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Human β-defensin-3 and nuclear factor-kappa B p65 synergistically promote the cell proliferation and invasion of oral squamous cell carcinoma

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is a usual oral cancer. Therefore, it's essential to identify targets for its early diagnosis and therapy. This research aimed to explore the roles of human β-defensin-3 (hBD-3) and nuclear factor-kappa B (NF-κB) p65 in the pathogenesis and progression of OSCC. The connection between NF-κB p65 and the carcinogenesis of oral cancer was analyzed by immunohistochemical staining. The relative expressions of hBD-3 and NF-κB p65 in OSCC cells were evaluated by qRT-PCR and Western blot. Afterward, hBD-3 was knocked down, and NF-κB p65 was overexpressed. The cell viability and invasion were tested via CCK-8 and Transwell experiment, and the expression of hBD-3, NF-κB p65, and its downstream molecules was evaluated by Western blot. The expression of NF-κB p65 was increased with the aggravation of the oral submucosal fibrosis. HBD-3 and NF-κB p65 were high-expressed in OSCC cells. The viability and invasion abilities of OSCC cells that knocked down hBD-3 were markedly decreased, while they were restored by the overexpression of NF-κB p65. The expressions of NF-κB p65 and c-myc were diminished while IκB and p21 were raised with the knockdown of hBD-3. After overexpression of NF-κB p65, the expression of hBD-3 and IκB did not change markedly, while c-myc was increased and p21 was decreased dramatically. HBD-3 and NF-κB p65 facilitate the proliferation and invasion of OSCC cells, and hBD-3 may promote this process by governing the expression of NF-κB p65 and its downstream c-myc and p21.

Introduction

About 90% of oral cancers are OSCC [1], and their incidence is increasing year by year [2]. Surgical resection is currently the preferred treatment for OSCC [3], combined with radiotherapy and chemotherapy [4]. Nevertheless, the long-term effect of these therapies is still not ideal due to the strong aggressiveness and high metastasis rate of OSCC [5]. Hence, seeking novel and efficiency targets for the early detection and treatment of OSCC is urgent.

Human β-defensin (hBD) is an important component of the human innate immune system, mainly expressed in some epithelial tissues, and plays an essential role in inflammatory diseases, injury repair, tumor formation, and metastasis [6,7]. There are mainly 6 kinds of hBD (hBD-1~6), which are mainly distributed in the skin, respiratory tract, tonsil epithelium, while oral mucosa epithelium mainly produces hBD-1~3 [8]. HBD-3 was initially isolated from the skin lesions of psoriasis patients, and its antibacterial properties were broader and stronger than hBD-1 and hBD-2 [9]. As a part of the natural immune barrier of the oral mucosal, hBD-3 is normally expressed low. When stimulated by external factors, the expression of hBD-3 in epithelial tissues would be remarkably increased, thus exerting its innate immune defense function [10]. Aberrant expression of hBD-3 has been reported in various cancer tissues, such as cervical cancer [11], colon cancer [12], head and neck cancer [13], and OSCC [14]. Overexpressed hBD-3 can affect the proliferation, invasion, cell cycle, apoptosis, migration, and other processes of tumor cells, hence being enrolled in the pathogenesis and development of tumors [15]. HBD-3 has been considered a potential tumor biomarker, and it is associated with a variety of oral precancerous lesions or precancerous states, such as oral lichen planus and oral leukoplakia [16]. NF-κB is a crucial nuclear transcription factor that participates in cell proliferation, differentiation, and apoptosis, which is tightly bound up with the progress of inflammatory tumors [17]. Recent

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study has shown that the hBD-3 can promote the growth of cervical cancer cells by activating NF-κB signaling pathway [11]. However, whether hBD-3 can participate in the progression of OSCC by regulating NF-κB-mediated inflammatory response is still indistinct.

In the present report, we assessed the predictability of NF-κB p65 in the carcinogenesis of oral cancer, then we examined the expression of hBD-3 and NF-κB p65 in oral mucosal epithelial cells and OSCC cell lines, and explored the roles and correlation of hBD-3 and NF-κB p65 in the occurrence and progression of OSCC, to provide a reference for the control and treatment of OSCC.

