

High cytoplasmic expression of SALL4 predicts a malignant phenotype and poor prognosis of breast invasive ductal carcinoma

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Sal-like 4 (SALL4) is significant for maintaining self-renewal and pluripotency in embryonic stem cells, cancer cells and perhaps even cancer stem cells. The expression of SALL4 has been recorded in various kinds of cancers and is deemed to have a clinical value for diagnosis. However, little information on SALL4 expression has been illustrated in breast cancer. In this study, the expression of SALL4 was scrutinized by immunohistochemical analysis in breast invasive ductal carcinoma in a large cohort of 160 patients. High cytoplasmic expression of SALL4 was detected in breast cancer tissues compared with normal adjacent tissues. High SALL4 expression was associated with advanced tumor invasion ($p = 0.019$), lymph node stage ($p = 0.027$), ER ($p = 0.030$), PR ($p = 0.037$), HER2 ($p = 0.019$) and TNBC ($p = 0.007$) in overall patients. Interestingly, in Kaplan–Meier analysis, breast cancer patients with high expression of SALL4 demonstrated a worse OS. Both univariate and multivariate analysis illustrated that examination of SALL4 was of great prognostic value in OS. Thus, our data showed that high cytoplasmic expression of SALL4 was considered to be an independent prognostic indicator for breast invasive ductal carcinoma.

Key words: breast cancer, SALL4, expressional analysis, immunohistochemical analysis, prognosis, biomarker

With at least 1.2 million new cases diagnosed and 500 000 deaths every year, breast cancer is known as the most common cancer in women and takes the second place of the leading cause of cancer related death in women all around the world [1, 2]. Being regarded as a high heterogeneous disease, breast cancer always results in different outcomes, some cancers displaying a slow tumor growth and splendid prognosis whilst other tumors presenting a clinical course with high aggression [3]. Although surgery and chemotherapeutic/radiologic interference could be feasible options for early stage breast cancer patients, the outcome is nonetheless gloomy for patients in advanced stage. Despite the fact that the

recognition of epidemiologic breast cancer risk factors have been enhanced, the prognostic value of most of acknowledged risk factors are still at low level. The current understanding of potential mechanisms of breast cancer, especially at molecular level, has proved inadequate to overcome the disease [4]. To enhance the therapeutic efficacy of breast cancer, it is imperative to understand profoundly the molecular pathogenesis of cancer cells as well as identify newer candidate genes/proteins which are crucial in the proliferating and metastatic process, are able to promote detection of tumor at high specificity and sensitivity rate and are possible to be recognized as newer treatment targets.

Sal-like 4 (SALL4), encoding a C2H2 zinc-finger transcription factor, is the human homologue of the *Drosophila* spalt homeotic gene [5]. SALL4 is supposed to play a significant role in the maintenance of pluripotency and self-renewal of embryonic stem cells, cancer cells and perhaps even cancer stem cells [6-9]. It was the first time that SALL4 was described

Abbreviations: SALL4 – Sal-like 4; ER, estrogen receptor; PR – progesterone receptor; HER2 – human epidermal growth factor receptor 2; TNBC – triple-negative breast cancer; OS – overall survival; MDS – myelodysplastic syndrome; AJCC – American Joint Committee on Cancer; ROC – receiver operating characteristic

as an oncogene when its expression was examined in leukemia [10]. SALL4 was shown to be constitutively expressed in human acute myeloid leukemia [10]. SALL4B transgenic mice developed myelodysplastic syndrome (MDS) – like features and subsequently acute myeloid leukemia [10]. Consecutively, the expression of SALL4 has been recorded in varied kinds of cancers and is deemed to have a clinical value for diagnosis in a few of them, including germ cell tumor, aggressive hepatocellular carcinoma, colorectal cancer, gastric cancer and extragonadal yolk sac tumors [11-15]. However, the information on expression and prognostic value of SALL4 in breast cancer was quite limited [16, 17]. More studies should be carried out to identify the exact role of SALL4 in the proliferation and even tumorigenesis of breast cancer. Hence, in this study, we aimed to investigate the prognostic value of SALL4 as a biomarker in a large cohort of breast cancer patients. We correlated the expression of SALL4 in 160 cases of breast invasive ductal carcinoma, the most common histological type of breast cancer.

