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Original Article

Expression of HAX1 and Ki-67 in breast cancer and its correlations with patient's clinicopathological characteristics and prognosis

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Abstract: Objective: This study aimed to investigate the HS-1-associated protein X-1 (HAX1) and Ki-67 expression in the breast cancer and its clinical significance. Methods: Breast cancer tissues and tumor-adjacent tissues were collected from 81 patients, and immunohistochemistry was conducted to detect the HAX1 and Ki-67 expression. The correlations of HAX1 expression with demographics, clinicopathological characteristics and prognosis were evaluated. Results: HAX1 was highly expressed in the breast cancer, and its expression was related to the degree of breast cancer differentiation (P=0.002), lymph nodes metastasis (P=0.008) and progesterone receptor (PR) (P=0.021), but not with the age, tumor size, histological type, estrogen receptor (ER), HER2 and p53. HAX1 expression was positively related to Ki-67 expression in the breast cancer (r^2=0.394, P=0.019). In addition, a higher HAX-1 expression was related to a lower 10-year survival rate. Conclusion: HAX1 is a new protein related to the breast cancer and probably plays an important role in the invasion and metastasis of breast cancer. Thus, HAX1 may be used as a potential target for the therapy of breast cancer.

Keywords: Breast cancer, HAX1, Ki-67, prognosis

Introduction

Breast cancer is currently the most common malignancy affecting women worldwide. It accounts for 25% of all cancers in women and caused 522,000 deaths worldwide in 2012 [1]. The estimated incidence of breast cancer is increasing worldwide and reported highest in developed countries, and breast cancer is currently the second leading cause of death for women in developed regions [2]. Although the multidisciplinary management has been used for the therapy of breast, the prognosis of some patients is still poor, especially for HER-2 positive or triple negative breast cancer patients [3]. Thus, it is imperative to identify a favorable target for the therapy of breast cancer.

HS-1-associated protein X-1 (HAX-1) was originally identified as a 35 kDa protein that interacts with HS-1, a Src kinase substrate, and was suggested to be involved in B cell signal transduction [4]. Due to its homology and perceived structural similarities to the anti-apoptotic protein Bcl-2, it was proposed early on that HAX-1 might represent a novel protein that regulates apoptosis and promotes cell survival, which has been confirmed in some in vivo and in vitro studies [5]. The involvement of HAX-1 in inhibition of apoptosis and promotion of cell migration-processes crucial to carcinogenesis and metastasis-suggests that overexpression of HAX-1 in cancer is likely to occur, as this could promote cell survival and enhance the invasive potential of malignant cells. Increasing evidence confirms that HAX-1 is highly expressed or over-expressed in various types of human malignancies [6, 7]. In addition, HAX is not only closely related to the pathogenesis of malignancies, but has relationship with other diseases including psoriasis and systemic sclerosis [8]. In this study, the HAX-1 expression was detected in the breast cancer tissues and adjacent normal tissues, and its correlations with demographics, clinicopathological characteristics and prognosis were further evaluated in 81 patients with breast cancer.

