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Original Article

The expression status of INHBA as a prognostic marker for human breast cancer

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Abstract: INHBA is reported to be up-regulated in various malignant tumours. However, the data on its expression pattern and its clinical relevance in breast cancer are unknown. The aim of this study is to investigate INHBA expression and its prognostic significance in breast cancer. INHBA expression at the mRNA level was examined by real-time quantitative polymerase chain reaction (RT-PCR) in 10 pairs of breast cancer tissues and their corresponding adjacent normal tissues. INHBA protein expression was analysed by using immunohistochemistry (IHC) on paraffin-embedded breast cancer samples and normal breast tissues. Statistical analyses were also performed to evaluate the clinicopathological significance of INHBA expression. The results showed that in 10 paired samples, the mRNA expression of INHBA was higher in breast cancer tissues than in the adjacent normal tissues. In the paraffin-embedded tissue samples, the expression of INHBA was higher in breast cancer than in the normal breast tissues. Compared with normal breast tissue samples, INHBA overexpression was detected in 51.59% (65/126) of patients. Overexpression of INHBA was significantly associated with clinical stage (P<0.001), N classification (P<0.001), differentiation (P=0.011), and decreased overall survival (P=0.001). In a multivariate analysis, INHBA expression was an independent prognostic factor for OS (overall survival) (Hazard ratio [HR] =0.305, 95% confidence interval [CI] 0.143-0.652; P=0.002). INHBA is up-regulated in breast cancer, and its expression is associated with clinical stage, N classification, differentiation and survival. INHBA may serve as a prognostic indicator for patients with breast cancer.

Keywords: Breast cancer, INHBA, overexpression, prognosis

Introduction

Breast cancer is one of the most prevalent malignant diseases among women with approximately 232,340 new cases and 39,620 breast cancer-related deaths predicted to occur among US women in 2013 [1]. Although there has been considerable development in achieving an early diagnosis through screening programmes and therapeutic strategies, the age-standardized mortality rate of breast cancer remains at 14.1 per 100,000 individuals [2]. To predict patient prognosis and guide treatment, researchers have evaluated various parameters such as hormone receptors and classical histological features. Recently, the expression of molecular markers such as HER2, Ki-67, EGFR, and TP53 has contributed to the improvements in predicting patient prognosis and developing more individualized treatment strategies for patients. Although the currently used biomarkers are valuable in breast cancer diagnosis and treatment, discovering new biomarkers related to breast cancer can help building a deeper and more comprehensive understanding as well as providing new treatment targets.

INHBA encodes inhibin βA, which is a subunit of both activin and inhibin, members of the transforming growth factor β (TGF-β) superfamily [3]. A group of functionally diverse yet structurally similar proteins constitute the TGF-β superfamily. These members play important roles in embryonic development and terminally differentiated tissues. Activin and inhibin participate in a variety of physiological processes including cell growth, proliferation, differentiation, metabolism, homeostasis, apoptosis and carcinogenesis [4] through autocrine, endocrine or parac-

Table 1. Correlation of INHBA expression with clinicopathologic features

Characteristics	Total (n=126)	INHBA expression		*P* value
		Low (n=89)	High (n=37)	
Age (years)				0.973
≥60	37 (29.37%)	18 (48.6%)	19 (51.4%)	
<60	89 (70.63%)	43 (48.3%)	46 (51.7%)	
Clinical stage				0.000
I	10 (7.94%)	8 (80%)	2 (20%)	
II	76 (60.32%)	48 (63.2%)	28 (36.8%)	
III	40 (31.75%)	5 (12.5%)	35 (87.5%)	
T classification				0.130
T1	26 (20.63%)	12 (46.2%)	14 (53.8%)	
T2	87 (69.05%)	46 (52.9%)	41 (47.1%)	
T3	13 (10.32%)	3 (23.1%)	10 (76.9%)	
N classification				0.000
N0	49 (38.89%)	43 (87.8%)	6 (12.2%)	
N1	39 (30.95%)	14 (35.9%)	25 (64.1%)	
N2	30 (23.81%)	4 (13.3%)	26 (86.7%)	
N3	8 (6.35%)	0 (0%)	8 (100%)	
Differentiation				0.011
Well	13 (10.32%)	11 (84.6%)	2 (15.4%)	
Moderate	94 (74.6%)	44 (46.8%)	50 (53.2%)	
Poor	19 (15.08%)	6 (31.6%)	13 (68.4%)	
Expression of ER				0.159
Negative	45 (35.71%)	18 (40%)	27 (60%)	
Positive	81 (64.29%)	43 (53.1%)	38 (46.9%)	
Expression of PR				0.440
Negative	54 (42.86%)	24 (44.4%)	30 (55.6%)	
Positive	72 (57.14%)	37 (51.4%)	35 (48.6%)	
Expression of HER2				0.081
Negative	90 (71.43%)	48 (53.3%)	42 (46.7%)	
Positive	36 (28.57%)	13 (36.1%)	23 (63.9%)	

