HOXA1 upregulation is associated with poor prognosis and tumor progression in breast cancer

JINTAO LIU¹, JINQUAN LIU² and XINYI LU¹

¹Department of Breast Surgery, Dalian Central Hospital Affiliated to Dalian Medical University, Dalian, Liaoning 116033; ²Department of Clinical Medicine, Datong University School of Medicine, Datong, Shanxi 037009, P.R. China

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Abstract. Breast cancer (BC) is the most commonly diagnosed cancer and the second leading cause of cancer-associated mortality among females worldwide. As a member of the homeobox (HOX) gene family, HOXA1 is involved in tumor progression and prognosis in several types of human cancer. However, the clinical significance and biological functions of HOXA1 in BC remains unknown. The current study assessed the expression of HOXA1 in BC tissues and cells via western blotting and reverse transcription-quantitative polymerase chain reaction. The association between HOXA1 expression and the clinicopathological features of patients with BC was analyzed using the Chi-square test. The overall survival of patients was calculated using the Kaplan-Meier method and examined using the log-rank test. Cell proliferation was examined via an MTT assay. Cell cycle distribution and cell apoptosis were analyzed using flow cytometry. The current study demonstrated that HOXA1 mRNA and protein expression was upregulated in BC. In addition, HOXA1 overexpression was associated with poor prognosis and advanced clinicopathological features in patients with BC. Furthermore, knockdown of HOXA1 significantly inhibited cell proliferation by enhancing cell apoptosis and cell cycle arrest in BC cells, which was accompanied with aberrant expression of cell cycle and apoptosis-associated proteins, cyclin D1, B-cell lymphoma 2 (Bcl-2) and Bcl-2-like protein 4. Taken together, the results suggested that HOXA1 may serve as a novel prognostic marker and therapeutic target in BC.

Introduction

Breast cancer (BC) is the most commonly diagnosed cancer and the second leading cause of cancer-associated mortality among women worldwide (1). In 2017, ~252,710 new cases of

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invasive BC and 40,610 BC-associated mortalities occurred among females in the US (2). In China, an estimated 272,400 new cases and 70,700 BC-associated mortalities were reported in 2015 (3). The current primary treatment strategy for BC is surgical resection followed by hormonal therapy, chemotherapy, radiotherapy and/or biological therapy (4). Although recent advances in early diagnosis and targeted therapies in BC have been achieved, the molecular mechanism of BC progression remains poorly understood and the prognosis of patients with advanced BC remains low. It is therefore important to identify novel biomarkers associated with clinical prognosis that are also involved in the progression of BC.

The homeobox (HOX) gene family encodes homeodomain-containing transcription factors that participate in various physiological processes, which include embryonic development, cell proliferation and differentiation (5). In vertebrates, the HOX gene family contains 39 Hox genes, which are organized into four different chromosome clusters, each containing 9-11 genes (5,6). As a member of the HOX gene family, HOXA1 was previously reported to be involved in tumor progression and prognosis in certain types of human cancer (7-9). Yuan et al (7) reported that elevated HOXA1 expression levels enhance cell proliferation and poor prognosis in gastric cancer. In addition, Wang et al (8) demonstrated that HOXA1 is upregulated in prostate cancer and knockdown of HOXA1 suppresses the growth, invasion and migration of prostate cancer cells. Furthermore, Zha et al (9) reported that HOXA1 overexpression correlates with poor prognosis in patients with hepatocellular carcinoma. However, whether HOXA1 is associated with the prognosis of patients with BC remains unknown.

