Homeodomain-containing gene 10 contributed to breast cancer malignant behaviors by activating Interleukin-6/Janus kinase 2/Signal transducer and activator of transcription 3 pathway

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ABSTRACT

Homeodomain-containing gene 10 (HOXC10) has been identified as an oncogene in various malignancies. Nevertheless, the role and function of HOXC10 in breast cancer (BC) remain unclear. RT-qPCR and Western blot were used to detect the mRNA and protein levels of genes, respectively. CCK-8, transwell, and TUNEL assays were performed to evaluate cell viability, invasion, migration, and apoptosis of BC cells in vitro. The xenograft model was established to examine the effect of HOXC10 on tumor growth in vivo. Our results indicated that HOXC10 expression was increased in BC and correlated with an unsatisfactory prognosis. Functional assays indicated that HOXC10 overexpression promoted cell proliferation and metastasis, and suppressed cell apoptosis of BC, while HOXC10 knockdown showed opposite trends. Furthermore, in vitro and in vivo assays uncovered that HOXC10 promoted the tumorigenesis of BC via the activation of IL-6/JAK2/STAT3 signaling. Overall, our study revealed that HOXC10 could function as a tumor promoter in BC by upregulating IL-6 levels to activate the JAK2/STAT3 signaling pathway.

Introduction

Breast cancer (BC) is the second most common cancer in the world and ranks first among the malignant tumors in women [1]. There are approximately 626,679 deaths and a total of 2.09 million new cases in 2018 alone [2] and the incidence of young women diagnosed with BC is increasing [3]. Although comprehensive treatments like surgery, chemotherapy, radiotherapy, targeted therapy, and biotherapy are used to treat BC, the overall survival rate is still unsatisfactory [4], mainly due to distant metastasis and recurrence [5]. Therefore, continuous researches on BC are needed to elucidate the underlying mechanism and discover more efficient treatments for BC patients.

Homeobox (HOX) genes are evolutionarily conserved genes that contribute to cell differentiation and embryonic development [6]. A total of 39 class I HOX genes exist in the human genome and are distributed in four genomic clusters (A, B, C, and D) which are located on 2, 7, 12, and 17 chromosomes, respectively [7]. A previous study reported that differential expression of HOX gene clusters can discriminate between tumor samples and healthy samples [7]. As a member of the HOX family, homeodomain-containing gene 10 (HOXC10) is associated with the progression of various human malignancies [8]. For instance, decreased HOXC10 level in liver cancer attenuates cell proliferation via interaction with miR-221 and activation of MAPK pathway [9]; HOXC10 exacerbates ovarian cancer malignancy by elevating epithelial–mesenchymal transition (EMT)-related gene Slug expression [10]; HOXC10 strengthens cell invasion and migration abilities in gastric cancer through the NF-kappaB pathway [11]. However, expression levels of HOXC10 in different types of cancers are diverse and its functions in cancers are also contradictory. HOXC10 has been reported to be upregulated in BC [12], while the detailed function of HOXC10 in BC remain to be clarified.

The present study aimed to explore the effects of HOXC10 on the proliferation, migration, invasion, and apoptosis in vitro and tumor growth in vivo. We
hypothesized that HOXC10 might act as an oncogene in BC progression. Our results may provide novel insights into the carcinogenesis of BC.

**Materials and methods**

**Patients and samples**

A total of 56 pairs of BC tissues and adjacent normal tissues were gained from patients with BC at the First People’s Hospital of Lianyungang Hospital. Specimens were stored in liquid nitrogen at −80°C instantly after collection from surgery for future use. This study was approved by the Ethics Committee of the First People’s Hospital of Lianyungang. All patients involved in this study signed written informed consent.

**Cell culture and treatment**

The human BC cell lines (MCF7, T47D, MDA-MB-453, and MDA-MB-231) and human breast epithelial cells (MCF10A) were acquired from the Chinese Academy of Sciences Cell Bank (Shanghai). Cells were cultured in DMEM (HyClone) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO₂. IL-6-neutralizing antibody (R&D Systems) was applied at a concentration of 0.5 µg/ml.

**Cell transfection**

Short hairpin RNA (shRNA) against HOXC10 (sh-HOXC10), sh-NC, HOXC10 overexpression plasmid (pcDNA3.1/HOXC10), and pcDNA3.1 vector were obtained from GenePharma (Shanghai). Transfection of these plasmids into BC cells was conducted via Lipofectamine 2000 (Invitrogen).