Patients and methods

Tissues and patients. 160 cases of breast cancer patients (median age, 51.0 years old; range, 29–83 years old) were recruited after randomization from January 2002 to October 2002. The specimens of carcinoma and matched adjacent noncancerous tissues were obtained immediately after tumor resections. All cases were examined pathologically to confirm the diagnosis of breast cancer, with the histological type of invasive ductal carcinoma. The carcinomas were evaluated by using the American Joint Committee on Cancer (AJCC), 7th edition, staging system. None of the patients had received chemotherapy or radiotherapy before surgery. All patients in the series underwent surgery, including radical mastectomy, modified radical mastectomy or dissection, from January 2002 to November 2002 with a follow up of 12 years after surgery (Overall survival was defined as the time from diagnosis to the date of death or when censored at the latest date if patients were still alive). Of the 160 breast cancer patients, 51 patients were censored as death during the 12 years of follow-up time. Patients with perioperative death were excluded from this study. The study was approved by the Research Ethics Committee of our hospital.

Immunohistochemical analysis and evaluation. Immunohistochemical analysis was employed to study the altered protein expression in 160 human breast invasive ductal carcinoma tissues and matched adjacent noncancerous tissues. The formalin-fixed, paraffin- embedded sections (5 mm thickness) were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions. The sections were subsequently submerged into EDTA (pH 8) and autoclave for 8 minutes (with temperature of 121 °C for the last 2 minutes) to retrieve the antigenicity. Endogenous peroxidase was quenched with 3% H₂O₂ for 15 minutes. After washing with phosphate buffer solution (PBS), the sections were incubated with the primary antibody

of SALL4 (1:1500; Abcam, ab29112), overnight at 4 °C. The sections were incubated with peroxidase-conjugated streptavidin for 30 min and the reaction products were visualized with diaminoben-zidine as a chromogen and counterstained with commercial hematoxylin. Negative controls were obtained by omitting the primary antibodies.

The brown granules in cytoplasm and nucleus were considered as staining of SALL4. Interestingly, SALL4 was predominantly observed in the cytoplasm with a diffuse distribution in the nucleus in both cancer and adjacent noncancerous tissues. In immunohistochemistry study, we focused on the cytoplasm staining of SALL4 instead of nuclear staining. For the assessment of immunostaining, the staining intensity was scored as follows [18, 19]: negative (score 0), bordering (score 1), weak (score 2), moderate (score 3) and strong (score 4). Staining extent was graded into five parts according to the percentage of elevated staining cells in the field: negative (score 0), 0–25% (score 1), 26–50% (score 2), 51–75% (score 3) and 76–100% (score 4). Cytoplasmic SALL4 expression was evaluated by multiplying the scores of staining intensity and extent. The merged overall score was subjected to further survival analysis. Immunohistochemical staining was assessed and scored by two independent pathologists (Jingshu Geng and Xiaoming Ning) who were blinded to the clinicopathological data. Their conclusions were in complete agreement in 86% (138/160) of the cases, suggesting that the scoring system was highly reproducible. If both of the pathologists agreed with the results they scored, the value was selected. If the results were completely different, two pathologists worked together to confirm the final score.

Statistical analysis. All statistical analyses were performed by using the SPSS software version 21.0. Differences were considered statistically significant when *p* values were ≤ 0.05. For survival analysis, optimal cutoff point for SALL4 expression was obtained by receiver operating characteristic (ROC) analysis. Correlation between expression levels was studied by using the chi-square test or Fisher's exact test. Cumulative overall survival (OS) curves were plotted by the Kaplan– Meier method and the relationship between each of the variables and survival was assessed by log-rank test in a univariate analysis. The parameters were then tested by multivariate Cox proportional hazards model, which was performed to identify independent variables for predicting survival.

