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Original Article

Elevated ASCL2 expression in breast cancer is associated with the poor prognosis of patients

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Abstract: Achaete scute-like 2 (ASCL2) is a member of the basic helix-loop-helix (bHLH) transcription factors, and is expressed mainly in intestinal stem cells under normal conditions. Recently, aberrantly elevated ASCL2 was detected in cancer tissues, but the clinical relevance of ASCL2 in breast cancers remains to be decided. In this study, we evaluated the expression of ASCL2 and its relationship to cancer progression in specimens from 191 cases of breast cancer patients with follow-up information. The results indicated that ASCL2 was highly expressed in cancer cells while it was undetectable in normal epithelial cells. Moreover, the expression of ASCL2 was positively correlated with breast tumor size, lymphatic metastasis and the active growth of tumor cells as shown by increased expression of Ki67. Kaplan-Meier analysis revealed that patients with higher levels of ASCL2 suffered higher tumor recurrent rate. Multivariable Cox-regression analysis showed that elevated expression of ASCL2 was an independent and unfavorable indicator of tumor relapse in breast cancer patients. Altogether, our study suggests that ASCL2 defines a subgroup of highly progressive breast cancer and serves as a marker to evaluate the risk of cancer relapse.

Keywords: ASCL2, breast cancer, proliferation, prognosis

Introduction

Breast cancer is the most common malignant tumor in females with 1,677,000 newly diagnosed cases and over 450,000 deaths annually [1]. Despite the development of novel treatment techniques, metastasis and relapse remain the main factors of therapeutic failure. This suggests the necessity to identify biomarkers for early discovery of breast cancer recurrence.

Achaete scute-like 2 (ASCL2), a basic helix-loop-helix (bHLH) transcription factor, is involved in the development and progression of several human cancers. ASCL2 forms dimer with HLH domain, then utilizes the basic domain of itself to combine with the E-box ('CANNTG') element for regulation of the transcription of downstream genes [2]. ASCL2 is mainly expressed in placental [3], large intestine, and intestine crypt base column cells (CBCs) with Lgr5 expression [4]. ASCL2 controls the differentiation of trophocyte lineage of normal human placenta. In mice, ASCL2 was found as an important transcriptional factor to maintain the stemness of CBCs [5]. High expression of ASCL2 in tumor tissues promotes the proliferation and invasion of tumor cells. Its expression is correlated with poorer prognosis of patients [6, 7]. ASCL2 is regulated by WNT signaling pathway which inhibits the expression of miR-200s in colorectal cancer [6, 8]. ASCL2 also cooperates with the chemokine receptor 4 (CXCR4) to promote the invasion and metastasis of cancer cells. However, the clinical relevance of ASCL2 in breast cancer is not documented. In the present study, we investigated the expression of ASCL2 in breast cancer and its relationship to the clinicopathological characteristics of patients.

Materials and methods

Patients and tissue specimens

The IHC cohort consisted of 191 patients diagnosed with breast cancer at Southwest Hospital

Table 1. Clinicopathological characteristics of breast cancer patients

Parameters	Cases
Age (years)	
<50	117
≥50	74
Tumor size (cm^3)	
<5	146
≥5	45
Subtype	
Lumina A	65
Lumina B	29
Basal like	76
Her2-enriched	21
Tumor differentiation	
Well	35
Middle	95
Poor	61
LNM	
Negative	81
Positive	110
Clinical stage	
I-II	157
III-IV	34
ER	
Negative	94
Positive	97
PR	
Negative	94
Positive	97
HER2	
Negative	163
Positive	28
Ki67	
<14%	129
≥14%	62

Abbreviation: LNM, Lymph node metastases.

of Third Military Medical University between 2005 and 2007. Patients were enrolled in this retrospective study based on the following criteria: diagnosis of invasive ductal carcinoma with histopathological assessment, no prior anticancer treatment (radiotherapy or chemotherapy), and the availability of complete clinicopathological and follow-up data. Tumor tissue specimens were grouped according to the seventh edition of WHO tumor-node-metastasis (TNM) classification. The clinical data and specimens were collected in accordance with the ethics committee of Southwest Hospital.

Immunohistochemistry staining

The tumor slides were deparaffinized, rehydrated, blocked and antigen retrieved with EDTA (pH = 8.0) using the PT-link system (DAKO, Denmark). The slides then incubated with ASCL2 antibody (dilution 1:200; Millipore, Billerica, Massachusetts, USA) at 4°C overnight. Finally, the sections were stained and visualized using the Real EnVision system (DAKO, Denmark).