In the current study, the expression of HOXA1 in BC tissue and adjacent non-cancerous tissue samples from patients with BC was examined. The prognosis of patients with BC with high and low HOXA1 mRNA expression levels was assessed. In addition, the association between HOXA1 expression and clinicopathological features of patients with BC was examined. Furthermore, the effect of HOXA1 expression on BC cell proliferation, apoptosis and cell cycle distribution was assessed. The present study demonstrated that HOXA1 was upregulated in BC. HOXA1 overexpression was associated with a poor prognosis and advanced clinicopathological features in patients with BC. In addition,

Correspondence to: Dr Jintao Liu, Department of Breast Surgery, Dalian Central Hospital Affiliated to Dalian Medical University, 826 Southwest Road, Dalian, Liaoning 116033, P.R. China E-mail: cancer1979@126.com

knockdown of HOXA1 significantly inhibited cell proliferation by enhancing cell apoptosis and cell cycle arrest in BC cells, which was accompanied with the aberrant expression of cell cycle and apoptosis-associated proteins, cyclin D1, B-cell lymphoma 2 (Bcl-2) and Bcl-2-like protein 4. The current study demonstrated that HOXA1 may act as a novel marker in BC prognosis and a potential therapeutic target for the treatment of BC.

Materials and methods

Tissue collection. BC tissue and adjacent non-cancerous tissue samples were collected from 45 female patients (age range, 40-72 years) who underwent surgical resection between January 2007 and December 2012 at Dalian Central Hospital Affiliated to Dalian Medical University (Dalian, China). Following surgery, fresh tissue samples were frozen in liquid nitrogen and stored at -80°C until further use. All samples were evaluated and subject to histological diagnosis according to the World Health Organization criteria by two pathologists (10). All patients had regular follow-ups (1-60 months; median follow-up time, 52.4 months) following surgical resection and overall survival was recorded. The clinicopathological features of each patient, including age, tumor size, tumor, node and metastasis (TNM) stage and lymph node metastasis, as well as biological parameters, including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER)-2 status were recorded (Table I). The current study was conducted according to the World Medical Association Declaration of Helsinki and approved by the Ethics Committee of Dalian Central Hospital Affiliated to Dalian Medical University. Written informed consent was obtained from each patient prior to surgery.

Cell culture. Human BC cell lines BT549, T47D, MDA-MB-231 and MCF7 as well as the human breast epithelial cell line MCF-10A were obtained from the Cell Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The four BC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin mixture (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). MCF-10A cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 100 U/ml penicillin/streptomycin mixture (both Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Cell transfection. Cells were seeded into six-well plates at 30-40% confluence and following a 12 h incubation, cells were transfected with small interfering RNA (siRNA) targeting HOXA1 (si-HOXA1) or scrambled negative control siRNA (si-NC) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. si-HOXA1 (5'-CAACAAGUACCUUAC ACGA-3') and si-NC (5'-UUCUCCGAACGUGUCACG UTT-3') were purchased from Shanghai GenePharma Co. Ltd. (Shanghai, China). Following 48 h of transfection, cells were harvested and transfection efficiency was detected by

Table	I. As	sociation	between	HOXA1	expression	and c	clinico-
pathol	ogica	al features	s of fema	le patient	ts with BC.		

		HOXA1 e		
Clinicopathological features	Cases (n)	Low (n=22)	High (n=23)	P-value
Age (years)				
≤50	18	8	10	0.626
>50	27	14	13	
Tumor size (cm)				
≤3	17	10	7	0.299
>3	28	12	16	
ER status				
Negative	20	11	9	0.463
Positive	25	11	14	
PR status				
Negative	19	11	8	0.302
Positive	26	11	15	
HER2 status				
Negative	18	10	8	0.465
Positive	27	12	15	
Lymph node status				
Negative	25	16	9	0.023ª
Positive	20	6	14	
TNM stage				
I-II	26	17	9	0.01ª
III	19	5	14	

^aP<0.05. BC, breast cancer; HOXA1, homeobox A1; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNM, tumor, node and metastasis.

reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis prior to functional assays.

