Thoracic Cancer

Open Access

Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

Oncogenic HBXIP enhances ZEB1 through Sp1 to accelerate breast cancer growth

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Keywords
Breast cancer; growth; HBXIP; Sp1; ZEB1.

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Received: 24 July 2018;
Accepted: 25 August 2018.

doi: 10.1111/1759-7714.12878

Thoracic Cancer **9** (2018) 1664–1670

Abstract

Background: There is abundant evidence to indicate that HBXIP functions as an oncoprotein and transcription co-activator during the development and promotion of cancers. In multiple cancers, ZEB1 serves as a transcription activator to regulate gene expression. We explored the roles of ZEB1 in HBXIP-induced breast cancer growth.

Methods: HBXIP regulation of ZEB1 was evaluated by reverse transcription PCR and immunoblotting. The stimulation of ZEB1 promoter by HBXIP and/or Sp1 was tested using luciferase reporter gene analysis. The alteration of cell proliferation mediated by HBXIP-induced ZEB1 was tested using methyl-thiazolyl-tetrazolium and 5-Ethynyl-2′-deoxyuridine (EdU) incorporation analysis. ZEB1 and HBXIP expression in human breast cancer tissues was analyzed using quantitative real-time PCR. The relationship between HBXIP and ZEB1 was confirmed by Pearson's correlation coefficient.

Results: We observed dose-dependent upregulation of ZEB1 by HBXIP in breast cancer cells. HBXIP can activate the ZEB1 promoter by interacting with transcription factor Sp1. Cell viability and EdU incorporation analysis showed that HBXIP could drive cell proliferation by enhancing ZEB1 in breast cancer. Using quantitative real-time PCR, ZEB1 overexpression and a positive relationship between ZEB1 and HBXIP were observed in clinical breast cancer samples.

Conclusion: Oncogenic HBXIP controls the transcription regulation of ZEB1 by co-activating Sp1, thereby accelerating breast cancer growth.

Introduction

Breast cancer is a major cause of mortality in women around the world.[1] Although HBXIP was first detected as a result of binding to HBX,[2] this protein is constitutively expressed in many tissues. HBXIP is one member of a regulator complex involved in amino acid-mediated mTORC1 activation.[3] Many investigations have indicated that HBXIP is an oncoprotein and can affect the progression of various cancers, including gastric adenocarcinoma; urothelial carcinoma of the bladder; and ovarian, breast, lung, and liver cancers.[4–13] Researchers have found that elevated HBXIP promotes breast cancer progression;[14–16] however, the mechanism of how HBXIP affects cancer development requires further investigation.

TCF8 gene-coded ZEB1 is a transcription factor that is involved in the malignancy of a variety of cancers. Overexpressed ZEB1 is related to the malignant development of prostate cancer and is thought to be a predictive biomarker for metastasis.[17,18] Elevated-ZEB1 accompanied by low E-cadherin is associated with the migration and invasion of endometrioid cancer cells.[19,20] There is a close correlation between high ZEB1 and the migration or metastasis of multiple cancers, especially colorectal cancer.[21–23] The balance among SNAIL, VDR, and ZEB1 might control the characteristics of colon cancer.[24] ZEB1 can induce

non-cancer stem cells (non-CSCs) to CSC plasticity and promotes carcinogenesis.[25] However, whether ZEB1 is implicated in HBXIP-induced breast cancer growth has not been explored.

In the current investigation, we clarify the modulation of HBXIP on ZEB1 in breast cancer. Interestingly, our data shows that HBXIP can stimulate ZEB1 expression by co-activating Sp1, resulting in increased cell proliferation of breast cancer. Our findings present the molecular mechanism of ZEB1 elevation by oncogenic HBXIP in breast cancer.

Methods

Plasmids and small interfering RNAs

The promoter region of ZEB1 was cloned from genomic DNA of MCF-7 cells and inserted into the KpnI/Hind III site of pGL3-Basic plasmid (Promega, Madison, WI, USA). pRL-TK was used as an internal vector in luciferase assay to normalize pGL3-Basic activity. Small interfering RNAs (siRNAs) targeting HBXIP, ZEB1, or Sp1 were purchased from RiboBio (Guangzhou, China).