ASCL2 scoring

The IHC staining scores were evaluated by two pathologists independently. Each slide was scored for the staining intensity (1-weak; 2-moderate; 3-strong) and the percentage of positive staining cells (0, <10%; 1, 11-30%; 2, 31-50%; 3, 51-80%; 4, >80%). The final IHC scores were semi-quantitatively calculated by multiplying the two factors. IHC score of 6 was chosen as the optimal cutoff value, which determined by relative risk analysis with X-tile software. Thus, patients with IHC scores <6 were defined as $ASCL2^{low}$ group and those with IHC scores above than 6 were defined as $ASCL2^{high}$ group.

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software (SPSS Inc). The Pearson χ^2 test was used to determine the correlation between ASCL2 expression and the clinicopathological features. Kaplan-Meier analysis with log-rank test was performed to illustrate the difference in the overall survival rates and disease-free survival rates of patients. Univariate and multivariate Cox-regression analysis with backward selection was applied to determine the hazard ratio of relevant survival factors. $P<0.05$ was considered statistically significant.

Results

Expression of ASCL2 in breast cancer tissues

This study included 191 breast cancer patients with the age range from 23 to 79 years old. Among the subjects, 110 (57.6%) cases showed lymph node metastasis and the other 81 (42.4%) did not. The cancers of 157 (82.2%) patients were diagnosed as stage I/II, and 34 (17.8%) were stage III/IV (**Table 1**). According to

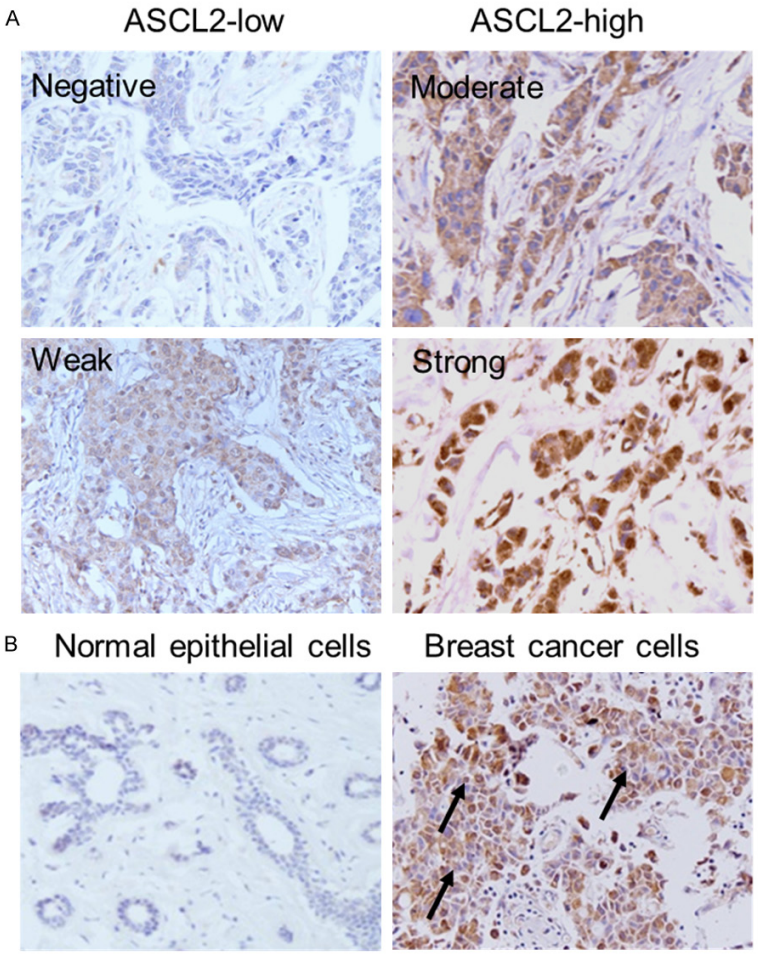

Figure 1. ASCL2 expression in breast tissues. A. IHC staining of ASCL2 in breast cancer patients with low or high expression levels. B. Negative immunoreactivity of ASCL2 in normal epithelial cells and positive staining in the cytoplasm of breast cancer cells. Representative cancer cells positive for ASCL2 in the cytoplasm are showed with black arrows. Magnification × 400.

the molecular profiles, the samples could be classified into Lumina A (65, 34%), Lumina B (29, 15.2%), Basal like (76, 39.8%), and Her2-enriched (21, 11%) breast cancer. The expression level of ASCL2 in breast cancer tissue was divided into high and low groups (**Figure 1A**). ASCL2 was mainly observed in the cytoplasm of cancer cells, while normal tissues adjacent to the cancer cells were negative (**Figure 1B**).