Results

Expression of SALL4 in human breast invasive ductal carcinoma and adjacent noncancerous tissues. Immunohistochemical analysis was employed to analyze the expression of SALL4 in breast invasive ductal carcinoma tissues compared with adjacent noncancerous tissues. As shown, SALL4 protein was found to be overexpressed in breast invasive ductal carcinoma tissues (Fig. 1 A1 and A2). Low or undetectable expression of SALL4 was observed in adjacent noncancerous tissues (Fig. 1 B1 and B2). To further assess survival analysis

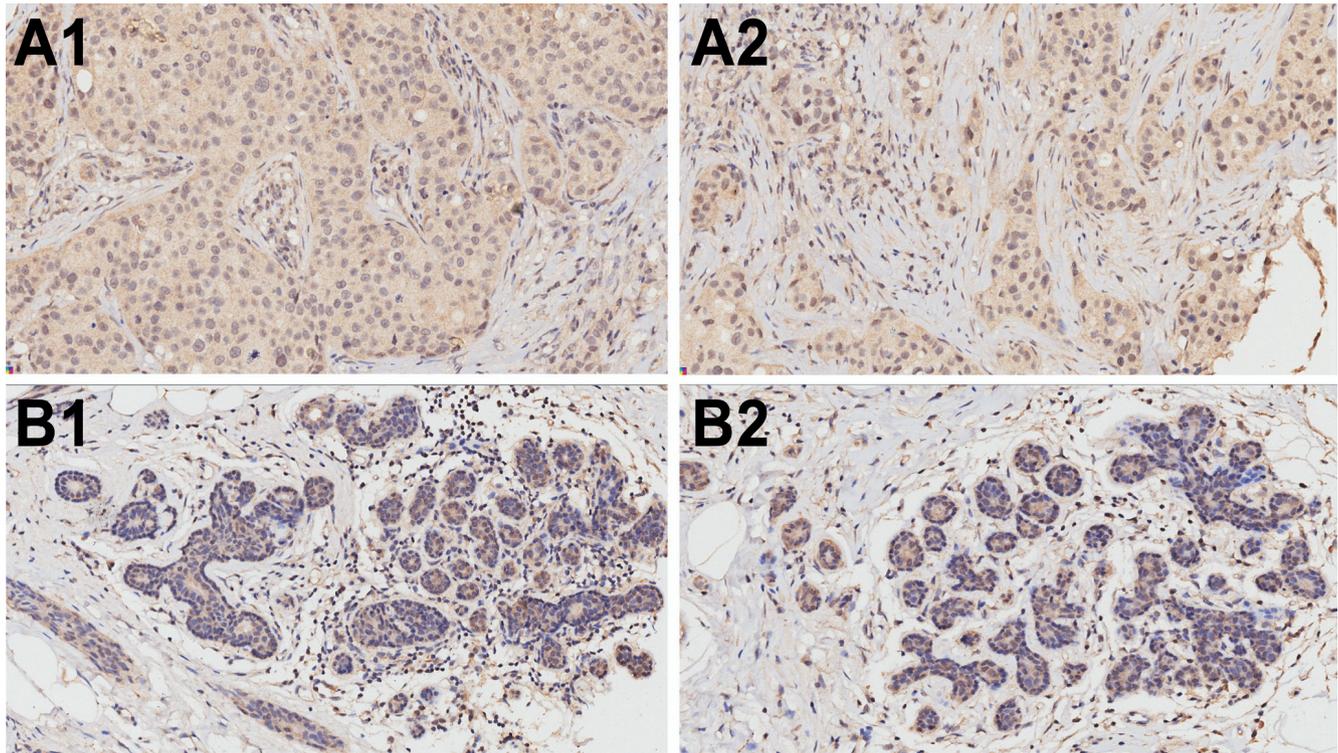


Figure 1. Immunohistochemical staining for SALL4 in breast invasive ductal carcinoma tissues and adjacent noncancerous tissues. (A1 and A2) SALL4 was overexpressed in breast invasive ductal carcinoma tissues (400 \times). The staining of SALL4 was predominantly observed in the cytoplasm with a diffuse distribution in the nucleus in breast invasive ductal carcinoma cells. (B1 and B2) SALL4 was moderately expressed in adjacent noncancerous tissues (400 \times). High SALL4 expression cases exhibited cytoplasmic signal accompanied by dispersed nuclear staining.

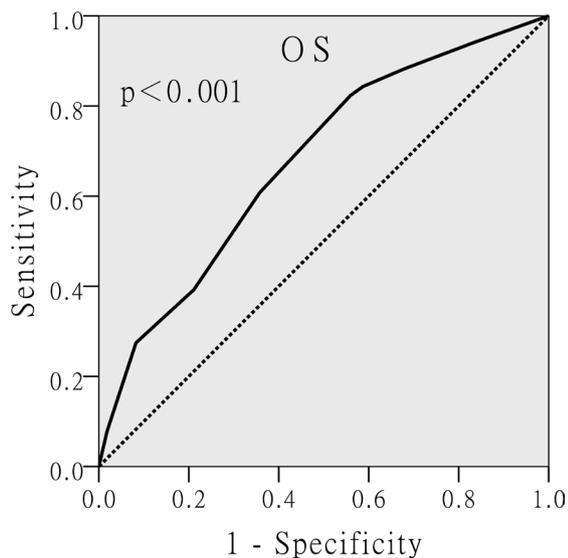


Figure 2. Receiver operating characteristic (ROC) curves analysis of SALL4 cutoff score in overall patients. SALL4 cutoff point for overall survival in breast invasive ductal carcinoma patients. At each immunohistochemical score, the sensitivity and specificity for the outcome being studied were plotted, thus generating a ROC curve. Area under the curve (AUC) for SALL4 expression score was as large as 0.677, whose 95% confidence interval ranged from 0.589 to 0.765, $p < 0.001$. SALL4 cutoff score for overall survival was 5.