**RT-qPCR**

Total RNA was extracted from tissues and cells using TRIZol® (Invitrogen) and reversely transcribed into cDNA using the cDNA Reverse Transcription kit (Invitrogen). Subsequently, ABI7300 system (Applied Biosystems) with SYBR-Green PCR Master Mix kit (Takara Bio) was used to perform qPCR. Expression levels were calculated using the 2^−ΔΔCq method and normalized to GAPDH.

**Western blot**

The protein was extracted using RIPA buffer (Beyotime), separated by 10% SDS-PAGE, and then transferred to PVDF membranes. The membrane was cultured with primary antibodies against HOXC10, Interleukin-6 (IL-6, ab233706, Abcam), Janus kinase 2 (JAK2, ab108596, Abcam), phospho-JAK2 (p-JAK2, ab195055, Abcam), Signal transducer and activator of transcription 3 (STAT3, ab68153, Abcam), phospho-STAT3 (p-STAT3, ab267373, Abcam), and GAPDH (ab8245, Abcam) at 4°C overnight, followed by incubation with secondary antibody for 1 h at 37°C. The enhanced chemiluminescent (ECL) reagent (Beyotime) was employed to visualize protein bands, and quantification was performed using IMAGE J software (National Institutes of Health) [13].

**CCK-8**

The transfected BC cells (1x10⁴ cells/well) were seeded into 96-well plates. At 0, 24, 48, and 72 h, 10 µl CCK-8 solution (Dojindo) was added to each well and incubated for 2 h. The absorbance was measured at 450 nm using a microplate reader [14].

**Transwell assay**

To determine cell invasion ability, transwell chambers (EMD Millipore) were coated with Matrigel (BD, USA) [15]. BC cells were seeded in serum-free DMEM in the upper chamber. Then 600 µL DMEM with 10% FBS was added to the lower chamber. After incubation for 24 h, the cells were fixed and stained. Finally, the invaded cell numbers were counted using a light microscope. The aforementioned steps were repeated to measure cell migration except that the membrane on the upper layer was not coated with Matrigel.

**TUNEL assay**

TUNEL assay was used to analyze cell apoptosis rate [16]. Briefly, BC cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. TUNEL reaction mixture was subsequently incubated with the cells for 1 h. Next, the TUNEL-stained cells were counterstained
Figure 1. HOXC10 is highly expressed in BC.
(a) GEPIA website showed the HOXC10 expression in breast invasive carcinoma samples and normal tissues. (b and c) RT-qPCR and IHC showed the HOXC10 expression in BC tissues and adjacent normal tissues. Kaplan-Meier analysis revealed the survival rate of BC patients with high or low expression of HOXC10. (e) RT-qPCR showed the HOXC10 expression in BC cell lines (MCF7, T47D, MDA-MB-453 and MDA-MB-231) and human breast epithelial cells (MCF10A). *P < 0.05; **P < 0.01.
with DAPI (2 μg/ml; Beyotime Institute of Biotechnology). Images were acquired from five randomly selected fields of view using a fluorescence microscope.

**In vivo assay**

Six Male BALB/c nude mice were randomly divided into 2 groups and maintained under pathogen-free conditions. Then, mice were inoculated with MCF7 cells (1×10⁶) with transfection of shHOXC10 or shNC by subcutaneous injection. Every 7 days, tumor volume was recorded, and mice were sacrificed after 4 weeks. The animal experiments were approved by the First People’s Hospital of Lianyungang.

**Immunohistochemistry (IHC)**

Tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 μm sections. Next, sections were incubated with HOXC10 or Ki67 antibodies at 4°C overnight. After incubation with secondary antibody, the sections were stained with diaminobenzidine and photographed under a microscope.

**Statistical analysis**

Each experiment is repeated no less than three times and the results are presented as the mean ± SD. SPSS 18.0 software (IBM) was used for statistical analysis. Comparisons between different groups are conducted by Student’s t-test and one-way ANOVA. The association between HOXC10 expression and clinicopathological characteristics of patients with BC was analyzed using χ² tests. Kaplan–Meier analysis and the log-rank test are employed to analyze survival rates. p < 0.05 is considered statistically significant.

**Results**

This study elaborated the HOXC10-mediated mechanism in BC. The results obtained from our research demonstrated that HOXC10 promoted BC malignancy via IL-6/JAK2/STAT3 pathway. These findings might offer a potential therapeutic target for BC treatment.