Materials and methods

Tissue samples

A total of 31 wax blocks of patients with oral submucosal fibrosis (OSF) confirmed by pathology were selected from the Pathology Department of Haikou People's Hospital from January 2017 to December 2020, including 15 cases in the early stage, 13 cases in the middle stage and 3 cases in the late stage. The age of the patients ranged from 21 to 70; There were 30 males and 1 female; 22 smoked cigarettes and 9 did not smoke cigarettes; 18 were drinking alcohol and 13 were not drinking alcohol; 31 were all HPV negative, and none developed oral cancer. None of them underwent any preoperative treatment and were not associated with other systemic diseases or mucosal diseases. This research program has been permitted by the Ethics Committee of Haikou People's Hospital.

Immunohistochemical (IHC) staining

The two-step IHC method was applied to assess the expression of NF-κB p65 and PDE4 in OSF (early, middle and late stage) tissue sections. After the dewaxing and rehydrating of paraffin sections, they were cleaned twice with PBS. Then the tissue antigen repair was performed, and the sections were blocked with a few drops of 3% H_2O_2. Furthermore, the sections were reacted with primary antibodies and secondary antibodies successively, and DAB solution (Aike Reagent, China) was dropped for color-developing. All the antibodies used were purchased from Abcam, including anti-NF-κB p65 (ab16502), anti-PDE4 (ab14613), and goat anti-rabbit IgG (ab97051). The slices were then washed with PBS, restained with hematoxylin (J&K Scientific, China), dehydrated, and sealed for microscopic examination.

Cell culture

The OSCC cell lines CAL27 and HN30 were acquired from Guangzhou Jennio Biotech (China), and oral mucosal epithelial cells (OMES) were provided by Shanghai Baiyi Biotechnology Center (China). These two kinds of OSCC cells were all cultured in DMEM (Gibco, US) that contained 10% fetal bovine serum (Hyclone, US) and 1% double antibodies (Absin, China). The cell culture environment is constant at 37 °C with 5% CO_2.

qRT-PCR

Total RNA of CAL27 and HN30 cells was collected using Trizol (Keybio, China). Then the cDNA was obtained using the reverse transcription kit (Biopike, China). The following were sequences of hBD-3 and NF-κB p65 primers: hBD-3 F: 5'-TAGCCTAACGTAATCGACTG-3', R: 5'-GACTAATGACCTACGTTCGAC-3'; NF-κB p65 F: 5'-GAGCTACATTGCAACTAGAC-3', R: 5'-CTATGACCTACGACTGATCC-3'. The qPCR reaction system was constructed using a 2 × SYBR Green qPCR kit (Dingguo Changsheng, China), and the reaction was conducted and analyzed by the Gentier 96E PCR analysis system (Tianlong, China). The tests were repeated independently three times.

Western blot

CAL27 and HN30 cells were digested and lysed, and the protein of cell lysate was quantified using the BCA kit (Absin, China). Then 15 μg of total protein was injected into the sample holes for SDS-PAGE. Furthermore, the dividual proteins in the gel were transferred to the PVDF membrane (Absin, China). After blocking with 1% bovine serum albumin, the membrane was incubated overnight with diverse primary antibodies at 4°C. Primary antibodies were as follows: anti-hBD-3 (Abcam, ab172703), anti-NF-κB p65 (Abcam, ab288751), anti-IκB (Abcam, ab97783), anti-c-Myc (Abcam, ab152146), anti-p21 (Abcam, ab227443), anti-GAPDH (Abcam, ab9484). On the second day, after incubating with secondary antibodies for 1 h, the protein bands could be visualized by developing. And Image J (NIH, USA) was used to analyze the intensity of protein bands.

Cell transfection

Oligonucleotide si-hBD-3 and si-NC were synthesized by Guangzhou Ribobio (China), si-hBD-3 F: 5'-UUCAUCGACCAUUACAGCUTT-3', R: 5'-CAGUCACGUUCAGUGACAATT-3', si-NC F: 5'-UGCAAGUUCAUGACUGATT-3', R: 5'-GAUUACCGAUGACUAACGTT-3'. si-hBD-3 or si-NC was transfected into CAL27 and HN30 cells following the procedure of Lipofectamine™2000 Transfection Reagent (Invitrogen, US). For the overexpression of NF-κB p65, the full length of NF-κB p65 was obtained by PCR amplification and inserted into the pcDNA3.1 plasmid (Miaoling, China) to construct the NF-κB p65 overexpression plasmid. Then the NF-κB p65- pcDNA3.1 or the empty pcDNA3.1 plasmid were transfected to CAL27 and HN30 cells using Lipofectamine™2000 reagent. Follow-up experiments could be performed 48 h after the transfection.