The correlation between ASCL2 expression and clinicopathological features

Next, we evaluated the relationship between ASCL2 expression and clinicopathological features (**Table 2**). According to IHC scores, 79 (41.4%) specimens were defined as $ASCL2^{high}$, and 112 (58.6%) specimens as $ASCL2^{low}$. The ASCL2 levels were positively correlated with tumor size (P = 0.027) (**Figure 2A**), lymph node metastasis (LNM) (P = 0.026) (**Figure 2B**), and cancer cell proliferation status as shown by increased Ki67 (P = 0.016) (**Figure 2C**). No significant correlation was observed between ASCL2 expression with age, clinical stage, molecular subtypes and differentiation (P>0.05) (**Figure 2D**, **2E**; **Table 2**).

The prognostic value of ASCL2 in breast cancer

Kaplan-Meier analysis and Cox-regression analysis showed that the survival of patients with $ASCL2^{high}$ cancers was significantly poorer than those with $ASCL2^{low}$ cancers (**Figure 3A** and **3B**). In addition, patients with $ASCL2^{low}$ showed decreased cancer recurrence than those with $ASCL2^{high}$ (26.67% vs 44.26%). Cox-regression analysis further showed that ASCL2 served as an unfavorable prognostic indicator (HR = 1.865, P = 0.003) to predict the cancer recurrence (HR = 1.278, P = 0.055) (**Tables 3** and **4**).

Discussion

Metastasis and relapse are two main reasons restricting the improvement for survival of breast cancer patients. The abnormal expressions of oncogenes and tumor-suppressor genes are involved in the tumorigenesis, progression and eventual prognosis. ASCL2 was found as an oncogene in human cancers, such as colorectal cancer [9] and non-small cell lung cancer [10]. ASCL2 exhibited a high expression in cancers of the colon, stomach, lung and ovary. Selective blockade of ASCL2 reduced cellular proliferation, migration and tumor

Table 2. The correlation between ASCL2 expression and clinicopathological characteristics in breast cancer patients

Parameters	Cases	ASCL2 expression		*P* value
		Low	High	
Age (years)				0.277
<50	117	65	52	
≥50	74	47	27	
Tumor size (cm^3)				0.027
<5	146	92	54	
≥5	45	20	25	
Subtype				0.687
Lumina A	65	42	23	
Lumina B	29	16	13	
Basal like	76	42	34	
Her2-enriched	21	12	9	
Tumor differentiation				0.191
Well	35	22	13	
Middle	95	60	35	
Poor	61	30	31	
LNM				0.026
Negative	81	55	26	
Positive	110	57	53	
Clinical stage				0.259
I-II	157	95	62	
III-IV	34	17	17	
ER				0.397
Negative	97	54	43	
Positive	94	58	36	
PR				0.742
Negative	97	58	39	
Positive	94	54	40	
HER2				0.066
Negative	163	100	63	
Positive	28	12	16	
Ki67				0.016
<14%	129	68	61	
≥14%	62	44	18	

Abbreviation: ASCL2, Achaete scute-like 2; LNM, Lymph node metastases.

growth in xenograft colon cancer experiments [5, 11]. Of note, lower ASCL2 gene expression levels were found in certain cancer subtypes, such as brain and gastric cancer, and melanoma [12]. In this study, we revealed that ASCL2 exhibited significant upregulation compared with normal tissues in a group of human breast cancer with worse clinical features and poorer overall and disease-free survival of patients.

Previous study showed that ASCL2 may form a complex with the Wnt pathway signal transducer β-catenin in order to synergistically activate the expression of downstream target genes [4, 8]. Moreover, ASCL2 modulates the plasticity between epithelial and mesenchymal characteristics in colon cancer [8] and promotes the invasion and metastasis of gastric cancer cells, and induces the resistance to 5-fluorouracil [13]. Interfering with the expression of ASCL2 inhibits the proliferation and invasion capabilities of colorectal cells with the downregulation of stemness-related genes Oct4, Bmi1, Lgr5, Sox and c-Myc [5]. Mechanistically, ASCL2 is a downstream target of PI3K-AKT signaling pathway, and binds to the promoter region of the chemokine receptor 4 (CXCR4) [14]. The activation of ASCL2-CXCR4 axis resulted in the increase of cancer cell metastasis and poorer outcome of colorectal cancer patients. Although the clinical significance of ASCL2 has not yet been reported, the elevated expression of CXCR4 is known to be correlated with increased lymph node metastasis and poor overall patient survival in breast cancer [15, 16]. Therefore, our data suggested that ASCL2-CXCR4 axis might also play a pivotal role in the progression of breast cancer.