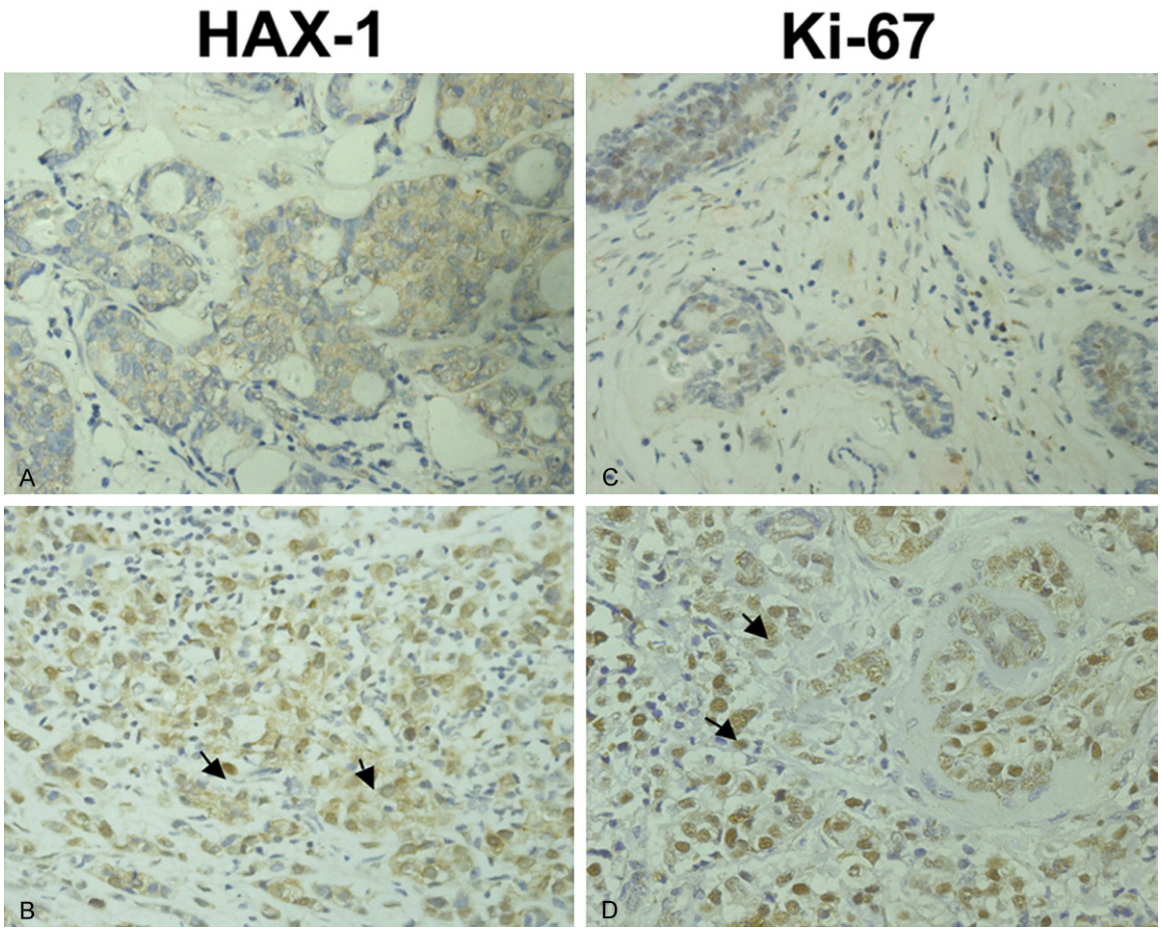

Figure 1. Immunohistochemisty for HAX1 and Ki67. The expressions of HAX1 and Ki67 in breast cancer tissues (B, D) were significantly higher than in adjacent normal tissues (A, C) Arrows: positive cells.

Subjects and methods

Subjects

The paraffin embedded specimens were collected from the breast cancer patients (n=81) who received surgical intervention and pathological examination in the Affiliated Hospital of Nantong University between 2002 and 2012. All the 81 patients were followed up after surgery, and physical examination, ultrasonography, CT and other examinations were conducted to determine the recurrence. The date of death was recorded. The pathological parameters were recorded according to WHO breast cancer classification: The age, degree of differentiation, tumor size, lymph node status, histological type and expressions of estrogen receptor (ER), progesterone receptor (PR), HER2, p53, HAX1 and Ki-67 were also recorded. The number of patients with pathological grade I, grade II and grade III breast cancer was 20, 38 and 23, respectively. The number of patients with and without lymph node metastasis was 48 and 33, respectively. In addition, 35 patients had the tumor diameter of ≤ 2.5 cm, and 46 had the tumor diameter of > 2.5 cm. Duct carcinoma and non-duct carcinoma were found in 59 and 22 patients, respectively. This study was approved by the Ethics Committee of Nantong University and informed consent was obtained before study.

Immunohistochemistry

The methods for immunohistochemistry were according to that previously reported [9].

The paraffin tissues were cut into 4-μm sections, and deparaffinized in 4% formaldehyde and gradient alcohol. The endogenous peroxidase was blocked with 0.3% hydrogen peroxide (H_2O_2). Sections were washed with double-distilled water, and then subjected to antigen retrieval at 100°C in citric acid for 5 min. After

Table 1. Expressions of HAX1 and Ki-67 and pathology of breast cancer

Parameter	N	HAX1 expression		P	Ki67		P
		Low ≤ 0.36	High > 0.36		Low ≤ 0.34	High > 0.34	
Age				0.102			0.586
≤ 50	34	14	20		18	16	
> 50	47	28	19		22	25	
Pathology typing				0.002*			0.007*
I	20	14	6		12	8	
III	38	23	15		23	15	
III	23	5	18		5	18	
Tumor size				0.702			0.899
≤ 2.5 cm	35	19	16		17	18	
> 2.5 cm	46	23	23		23	23	
Axillary lymph node				0.008*			0.044*
No	33	23	10		20	13	
Nx	48	19	29		20	28	
Tissue typing				0.088			0.152
Tube	59	34	25		32	27	
Others	22	8	14		8	14	