**HOXC10 expression is augmented in BC**

According to data from GEPIA website [17], HOXC10 expression was remarkably upregulated in breast invasive carcinoma (Figure 1(a)). To validate the result in BC, RT-qPCR and IHC were employed to compare HOXC10 expression between BC tissues and non-tumor tissues. Both assays indicated that the expression of HOXC10 was significantly upregulated in BC tissues (Figure 1(b,c)). Moreover, high HOXC10 expression was associated with a shorter survival time of BC patients (Figure 1(d)). Next, analysis of clinical pathological features identified that high levels of HOXC10 was positively correlated with tumor sizes, lymph node metastasis, TNM stage, and distant metastases (Table 1). Furthermore, the expression of HOXC10 was highly expressed in BC cell lines, particularly in MCF7 and MDA-MB-231 in comparison with that in MCF10A (Figure 1(e)). The aforementioned results implicated that HOXC10 expression was elevated in BC and the high HOXC10 expression contributed to poor prognosis.

**HOXC10 overexpression facilitates cell proliferation and metastasis in BC**

To explore the role of HOXC10 in BC, overexpression HOXC10 plasmid was transfected into MCF7 and MDA-MB-231 cells. As shown in Figure 2(a,b), mRNA and protein levels of HOXC10 were both elevated after the transfection. Next, functional assays were performed to determine the effect of...
HOXC10 overexpression on BC cellular activities. As presented in Figure 2(c), CCK-8 assay revealed that cell proliferation was significantly enhanced. In transwell assays, cell invasion and migration abilities were both increased by HOXC10 overexpression (Figure 3(d,e)). TUNEL assay revealed that HOXC10 overexpression notably reduced apoptosis of BC cells (figure 3(f)). All in all, HOXC10
overexpression promoted cell proliferation and metastasis of BC.

**HOXC10 knockdown suppresses cell growth and metastasis in BC**

With the purpose of re-validate the role of HOXC10 in BC, the influence of HOXC10 knockdown was evaluated. The HOXC10 level in BC cells was reduced following HOXC10 knockdown (Figure 3(a,b)). Subsequently, CCK-8, transwell, and TUNEL assays showed that silence of HOXC10 inhibited the abilities of cell proliferation, invasion, and migration but increased cell apoptosis (Figure 3(c-f)). The aforementioned results demonstrated that BC cell growth and metastasis could be inhibited by HOXC10 knockdown.

*Figure 3. HOXC10 knockdown suppresses cell growth and metastasis in BC.
(a, b) RT-qPCR and Western blot showed the HOXC10 expression in MCF7 and MDA-MB-231 cells transfected with sh-NC and sh-HOXC10. (c-f) The viability, migration, invasion and apoptosis of MCF7 and MDA-MB-231 cells following HOXC10 depletion were evaluated by CCK-8, transwell and TUNEL assay. *P < 0.05; **P < 0.01; ***P < 0.001.
HOXC10 regulates IL6-JAK2/STAT3 pathway in BC

IL-6/JAK2/STAT3 pathway has been reported to participate in the tumorigenesis of BC. For instance, P16 promoted BC progression via IL-6/JAK2/STAT3 pathway (23). TrkB triggered the potential of BC metastasis through activating IL-6-JAK2/STAT3 pathways (24). Therefore, the expressions of IL6, p-JAK2, and p-STAT3 were measured by Western blot. It was found that HOXC10 overexpression upregulated the expression of IL-6 (Figure 4(a)), while HOXC10 knockdown downregulated IL-6 expression (Figure 4(b)). In addition, HOXC10 expression was positively correlated with IL-6 in the BC tissues (Figure 4(c)). Moreover, the upregulation of HOXC10 increased the levels of p-JAK2 and p-STAT3 (Figure 4(d)), while HOXC10 silencing exhibited an opposite result (Figure 4(e)). These data indicated that HOXC10 could positively regulate IL-6 expression and activate the JAK2/STAT3 pathway in BC.

HOXC10 facilitates BC development via IL6-JAK2/STAT3 pathway

To validate whether HOXC10 facilitated BC malignancy via the IL6-JAK2/STAT3 signaling pathway, MCF7 and MDA-MB-231 were first
overexpressed with HOXC10 then treated with an IL-6-neutralizing antibody. As presented in CCK-8 assay, increased HOXC10 level remarkably augmented cell viability but the addition of the IL-6-neutralizing antibody partially reversed the augmentation (Figure 5(a)). Similarly, in transwell assays, notably strengthened invasion and migration abilities of BC cells caused by overexpressing HOXC10 were partially restored by IL-6-neutralizing antibody treatment (Figure 5(b,c)). Moreover, TUNEL assay indicated that HOXC10 overexpression significantly reduced BC cell apoptosis while IL-6-neutralizing antibody abrogated this effect (Figure 5(d)). To summarize, HOXC10 promoted BC progression via IL6-JAK2/STAT3 signaling in vitro.
**HOXC10 silencing attenuates BC tumorigenesis via IL6-JAK2/STAT3 pathway in vivo**