and avoid the problems of multiple cutoff point selection, ROC curve analysis was employed to determine a cutoff score for cytoplasmic SALL4 expression in overall patients. As shown in Figure 2, the SALL4 cutoff score for OS in overall patients ($n = 160$) was 5 ($p < 0.001$). We thus selected a cytoplasmic SALL4 expression score of 5 (>5 denoted as high expression vs. ≤ 5 denoted as low expression) as the uniform cutoff point for survival analysis. With the cytoplasmic SALL4 expression score of 5 defined as the cutoff point, the high expression rate of SALL4 was 64.4% in breast cancer tissues ($n=160$, Fig. 1A) and 35.6% in adjacent noncancerous tissues ($n=160$, Fig. 1B) respectively (Figure 3, $p < 0.001$).

SALL4 expression and clinical features. ROC-derived SALL4 cutoff score of 5 segregated overall patients into high (103/160, 64.4%) and low (57/160, 35.6%) subgroups. As shown in Table 1, high expression of SALL4 was mostly found in poorly differentiated breast invasive ductal carcinoma tissues (27/35, 77.1%, in Grade 3 vs. 48/70, 68.6%, in G2 vs. 28/55, 50.9%, in G1, $p = 0.025$). Further correlation analysis demonstrated that SALL4 was significantly positively associated with AJCC stage ($p = 0.008$, Table 1). In addition, high cytoplasmic SALL4 expression is associated with advanced tumor invasion ($p = 0.019$, Table 1), lymph node stage ($p = 0.027$, Table 1), ER status ($p = 0.030$, Table 1), PR status ($p = 0.037$, Table 1),