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.

rine mechanisms [5]. Activins are known to induce cellular responses through activin receptors and the SMAD2/3 pathway, and this activity can be inhibited by antagonizing activins via competition for receptor binding or β-glycan. Several studies have reported INHBA overexpression in various malignant tumours such as tongue squamous cell carcinoma, oesophageal adenocarcinoma, lung cancer, and gastric cancer [6, 7]. However, its clinical significance in breast cancer has not been systemically evaluated. Our present study examined INHBA expression in breast cancer tissue samples and elucidated its clinicopathological and prognostic significance.

Materials and methods

Patients and specimens

This study was conducted in a total of 126 paraffin-embedded primary breast cancer samples that were histopathologically diagnosed and excised via curative resection at the Third Affiliated Hospital of Sun Yat-sen University between March 2001 and December 2012. None of the patients received any type of neoadjuvant therapy, and all of them underwent curative surgery. The clinical information of these samples is summarized in **Table 1**. The follow-up time of the breast cancer cohort ranged from 2 to 131 months, and the median follow-up time was 111 months. Of these 126 breast cancer patients, paired adjacent non-cancerous tissues (adjacent non-cancerous tissue was defined as at least 2 cm distance from the edge of tumour) were obtained in 10 patients. In addition, 20 normal breast tissue samples were obtained from patients who underwent mammaplasty.

The clinicopathological classification and staging were determined according to the AJCC (American Joint Committee on Cancer Seventh Edition) criteria. Patient consent for the use of these clinical specimens for research purposes was gained prior to experimentation, and the protocol was approved by the internal Institutional Research Ethics Committee. The 10 pairs of breast cancer and adjacent non-cancerous tissues were collected immediately after operation for real-time PCR.

Real-time PCR (RT-PCR) analysis

Total RNA samples were extracted from primary breast tumour materials using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Extracted RNA was pretreated with RNase-free DNase, and 2 μg of RNA from each sample was used for cDNA syn-

thesis. For the PCR amplification of INHBA cDNA, an initial amplification step using INHBA-specific primers was performed with denaturation at 95°C for 10 min followed by 28 cycles of denaturation at 95°C for 60 s, primer annealing at 58°C for 30 s, and primer extension at 72°C for 30 s. Upon completion of the cycles, a final extension at 72°C for 5 min was performed before the reaction mixture was stored at 4°C. Then, real-time PCR was performed to determine the fold increase of INHBA mRNA in each of the pairs of breast tumours and normal breast tissue from the same patient. The primer sequences were as follows: *INHBA* fragments, 5'-CCTCGGAGATCATCACGTTT-3' (forward) and 5'-CCCTTTAAGCCCACTTCCTC-3' (reverse); and GAPDH, 5'-TGTTGCCATCAATGACCCC-3' (forward), 5'-CTCCACGACGTACTCAGC-3' (reverse). The primers were designed by Primer Express v 2.0 software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, and all experiments were performed in triplicate.

Immunohistochemical analysis

Immunohistochemical (IHC) staining was performed to study changes in protein expression in 126 human breast cancer tissues and 20 normal breast tissues. Briefly, 4-μm-thick paraffin sections of the tissue were deparaffinized with xylene and then rehydrated. Antigen retrieval was performed by submerging the slides into EDTA antigen retrieval buffer and heating in a microwave. To quench endogenous peroxidase activity, the slides were treated with 3% hydrogen peroxide in methanol and then incubated with 1% bovine serum albumin to block nonspecific binding. Afterwards, the sections were incubated with anti-INHBA rabbit polyclonal antibody (1:100, Abcam) at 4°C overnight. Normal goat serum was used as a negative control. The tissue sections were incubated with a biotinylated anti-rabbit secondary antibody (Abcam) after 3 washes followed by incubation with a streptavidin-horseradish peroxidase complex (Abcam). The slides were immersed in 3-amino-9-ethyl carbazole, counterstained with 10% Mayer's haematoxylin, dehydrated and finally mounted in Crystal Mount.