According to the American Society of Clinical Oncology (ASCO), biomarkers for breast cancer mainly include the carcinoembryonic antigen (CEA), caner antigen 125 (CA125), and hormone receptors. The estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) have been widely used in diagnosis, prognosis evaluation, therapy decision and post-therapy surveillance [17-19]. However, the traditional biomarkers have been found to be insufficient in the examine sensitivity and specificity [20]. Combined detection arise as the promising measure to improve the accuracy.

Recently, CXCR4, STAT3, SOX9 and other biomarkers have gained widespread recognition, and our study revealed that ASCL2 was more highly expressed in aggressive breast cancers. In addition, despite the molecular subtypes of breast cancer are largely distinct with each other in patient survival and treatment procedure, we did not found correlations between subtypes of breast cancers, which implied that the ASCL2 could play more fundamental roles

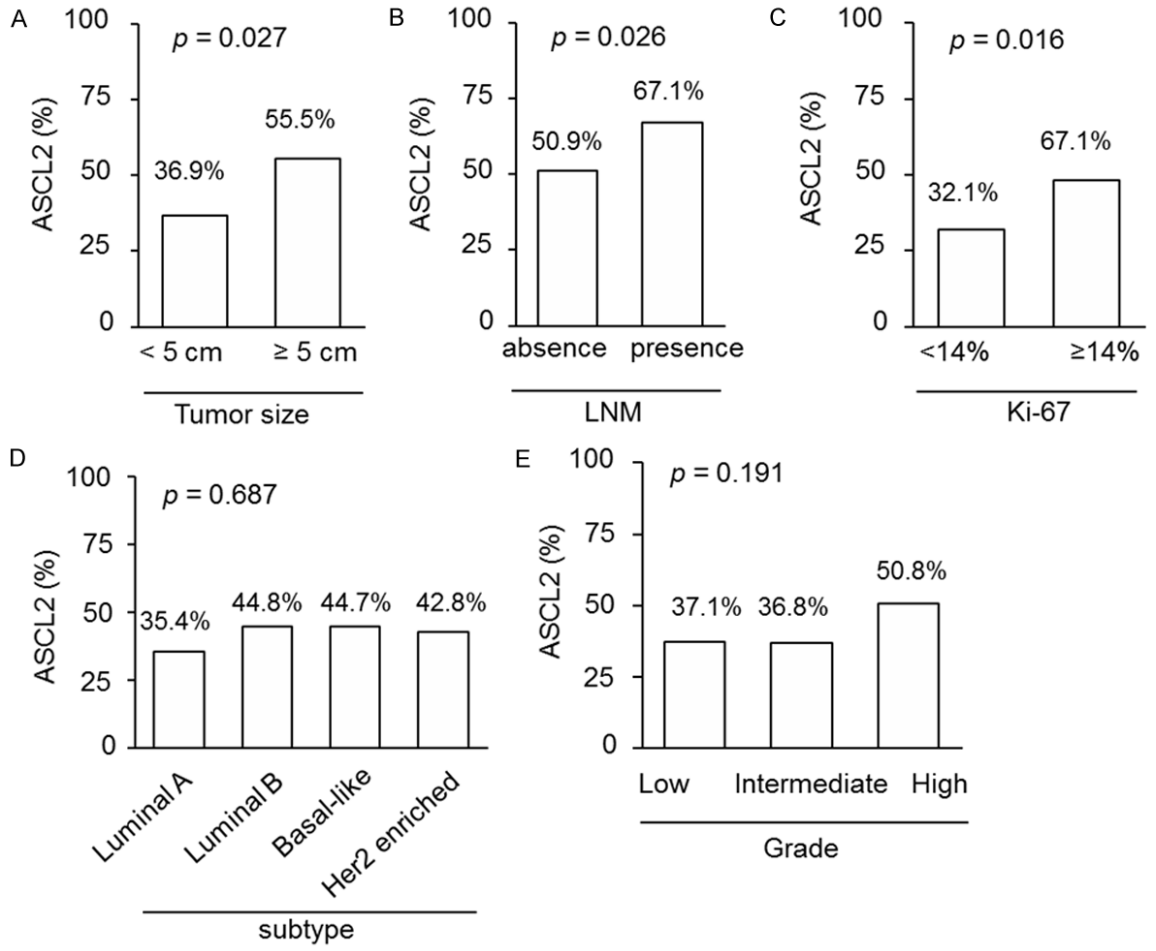

Figure 2. The correlation between ASCL2 expression and clinical pathological characters of breast cancer patients. (A-C) Patients were designated as having high or low ASCL2 expression levels. Significant differences in lymph node metastasis (A), tumor size (B) and proliferation index indicated by Ki67 (C). (D, E) No significant correlation was observed between ASCL2 expression with subtype and differentiation (P>0.05).