Cell culture

Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum was utilized to maintain MCF-7 and MCF-7-HBXIP (stably HBXIP-expressed MCF-7 cells) according to ATCC protocol.

Reverse-transcription and quantitative real-time PCR

Total RNA from clinical breast cancer tissues or breast cancer cells was obtained using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). PrimeScript RT Master Mix (TaKaRa Biotechnology, China) was applied in reverse transcription reactions. TransStart Top Green qPCR SuperMix (TransGen Biotech, China) was utilized for quantitative real-time PCR analysis. ZEB1 or HBXIP was normalized by glyceraldehyde 3-phosphate dehydrogenase.

Immunoblotting assay

Radioimmunoprecipitation assay buffer was used to lyse breast cancer cells and extract the total protein. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the total protein was transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The primary antibodies included anti-ZEB1 or anti-β-actin (Abcam, Cambridge, MA, USA).

Luciferase reporter analysis

The cells were seeded on 24-well plates and the indicated plasmids and/or siRNAs were then introduced. The Dual-Luciferase Reporter Assay System (Promega) was used to analyze luciferase activity. pRL-TK served as an internal control to normalize the activity of firefly luciferase.

Chromatin immunoprecipitation assay

MCF-7-HBXIP cells were transfected using si-Ctrl or si-Sp1 and were used to perform chromatin immunoprecipitation assay (EpiGentek, Farmingdale, NY, USA), following the manufacturer's instructions. Anti-HBXIP antibodies were used to immunoprecipitate protein-DNA complexes. Anti-RNA polymerase II or mouse immunoglobulin G served as positive and negative control antibodies, respectively. PCR assay was used to analyze DNA from these samples. The fragment of ZEB1 promoter including the Sp1 sites was amplified from the immunoprecipitated DNA samples with the specific primers.

Cell proliferation evaluation

Ninety-six or 12-well plates were used to seed cells. The cells were treated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and 5-Ethynyl-2′-deoxyuridine (EdU). For MTT assay, the cells were analyzed every second day for three days post transfection. For EdU analysis, images were captured by fluorescence microscopy using a filter at a certain wavelength.

Patient samples

Twenty-six pairs of breast tumor and peritumor samples were obtained from the China–Japan Union Hospital of Jilin University (Changchun, China). The research ethics committee approved the study protocol. Written consent was obtained from all patients for their tissues to be used for research. The patients' clinical details are shown in Table S1.

Statistical methods

A two-tailed Student's *t*-test was applied to assess statistical significance: $P < 0.001$***, $P < 0.01$**, or $P < 0.05$*. The relationship between HBXIP and ZEB1 in human breast cancer samples was evaluated by Pearson's correlation coefficient.

Results

ZEB1 is induced by oncoprotein HBXIP in breast cancer MCF-7 cells

HBXIP can enhance breast cancer progression by modulating many tumor-related factors.[26,27] ZEB1 is involved in the malignancy of a variety of cancers. In this study, we investigated whether HBXIP affects the expression of ZEB1 in breast cancer. After HBXIP was overexpressed, quantitative real-time PCR and immunoblotting assays were utilized to determine the HBXIP-induced effect on ZEB1. Notably, we observed that HBXIP could dose-dependently augmented the messenger RNA (mRNA) and protein levels of ZEB1 (Fig 1a,b). Meanwhile, HBXIP silencing markedly inhibited the level of ZEB1 when HBXIP-ovexpressed MCF-7 cells were transfected by HBXIP siRNAs (Fig 1c,d). Our data imply that oncogenic ZEB1 is upregulated by HBXIP in breast cancer.