Notes: *P < 0.05.

being allowed to cool to room temperature, sections were washed three times with phosphate buffer solution (PBS), and blocked in 10% calf serum. After washing with PBS, sections were incubated at 36°C for 120 min with polyclonal rabbit anti-human HAX1 antibody and Ki-67 antibody (1:100, all were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) independently. Following washing with PBS thrice, sections were incubated for 30 min with biotinylated anti-rabbit secondary antibody IgG and subsequently washed in PBS buffer thrice. Visualization was done in DAB, and counterstaining was performed with hematoxylin. Sections were observed and protein expression was determined after mounting with gelatin glycerin.

Result evaluation

HAX1 or Ki-67 positive cells had brown granules in the cytoplasm and nucleus. Four fields were randomly selected under a light microscope from each section at a magnification of ×400, and 400 cells were counted in each field. The proportion of positive cells was calculated as the 1abel index (LI): LI= the number of positive cells/total cell count.

Statistical analysis

Kaplan-Meier method was used for survival analysis, and Cox regression analysis was used for the comparison of survival rate. Correlation analysis was used for the evaluation of correlations of HAX1 expressions with Ki-67 expression, demographics, clinicopathological characteristics and prognosis. Statistical analysis was performed with SPSS version 15.0 and a value of $P < 0.05$ was considered statistically significant.

Results

Expressions of HAX1 and Ki-67 in breast cancer tissues and adjacent normal tissues

HAX1 and Ki-67 were highly expressed in the breast cancer. HAX1 expression was mainly found in the cytoplasm, and Ki-67 was mainly expressed in the nucleus. The expressions of HAX1 and Ki-67 in the breast cancer were significantly higher than in the adjacent normal tissues (**Figure 1**).

Correlation between HAX1 and Ki-67 expression and clinicopathological characteristics

Patients were divided into high expression group and low expression group (cut-off value of LI: HAX1=0.36 and Ki67=0.34). The relationship between HAX1 and Ki-67 expression and clinicopathological characteristics was evaluated. Results showed HAX1 expression was related to the degree of breast cancer differen-

Table 2. Relationship between HAX1 expression and molecular markers of breast cancer

Parameter	N	HAX1		P	Ki67		P
		Low ≤ 0.36	High > 0.36		Low ≤ 0.34	High > 0.34	
ER				0.102			0.031*
Negative	34	14	20		12	22	
Positive	47	28	19		28	29	
PR				0.021*			0.311
Negative	37	14	23		16	21	
Positive	44	28	16		24	20	
HER2				0.551			0.091
Negative	36	20	16		14	22	
Positive	45	22	23		26	19	
P53				0.586			0.223
Negative	39	19	20		22	17	
Positive	42	23	19		18	24	
Ki67				0.019*			
Low expression	40	26	14				
High expression	41	16	25				
HAX1							0.019*
Low expression	42				26	16	
High expression	39				14	25	

Notes: *P < 0.05

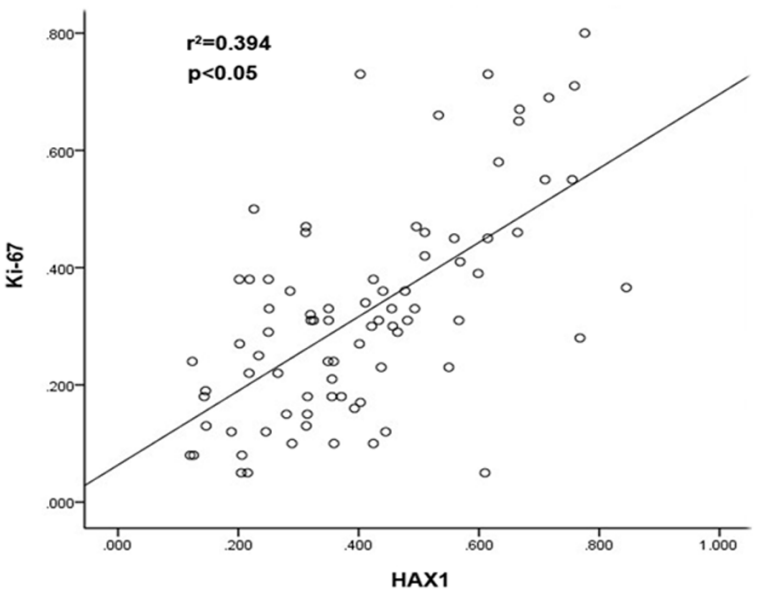

Figure 2. Correlation analysis of HAX1 expression and Ki-67 expression, (r^2=0.394, P=0.019).