RNA extraction and RT-qPCR. Total RNA was extracted from tissue samples or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a PrimeScriptTM RT-PCR kit (Takara Bio, Inc., Otsu, Japan) for 15 min at 37°C and terminated by heating the samples at 85°C for 5 sec. HOXA1 expression was detected by qPCR, which was performed using SYBR-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primer pairs were used for qPCR: HOXA1 forward, 5'-CGGCTTCCTGTG CTAAGTCT-3' and reverse, 5'-TTCATTGTGCCATCCATC AC-3'; and β-actin forward, 5'-TTAGTTGCGTTACACCCT TTC-3' and reverse, 5'-ACCTTCACCGTTCCAGTTT-3'. The reaction conditions for PCR were as follows: 95°C for 3 min and 40 cycles of 95°C for 30 sec and 60°C for 30 sec. HOXA1 mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (11) and normalized to the internal control β -actin. RT-qPCR was repeated three times.

Western blot analysis. Total protein was extracted from tissue samples or cells using radioimmunoprecipitation assay buffer (Pierce; Thermo Fisher Scientific, Inc.) supplemented with protease inhibitor (78438; 1:100; Thermo Fisher Scientific, Inc.). Total protein was quantified using a bicinchoninic acid assay and equal amount of protein (30 μ g per lane) was separated via SDS-PAGE on a 10% gel. Separated proteins were transferred to polyvinylidene fluoride membranes and blocked with 5% non-fat milk at room temperature for 1 h. Membranes were washed three times and incubated with primary antibodies against HOXA1 (ab230513; 1:1,000), β-actin (ab8227; 1:2,000), cyclin D1 (ab40754; 1:2,000; all Abcam, Cambridge, UK), B-cell lymphoma 2 (cat. no. 4223, 1:1,000) and Bcl-2-like protein 4 (Bax; cat. no. 2774, 1:1,000; both Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. Following primary incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies [horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin G (IgG); sc-2005 and goat anti-rabbit IgG-HRP; sc-2004; 1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA] for 2 h at room temperature. Protein bands were visualized using the enhanced chemiluminescence detection system (Pierce; Thermo Fisher Scientific, Inc.) with an ECL kit (EMD Millipore, Billerica, MA, USA). Protein expression was quantified using β -actin as an internal control. Relative protein expression was analyzed using ImageJ software 1.4 (National Institutes of Health, Bethesda, MD, USA). Each experiment was performed in triplicate.

Cell proliferation. To examine the effect of HOXA1 on BC cell proliferation, MDA-MB-231 and MCF7 BC cell lines were transfected with si-HOXA1 and cell proliferation was assessed using the MTT assay. Briefly, transfected cells were seeded into 96-well plates at $3x10^3$ cells/well and cultured at 37° C. Following incubation for 24, 48, 72 and 96 h, 100 μ l full DMEM medium containing 0.5 mg/ml MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into each well and incubated for a further 4 h at 37° C. Subsequently, the medium was removed and 150 μ l dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan crystals. The absorbance was measured at 490 nm using a microplate reader (Molecular Devices, USA). Each experiment was performed in triplicate.

Cell cycle analysis. Cell cycle distribution was determined by propidium iodide (PI) staining. Following 48 h transfection with si-NC or si-HOXA1, MDA-MB-231 and MCF7 cells were seeded into 6-cm dishes at a confluence of 30% and incubated for 48 h. Cells were subsequently collected and fixed with 70% ethanol overnight at 4°C. Cells were washed twice with cold PBS and stained with PI (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature in the dark. The proportion of cells in each phase was analyzed using a BD FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). Each experiment was performed in triplicate.

Apoptosis assay. Cell apoptosis was analyzed by using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Pharmingen; BD Biosciences, San Jose, CA, USA). Following 48 h transfection with si-NC or si-HOXA1, cells were collected via centrifugation at a speed of 50 x g for 5 min at room temperature and resuspended in 200 μ l binding buffer. Cells were subsequently stained with 5 μ l Annexin V-FITC and 5 μ l PI together for 15 min at room temperature in the dark. Apoptotic cells were detected using a BD FACSCalibur Flow cytometer (BD Biosciences) and the rate of apoptosis was analyzed using SPSS software (version 18.0; SPSS, Inc.). Each experiment was performed in triplicate.

