International Journal of Biochemistry and Cell Biology 95 (2018) 1–8

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

CBX4 exhibits oncogenic activities in breast cancer via Notch1 signaling

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ARTICLE INFO

Keywords:
CBX4
miR-137
Notch1
Breast cancer

ABSTRACT

Polycomb chromobox (CBX) proteins are involved in gene silencing to function as oncogenes or tumor suppressors through the polycomb repressive complex (PRC1). CBX4 has been implicated in the progression of human cancers, but its role and clinical significance in breast cancer remain unclear. Here, we show that CBX4 is up-regulated in breast cancer and exerts oncogenic activities via miR-137-mediated activation of Notch1 signaling pathway. CBX4 expression was increased in breast cancer, compared with the nontumorous tissues. High CBX4 expression was closely correlated with tumor metastasis, advanced clinical stage and poor overall survival in a cohort of 179 patients with breast cancer. *In vitro* studies demonstrated that CBX4 overexpression enhanced, whereas CBX4 knockdown inhibited cell growth and migration. Mechanistically, in a PRC1-dependent manner, CBX4 inhibited the promoter activity of miR-137 and suppressed its expression. miR-137 decreased the expression of Notch1, Jag1 and Hey2 via targeting their 3′-UTRs. The suppression of Notch1 by siRNA or overexpression of miR-137 markedly attenuated CBX4-promoted phenotypes. Collectively, these findings indicate that CBX4 promotes breast cancer via miR-137-mediated Notch1 signaling. Our data, therefore, suggest that CBX4 serve as a prognostic biomarker and that targeting CBX4/miR-137 axis may provide therapeutic potent in the treatment of breast cancer.

1. Introduction

Polycomb group (PcG) proteins function as transcriptional repressors to exhibit pivotal roles in chromatin modulation, stem cell maintenance, cell differentiation and tumorigenesis in mammals (Morey and Helin, 2010; Mozgova and Hennig, 2015). PcG proteins are comprised of structurally diverse and functionally related proteins, such as EZH2 and BMI1, to form the polycomb repressive complex (PRC), PRC1 and PRC2 which may cooperate with each other for gene silencing (Martin-Perez et al., 2010; Vandamme et al., 2011). However, a clear picture of the exact regulatory events of PcG complexes in cancers is hindered by the multiplicity of PcG complexes and the distinct functions of PcG proteins. The polycomb chromobox (CBX) proteins, including CBX2, CBX4, CBX6, CBX7, and CBX8, interact with the core PRC1 complex to perform distinct functions in different tissues (Di Croce and Helin, 2013; Zhou et al., 2016). Unique among CBX proteins, CBX4 (also known as hPC2) exerts small ubiquitin-related modifier (SUMO) E3 ligase activity (Li et al., 2007; Soria-Bretones et al., 2017). CBX4 facilitates the differentiation of haematopoietic stem cells (Klauke et al., 2013). CBX4 maintains the epithelial lineage identity via repression of the selected nonepidermal lineage and cell cycle inhibitor genes (Mardaryev et al., 2016). In human cancers, CBX4 manifests both oncogenic and anti-tumor activities, dependent on the cellular context. Overexpression of CBX4 in hepatocellular carcinoma is correlated with poor prognosis, promotes VEGF-mediated angiogenesis via repressing HIF-1α expression, and represents a potent therapeutic strategy (Jiao et al., 2015; Li et al., 2014; Wang et al., 2013). The oncogenic property of CBX4 was also demonstrated in osteosarcoma (Yang et al., 2016). On the other hand, CBX4 suppresses the tumor metastasis in colorectal cancer via blocking Runx2 expression via recruiting HDAC3 to the Runx2 promoter (Wang et al., 2016). However, the role of CBX4 and its clinical significance in breast cancer are still unclear.

The evolutionarily conserved Notch signaling pathway has been implicated in the regulation of cancer initiation and progression. Four receptors (Notch1, 2, 3, 4) and five ligands (DLL-1, 3, 4 and Jag1, 2) are identified in Notch signaling pathway (Radtke and Raj, 2003). Cell-cell

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https://doi.org/10.1016/j.biocel.2017.12.006
Received 30 September 2017; Accepted 6 December 2017
Available online 08 December 2017

contact may activate the Notch signaling pathway through ligand-receptor binding to form the cleavage of the intracellular domain of Notch (NICD), yielding the active form of the Notch receptor (Roy et al., 2007). The NICD translocates to the nucleus to cooperate with the transcription complex to trigger the downstream effectors, such as HES/Hey genes (Nowell and Radtke, 2017). Specially, Notch1 is cleaved by ADAM and the intramembrane g-secretase complex to generate NICD1 that converts the DNA-binding protein RBP-J from a transcriptional repressor into an activator (Tani et al., 2001). Deregulation of Notch1 signaling contributes to the tumorigenesis and malignant progression of breast cancer. Elevated expressions of Notch1 and Jag1 have been found in breast cancer to correlate with poor outcomes (Reedijk et al., 2005). Aberrant activation of Notch1 signaling promotes the breast cancer via maintaining the breast cancer stem cells and resisting the chemotherapeutic therapy (Licciardello et al., 2015; Xie et al., 2017).

In this study, the expression of CBX4 and its clinical implication in breast cancer were examined and revealed. The effect of CBX4 on the cell growth and migration was determined. The underlying mechanism of CBX4 biological function in breast cancer was investigated. Our data suggest CBX4 is overexpressed in breast cancer and exerts oncogenic activities via the miR-137-mediated activation of Notch1 signaling pathway.