HBXIP stimulates ZEB1 promoter through Sp1

To investigate how HBXIP increases ZEB1 in breast cancer cells, we predicted the promoter of ZEB1 (−448/+137) using GeneCopoeia (http://www.genecopoeia.com/) and JASPAR (http://jaspar.binf.ku.dk/cgi-bin/jaspar_db.pl). The promoter of ZEB1 was cloned into a pGL3-basic vector and its activity was tested. After HBXIP was overexpressed, luciferase reporter analysis showed that ZEB1 promoter activity in MCF-7 cells was induced by HBXIP (Fig 2a) and suppressed by HBXIP siRNAs in dose-dependent manners (Fig 2b). We then used ALGGEN-PROMO (http://alggen.lsi.upc.es/) to identify the transcription factors that might bind to the promoter of ZEB1. We found that there is a putative binding site of transcription factor Sp1 in the promoter of ZEB1, suggesting that Sp1 might be a transcription factor for ZEB1 activation. We used Sp1 siRNAs to study the effect of Sp1 on ZEB1 transcription induced by HBXIP in MCF-7 cells. Interestingly, Sp1 silencing could significantly abrogate HBXIP-increased

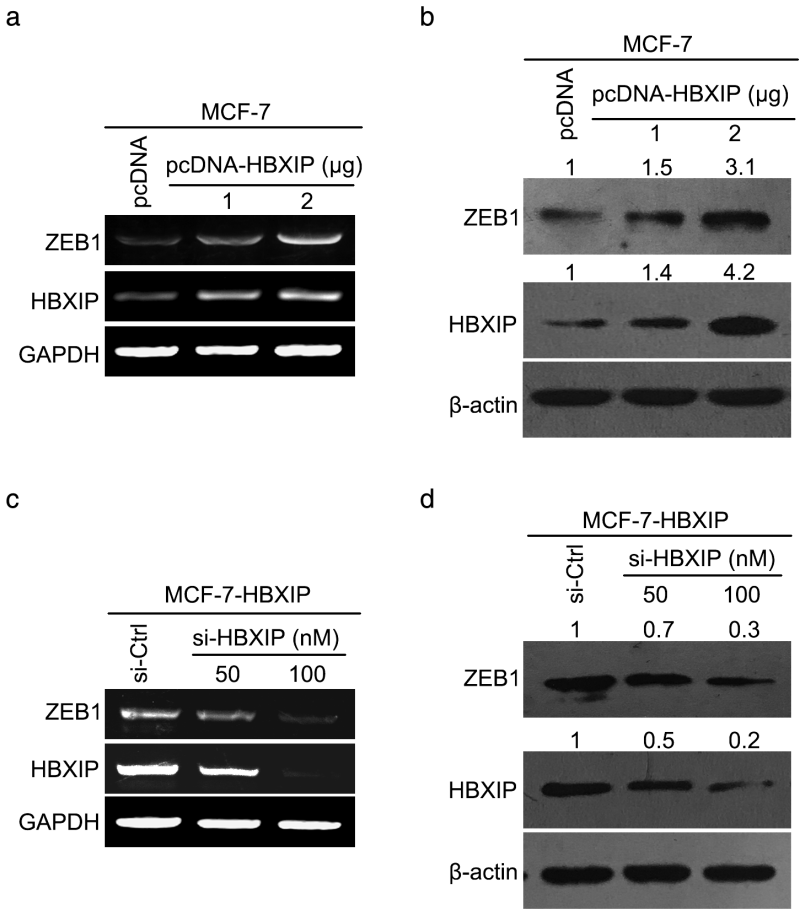

Figure 1 ZEB1 is induced by oncoprotein HBXIP in breast cancer MCF-7 cells. ZEB1 expression in MCF-7 cells (**a**,**b**) with HBXIP overexpression and (**c**,**d**) with HBXIP silencing were tested by reverse transcriptase-PCR and immunoblotting assay. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; si, small interfering.