Statistical analysis. Data are presented as the mean \pm standard deviation of three replicates. Statistical analyses were performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA) and GraphPad Prism software (version 6.0; GraphPad Software, La Jolla, CA, USA). Expression levels of HOXA1 mRNA in BC tissue and adjacent non-cancerous tissue samples were compared using the Wilcoxon signed-rank nonparametric test. Student's t-test was used to analyze differences between two groups. Tukey's analysis following one-way analysis of variance was used to analyze differences among multiple groups. The association between HOXA1 expression and clinicopathological features of patients with BC was analyzed using the Chi-square test. The overall survival of patients was calculated using the Kaplan-Meier method, and the differences between survival curves were examined using the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

HOXA1 upregulation is associated with poor prognosis in patients with BC. To investigate the expression of HOXA1 in BC, the relative HOXA1 protein expression in BC tissue and adjacent non-cancerous tissue samples from patients with BC was determined by western blot analysis. HOXA1 protein expression was significantly increased in BC tissue compared with adjacent non-cancerous tissue samples (P<0.05; Fig. 1A). In addition, the relative HOXA1 mRNA expression levels in BC tissue and adjacent non-cancerous tissues from patients with BC were determined by RT-qPCR. HOXA1 mRNA expression levels were significantly increased in BC tissue compared with adjacent non-cancerous tissue samples (P<0.05; Fig. 1B). Patients with BC were divided into two groups: High (n=23) and low (n=22) HOXA1 expression based on the median mRNA expression level of HOXA1 (3.782 relative to β-actin) detected in BC tissue samples from patients with BC. To determine the effect of HOXA1 expression level on the survival of patients with BC, survival curves were plotted using the Kaplan-Meier method and the differences between survival curves were examined using the log-rank test. The overall survival of patients in the high HOXA1 expression group was significantly decreased compared with patients with BC in the low HOXA1 expression group (P=0.0344; Fig. 1C). These results suggested that HOXA1 was upregulated in patients with BC and HOXA1 upregulation may be associated with poor prognosis.



Figure 1. HOXA1 upregulation is associated with poor prognosis in patients with BC. (A) Relative HOXA1 protein expression levels were determined by western blot analysis in BC tissue and adjacent non-cancerous tissue samples from patients with BC. (B) Relative HOXA1 mRNA expression levels were determined by reverse transcription-quantitative polymerase chain reaction in BC tissue and adjacent non-cancerous tissue samples from patients with BC. (C) Overall survival of patients with BC with high or low HOXA1 expression was analyzed using the Kaplan-Meier method. Data are presented as the mean \pm standard deviation. *P<0.05 vs. Normal. HOXA1, homeobox A1; BC, breast cancer; T, breast cancer tissue; N, adjacent non-cancerous tissue.

HOXA1 upregulation is associated with tumor progression in BC. The association between HOXA1 expression and clinicopathological features of patients with BC was examined (Table I). The mRNA expression level of HOXA1 was significantly associated with lymph node metastasis (P=0.023) and TNM staging (P=0.01); however, no association with age, tumor size, and ER, PR or HER2 status was identified (P>0.05). These results suggested that HOXA1 upregulation may be associated with tumor progression in BC.

HOXA1 siRNA effectively silences HOXA1 gene expression in BC cells. To further confirm HOXA1 overexpression in BC, the mRNA and protein expression levels were determined in BC cell lines BT549, T47D, MDA-MB-231 and MCF7 and the normal breast epithelial cell line MCF-10A. HOXA1 mRNA expression levels were significantly increased in BC cell lines compared with MCF-10A (P<0.05; Fig. 2A and B). Similarly, western blot analysis revealed that HOXA1 protein expression levels were increased in BC cell lines compared with MCF-10A (Fig. 2B). To investigate the functional role of HOXA1 in BC progression, BC cell lines MDA-MB-231 and MCF7 were transfected with si-HOXA1 or si-NC and transfection efficiency was confirmed by RT-qPCR and western blot analysis. The results demonstrated that si-HOXA1 significantly downregulated HOXA1 mRNA (P<0.05) and markedly reduced protein expression in BC cell lines compared with the si-NC control (Fig. 2C and D).