Table 1. Association of SALL4 expression with patients' characteristics in breast invasive ductal carcinoma

Variables	No.(160)	SALL4			P
		High group	Low group	%	
Age (years)					0.819
< 40	27	17	10	62.96	
40-60	86	54	32	62.79	
>60	47	32	15	68.09	
Family history of tumor					0.07
Yes	40	21	19	52.50	
No	120	82	38	68.33	
Differentiation grade					0.025
G 1	55	28	27	50.91	
G 2	70	48	22	68.57	
G 3	35	27	8	77.14	
pT classification					0.019
T1	37	17	20	45.95	
T2	108	74	34	68.52	
T3	15	12	3	80.00	
pN classification					0.027
N0, N1	115	68	47	59.13	
N2, N3	45	35	10	77.78	
AJCC stage					0.008
I	16	5	11	31.25	
II	94	61	33	64.89	
III	50	37	13	74.00	
Estrogen receptor					0.03
Positive	103	60	43	58.25	
Negative	57	43	14	75.44	
Progesterone receptor					0.037
Positive	89	51	38	57.30	
Negative	71	52	19	73.24	
Human epidermal growth factor receptor 2					0.019
Positive	49	25	24	51.02	
Negative	111	78	33	70.27	
Cancer subtype					0.007
TNBC	29	25	4	86.21	
non-TNBC	131	78	53	59.54	

HER2 status ($p = 0.019$, Table 1) and cancer subtype (TNBC vs. non-TNBC) ($p = 0.007$, Table 1) in overall patient. We failed to detect that SALL4 was correlated with other patients' characteristics, including age and tumor family history.

SALL4 expression and survival analysis: Univariate survival analysis. Kaplan-Meier analysis showed that high expression of SALL4 strongly predicted an inferior OS in overall patients ($p < 0.001$ for OS, Fig. 4A). Further analysis was performed with regard to cytoplasmic SALL4 expression in subsets of breast cancer patients within each clinical stage (stage I was not included due to few patients). The results demonstrated that high cytoplasmic expression of SALL4 could be supposed as a poor prognostic factor in each stage of breast cancer patients: stage II ($p = 0.036$ for OS, Fig. 4 B) and stage III ($p = 0.038$ for OS, Fig. 4C). In univariate Cox

regression models, tumor differentiation grade ($p = 0.001$), tumor invasion ($p = 0.011$), lymph node stage ($p = 0.001$), AJCC stage ($p = 0.003$), ER status ($p = 0.024$), PR status ($p = 0.024$), HER2 status ($p = 0.023$), cancer subtype (TNBC vs. non-TNBC) ($p < 0.001$) and SALL4 ($p = 0.001$) were found to achieve statistical significance (Table 2).

Multivariate Cox regression analysis. The cytoplasmic expression of SALL4 as well as other parameters were examined in multivariate Cox analysis (Table 2). SALL4 was indeed found to be a significant independent prognostic factor for OS (hazard ratio, 0.417; 95% CI, 0.189–0.921; $p = 0.030$; Table 2). Moreover, histology differentiation was also identified as an independent prognostic parameter for OS in overall patients (Table 2). Of other parameters, cancer subtype (TNBC vs. non-TNBC) was evaluated as an independent prognostic fac-