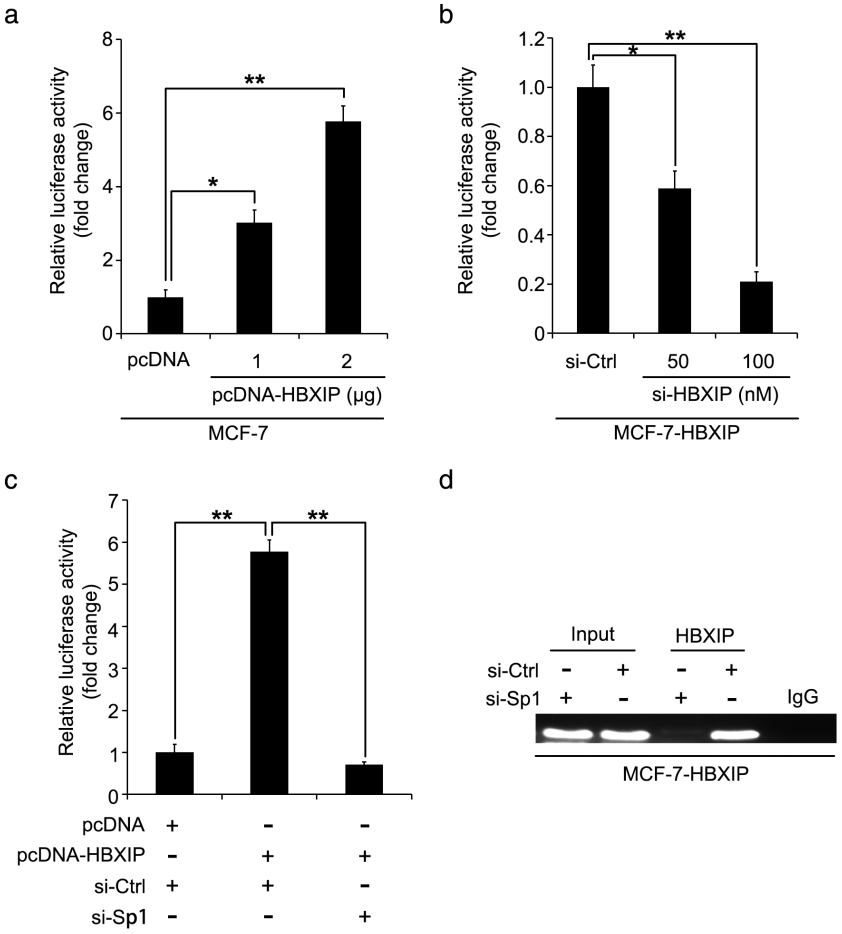

Figure 2 HBXIP stimulates ZEB1 promoter through Sp1. Luciferase activity of ZEB1 promoter in (**a**,**b**) HBXIP-overexpressed or HBXIP-silenced MCF-7-HBXIP cells and (**c**) in MCF-7 cells with HBXIP and/or Sp1 silencing was analyzed. (**d**) In MCF-7-HBXIP cells, binding of HBXIP and Sp1 to the ZEB1 promoter region was examined by chromatin immunoprecipitation assay. $*P < 0.05$, $**P < 0.01$. IgG, immunoglobulin G; si, small interfering.

activities of ZEB1 promoter (Fig 2c). Finally, chromatin immunoprecipitation assay data showed that HBXIP was capable of co-activating transcription factor Sp1 to interact with ZEB1 promoter (Fig 2d). Collectively, these results indicate that Sp1 takes part in ZEB1 activation induced by HBXIP.

HBXIP-upregulated ZEB1 enhances breast cancer cell proliferation

To determine whether ZEB1 is involved in HBXIP-promoted breast cancer growth, cell viability and EdU incorporation assay were used to evaluate changes in the proliferation ability of breast cancer cells treated with HBXIP, si-ZEB1, or HBXIP/si-ZEB1. Cell viability assay demonstrated that HBXIP could accelerate cell proliferation, while ZEB1 siRNAs could markedly suppress cell proliferation. Notably, ZEB1 siRNAs obviously abolished HBXIP-accelerated cell proliferation (Fig 3a). Meanwhile, EdU assay revealed that ZEB1 knockdown could block HBXIP-driven cell growth (Fig 3b). Therefore, we concluded that HBXIP could accelerate cell proliferation by upregulating ZEB1 in breast cancer.

