	0.509-1.288	0.372			
Tumor size	1.332	0.744-2.386	0.334			
LNM	3.234	1.917-5.456	<0.001	2.194	1.267-3.799	0.005
BCSCs	11.450	7.006-18.714	<0.001	7.072	4.170-11.996	<0.001
ER	0.406	0.253-0.650	<0.001	1.345	0.692-2.613	0.382
PR	0.309	0.194-0.492	<0.001	0.470	0.248-0.889	0.020
HER2	2.131	0.978-4.646	0.057			
Ki67	2.995	1.740-5.153	<0.001	1.491	0.823-2.703	0.188
P53	3.155	1.991-4.999	<0.001	1.790	1.079-2.970	0.024
Sox9	5.126	3.063-8.578	<0.001	2.476	1.404-4.367	0.002

Table 4. Prognostic factors in the Cox model of proportional hazards for DFS

Variable	Univariate			Multivariate		
	RR	95% CI	*P*	RR	95% CI	*P*
Age (years)	1.074	0.729-1.580	0.719			
Tumor size	1.319	0.809-2.149	0.267			
LNM	3.590	2.300-5.602	<0.001	2.908	1.840-4.597	<0.001
BCSCs	5.961	4.037-8.803	<0.001	3.877	2.556-5.881	<0.001
ER	0.508	0.344-0.750	0.001	1.530	0.888-2.638	0.126
PR	0.371	0.252-0.546	<0.001	0.436	0.259-0.734	0.002
HER2	1.673	0.813-3.442	0.162			
Ki67	2.489	1.612-3.842	<0.001	1.748	1.100-2.779	0.018
P53	2.123	1.438-3.134	<0.001	1.361	0.896-2.067	0.149
Sox9	3.802	2.534-5.705	<0.001	2.420	1.555-3.766	<0.001

Statistical analysis

Statistical software (SPSS version 17.0) was used to analyze the results. The relationship between Sox9, BCSCs, and clinicopathological features was assessed by the Chi-square test. OS and DFS were estimated by the Kaplan-Meier method. Factors affecting OS and DFS were assessed by Cox univariate and multivariate regression analyses. A *P* value<0.05 was considered statistically significant.

Results

Patient characteristics

A total of 420 breast cancer specimens were used to analyze the potential role of Sox9 and BCSCs. Patients ranged in age from 28 to 76 years. A total of 99 (23.6%) patients had tumors smaller than 2 cm, and 321 (76.4%) patients had tumors larger than 2 cm. Lymph node metastases were present in 209 patients (49.8%) and absent in 211 (50.2%). A total of 82 (19.5%) patients were positive for BCSCs and 338 (80.5%) patients were negative. The demographic characteristics of the study subjects are shown in **Table 1**.

Relationship between Sox9 and clinicopathological features including BCSCs

The status of Sox9 protein and BCSCs was detected by immunohistochemistry (**Figure 1**). The results of relationship between Sox9 and clinicopathological features including BCSCs are shown in **Table 2**. The expression levels of Sox9 were correlated with LNM (*P*=0.012), BCSCs (*P*<0.001), ER (*P*<0.001), PR (*P*=0.001), Ki67 expression (*P*<0.001), and p53 (*P*=0.001).

Univariate and multivariate analyses

The effects of all the factors on prognosis were evaluated by univariate and multivariate survival analyses. Univariate analysis showed that

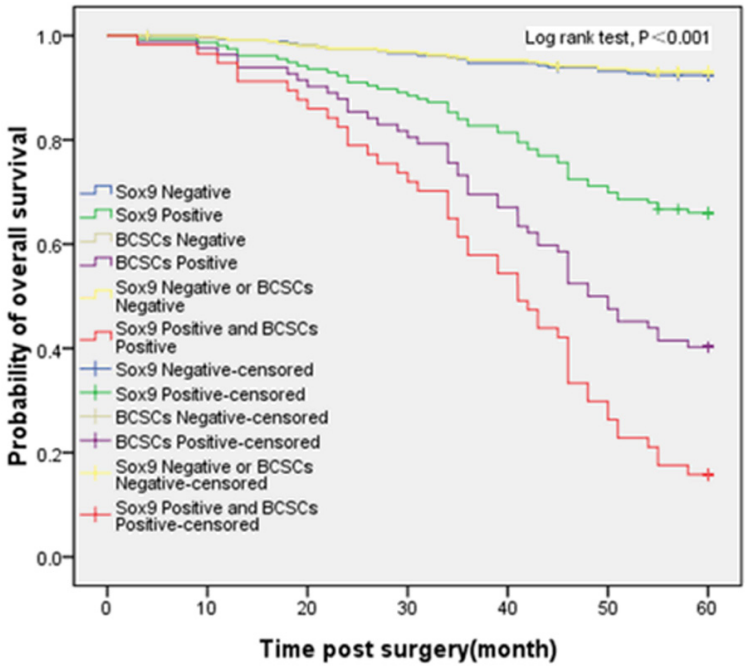

Figure 2. Kaplan-Meier analysis of OS based on Sox9 and BCSC expression levels in 420 breast cancer patients.