CCK-8 assay

This assay was carried out to evaluate the cell viability according to the working manual of Cell Counting Kit-8 (Sangon Biotech, China). Briefly, 5×10^5 of CAL27 or HN30 cells were inoculated in 96-well plates. When all cells were completely adherent to the plate, 10 μL of CCK-8 reagent was injected to treat cells for another 2 h. Finally, the OD_{450} was measured by the microplate reader (Tecan, Swiss).

Transwell invasion assay

The basement membrane of the Transwell chamber (Corning, US) was pre-coated with Matrigel (Corning, US) one day before the experiment, and the chambers were seated in 24-well plates. Then, the transfected CAL27 and HN30 cells were placed in the upper chamber, and 500 μL/well of the complete DMEM was injected into the lower chamber. After 24 h of culture, the chamber was taken out and stained with 0.1% crystal violet (Aladdin, China). Then the remaining cells on the upside of the basement membrane were erased with Q-tips, and the cells that invaded the bottom side were observed with the microscope (Nikon, Japan).

Statistical analysis

All quantitative data were shown as Mean ± Standard deviation. The SPSS software (V 19.0) was used for the statistical analysis. The comparison between the two groups was conducted using Student's *t*-test, and one-way ANOVA was used for three groups. $P < 0.05$ was considered statistically significant.

Results

NF-κB p65 is correlated with carcinogenesis of the oral mucosa

It is unclear whether NF-κB p65 is associated with oral precancerous

lesions. Therefore, we examined its expression in clinical tissues of patients with early, intermediate, and advanced OSF by IHC assay. PDE4, an enzyme that can specifically hydrolyze c-AMP, has been proved to be abundant in various tumor cells, and its level is positively correlated with the carcinogenesis process [18]. Therefore, we selected PDE4 as the positive control. The results revealed that NF-κB p65 and PDE4 in OSF tissues gradually increased with the progression of OSF (Fig. 1A and B), suggesting that NF-κB p65 is correlated with carcinogenesis of the oral mucosa.

hBD-3 and NF-κB p65 are highly expressed in OSCC cell lines

Next, we investigated the relative expression capacity of hBD-3 and NF-κB p65 in OSCC cells. On the one hand, the hBD-3 and NF-κB p65 mRNA expression in CAL27 and HN30 cells was found significantly higher than that in OMES (Fig. 2A). On the other hand, the protein level of hBD-3 and NF-κB p65 exhibited similar results, they were low expressed in OMES, but obviously higher in CAL27 and HN30 cells (Fig. 2B).

hBD-3 facilitates the proliferation and invasion of OSCC cells

To further investigate the role of hBD-3 in the occurrence and development of OSCC, we used siRNA technology to knock down hBD-3 expression in CAL27 and HN30 cells. The knockdown efficiency of si-hBD3-#1 in CAL27 and HN30 cells was about 92.6% and 52.2%, respectively, and si-hBD3-#2 was about 59.5% and 58.7%, respectively (Fig. 3A). si-hBD3-#2 was chosen for further experiments. Next, we evaluated the function of hBD-3 on the viability of CAL27 and HN30 cells by CCK-8 assay. The results showed that 72 h after hBD-3 was knocked down, cell viability was dramatically inhibited (Fig. 3B). In addition, we found that the invasion ability of CAL27 and HN30 cells were obviously decreased after the knockdown of hBD-3 (Fig. 3C). The above-mentioned results illustrated that hBD-3 could facilitate proliferation and invasion of OSCC cells.

NF-κB p65 reverses the effects of hBD-3-knockdown on OSCC cells

Additionally, we proposed to clarify the role of NF-κB p65 in the progression of OSCC. To this end, we overexpressed NF-κB p65 in CAL27 and HN30 cells in which hBD-3 was knocked down. We found that the cell viability of CAL27 and HN30 cells that knocked down hBD-3 and co-overexpressed NF-κB p65 was markedly enhanced compared with the group that only knocked down hBD-3 (Fig. 4A, $P < 0.05$). Besides, knockdown of hBD-3 and co-overexpression of NF-κB p65 also restored the invasive ability of CAL27 and HN30 cells (Fig. 4B and C). The above results signal that NF-κB p65 can reverse the effects of knockdown of hBD-3 on OSCC cells, it is a potential promoter for the progression of OSCC.