2. Materials and methods

2.1. Patients and specimens

Thirty pairs of fresh tissues were obtained at First Affiliated Hospital of NanChang University, and subjected to quantitative real-time PCR and Western blot. A cohort of 179 paraffin-embedded samples, along with the clinicopathological information, was collected from patients received surgical resection between January 2000 and December 2010. None of the patients had received adjuvant therapies before surgery. The use of tissues for this study has been approved by the Institute Research Medical Ethics Committees of The First Affiliated Hospital of Nanchang University.

2.2. Cell lines and transfection

The breast cancer cell lines (MCF7 and SKBR3), purchased from the Cell Resource Center, Chinese Academy of Science Committee (Shanghai, China), were cultured with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT). Following the instructions for Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA), cells were transfected with CBX4 overexpressing vector or CBX4 siRNA/shRNA for 24 h (sc-38193, santa-cruz biotechnology) and then selected in G418-contained medium for another 2 weeks to construct stable cell lines.

2.3. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as described in our previous study. Primers used in this study were designed as the following: CBX4, forward: 5′-AGTGGAGTATCTGGTGAAATGGA-3′ and reverse: 5′-TCCTGCCTTTCC CTGTTCTG-3′; Notch1: forward: 5′-GCCGCCTTTGTGCTTCTGTTC-3′ and reverse: 5′-CCGGTGGTCTGTCTGGTCGTC-3′; Jag1: forward: 5′-GTCCATGCAGAACGTGAACG-3′ and reverse: 5′-GCGGGACTGATAC TCCTTGA-3′; HEY2, forward: 5′-TGGGGAGCGAGAACAATTAC-3′ and reverse: 5′-TTTTCAAAAGCTGTTGGCACT-3′; DLL4, forward: 5′-GTCTC CACGCCGGTATTGG-3′ and reverse: 5′-CAGGTGAAATTGAAGGGC AGT-3′; HES1, forward: 5′-TCAACACGACACCGGATAAAC-3′ and reverse: 5′-GCCGCGAGCTATCTTTCTTCA-3′; β-actin, forward: 5′-TGGCA CCCAGCACAATGAA-3′ and reverse: 5′-CTAAGTCATAGTCCGCCTAGA AGCA-3′.

2.4. Western blot

HCC fresh tissue were lysised by 6X sodium dodecyl sulfate (SDS) loading buffer and then fractionated by SDS-PAGE. The proteins were transferred to PVDF membrane which was then incubated with a primary specific antibody in 5% of non-fat milk, followed by a horse radish peroxidase (HRP)-conjugated anti-mouse second antibody. ECL detection reagent (Amersham Life Science, Piscataway, NJ, USA) was used to reveal the results. Antibodies for CBX4, Jag1, Hey2 and β-actin were purchased from Santa Cruz Biotechnology. Antibodies for p-ERK, ERK, p-AKT, AKT, Notch1 and NICD were obtained from Cell Signaling Technology.

2.5. Immunohistochemistry (IHC) and evaluation

Tissue microarray (TMA) consisting of 179 breast cancer samples and adjacent nontumorous liver tissues was constructed. Formalin-fixed and paraffin-embedded HCC sections were dewaxed in xylene and graded alcohols, hydrated, and washed in PBS. After pretreatment in a microwave oven, endogenous peroxidase was inhibited by 3% hydrogen peroxide in methanol for 20 min, followed by avidin–biotin blocking using a biotin-blocking kit (DAKO, Darmstadt, Germany). Slides were then incubated with CBX4 antibody overnight in a moist chamber at 4 °C, washed in PBS and incubated with biotinylated goat anti-rabbit/mouse antibodies. Slides were developed with DAB and counterstained with hematoxylin. Semi-quantitative IHC detection was used to determine the CBX4 protein levels. Using the H-score method, we multiplied the percentage score by the staining intensity score. The percentage of positively stained cells was scored as "0″ (0%), "1″ (1%–25%), "2″ (26%–50%), "3″ (51%–75%), or "4″ (76%–100%). Intensity was scored as "0″ (negative staining), "1″ (weak staining), "2″ (moderate staining), or "3″ (strong staining). For each case, 1000 cells were randomly selected and scored. The scores were independently decided by 2 pathologists.

2.6. MTT

Cells were seeded in 96-well plates and cultured for 5 days. Then, 20 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (5 mg/ml, AMRESCO, Solon, OH, USA) was added for 4 h at 37 °C. The formazan crystals were dissolved in DMSO (120 μl/well). The absorbance at 490 nm of each sample was measured. The cell growth rates were calculated according to the absorbance values.

2.7. Transwell assay

A total of 3×10^4 cells were seeded in the upper transwell chamber (Corning; Lowell, MA) with serum-free medium. After culture for 24 h, the migrated cells on the lower membrane were stained with 0.5% crystal violet and counted. The percentages of relative migrated cells were shown.

2.8. Foci formation

Stable cells were seeded into 6-well plates with or without 0.6% Bacto agar (Sigma-Aldrich) for 3 weeks. Colonies composing more than 50 cells were stained with 0.5% crystal violet and counted. Triplicate independent experiments were performed.

2.9. Luciferase reporter assay

For the binding of CBX4 to the miR-137 promoter, MCF7 cells co-transfected with CBX4 and the pmiR-RB-REPORT-miR-137 promoter were collected. Luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega, CA, USA).