To evaluate the immunostaining, the intensity of immunostaining was viewed and scored separately by two pathologists who were blind to the histopathological characteristics and patient information corresponding to the samples. Scores given by the two independent pathologists were averaged for further comparative evaluation of INHBA expression. The intensity of INHBA staining was graded according to the following criteria: 0, no staining; 1, weak staining = light yellow; 2, moderate staining = yellow brown; and 3, strong staining = brown. The percentage of stained tumour cells was scored as follows: 0, no positive tumour cells; 1, 1-25% positive tumour cells; 2, 26-50% positive tumour cells; 3, 51-75% positive tumour cells; and 4, >75% positive tumour cells.

The staining score was calculated as the product of the percentage of positive tumour cells and the staining intensity score. The expression levels of INHBA were defined as follows: "-" (score 0, negative), "+" (score 1-4, weakly positive), "++" (score 5-8, positive), and "+++" (score 9-12, strongly positive). The cut-off values for INHBA were chosen on the basis of heterogeneity using the log-rank test with respect to overall survival (OS). The optimal cut-off value was estimated as follows: a staining index score of ≥8 was used to define tumours with high INHBA expression, and a score <8 indicated low INHBA expression.

Statistical analysis

The time from the date of each patient's randomization to either their date of death due to any cause or the censoring of the patient at the last follow-up date was defined as the OS. All of the statistical analyses were conducted using SPSS 20.0 statistical software packages. The difference in INHBA expression between breast cancer tissue and normal breast tissues was analysed by the chi-square test. Survival curves were plotted by using the Kaplan-Meier method and compared using the log-rank test. The relationship between INHBA expression and other clinicopathological characteristics was analysed by the chi-square test and Fisher's exact test. Bivariate correlations between the clinicopathological characteristics were calculated by Spearman's rank correlation coefficients. The clinicopathological characteristics used to predict patient prognosis in clinical practice were evaluated by univariate and multivariate Cox regression analyses. The chosen Cox model for the univariate analysis was the enter method and for the multivariate analysis was the for-

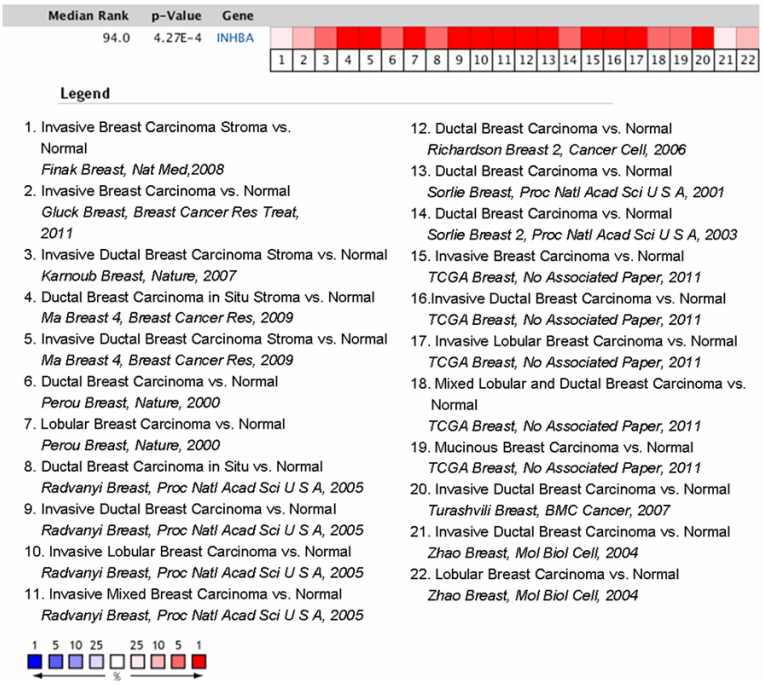

Figure 1. INHBA expression is up-regulated in human breast cancer samples from the Oncomine database. Oncomine heat map of INHBA gene expression in clinical breast cancer samples compared with normal breast tissues.