Xenograft experiments were performed to validate the effect of HOXC10 on BC progression in vivo, and results showed that the volume and weight of tumor were reduced in shHOXC10 group compared to control group (Figure 6(a, b)). Moreover, IHC results demonstrated that the level of Ki-67 was decreased in the HOXC10 knockdown group (Figure 6(c)). In addition, the levels of IL-6, p-JAK2, and p-STAT3 were decreased in shHOXC10 group compared with shNC group (Figure 6(d)). In sum, these results demonstrated that HOXC10 contributed to the tumorigenesis of BC through IL6-JAK2/STAT3 pathway in vivo.

**Discussion**

With the rapid development of next-generation sequencing technology, an increasing number of protein-coding genes or non-coding RNAs have been discovered, which are further identified as biomarkers for diagnosis, treatment, and prognosis of various human cancers, including BC [18–20]. For example, RBMS2 expression is low expressed in BC and suppresses BC progression [21]. Level of HuR is elevated at all stages of BC and leads to unsatisfactory clinical outcomes [22]. Increased expression of SIPL1 in BC augments the risk of BC [23]. HOXC10 was previously detected to be highly expressed in BC [12], but its functions and mechanism in BC remain largely unknown. The present research investigated HOXC10 expression as well as the molecular mechanism of HOXC10 in BC. Our data indicated that HOXC10 expression was increased in BC and correlated with an unsatisfactory prognosis. Furthermore, HOXC10 was overexpressed or knocked down to evaluate the effect of HOXC10 expression changes on malignant behaviors of BC. The results indicated that increased or decreased HOXC10 levels could promote or inhibit BC cell growth and metastasis, respectively.

The IL6-JAK2/STAT3 signaling is that IL-6 receptors activate JAK2, which in turns phosphorylate STAT3 [24]. Previous studies have disclosed that IL6-JAK2/STAT3 pathway is closely related to multiple cellular behaviors and is one of the most typical oncogenic signaling pathways in cancers. Jiang et al. described IL6 signaling regulated by NFAT1 aggravated glioma phenotypes [25]. Ni et al. reported that miR-515-5p targeted IL6-JAK/STAT3 signaling and inhibited migration and invasion of HCC [26]. Yao et al. demonstrated that IL-6-JAK/STAT3 pathway was correlated with unsatisfactory prognosis in glioma patients [27]. In our study, HOXC10 could positively regulate IL-6 expression and activate the JAK2/STAT3 signaling in BC. The mechanistic investigation demonstrated that IL-6-neutralizing antibody reversed the impact of HOXC10 overexpression on BC progression. Finally, in vivo assay demonstrated that HOXC10 knockdown inhibited tumor growth via IL-6/JAK2/STAT3 axis in BC.
Conclusion

Our results demonstrated that HOXC10 was significantly elevated in BC, and HOXC10 could augment the progression of BC through IL6-JAK2/STAT3 pathway. These findings might provide novel therapeutic directions for BC treatment. In the future, BC cells transfected with shHOXC10 and additional treated with IL-6 will be used to further validate the molecular mechanisms of HOXC10 in BC.