Knockdown of HOXA1 inhibits BC cell proliferation. MTT assays were performed to investigate the effect of HOXA1 knockdown on cell proliferation in BC cell lines MDA-MB-231 and MCF7. Knockdown of HOXA1 significantly inhibited BC cell proliferation compared with si-NC at \geq 72 h (P<0.05; Fig. 3A and B). In addition, PI staining was used to assess cell cycle distribution using flow cytometry in BC cell lines MDA-MB-231 and MCF7 following transfection with si-HOXA1 or si-NC. The percentage of cells in G1 phase was significantly increased, whilst those in S phase were significantly decreased in BC cells following HOXA1 knockdown (P<0.05; Fig. 3C and D). These results suggested that knockdown of HOXA1 may inhibit BC cell proliferation partly by suppressing cell cycle progression.

Knockdown of HOXA1 promotes BC cell apoptosis. Cell apoptosis was analyzed in BC cell lines MDA-MB-231 and MCF7 following transfection with si-HOXA1 or si-NC. Flow cytometry revealed that knockdown of HOXA1 significantly enhanced BC cell apoptosis (P<0.05; Fig. 4A and B). In addition, western blot analysis was used to determine the expression level of cell cycle and apoptosis-associated proteins cyclin D1, Bax and Bcl-2 following transfection with si-HOXA1 or si-NC. The protein expression levels of cyclin D1 and Bcl-2 markedly decreased, whilst the levels of Bax markedly increased in BC cells following HOXA1 knockdown (Fig. 4C and D). Taken together, these results suggested that knockdown of HOXA1 may inhibit BC cell proliferation partly by inducing cell apoptosis.

Discussion

BC is the most common malignancy among females worldwide and as the incidence of BC has been increasing over the past



Figure 2. si-HOXA1 effectively silences HOXA1 gene expression in BC cell lines. (A) Relative HOXA1 mRNA expression levels determined by reverse transcription-quantitative polymerase chain reaction in BC cell lines MDA-MB-231, BT549, MCF7 and T47D and the normal breast epithelial cell line MCF-10A. (B) Relative HOXA1 protein expression was determined by western blot analysis in BC cell lines (MDA-MB-231, BT549, MCF7 and T47D) and the normal breast epithelial cell line MCF-10A. (C) Relative HOXA1 mRNA expression level in BC cell lines MDA-MB-231 and MCF7 following transfection with si-HOXA1. (D) Relative HOXA1 protein expression in BC cell lines MDA-MB-231 and MCF7 following transfection with si-HOXA1. (D) Relative HOXA1 protein expression in BC cell lines MDA-MB-231 and MCF7 following transfection with si-HOXA1. Data are presented as the mean ± standard deviation. *P<0.05 vs. MCF-10A and *P<0.05 vs. si-NC. HOXA1, homeobox A1; BC, breast cancer; si, small interfering RNA; NC, negative; si-HOXA1, siRNA targeting HOXA1.



Figure 3. Knockdown of HOXA1 inhibits BC cell proliferation. Cell proliferation was examined in (A) MDA-MB-231 and (B) MCF7 BC cell lines by MTT assay following transfection with si-HOXA1. Cell cycle distribution was examined in (C) MDA-MB-231 and (D) MCF7 BC cell lines by flow cytometry following transfection with si-HOXA1 or si-NC. Data are presented as the mean ± standard deviation. *P<0.05 vs. si-NC. HOXA1, homeobox A1; BC, breast cancer; si, small interfering RNA; NC, negative; si-HOXA1, siRNA targeting HOXA1.



