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Research paper

CBX3 promotes tumor proliferation by regulating G1/S phase via p21 downregulation and associates with poor prognosis in tongue squamous cell carcinoma

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ABSTRACT

Chromobox protein homolog 3 (CBX3), a core component of the heterochromatin proteins 1, is recently proved to be involved in human cancerogenesis and associated with the prognosis of patient. However, the role of CBX3 in Tongue squamous cell carcinoma (TSCC) remains unclear. In the present study we found that CBX3 was upregulated in TSCC tissues when compared to adjacent non-tumor tissues, and multivariable analysis showed that high CBX3 expression was associated with clinical stage and cervical node metastasis, which was an independent prognostic indicator of TSCC. Furthermore, Kaplan-Meier survival analysis and log-rank test showed that TSCC patients with high CBX3 expression had a poorer rate of OS compared to patients with low CBX3 expression. Moreover, knocking down CBX3 inhibited TSCC cells proliferation both in vitro and in vivo, while overexpressing CBX3 promoted TSCC cells proliferation. In addition, CBX3 depletion resulted in cell cycle delay at the G1/S phase via the p21 pathway. In summary, we identifies CBX3 as a potential novel oncogene in TSCC, which may act as a biomarker and target in the diagnosis and treatment of this killer disease.

1. Introduction

Tongue squamous cell carcinoma (TSCC) is the most prevalent oral cavity malignancy, representing one-third of all oral cancers worldwide (Siegel et al., 2017). Both the incidence and associated mortality rates of TSCC have been increasing rapidly during the past five years. This increase is likely due to several well-known etiologic risks for carcinogenesis, including human papillomavirus infection, alcohol consumption, and tobacco intake (Chi et al., 2015; Duz et al., 2016). As one of the most representative and aggressive subtypes of oral cancer, TSCC often has a high frequency of local invasion and occult lymph node metastasis, even in cases of small primary lesions. Despite the advances in diagnostic and therapeutic techniques, the overall five-year survival rate (< 50%) has remains almost unchanged in the past few decades (Li et al., 2013). Therefore, it is necessary and urgent to identify novel biomarkers and therapeutic targets to aid the clinical management of patients with TSCC.

Both genetic and epigenetic alterations are characteristic of human cancer, contributing greatly to carcinogenesis and progression (Salas et al., 2017). Accumulating evidence showed that epigenetic modulation plays a key role in driving tumorigenesis by serving as oncogenes or tumor suppressing genes. Moreover, epigenetic modulators may become novel anti-cancer therapeutic targets due to their potentially reversible nature of epigenetic changes (Ronnekleiv-Kelly et al., 2017). The methyl groups of histone H3 at lysine 9 (H3K9) are the hallmark of epigenetic silencing in the chromatin. Recent studies reveal that the histone lysine-specific demethylase (LSD1), a critical chromatin modifier for the demethylation of H3K9, promotes cancer cell proliferation and associated with unfavorable prognosis; this suggests that H3K9 methylation play key roles in tongue cancer tumorigenesis (Yuan et al., 2015).

Members of the heterochromatin protein 1 (HP1) family are conserved chromatin binding proteins that interact with the methylated H3K9 promoter region to silence gene expression in heterochromatin (Canzio et al., 2014). The abnormal expression of an HP1 gene could lead to a plethora of human diseases, including organism defect and cancer progression (Dialynas et al., 2008). In humans, there are three main HP1 paralogs: HP1α, HP1β and HP1γ, encoded by the CBX5,

Abbreviations: CBX3, Chromobox protein homolog 3; TSCC, Tongue squamous cell carcinoma; H3K9, histone H3 at lysine 9; HP1, heterochromatin protein 1; IHC, immunohistochemistry

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CBX1, and CBX3 genes respectively. Unlike the other two paralogs, CBX3/HP1γ is found in both heterochromatin and euchromatin areas, which suggests that it may be associated with either transcriptional repression or activation (Minc et al., 2000). Previous studies show that CBX3 is overexpressed in various kinds of cancers such as prostate, colon, and lung cancer (Slezak et al., 2013; Takanashi et al., 2009; Zhou et al., 2014a), and always indicates a poor prognosis. However, the relationship between the expression level of CBX3 and the clinical pathologic parameters in TSCC remains unclear.

In this present study, we evaluated the expression of CBX3 in TSCC tissues and cell lines and explored its influence on cell proliferation both in vivo and in vitro. This work suggested that CBX3 depletion by lentivirus-mediated short hairpin RNA (shRNA) inhibited cell proliferation by upregulating p21, while overexpressing CBX3 promoted TSCC cells proliferation. We also found that CBX3 silencing could affect the cell cycle distribution and caused cell cycle arrest at the G1/S phase in TSCC cells. In sum, these results indicate that CBX3 is a positive regulator of TSCC pathogenesis.

2. Materials and methods

2.1. Oncomine database analyses

To determine the outline of the CBX3 expression pattern, we investigated the CBX3 mRNA levels of human TSCC in the Oncomine database (http://www.oncomine.org), which is publicly available.