Highly expressed ZEB1 is associated with elevated HBXIP in human breast cancer

Studies have shown that highly expressed HBXIP leads to the promotion of breast cancer development.[28,29] Herein, we evaluated the relationship between ZEB1 and HBXIP in regard to breast cancer malignancy. The levels of ZEB1 and HBXIP in clinical breast cancer samples were examined. ZEB1 was highly expressed in breast cancer samples (Fig 4a). Furthermore, quantitative real-time PCR revealed a positive correlation between HBXIP and ZEB1 ($R^2 = 0.6525$, $P < 0.01$) (Fig 4b). Our data imply that high ZEB1 expression is correlated with high HBXIP in breast cancer.

Discussion

Breast cancer is a major cause of cancer-related death in women.[30] Previous studies have reported that HBXIP plays

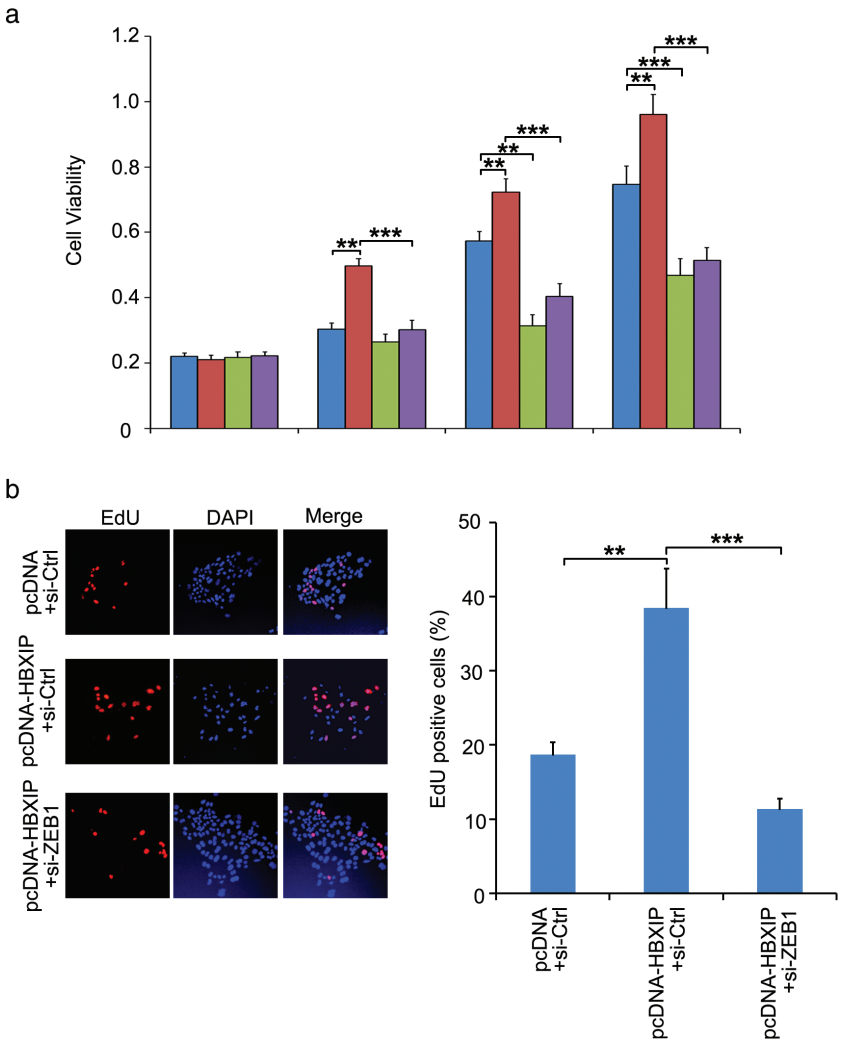

Figure 3 HBXIP-upregulated ZEB1 enhances breast cancer cell proliferation. (**a**,**b**) Cellular proliferative ability was assessed through cell viability or 5-Ethynyl-2′-deoxyuridine (EdU) analysis in MCF-7 cells with indicated treatment. (■) pcDNA+ small interfering-control (si-Ctrl), (■) pcDNA-HBXIP+si-Ctrl, (■) pcDNA +si-ZEB1, (■) pcDNA-HBXIP+si-ZEB1. $^{**}P < 0.01$, $^{***}P < 0.001$.