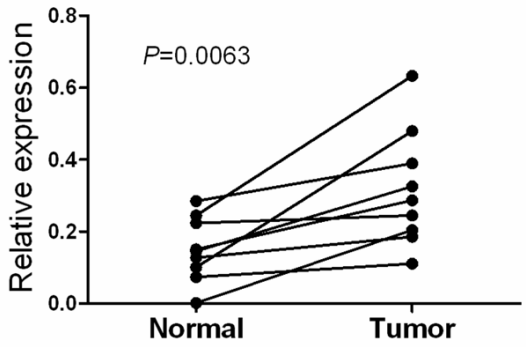

Figure 2. Expression levels of INHBA mRNA in breast cancer and corresponding adjacent non-cancerous tissues. Expression levels of INHBA mRNA in ten paired breast cancer and adjacent non-cancerous tissues as determined by real-time PCR. Normal, adjacent non-cancerous tissues. Tumour, breast cancer tissues.

ward method. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

INHBA is overexpressed in breast cancer tissues

To determine whether the INHBA expression levels were differential between breast cancer and normal breast tissues, we first queried the Oncomine database; the meta-analysis showed that INHBA expression was significantly higher in breast cancer than in corresponding normal tissues with a median rank of 94 and a *P*-value of 4.27E-4 (**Figure 1**). To confirm this result, we performed RT-PCR on 10 breast tumour samples and adjacent non-cancerous tissues. As illustrated in **Figure 2**, INHBA mRNA was expressed at higher levels in all of the 10 breast cancer tissues than in the corresponding adjacent non-cancerous tissues with the differential expression levels ranging from 1.9- to 67.2-fold. The immunostaining results show that overexpression of INHBA was observed in 51.59% (65/126) breast cancer patients. INHBA protein staining was weak or nonexistent in the normal breast tissues with only 10% (2/20) of the normal breast tissue samples showing any staining. The difference in immunostaining between the breast cancer group and normal breast tissue group was statistically significant (X^2=12.022, P=0.001).

INHBA overexpression is associated with breast cancer clinical features

To better understand the potential roles of INHBA in breast cancer development and progression, we investigated the status of INHBA expression in 126 paraffin-embedded archived breast cancer tissues by immunohistochemical staining, including 10 stage I tumours, 76 stage II tumours, and 40 stage III tumours. Among the 126 samples, high levels of INHBA protein expression were detected in 65 samples (51.59%), and either weak or nonexistent staining was observed in 61 tumour samples (48.41%, **Table 1**). As shown in **Figure 3**, INHBA was highly expressed in breast cancer tissues. In contrast, either no signal or a weak signal was detected in normal breast tissues. The subcellular localization of INHBA was mainly in the cytoplasm.

We further analysed the correlation between INHBA expression and the clinicopathological