2.2. Patients and tissue specimens

Paraffin-embedded specimens from 98 TSCC patients, who underwent radical tumor resection and neck dissection at the Sun Yat-Sen University Cancer Center between 2011 and 2012, were enrolled for immunohistochemistry (IHC) analysis. Fresh TSCC tissues and adjacent non-tumor tissues from the same patients were stored in liquid nitrogen for quantitative RT-PCR and western blot experiments. All samples were classified according to TNM staging based on American Joint Committee on Cancer (AJCC), 7th edition (Edge and Compton, 2010). In addition, a pathologist reviewed all samples to verify the diagnosis, histological grade, and stage. The Medical Ethics Committee of The Sun Yat-Sen University Cancer Center approved this study. All patients provided informed consent.

2.3. Immunohistochemistry analysis and assessment

Immunohistochemistry (IHC) studies were performed on formalin-fixed, paraffin-embedded specimens following routine procedure. The slides were heated for 3–4 h at 65 °C, deparaffinized in xylene, hydrated in an alcohol gradient from 100%, 95%, and 80% to 70%, and treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. In order to complete antigen retrieval researchers boiled the slides in citric acid buffer (10 mM, pH 6.0) for 15 min in a pressure cooker. In order to block nonspecific binding, slides were incubated with 10% normal goat serum for 15 min at room temperature, followed by incubation with a rabbit anti-CBX3 antibody (diluted 1:400 in PBS; Proteintech, Taipei, Taiwan) overnight at 4 °C. The slides were washed with PBS thrice, and then incubated with secondary anti-rabbit antibody at a concentration of 1:100 at 37 °C for 30 min. The slides were then immersed in a 3,3′-diaminobenzidine (DAB) solution for 5 min and counterstained with 10% Meyer's hematoxylin for 3 min, followed by polarization with 70% ethyl alcohol containing 0.1% hydrochloric acid for 10 s. PBS replaced the primary antibody as a negative control, whereas IHC-positive CBX3 staining slides of colon cancer were acted as the positive control.

Two pathologists, blind to the relevant clinicopathological data, scored immunoreactivity in terms of positively-stained tumor cell proportion and the intensity of stained cancer cells. The estimated fraction of positively-stained tumor cells was defined as the proportion score (0, none; 1, 10%; 2, 11–50%; 3, 51–80%; 4, > 80%), whereas the estimated staining intensity was defined as the intensity score (0, no staining; 1, weak; 2, moderate; 3, strong) with the aggregate score ranging from 0 to 12, as described in other studies (Zheng et al., 2016). An aggregate score of ≥6 indicated overexpression of CBX3, whereas < 6 indicated low CBX3 expression.

2.4. Cell culture

Normal oral mucosa epithelial cell line (NOK) and four human TSCC cell lines (SCC9, SCC25, Tca8113, Cal27) were used in this study. NOK and Tca8113 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology (SIBCB, Shanghai, China), while the other three cancerous cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cancerous cell lines were cultured at 37 °C under a 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL).NOK cells were maintained in defined keratinocyte medium-SFM (Gibco, USA).

2.5. Western blot analysis

After being ground in liquid nitrogen, frozen tissues were lysed in RIPA reagent (Beyotime, Shanghai, China) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland) and PMSF (Roche). The lysates were centrifuged at 12,000*g* for 15 min at 4 °C to obtain supernatant. Researchers extracted total proteins of cultured cells following the same procedures mentioned above, except the grinding step, and quantified the proteins using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

The protein contents were separated by 10% SDS-PAGE, and transferred onto a PVDF membrane at 350 mA for 1 h at room temperature. The membrane was blocked with 5% nonfat milk for 1 h, and then incubated with primary antibodies overnight at 4 °C. The membranes were washed in phosphate buffer saline containing 0.1% Tween (PBST) thrice, followed by incubation with a secondary antibody for 1 h at room temperature. The signals were detected by using Luminol Reagent (Millipore, USA). The following antibodies were used: CBX3 (1:500, Proteintech, Taiwan); p21, p16, cdc25A, CDK2, CDK6 and CCND1 (1:1000, Cell Signaling Technology, MA, USA); GAPDH (1:10,000, Santa Cruz, USA).

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total RNA was extracted from frozen tissues and cells using TRIzol reagent (Invitrogen Life Technologies, USA), following the instructions of the manufacturer. Approximately 1 μg RNA out of total 20 μg was applied for reverse transcribing to acquire complementary DNA (cDNA) with random primers using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The qRT-PCR was conducted using UtraSYBR Mixture (CWBIO, China) in triplicate on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ Method with GAPDH as a control and for normalization. The primer sequences are listed in Table 1.

2.7. Construction of stable cell lines and transfection of small interfering RNAs (siRNAs)

Lentivirus for CBX3 overexpression and lentiviruses harboring a shRNA sequence targeting CBX3 or non-targeting control (NC) sequences were purchased from HANBIO (Shanghai, China). The virus vector was pHBLV-CMVIE-Zs Green-T2A-Puro. The targeting oligonucleotides were 5′-GUGUAGUGAAUGGGAAAGU-3′. The lentivirus with