Figure 4. Knockdown of HOXA1 promotes BC cell apoptosis. Cell apoptosis was analyzed in (A) MDA-MB-231 and (B) MCF7 BC cell lines by flow cytometry following transfection with si-HOXA1. Relative protein expression of cyclin D1, Bax and Bcl-2 was determined by western blot analysis in (C) MDA-MB-231 and (D) MCF7 BC cell lines following transfection with si-HOXA1. Data are presented as the mean ± standard deviation. *P<0.05 vs. si-NC. HOXA1, homeobox A1; BC, breast cancer; si, small interfering RNA; NC, negative; si-HOXA1, siRNA targeting HOXA1; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-like protein 4; PI, propidium iodide; FITC, fluorescein isothiocyanate.

years, it is considered to be a serious health risk (12,13). Thus, a better understanding of the molecular mechanisms involved in BC initiation and progression as well as validation of novel prognostic biomarkers may have a potential clinical significance for patients with BC. Previous studies demonstrated that several HOX genes, including HOXA5, HOXB9 and HOXC8 were aberrantly expressed in BC and served roles in tumor progression in BC (14-16). Additionally, HOXA1 is involved in the development and prognosis of several types of human cancer, including gastric cancer, prostate cancer and hepatocellular carcinoma (7-9). However, the clinical significance of HOXA1 in BC remains unknown.

In the current study, the mRNA and protein expression levels of HOXA1 in BC tissue and adjacent non-cancerous tissue samples were determined via RT-qPCR and western blot analysis, respectively. The results demonstrated that both HOXA1 mRNA and protein expression levels were significantly upregulated in BC tissue compared with non-cancerous tissue samples from patients with BC. Survival analysis indicated that patients with high HOXA1 expression had a significantly poorer overall survival compared with patients with low HOXA1 expression. In addition, the association between HOXA1 expression and clinicopathological features of patients with BC was examined and HOXA1 expression was associated with lymph node metastasis and TNM staging. No association with age, tumor size and ER, PR or HER2 status was identified. Taken together, these results suggested that HOXA1 upregulation may be associated with poor prognosis and tumor progression in BC.

To investigate the biological function of HOXA1 in BC, BC cell lines MDA-MB-231 and MCF7 were transfected with si-HOXA1 or si-NC. Knockdown of HOXA1 significantly inhibited cell proliferation and induced apoptosis and cell cycle arrest in BC cells. Furthermore, the expression of cell cycle and apoptosis-associated proteins, cyclin D1, Bax and Bcl-2 were examined. Western blot analysis revealed that the protein expression levels of cyclin D1 and Bcl-2 decreased, whilst the level of Bax increased following knockdown of HOXA1 in BC cells. Together, these results suggest that HOXA1 may be involved in the development of BC.

The underlying mechanism of HOXA1 in BC progression was not investigated in the current study and remains unclear. A recent study demonstrated that HOXA1 activates nuclear factor (NF)- κ B and operates upstream of the NF- κ B inhibitor, I κ B to modulate the tumor necrosis factor- α /NF- κ B signaling pathway (17). In addition, a previous study reported

that HOXA1 regulates tumor progression in gastric cancer via cyclin D1 (7). Furthermore, previous studies demonstrated that in different types of cancer, HOXA1 is regulated by several microRNAs (miR), including miR-99a, miR-30c and miR-100 (18-20). Therefore, further studies are required to investigate the underlying mechanism of HOXA1 in BC.

In conclusion, the current study demonstrated that HOXA1 mRNA and protein expression levels were significantly upregulated in BC. In addition, HOXA1 overexpression was associated with poor prognosis and tumor progression in BC. Furthermore, knockdown of HOXA1 significantly inhibited cell proliferation by inducing apoptosis and cell cycle arrest in BC cells. Together, these findings suggested that HOXA1 may serve as a novel prognostic marker and a potential therapeutic target in BC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JintL designed the current study. JintL and JinqL performed the experiments. JingL and XL collected and analyzed the data. JintL and XL wrote and revised the manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of Dalian Central Hospital Affiliated to Dalian Medical University. Written informed consent was obtained from each patient.

Patient consent for publication

Patients provided written informed consent for the publication of any associated data from their samples.

Competing interests

The authors declare that they have no competing interests.

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