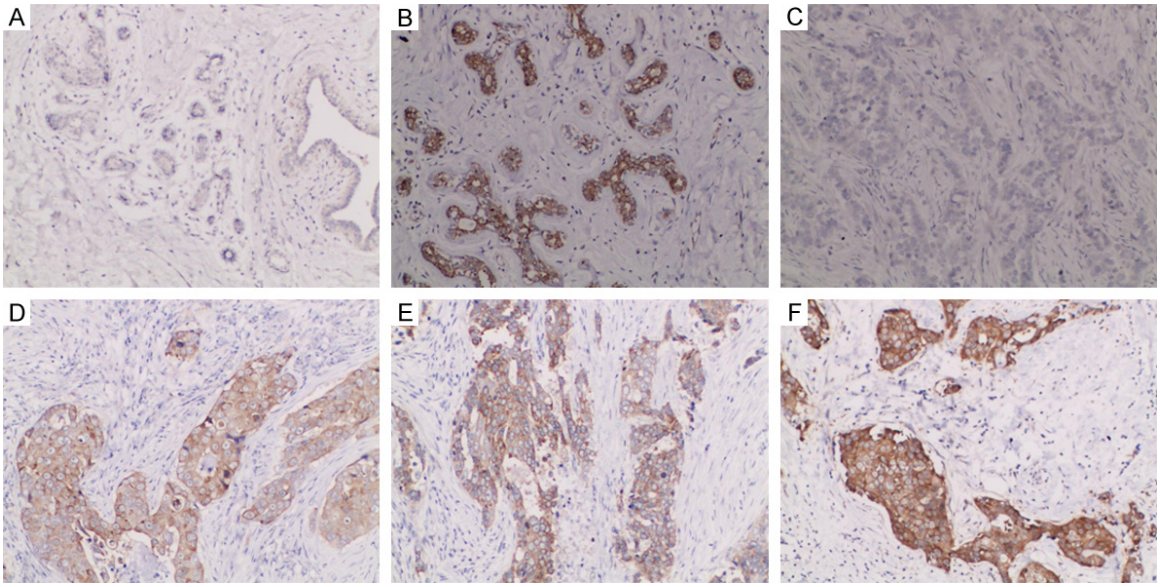

Figure 3. Expression analysis of INHBA protein as determined by immunohistochemistry. INHBA expression was mainly localized in the cytoplasm of breast tumour cells. INHBA expression is either weak or nonexistent in normal breast epithelial cells. A. Negative staining of INHBA in normal breast tissues. B. Positive staining of INHBA in normal breast tissues. C. Negative staining of INHBA in breast cancer tissues. D. "+" (score 1-4, weakly positive) expression of INHBA in breast cancer tissues. E. "++" (score 5-8, positive) expression of INHBA in breast cancer tissues. F. "+++" (score 9-12, strongly positive) expression of INHBA in breast cancer tissues.

characteristics of patients. As summarized in **Table 1**, there were no significant correlations between the expression of INHBA protein and patient age, T classification, oestrogen receptor (ER) expression levels, progesterone receptor (PR) expression levels or human epidermal growth factor receptor-2 (HER2) levels in patients with breast cancer. However, INHBA expression was markedly associated with clinical stage (P<0.001), N classification (P<0.001) and differentiation status (P=0.011).

Association between INHBA expression and patient survival

Survival analysis showed a clear negative correlation between INHBA protein expression level and the OS of patients with breast cancer (P=0.001, **Figure 4A**). In addition, Cox regression revealed that INHBA expression and PR expression were independent prognostic factors for OS (**Table 2**). Furthermore, we analysed the prognostic value of INHBA in selective patient subgroups stratified by clinical stage, N classification and differentiation. The expression of INHBA was strongly associated with OS duration in patients with well-differentiated tumour (**Figure 4F**, log-rank test, P<0.001), but not in patients with poorly differentiated tumour (**Figure 4G**, log-rank test, P=0.553). The expression of INHBA was also strongly associated with OS duration of the patients with both N0 tumours (**Figure 4D**, log-rank test, P=0.026) and N1-3 tumours (**Figure 4E**, log-rank test, P=0.004). However, when evaluated according to clinical stage, the impact on outcomes associated with the expression of INHBA was not statistically significant in both the stage I-II subgroup (**Figure 4B**, log-rank test, P=0.06) and the stage III subgroup (**Figure 4C**, log-rank test, P=0.083).

Discussion

Inhibin βA is a subunit of both activin and inhibin, which are two tightly related glycoproteins with opposite biological effects, and are members of the TGF-β superfamily [8-10]. Activins and inhibins produce opposing effects during different stages of cell growth, proliferation and differentiation by acting on the hypothalamic-pituitary-gonadal axis [11]. To initiate the activin cascade pathway, activin needs to bind with a complex of type I and type II single transmembrane serine/threonine kinase receptors. This interaction can trigger phosphorylation of the receptor and initiate activation of Smad proteins. The activated Smad protein complex