c-myc and p21 may be downstream signaling molecules of hBD-3 and NF-κB p65

To elucidate how hBD-3 and NF-κB p65 regulate OSCC, we further investigated the expression of downstream signaling molecules of NF-κB p65 in CAL27 cells. After the knockdown of hBD-3, the protein content of IκB and p21 was notably increased, and c-myc and NF-κB p65 were decreased. When NF-κB p65 was simultaneously overexpressed, the protein content of c-myc was dramatically increased, p21 was decreased, while the level of IκB was not significantly changed (Fig. 5A and B). These results demonstrate that c-myc and p21 may be downstream signaling molecules of hBD-3 and NF-κB p65.

Discussion

At present, the 5-year survival rate of OSCC patients is less than 50%, which can be improved by early detection and treatment [19]. OSCC usually develops from precancerous lesions, such as oral erythema, oral leukoplakia, oral lichen planus, and OSF [20]. OSF is a chronic mucosal inflammatory disease with unknown etiology, and it is considered a precancerous lesion of oral mucosa with certain carcinogenic potential and is recognized by the World Health Organization [21]. It has been reported that about 3% ~ 19% of OSF patients may develop oral cancer, and this probability is increasing year by year [22]. Recently, studies have found that the NF-κB pathway is strongly linked to the occurrence of tissue fibrosis, and its abnormal activation can promote the formation of inflammatory fibrosis [23]. NF-κB has a certain relationship with oral precancerous lesions and the occurrence and metastasis of OSCC [24]. In this study, the expression of NF-κB p65 in the oral mucosa of OSF patients was found to increase with the aggravation of OSF, which indicated that NF-κB p65 was closely related to the carcinogenesis of the oral mucosa. In the process of normal oral mucosal carcinogenesis, activation of NF-κB p65 may accelerate the expression of genes that accelerate cell proliferation and inhibit apoptosis, thereby causing malignant

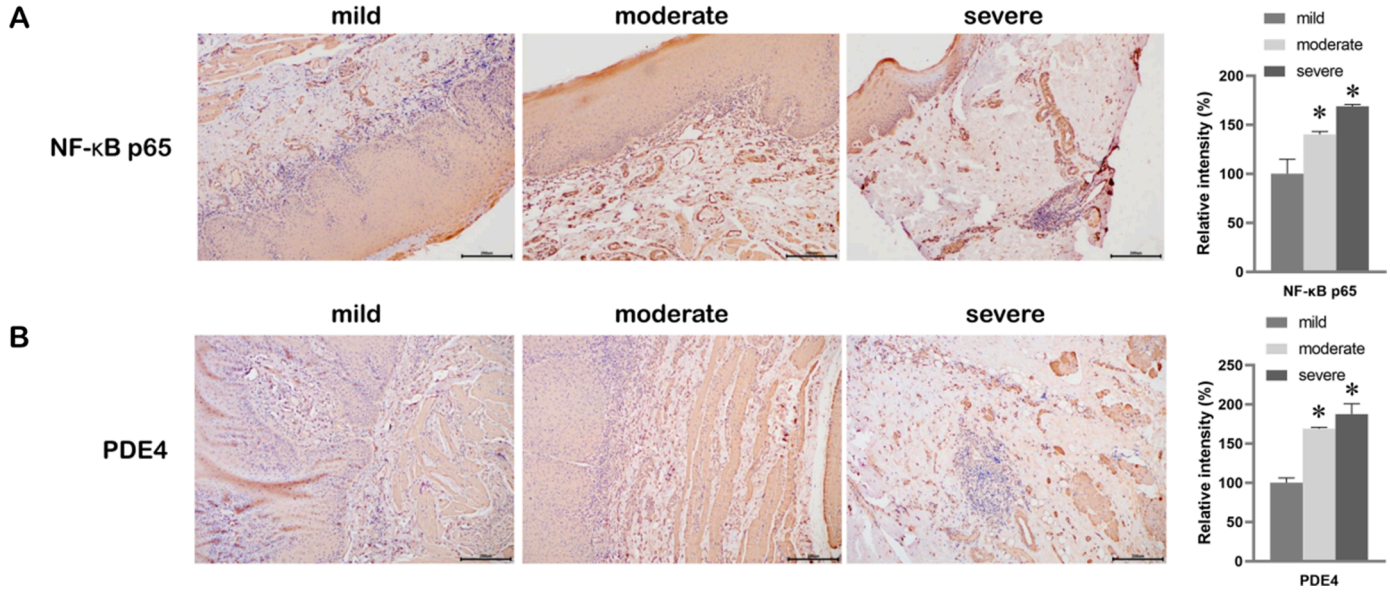

Fig. 1. Immunohistochemical staining of A NF-κB p65 and B PDE4 in oral mucosa tissues of OSF patients with mild, moderate, and severe degrees.