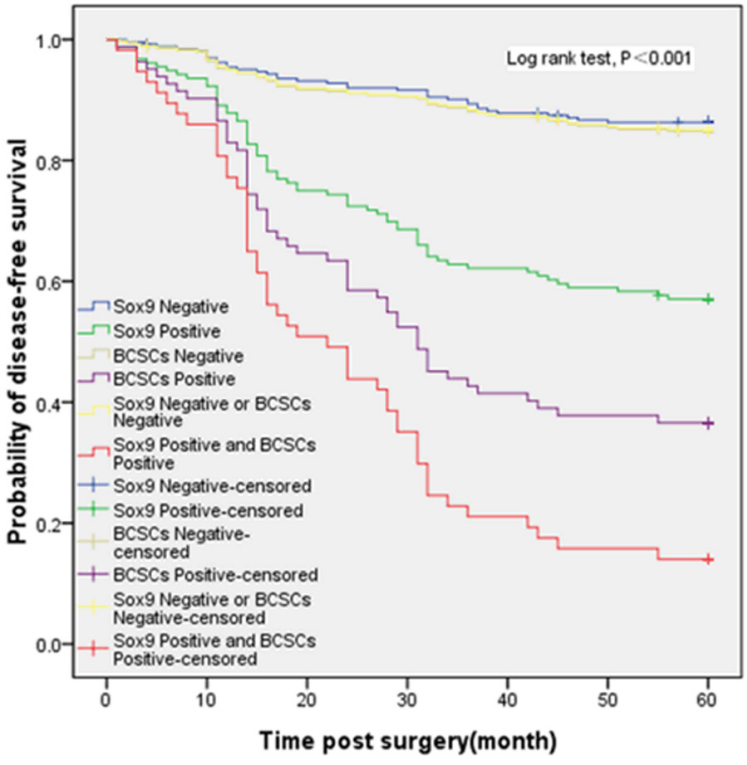

Figure 3. Kaplan-Meier analysis of DFS based on Sox9 and BCSC expression levels in 420 breast cancer patients.

Sox9, BCSCs, lymph node metastasis, ER, PR, Ki-67 expression and p53 were significant prognostic indicators for OS and DFS. Further results of multivariate analysis indicated that Sox9 and BCSCs were independent prognostic factors (**Tables 3** and **4**).

Kaplan-Meier survival analysis

Data showed that patients with high expression levels of Sox9 had poor OS and DFS. Moreover, patients with positive BCSC expression also had poor OS and DFS; patients with both upregulated Sox9 and positive BCSCs had the worst OS and DFS (**Figures 2** and **3**). The results indicated the presence of synergy between Sox9 and BCSCs, and that Sox9 may play an important role in the regulation of stemness in BCSCs.

Discussion

To study the relationship between Sox9 and BCSCs and their influence on the prognosis of breast cancer, we analyzed data of 420 cases of breast cancer tissue microarray. The results indicated that Sox9 expression was associated with LNM, ER, PR, Ki67, p53 and BCSCs in breast cancer tissues. The expression levels of Sox9 were closely associated with the recurrence of breast cancer. High expression levels of Sox9 and positive BCSC status correlated with poor OS and DFS in these patients; moreover, patients with both high expression of Sox9 and BCSCs had the worst prognosis. To assess the importance of each prognostic, univariate and multivariate analyses were performed. The results showed that Sox9 expression, LNM, and BCSCs were independent predictors of survival in these patients. ER, PR, Ki67, p53 and LNM are associated with a malignant state in breast cancer [17-19]. These factors might be

associated with Sox9 and together caused shorter OS and DFS. Combined assessment of Sox9 expression and clinicopathological features was a predictor of prognosis in the present study and should be considered to predict the prognosis of patients with breast cancer.

Sox9 expression was correlated with the presence of BCSCs. Sox9 and Slug play critical roles in BCSCs and in promoting breast cancer cell metastasis, while knockdown of Sox9 inhibits metastasis in the lungs of mice [20]. Sox9 may regulate stem cells and in turn affect the prognosis of patients with breast cancer. Our study confirmed the relationship between Sox9 and BCSCs at an organizational level and their influence on the prognosis of breast cancer. Whether Sox9 could be a new therapeutic target in breast cancer needs to be further analyzed.

The present study had several limitations. Patients enrolled in our study were all Chinese, and samples were obtained from a single center. In addition, Sox9 is only one of several genes with an effect on BCSCs [21]; Therefore, the potential connections with other genes needs to be further analyzed.

In conclusion, our data indicated that Sox9 is associated with LNM, ER, PR, Ki67, p53 and BCSCs. High Sox9 expression was associated with BCSCs and they both predicted poor OS and DFS in breast cancer patients. Sox9 could be a prognostic biomarker and a therapeutic target for eliminating BCSCs.

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Disclosure of conflict of interest

None.

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