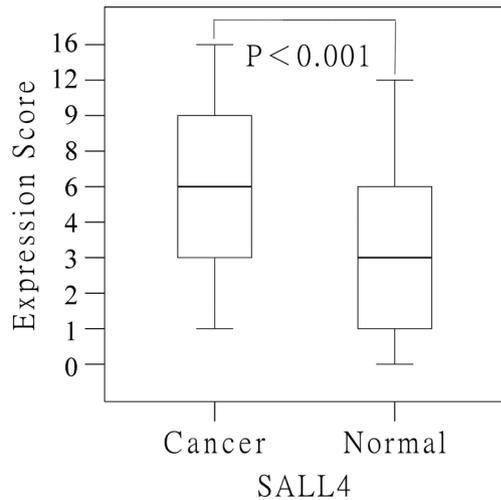


Figure 3. Immunohistochemical analysis of proteins expression in breast invasive ductal carcinoma tissues (n = 160) and adjacent noncancerous tissues (n = 160). Compared with the adjacent noncancerous tissues, breast cancer tissues expressed significantly high SALL4 (P < 0.0001).

tor for OS in overall patients (Table 2). However, we failed to detect that other factors, including age, family history of tumor, tumor invasion, lymph node stage, AJCC stage, ER status, PR status and HER2 status, were independent prognostic factors for breast cancer, implying that a larger number of cases might be needed in future studies.

Discussion

SALL4, belonging to SALL gene family and acting as a zinc-finger transcription factor, has been suggested to be involved in tumorigenesis and identified as a novel prognostic biomarker for some tumors. SALL4 was reported to be involved in three signaling pathways, associating with self-renewal and differentiation, including PTEN [20], Wnt/beta-catenin [10] and Bmi-1 [21, 22]. SALL4 was also supposed to sustain the

stemness of embryonic stem cells through establishing a regulatory signal transmission network with Sox2, Nanog and Oct4 [7, 9, 23-26]. These previous researches have revealed that SALL4 plays a significant role in preserving the pluripotency, self-renewal and even decision of cell fate in embryonic stem cells [6, 23-25, 27]. SALL4 has been demonstrated to be involved in normal hematopoiesis. The dysfunction of SALL4 signaling was connected with leukemogenesis. SALL4 was discovered to be expressed in and also lead to acute myeloid leukemia highly possible through the signaling pathway of Wnt/beta-catenin [10]. In addition, a novel association between SALL4 and Bmi-1 was found in adjusting self-renewal and pluripotency of normal and leukemic stem cells [21]. Furthermore, the anomalous expression of SALL4 has also been investigated in solid tumors. SALL4, as a specific and sensitive marker, has been proved to be beneficial in screening out metastatic germ cell tumors, especially metastatic yolk sac tumors [11]. SALL4 was demonstrated to play an oncogenic role in gastric cancer by modulating cell stemness and epithelial-mesenchymal transition and supposed to be a new target for diagnosis and therapy for human gastric cancer [15]. The over expression of SALL4 has been observed in human liver cancer [28]. SALL4 was also considered as a marker for an aggressive phenotype of liver cancer in a progenitor subclass, and most likely a novel target with therapeutic purpose for hepatocellular carcinoma [12]. However, when it comes to the expression of SALL4 in breast cancer, very few studies have been focused on. The levels of SALL4 mRNA were detected to rise up to 86.1% (31/36) in early-stage breast cancer tissue samples [16]. In cell culture and behavior analyses, SALL4 was characterized as a dispersion factor by suppressing adhesion gene CDH1 expression and maintaining the dispersion status of basal-like breast cancer [17]. The expression dynamics of SALL4 and its prognostic significance in breast cancer remain widely unknown.

In the present study, the expression pattern of SALL4 has been detected in breast invasive ductal carcinoma and adjacent noncancerous tissues. The staining of SALL4 was

Table 2. Results of univariate and multivariate Cox proportional-hazards analysis in overall patients