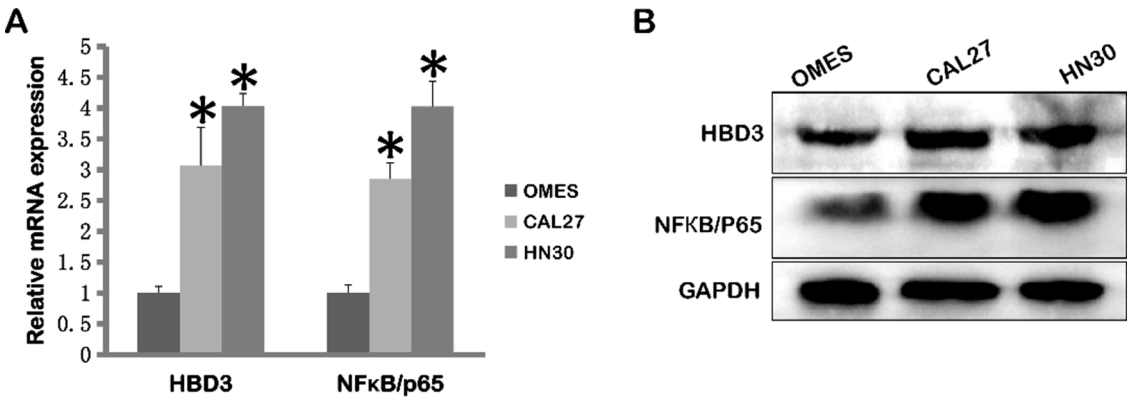

Fig. 2. A qRT-PCR and B Western blot analysis of the expression of hBD-3 and NF-κB p65 in OMES, CAL27, and HN30 cells. * $P < 0.05$.