tiation (P=0.002), lymph nodes metastasis (P=0.008) and PR (P=0.021), but not to the age (P=0.102), tumor size (P=0.702), histological type (P =0.088), ER (P=0.102), HER2 (P=0.551), and p53 (P=0.586). Ki-67 expression was related to the degree of breast cancer differentiation (P=0.007), lymph node metastasis (P=0.044) and PR (P=0.031), but not to the age (P=0.586), tumor size (P=0.899), histological type (P=0.152), ER (P=0.311), HER2 (P=0.091) and p53 (P=0.223) (**Tables 1** and **2**).

Correlation between HAX1 expression and Ki-67 expression

Results showed the HAX1 expression was positively related to Ki-67 expression in the breast cancer (**Figure 2**).

Correlation between survival and clinicopathological characteristics

Breast cancer patients were divided into survival group and death group, and the correla-

Table 3. Relationship between survival rate and clinicopathological characteristics of breast cancer patients

Parameters	N	Survival period		P
		Survival	Death	
Age				0.512
≤ 50	34	20	14	
> 50	47	31	16	
Pathology typing				0.003*
I	20	16	4	
II	38	27	11	
III	23	8	15	
Tumor size				0.362
≤ 2.5 cm	35	25	8	
> 2.5 cm	46	27	19	
Axillary lymph node				0.048*
No	33	25	8	
Nx	48	26	22	
ER				0.849
Negative	34	21	13	
Positive	47	30	17	
PR				0.890
Negative	37	23	14	
Positive	44	28	16	
HER2				0.758
Low expression (0-1+)	36	22	14	
High expression (2-3+)	45	29	16	
P53				0.260
Negative	39	27	12	
Positive	42	24	18	
Ki67				0.027*
Low expression	40	30	10	
High expression	41	21	20	
HAX1				0.036*
Low expression	42	31	11	
High expression	39	20	19	

Notes: *P < 0.05.

tion between survival and clinicopathological characteristics was further evaluated. Results showed the degree of breast cancer differentiation (P=0.003), lymph node metastasis (P=0.048), high Ki-67 expression (P=0.027) and high HAX1 expression (P=0.036) was related to the prognosis, but the age (P=0.512), tumor size (P=0.362), ER (P=0.849), PR (P=0.890), HER2 (P=0.758) and p53 (P=0.260) had no relationship with the prognosis. This indicates that both HAX1 and Ki-67 are indicative of the invasion and metastasis of breast cancer (**Table 3**).

Correlation between HAX1 expression and 10-year survival rate

The 10-year survival rate was calculated with Kaplan-Meier method, and results showed that there was significant difference in the 10-year survival rate between high HAX1 expression group and low HAX1 expression group (**Figure 3**).

Discussion

Breast cancer isa malignant tumor significantly affecting the health of women, and has been the second cause of death in women of developed countries [1]. Although the diagnosis and therapy of breast cancer have been improved to a certain extent with the development of medical technology, the prognosis of some breast cancer patients is still poor, especially for those with HER-2 positive or triple negative breast cancer [3]. Thus, it is important to identify new target for the early diagnosis and targeted therapy of breast cancer.

In this study, we investigated the HAX1 expression in the breast cancer and explored its clinical significance in the breast cancer patients. HAX-1 was originally identified as a protein involved in the maturation of T cells. Based on its apparent homology with the anti-apoptotic protein, B cell lymphoma/leukemia-2 (Bcl-2), HAX-1 was suggested to be involved in the regulation of apoptosis or programmed cell death [4]. In addition, increasing studies confirm that HAX is able to increase the cell proliferation, migration, adhesion and invasion [10, 11]. The involvement of HAX-1 in inhibition of apoptosis and promotion of cell migration implies that over-expression of HAX-1 in cancer is likely to occur, as this could promote cell survival and enhance the invasive potential of