a crucial part in the development of breast cancer. Many cancer-associated factors can be regulated by HBXIP in the progression of breast cancer.[31,32] Glucose metabolism reprogramming in breast cancer can be induced by HBXIP to promote cell growth.[16] ZEB1, an oncogenic transcription factor, is implicated in the malignancies of many types of cancers. Highly expressed ZEB1 is considered a biomarker for metastasis in prostate cancer.[17,18] ZEB1 overexpression is associated with tumor progression, particularly in colorectal carcinomas.[21–23] ZEB1 can induce non-CSCs to CSC

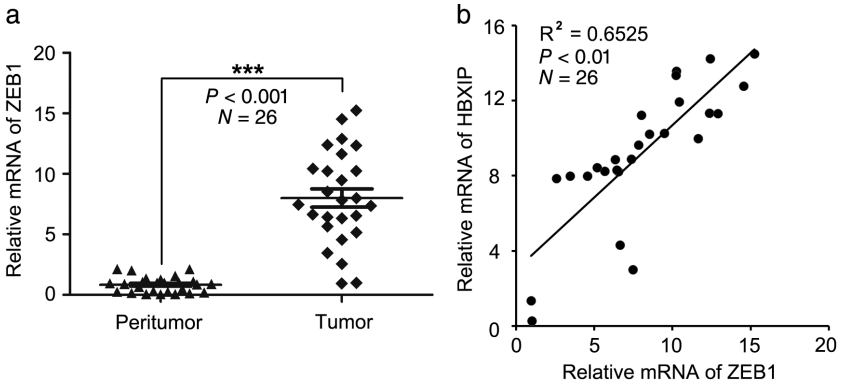

Figure 4 Highly expressed ZEB1 is associated with elevated HBXIP in human breast cancer. (**a**) ZEB1 expression was analyzed by quantitative real-time PCR in 26 pairs of clinical breast tumor and peritumor samples. (**b**) The relationship between HBXIP and ZEB1was assessed using Pearson's correlation coefficient. $^{***}P < 0.001$. mRNA, messenger RNA.

plasticity for tumorigenicity.[25] However, it is not yet known whether ZEB1 plays a part in HBXIP-induced breast cancer.

To explore whether HBXIP affects ZEB1 levels, we performed overexpression and RNA interference of HBXIP in breast cancer cells. We found that oncogenic HBXIP can increase ZEB1 expression. When the cells were transfected by HBXIP siRNAs, the ZEB1 level was decreased, suggesting that ZEB1 could be regulated by HBXIP. To determine how ZEB1 is modulated, we cloned the promoter of ZEB1. We found that HBXIP could stimulate ZEB1 transcription. Because HBXIP is an oncogenic transcription co-activator, we identified the transcription factors that might be responsible for ZEB1 activation. We first proved that Sp1 could directly bind to the promoter of ZEB1 to stimulate its transcription. Our data indicated that HBXIP induced ZEB1 expression by co-activating Sp1. In regard to function, ZEB1 played a key role in HBXIP-enhanced cell growth in breast cancer. We analyzed the levels of HBXIP or ZEB1 in human clinical breast cancer samples, and observed an obviously positive relationship between HBXIP and ZEB1. Our results show that HBXIP can induce ZEB1 via Sp1 to accelerate the growth of breast cancer cells.

In our present investigation, we found that HBXIP upregulates ZEB1 expression by transcription factor Sp1, leading to accelerated breast cancer growth. We present a novel mechanism of ZEB1 regulation in breast cancer.

Acknowledgments

This study was supported by the Department of Science and Technology of Jilin province (No. 20160204022YY), the Norman Bethune Program of Jilin University (No. 2015218), the International Cooperation Project funded by the Department of Science and Technology of Jilin Province (No. 20160414007GH, 20160414047GH), the Health Appropriate Technology Poverty Alleviation Project of Jilin Province (No. 2017FP022), and the Health Management Model Innovation Project of Jilin Province (No. 2016G014).

Disclosure

No authors report any conflict of interest.

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Supporting Information

Additional Supporting Informationmay be found in the online version of this article at the publisher's website:

Table S1. Clinical characteristics of 26 breast cancer tissue samples