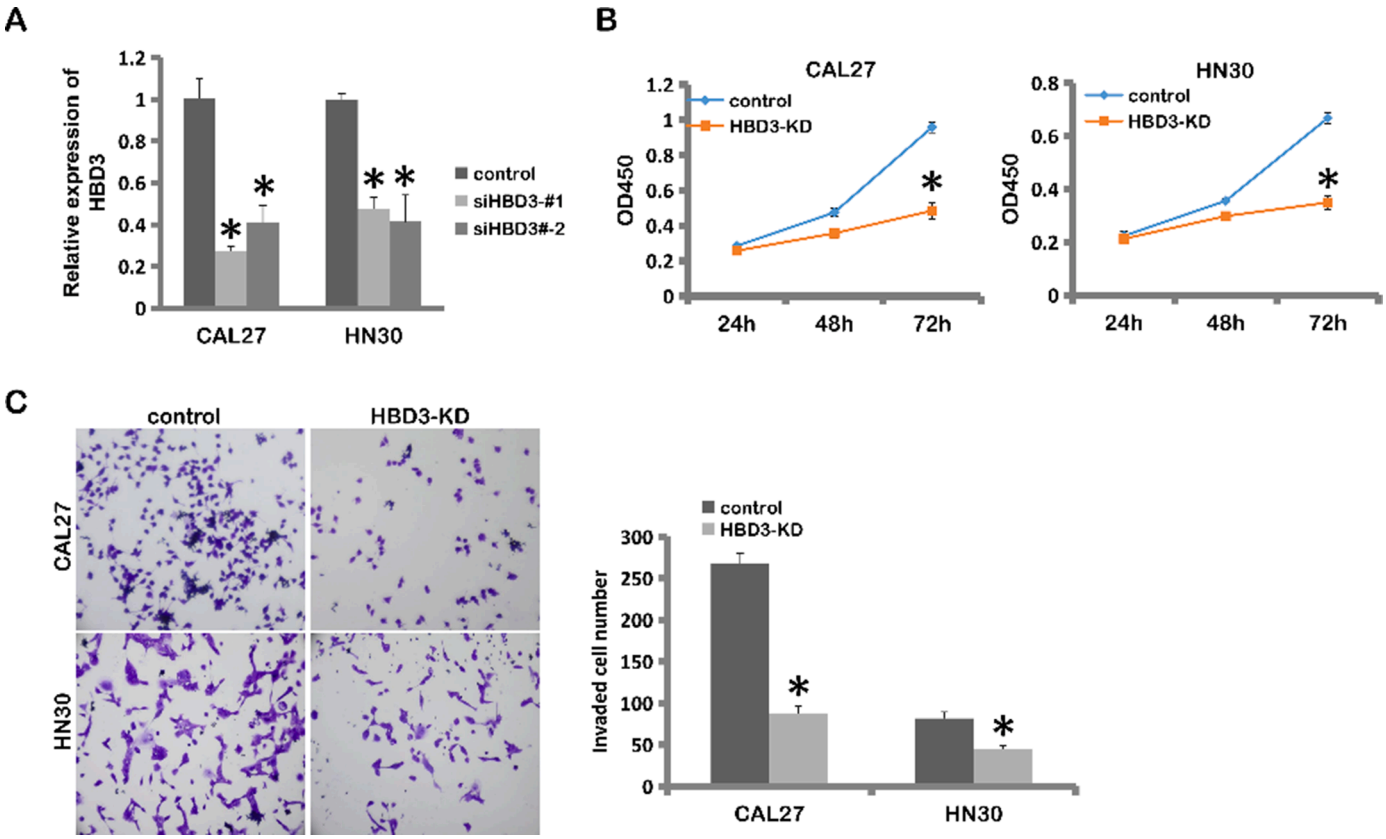

Fig. 3. Effects of the knockdown of hBD-3 on proliferation and invasion of OSCC cells. A The efficiency of si-hBD-3 was verified by qRT-PCR. B Cell viability was tested by CCK-8 assay 48 and 72 h after the transfection. C The invasion ability of the transfected OSCC cells was evaluated by Transwell assay. * $P < 0.05$.

transformation of oral mucosal cells to OSCC cells.

HBD-3 is also closely linked to the evolution of tumors [12]. Marco et al. found that hBD-3 is frequently overexpressed in OSCC by analyzing the expression profile of hBD-3 in cancerous and non-cancerous specimens of OSCC patients, and hBD-3 may be related to the pathogenesis of OSCC [7]. While Joly et al. found that the expression of hBD-1 and hBD-2 basal messenger RNAs was significantly decreased in OSCC by real-time polymerase chain reaction experiments [25]. Mburu et al. confirmed that hBD-3 can promote the metastasis of head and neck squamous cell carcinoma by up-regulating the expression of CCR7 through the NF-κB pathway [26]. The correlation between hBD-3 and NF-κB p65 in the pathogenesis and progression of OSCC has not been reported. Here, we found that the knockdown of hBD-3 reduced the proliferative and invasive abilities of OSCC cells, which was in keeping with the findings of Shuyi et al. [14]. To elucidate the correlation between hBD-3 and NF-κB p65 in OSCC, we overexpressed NF-κB p65 in CSCC cells while knocking down hBD-3. The inhibitory effect of hBD-3-knockdown on OSCC cells was reversed by overexpressing NF-κB p65 to a great extent. The decreased expression of NF-κB p65 was also observed in OSCC cells after the knockdown of hBD-3, suggesting that hBD-3 may be the activator of NF-κB p65.

Furthermore, we detected the expression levels of the downstream signaling molecules, including IκB, c-myc, and p21. Although the expressions of IκB, c-myc, and p21 were significantly up-regulated or down-regulated after knockdown of hBD-3, the expression of IκB did not change significantly after overexpression of NF-κB p65, which suggests that IκB is not the common downstream target of hBD-3 and NF-κB p65, and c-myc and p21 may be the key downstream signaling molecules that