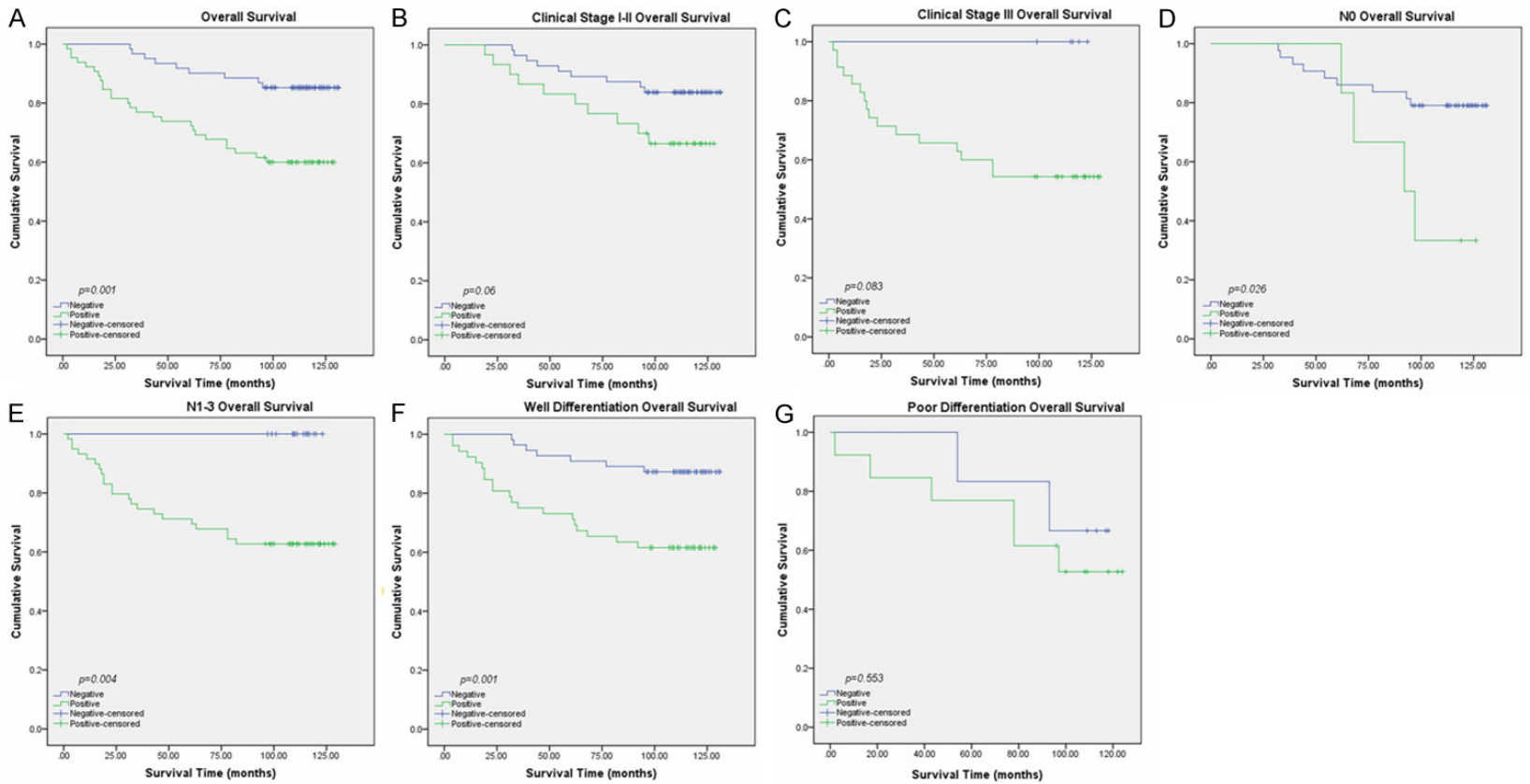

Figure 4. Kaplan-Meier curves of the univariate analysis (log-rank). A. OS rates for patients with high INHBA expression versus those with low INHBA expression levels. B. OS rate for early clinical stage cancer (stage I/ II) patients with high INHBA expression versus those with low INHBA expression. C. OS rate for late stage (stage III) patients with high INHBA expression versus those with low INHBA expression. D. OS rate for patients without lymphatic metastasis (N0) with high INHBA expression versus those patients with low INHBA expression. E. OS rate for patients with lymphatic metastasis (N1-3) with high INHBA expression versus those patients with low INHBA expression. F. OS rate for patients with well-differentiated tumours with high INHBA expression versus those patients with low INHBA expression. G. OS rate for patients with poorly differentiated tumours with high INHBA expression versus those patients with low INHBA expression.

Table 2. Cox-regression analysis of various prognostic parameters in patients for all patients

Factor	Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age				
<60	Reference			
≥60	0.794 (0.395-1.597)	0.518	—	—
Clinical stage				
I	Reference	0.045	—	—
II	2.612 (0.349-19.573)	0.35	—	—
III	5.436 (0.721-41.011)	0.101		
T classification				
T1	Reference	0.296		
T2	1.601 (0.613-4.182)	0.337	—	—
T3	2.682 (0.776-9.269)	0.119	—	—
N classification				
N0	Reference	0.123		
N1	0.694 (0.277-1.739)	0.435	—	—
N2	1.695 (0.759-3.784)	0.198	—	—
N3	2.407 (0.784-7.389)	0.125	—	—
Differentiation				
Well	Reference	0.187		
Moderate	4.036 (0.548-29.743)	0.171	—	—
Poor	6.255 (0.782-50.025)	0.084	—	—
Expression of ER				
Negative	Reference			
Positive	2.584 (1.328-5.028)	0.005	—	—
Expression of PR			Reference	
Negative	Reference		2.967 (1.475-5.970)	0.002
Positive	2.996 (1.490-6.025)	0.002		
Expression of HER2			—	—
Negative	Reference			
Positive	0.994 (0.477-2.069)	0.987	—	—
HNHBA expression				
Low	Reference		Reference	
High	0.302 (0.142-0.645)	0.002	0.305 (0.143-0.652)	0.002