Variable	Univariate for death			Multivariate for death		
	Risk ratio	95%CI	p	Risk ratio	95%CI	p
Age	1.338	(0.876 , 2.043)	0.178	1.148	(0.749 , 1.759)	0.526
Family history of tumor	0.812	(0.417 , 1.584)	0.542	0.786	(0.381 , 1.620)	0.513
Differentiation grade	3.447	(1.656 , 7.175)	0.001	1.648	(1.036 , 2.622)	0.035
Tumor invasion	2.080	(1.179 , 3.670)	0.011	0.738	(0.265 , 2.057)	0.562
Lymph node stage	2.561	(1.474 , 4.450)	0.001	0.732	(0.092 , 5.846)	0.769
AJCC stage	2.323	(1.340 , 4.027)	0.003	0.598	(0.059 , 6.053)	0.663
Estrogen receptor	0.531	(0.306 , 0.920)	0.024	0.215	(0.045 , 1.016)	0.052
Progesterone receptor	0.527	(0.302 , 0.917)	0.024	0.669	(0.270 , 1.659)	0.386
Human epidermal growth factor receptor 2	0.433	(0.211 , 0.891)	0.023	0.792	(0.336 , 1.865)	0.594
Triple-negative breast cancer	6.593	(3.699 , 11.750)	<0.001	0.033	(0.006 , 0.188)	<0.001
SALL4	3.447	(1.656 , 7.175)	0.001	0.417	(0.189 , 0.921)	0.030

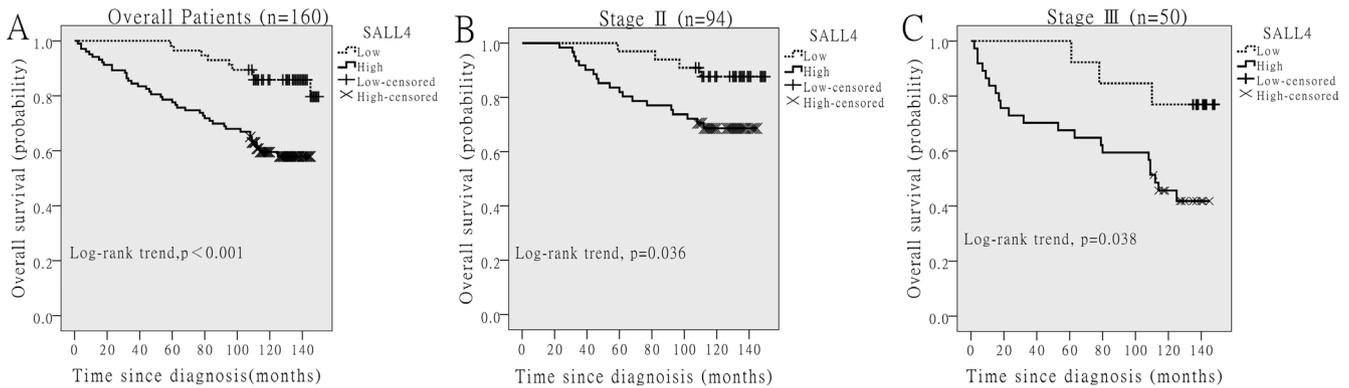


Figure 4. Kaplan-Meier survival analysis of SALL4 expression in breast invasive ductal carcinoma patients. (A) Probability of OS in breast cancer patients with all stages: high expression, $n = 103$; low expression, $n = 57$. High expression of SALL4 was closely correlated with poor OS in overall patients (chi-square statistic = 12.355, $p < 0.001$). (B) Probability of OS in breast cancer patients with AJCC stage II: high expression, $n = 61$; low expression, $n = 33$. High expression of SALL4 acquired an inferior OS in patients with AJCC stage II (chi-square statistic = 4.390, $p = 0.036$). (C) Probability of OS in breast cancer patients with AJCC stage III: high expression, $n = 37$; low expression, $n = 13$. High expression of SALL4 was also associated with a poor OS in patients with AJCC stage III (chi-square statistic = 4.320, $p = 0.038$).