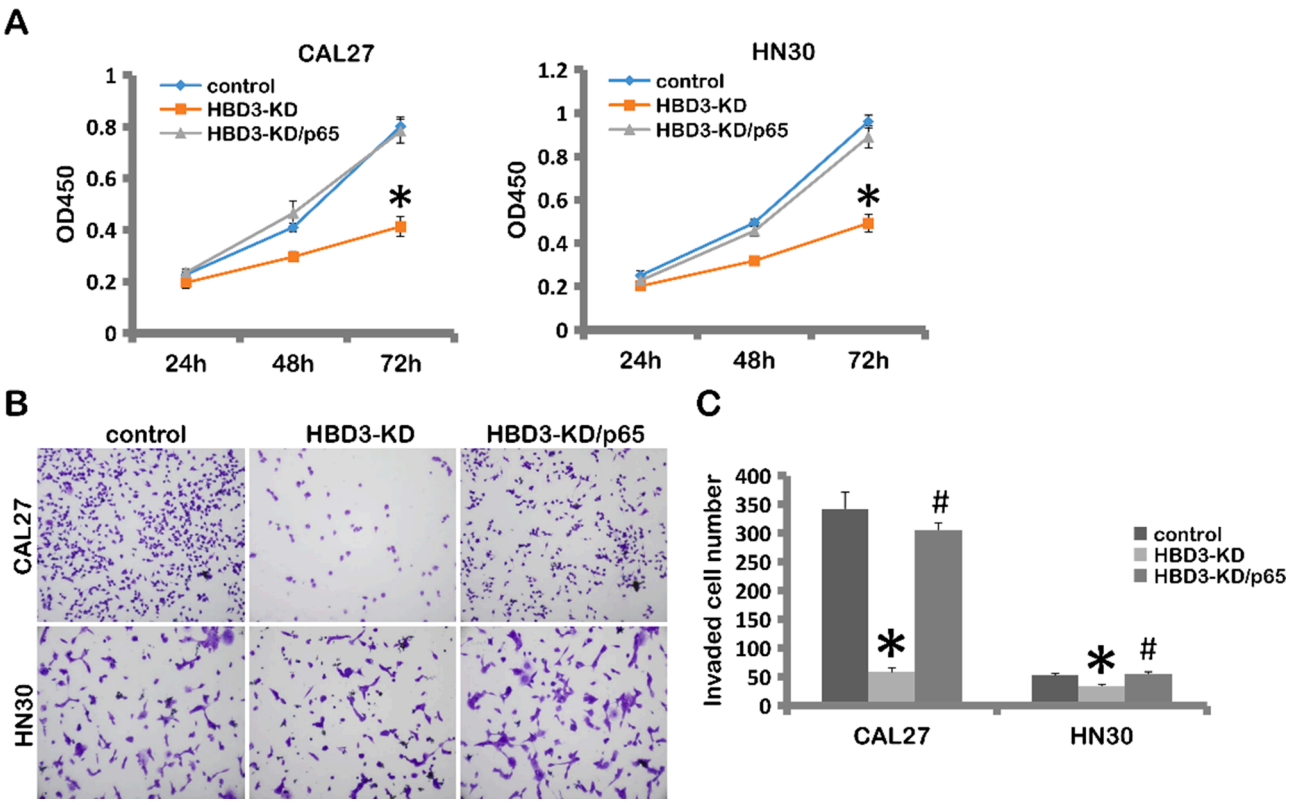

Fig. 4. Overexpression of NF-κB p65 reversed the effect of si-hBD-3. A The proliferation ability and B, C the invasion ability of OSCC cells in control, hBD-3 knockdown, and simultaneous overexpression of NF-κB p65 group was evaluated by CCK-8 and Transwell assay, respectively. * $P < 0.05$ *vs* control group, # $P < 0.05$ vs HBD3-KD group.