then translocates into the nucleus where it can bind to the promoter of target genes and regulate gene transcription and cellular function [12]. However, inhibin exerts an opposing function by binding to type II receptors, which are mediated by the coreceptor betaglycan.

Many factors can influence the activin signalling pathway at the extracellular, membrane and intracellular phases. The interaction of the disulfide-linked homodimer of INHBA constitutes activin A, which was originally reported in 1978 for its role in the hypothalamic-pituitary-gonadal axis [13, 14]. Moreover, when combined with the β and α isoforms, INHBA forms activin AB and inhibin A, respectively, [15].

Activin A was identified as having an important role in embryonic stem cell differentiation [16] and tumourigenesis [17, 18]. Since then, many researchers have reported that the overexpression of activin A is association with oesophageal [17], lung [19], gastric [6], pancreatic [20], prostate [21], colon [22], ovarian [23, 24], endometrial and cervical cancers [25]. In accordance with these findings, the overexpression of INHBA also has been reported in various tumours such as tongue squamous cell

carcinoma, oesophageal adenocarcinoma, lung cancer, and gastric cancer [6, 7]. However, to the best of our knowledge, there are no reports that focus on the relationship of INHBA and breast cancer.

Based on the prevailing theory and our findings above, INHBA expression is likely associated with tumourigenesis and progression. In agreement with the aforementioned discoveries, our present study identified that INHBA expression was significantly elevated in breast cancer. Our results clearly showed that breast cancer lesions displayed higher INHBA expression at the mRNA and protein levels compared with non-cancerous tissues. Therefore, we consider INHBA as an important molecular marker of breast cancer that can help increase the precision of diagnoses.

We further analysed the relationship between the expression of INHBA and the clinical characteristics of patients with breast cancer. There was a significant correlation between INHBA expression and the clinical stage, N classification and differentiation. Meanwhile, there were no significant correlations between the expression of INHBA protein and patient age, T classification, oestrogen receptor (ER) expression levels, progesterone receptor (PR) expression levels or human epidermal growth factor receptor-2 (HER2) levels. However, the relationship between the expression of INHBA and clinical outcomes seems to be diverse in different cancers. Some researchers have even reported decreased expression of INHBA in carcinomas. For example, J Hofland et al. demonstrated lower expression of INHBA in adrenocortical carcinomas tissues [26]. Our data demonstrate that INHBA is an indicator of poor prognosis in breast cancer as measured by disease-specific and metastasis-free survival. To this point, the prognostic implication of INHBA in breast cancer has not been investigated. Our data demonstrate that INHBA is an indicator of poor prognosis in breast cancer. Multivariate analysis revealed that INHBA expression might be an independent prognostic indicator for OS in breast cancer patients (**Table 2**). This finding indicates the possibility of using high expression levels of INHBA as a predictor for patient prognosis and survival. Interestingly, a subgroup analysis revealed that among patients who had well-differentiated tumours, patients overexpressing INHBA had a significantly poor prognosis. And among patients with or without lymph node metastasis, overexpressing of INHBA was related to a significantly poor prognosis.

In conclusion, to the best of our knowledge, this is the first report addressing INHBA expression and its clinicopathological and prognostic significance in breast cancer. Our findings suggest that INHBA is up-regulated in breast cancer and is associated with clinical stage, N classification and differentiation. Multivariate analysis revealed that INHBA might be an independent biomarker for the prediction of breast cancer prognosis and survival. Therefore, testing INHBA protein levels may be helpful for stratifying patients for implementing a novel therapeutic strategy and establishing rational treatment selection criteria for breast cancer patients. Further investigation is also needed to investigate the molecular mechanism of INHBA involvement in the development and progression of breast cancer.

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Disclosure of conflict of interest

None.

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