mainly found in the cytoplasm with a diffuse distribution in the nucleus. The result of predominantly cytoplasmic staining of SALL4 was dissimilar with most studies of SALL4 in other carcinomas which observed largely nuclear staining of SALL4 [15, 29, 30]. Under some circumstances, some kinds of transcription factors may be located in the cytoplasm prior to activation, and become relocated in the nucleus in response to the appropriate signal [31]. We hypothesize that SALL4, as a transcription factor, may be able to shuttle between nucleus and cytoplasm, which needs to be further verified by more studies. With similarity to the results in previous studies [29, 32], the expression level of SALL4, correlating tightly with cancer differentiation, was found to be higher in cancer tissues with poor differentiation (Fig. 1; Table 1). In addition, SALL4 was further confirmed as an independent biomarker with prognostic value for OS in overall patients (Fig. 4; Tables 2). Simultaneously, inferior prognostic influence of high SALL4 expression was also displayed in breast cancer patients among each clinical stage (Fig. 4), suggesting that detection of SALL4 expressing status could be validated to be an extra efficient tool in sorting out those breast invasive ductal carcinoma patients with higher risk of tumor recurrence and progression. As a result of the limitation of the data of triple-negative breast cancer patients involved in our study, a precise prognostic value of the SALL4 expression remains uncertain for this significant subtype of breast cancer and much more studies are nevertheless needed. To sum up, our discoveries in this research presented evidence that elevated cytoplasmic SALL4 expression might indicate a phenotype of breast cancer with increased risk of malignance and worse prognosis. With no other histological type, such as invasive lobular carcinoma, involved in our research, a larger cohort of clinicopathological study on the prognostic value of SALL4 in breast cancer is still of utmost necessity.

SALL4 administers the survival of cells through aiming at a series of genes involved in pro-apoptotic and anti-apoptotic signaling pathways [33]. In addition, the expression of this kind of genes is administered by SALL4 in embryonic stem cells [9]. In breast cancer cells, the overexpression of SALL4 might play an essential role in sustaining the survival of tumor cell by transcriptionally repressing genes in pro-apoptotic pathways, such as PTEN. Coincidentally, the expression level of PTEN in breast cancer tissues was lower or negative compared with matched adjacent noncancerous tissues [34-36]. The reason for deficient expression of PTEN protein in breast cancer tissues could be determined by varied mutations of the PTEN gene. On the basis of our findings, we hypothesize that overexpression of SALL4 probably plays a novel role in repressing the transcript of PTEN and contributing to down-regulation of PTEN in breast cancer cells. First of all, down-regulation of SALL4 could lead to apoptosis [22], possible through activating some genes, such as PTEN, which are in pro-apoptotic pathway. It has been found that, by targeting and adjusting a series of genes involved in signaling pathways of both pro- and anti-apoptosis, SALL4 regulates cell survival in human acute promyelocytic leukemia cells [22]. Additionally, PTEN was regarded as one of the most significant tumor-suppressing genes which have close association with breast cancer risks [37, 38]. Thus, overexpression of SALL4 might regulate cells survival of breast cancer by restraining the process of apoptosis through inhibiting PTEN transcription. What's more, PTEN restrains the PI3K/AKT/mTOR signaling pathway through splitting a phosphate group from the second messenger PIP-3 activated by PI3K [39-41]. It is our hypothesis that SALL4 might play a role in the PI3K/AKT/mTOR pathway by tightly connecting with PTEN. Interestingly, the initiation of process of autophagy is partly regulated through the PI3K/AKT/mTOR pathway [42]. Therefore, we also hypothesize that SALL4 might have a close association

with autophagy through regulating the PI3K/AKT/mTOR pathway, which needs to be proved further by more in vitro and in vivo studies.

In conclusion, our research suggested that SALL4 served as an independent marker with prognostic value and even a therapeutic target for patient survival in breast invasive ductal carcinoma and the cytoplasmic expression of SALL4 could be of help in determining the outcome of patients with breast cancer in clinical practice. Our data demonstrate a novel understanding of the connection between SALL4 and tumor differentiation in tumor progression and may shed light on the illustration of tumor pathophysiology of breast cancer.

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