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References

[1] Wu X, Chen C, Luo B, et al. Nuclear ING3 expression is correlated with a good prognosis of breast cancer. Front Oncol. 2020;10:589009.
[2] Gargiulo L, Rivero EM, Di Siervi N, et al. Agonist effects of propranolol on non-tumor human breast cells. Cells. 2020. 9(4):1036.
[3] Houghton LC, Howland RE, McDonald JA. Mobilizing breast cancer prevention research through smartphone apps: a systematic review of the literature. Front Public Health. 2019;7:298.
[4] Du L, Li X, Zhen L, et al. Everolimus inhibits breast cancer cell growth through PI3K/AKT/mTOR signaling pathway. Mol Med Rep. 2018;17:7163–7169.
[5] Wang Z, Li TE, Chen M, et al. miR-106b-5p contributes to the lung metastasis of breast cancer via targeting CNN1 and regulating Rho/ROCK1 pathway. Aging (Albany NY). 2020;12:1867–1887.
[6] Xie X, Xiao Y, Huang X. Homeobox C10 knockdown suppresses cell proliferation and promotes cell apoptosis in osteosarcoma cells through regulating caspase 3. Onco Targets Ther. 2018;11:473–482.
[7] Adato O, Orenstein Y, Kopolovic J, et al. Quantitative analysis of differential expression of HOX genes in multiple cancers. Cancers (Basel). 2020;12(6):1572.
[8] Dai BW, Yang ZM, Deng P, et al. HOXC10 promotes migration and invasion via the WNT-EMT signaling pathway in oral squamous cell carcinoma. J Cancer. 2019;10:4540–4551.
[9] Ma K, Zhao C, Guo K, et al. Low HOXC10 expression in liver cancer regulates proliferation via a mechanism involving miR-221 and the MAPK signaling pathway. Oncol Lett. 2020;20:127.
[10] Peng Y, Li Y, Li Y, et al. HOXC10 promotes tumour metastasis by regulating the EMT-related gene Slug in ovarian cancer. Aging (Albany NY). 2020;12:19375–19398.
[11] Yao S, He L, Zhang Y, et al. HOXC10 promotes gastric cancer cell invasion and migration via regulation of the NF-κB pathway. Biochem Biophys Res Commun. 2018;501:628–635.
[12] Ansari KI, Hussain I, Kasiri S, et al. HOXC10 is over-expressed in breast cancer and transcriptionally regulated by estrogen via involvement of histone methylases MLL3 and MLL4. J Mol Endocrinol. 2012;48:61–75.
[13] Zhang Q, Wang Z, Cheng X, et al. lncRNA DANCR promotes the migration an invasion of trophoblast cells through microRNA-214-5p in preeclampsia. Bioengineered. 2021;12(2):9424–9434.
[14] Zhou X, Wang Z, Xu B, et al. Long non-coding RNA NORAD protects against cerebral ischemia/reperfusion injury induced brain damage, cell apoptosis, oxidative stress and inflammation by regulating miR-30a-5p/YWHAG. Bioengineered. 2021;12:9174–9188.
[15] Zhang M, Yang L, Hou L, et al. LncRNA SNHG1 promotes tumor progression and cisplatin resistance through epigenetically silencing miR-381 in breast cancer. Bioengineered. 2021;12:9239–9250.
[16] Sun M, Geng D, Li S, et al. LncRNA PART1 modulates toll-like receptor pathways to influence cell proliferation and apoptosis in prostate cancer cells. Biol Chem. 2018;399:387–395.
[17] Tang Z, Li C, Kang B, et al. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res. 2017;45(W1):W98–w102.
[18] You ZP, Zhang YL, Li BY, et al. Bioinformatics analysis of weighted genes in diabetic retinopathy. Invest Ophthalmon Vis Sci. 2018;59:5558–5563.
[19] Jiang N, Meng X, Mi H, et al. Circulating LncRNA XLOC_009167 serves as a diagnostic biomarker to predict lung cancer. Clin Chim Acta. 2018;486:26–33.
[20] Su J, Zhang X, Zhao Q, et al. PD-1 mRNA expression in peripheral blood mononuclear cells as a biomarker for different stages of primary gouty arthritis. J Cell Mol Med. 2020;24(16):9323–9331.
[21] Sun X, Hu Y, Wu J, et al. RBMS2 inhibits the proliferation by stabilizing P21 mRNA in breast cancer. J Exp Clin Cancer Res. 2018;37:298.
[22] Zhang Z, Huang A, Zhang A, et al. HuR promotes breast cancer cell proliferation and survival via binding
to CDK3 mRNA. Biomed Pharmacother. 2017;91:788–795.

[23] De Melo J, Tang D. Elevation of SIPL1 (SHARPIN) increases breast cancer risk. PLoS One. 2015;10: e0127546.

[24] Dambal S, Alfaqih M, Sanders S, et al. 27-hydroxycholesterol impairs plasma membrane lipid raft signaling as evidenced by inhibition of IL6-JAK-STAT3 signaling in prostate cancer cells. Mol Cancer Res. 2020;18:671–684.

[25] Jiang Y, Han S, Cheng W, et al. NFAT1-regulated IL6 signalling contributes to aggressive phenotypes of glioma. Cell Commun Signal. 2017;15(1):54.

[26] Ni JS, Zheng H, Ou YL, et al. miR-515-5p suppresses HCC migration and invasion via targeting IL6/JAK/STAT3 pathway. Surg Oncol. 2020;34:113–120.

[27] Yao Y, Ye H, Qi Z, et al. B7-H4(B7x)-mediated cross-talk between glioma-initiating cells and macrophages via the IL6/JAK/STAT3 pathway lead to poor prognosis in glioma patients. Clin Cancer Res. 2016;22:2778–2790.