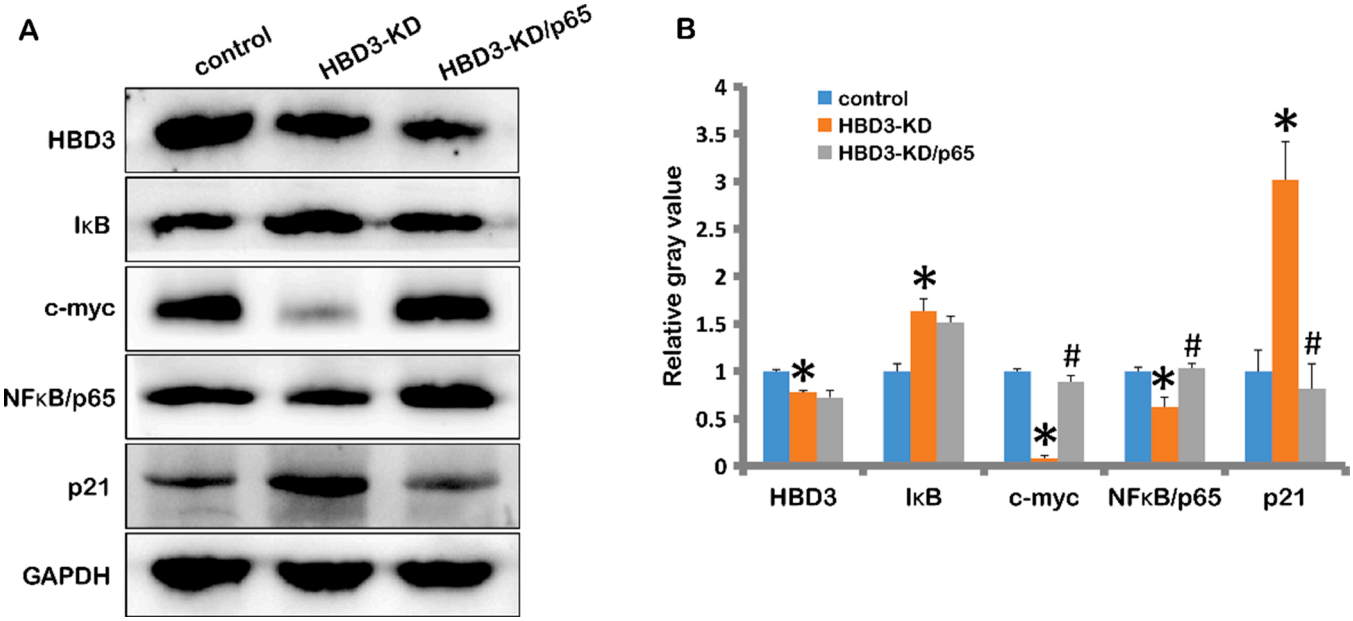

Fig. 5. A Western blot analysis and B relative gray analysis of the changes in protein expression of hBD-3 and signaling molecules of NF-κB p65 pathway in OSCC cells. * $P < 0.05$ *vs* control group, # $P < 0.05$ vs HBD3-KD group.

enrolled in the regulation of pathogenesis and progress of OSCC by the hBD-3 and NF-κB p65. The study by Li et al. pointed out that high-expressed c-myc was found in OSCC tissues and cell lines, and it could accelerate the progress of OSCC [27]. As a negative regulator of the cell cycle, abnormal expression of p21 can lead to cell cycle disorder and ultimately lead to tumorigenesis [28]. Research shows that NF- κB plays an important role in immune signals [29], and plays an important role in the production of neutrophils through the regulation of granulocyte colony-stimulating factor receptor (G-CSFR) [30–35]. However, whether hBD-3 and NF- κB p65 Whether governing OSCC progression through immune signaling pathways remains to be further explored. However, we did not investigate them in depth in this research. In the

future, we will investigate how hBD-3 and NF-κB p65 regulate downstream targets to promote the progression of OSCC.

Conclusion

In conclusion, in the current study, we discovered that NF-κB p65 is heavily associated with the carcinogenesis of OSF. The knockdown of hBD-3 inhibited the proliferation and invasion of OSCC cells, which can be reversed distinctly when NF-κB p65 was overexpressed. hBD-3 and NF-κB p65 may facilitate the occurrence and progression of OSCC by regulating the expression of c-myc and p21.

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Ethics approval and consent to participate

This article follows the applicable consort guidelines and was drafted following the recommendations of the STROBE Statement. The experimental protocols were approved by the Ethics Committee of the Affiliated Haikou Hospital of Xiangya Medical College of Central South University. This paper has not been published elsewhere in whole or in part. All authors have read and approved the content, and agree to submit it for consideration for publication in your journal. There are no ethical conflicts involved in this article. Informed consent was obtained from all individual participants included in the research.

Data availability

All the data during the current study are included in the article or uploaded as supplementary information.

CRediT authorship contribution statement

Yongxiu Du: Methodology, Investigation, Data curation, Formal analysis, Visualization, Funding acquisition, Writing – original draft. **Yanlan Yang:** Data curation, Formal analysis, Writing – review & editing. **Wenbo Zhang:** Data curation, Formal analysis. **Chenxi Yang:** Data curation, Formal analysis. **Pu Xu:** Conceptualization, Data curation, Formal analysis, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101582.

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