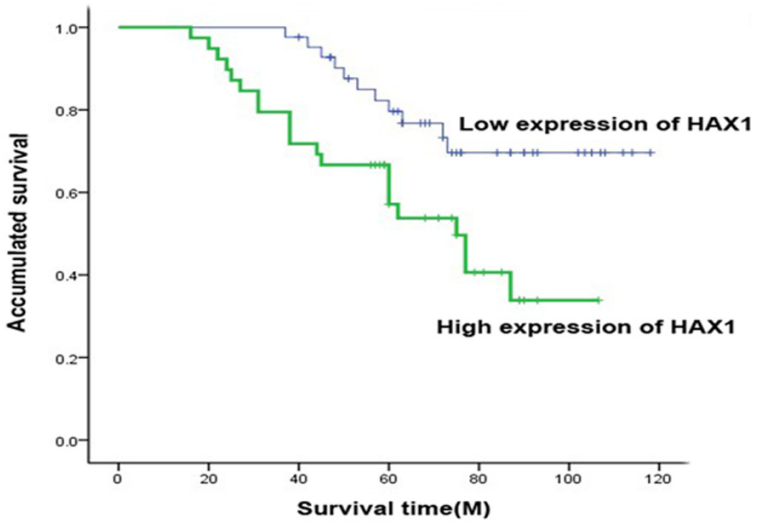

Figure 3. Overall survival rate within 10 years of patients stratified according to HAX1 expression.

malignant cells, which has been confirmed in clinical and experimental studies. Immunohistochemical analyses demonstrated significant up-regulation of HAX-1 in various human premalignant epithelial dysplastic and oral squamous cell carcinoma (SCC) samples [12], as well as in breast cancer tissues [13]. In addition, HAX-1 expression correlated with the size and grade of the tumor, with higher amounts of HAX-1 detected as the disease progressed, at least for breast cancer [13]. In the study of Solomon et al, the HAX-1 expression was measured in two breast adenocarcinoma cell lines, namely MCF7 (estrogen-receptor positive) and MDA-MB-231 (estrogen receptor negative), the skin squamous cell cancer line SCC13, and the colon adenocarcinoma cell line LS174T [5]. Results revealed a significant up-regulation of the prototypical ~35 kDa protein in skin and colon, but not breast cancer. Moreover, there was the existence of additional HAX-1 proteins, with sizes of ~25, ~50, ~55, ~65, and ~75 kDa. Of those, the ~55 and ~75 kDa isoforms were over-expressed in all or select types of cancer, respectively, while the ~25, ~50, and ~65 kDa isoforms are abundantly expressed in non-malignant breast epithelium, but significantly down-regulated or even abrogated during breast cancer formation. This finding suggests that selected HAX-1 isoforms may function in an opposite manner to the anti-apoptotic variant 001, and potentially promote cell death.

Our results showed HAX-1 expression increased significantly in the breast cancer tissues as compared to adjacent normal tissues. Ki-67 is a non-histone nucleoprotein locating in the nucleus with a short half life. It has been used as a marker of cell proliferation [14] and found to be highly related to the development, metastasis and prognosis of malignant tumor [15, 16]. Our results also indicated a positive relationship between HAX-1 expression and Ki-67 expression, suggesting the pro-tumor effect of HAX-1.

In addition, we also evaluated the relationship of HAX-1 expression with clinicopathological characteristics. Results revealed that HAX1 expression was closely related to the degree of breast cancer differentiation (P=0.002) and the lymph node metastasis (P=0.017), suggesting that high HAX1 expression is associated with the invasion and metastasis of breast cancer. Moreover, the 10-year survival rate in patients with HAX1 over-expression was significantly lower than that in those with low HAX1 expression, indicating that high HAX1 expression predicts a poor prognosis in breast cancer patients.

There were limitations in this study. This was a retrospective study, and only 81 patients were recruited. Although modified radical mastectomy was performed in all the patients, the post-operative adjunctive therapies might vary between individuals.

In conclusion, our findings indicate that HAX1 expression is up-regulated in the breast cancer tissues, which is related to the degree of breast cancer differentiation, lymph nodes metastasis, PR and Ki-67 expression. Moreover, high HAX1 expression in the breast cancer predicts a poor prognosis. These findings provide evidence on the role of HAX1 expression in the occurrence and development of breast cancer and present us a new target for the therapy of breast cancer.

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Disclosure of conflict of interest

None.

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