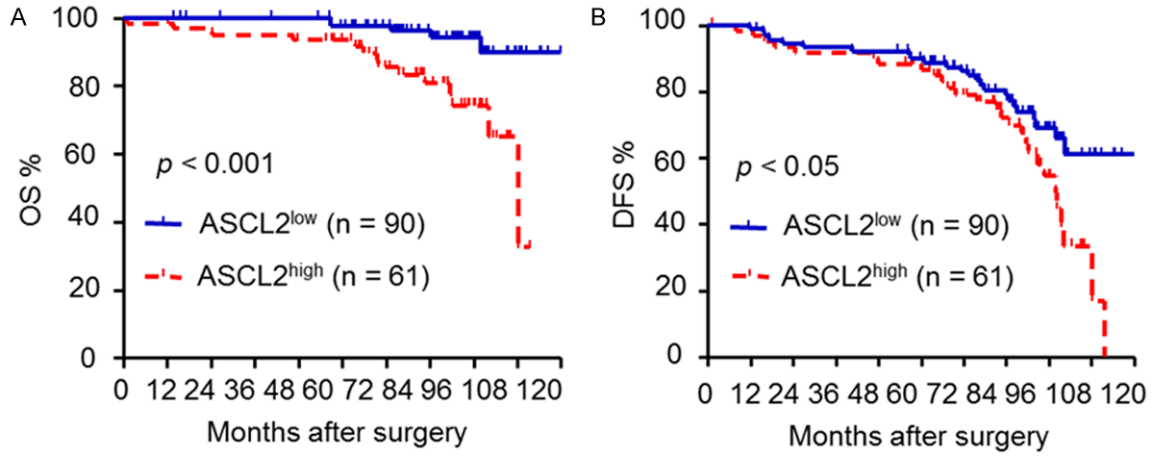

Figure 3. Clinical significance of ASCL2 in breast cancer cells. (A, B) Differences in overall survival (OS) (A) and disease-free survival (DFS) (B) between high and low ASCL2 in tumors of patients.

Table 3. Univariate/Multivariate analyses of the relationship between ASCL2 expression and overall survival of breast cancer patients

Factors	Univariate			Multivariate		
	HR	95% CI	*P* value	HR	95% CI	*P* value
ASCL2 (High vs Low)	1.896	1.230-2.923	0.004	1.865	1.229-2.828	0.003
Age (≥50 vs <50)	0.973	0.389-2.434	0.953			
Tumor Size (≥2 vs <2)	1.953	0.935-4.082	0.075			
LNM (Positive vs Negative)	1.713	0.640-4.587	0.284			
Location (Right vs Left)	1.270	0.515-3.130	0.604			
PR (Positive vs Negative)	0.378	0.148-0.965	0.042	0.374	0.146-0.956	0.040
Her2 (Positive vs Negative)	0.716	0.165-3.103	0.655			

Abbreviation: ASCL2, Achaete scute-like 2; HR, Hazard ratios; CI, Confidence interval; LNM, Lymph node metastases.

Table 4. Univariate/Multivariate analyses of the relationship between ASCL2 expression and disease-free survival of breast cancer patients

Factors	Univariate			Multivariate		
	HR	95% CI	*P* value	HR	95% CI	*P* value
ASCL2 (High vs Low)	1.289	1.00-1.661	0.050	1.278	0.995-1.640	0.055
Age (≥50 vs <50)	0.627	0.346-1.136	0.124	0.444	0.233-0.847	0.014
Tumor Size (≥2 vs <2)	1.677	1.077-2.611	0.022			
LNM (Positive vs Negative)	2.480	1.313-4.681	0.005			
Location (Right vs Left)	1.276	0.736-2.212	0.386			
PR (Positive vs Negative)	0.427	0.241-0.756	0.004	0.311	0.168-0.575	0.000
Her2 (Positive vs Negative)	1.652	0.826-3.304	0.156			

Abbreviation: ASCL2, Achaete scute-like 2; HR, Hazard ratios; CI, Confidence interval; LNM, Lymph node metastases.

during the tumorigenesis and progression of breast cancer. Altogether, ASCL2 might serve as a biomarker to evaluate the prognosis of breast cancer patients.

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Disclosure of conflict of interest

None.

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