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Original Article

Co-expression of HIF-1 and TLR3 is associated with poor prognosis in oral squamous cell carcinoma

Shengwei Han[1,2], Xiteng Yin[1,2], Yufeng Wang[1,2], Wenguang Xu[1,2], Wei Cheng[1,3]

[1]Central Laboratory of Stomatology, [2]Departments of Oral and Maxillofacial Surgery, [3]Dental Implantology, Nanjing Stomatological Hospital, Medical School of Nanjing University, Nanjing, China

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Abstract: This study investigates the prognostic impact of the expression of hypoxia inducible factor (HIF)-1α and Toll-like receptor (TLR) 3 detected by immunohistochemistry in oral squamous cell carcinoma (OSCC). The study also evaluates the treatment outcome by inhibition of the HIF-1α and TLR 3 pathway (nuclear factor [NF]-κB) in an OSCC transplantation model in nude mice. Statistical analysis of immunohistochemical results with clinical findings that included overall survival outcomes was performed for 90 OSCC patients. Forty nude mice were divided into four groups (control; inhibition of HIF-1α; inhibition of NF-κB; and inhibition of HIF-1α and NF-κB). Tumor weight and immunohistochemical results of each group were compared. The results show that co-detection of low HIF-1α/TLR3 expression is significantly correlated with a better prognosis for OSCC patients. Use of an inhibitor of the HIF-1 and TLR3 pathway in an OSCC transplantation model shows a good treatment outcome.

Keywords: HIF-1, TLR-3, NF-κB, oral squamous cell carcinoma, prognosis

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors in head and neck [1, 2]. OSCC is characterized by a low survival rate despite advancements in diagnosis and treatment [2-4]. Recent research is focused on tumor microenvironment which is connected to patient outcome. Tumor hypoxia and inflammation are two important features of the tumor environment.

Tumor hypoxia results from insufficient blood supply that supports tumor proliferation. Hypoxia inducible factor (HIF)-1 is a key mediator in cell response to hypoxia [5, 6]. It consists of an oxygen-regulated HIF-1α subunit and constitutively expressed HIF-1β subunit. HIF-1 is believed to be related to tumor progression and radiotherapy resistance [7]. Our previous report shows that expression of HIF-1α is associated with poor clinical prognosis in OSCC patients [8].

Tumor hypoxia is an important aspect of the tumor microenvironment. Another aspect of the tumor microenvironment is tumor inflammation [9]. Usually, the inflammatory response could help in tissue healing. However, its dysregulation could lead to tumorigenesis [10]. Nuclear factor κB (NF-κB) is a key transcription factor during the inflammation response that is believed to be a critical link between inflammation and cancer [11]. Its signal can be mediated through Toll-like receptors (TLRs). TLRs are a family of transmembrane receptors that could recognize the conserved patterns of microbial structures [12]. It is believed that TLRs are expressed only in immune cells and could play key roles in host defense against infection by recognizing a range of chemicals produced by bacteria, viruses, and protozoa [13]. However, recent evidence indicates that various cancers and cancer cell lines may also express TLRs. TLR3, TLR4, and TLR9 are found in breast carcinoma, prostate carcinoma, and colon cancer [14-16]. TLR7 is found in esophageal squamous cell carcinomas [17]. To sum up, the expression of TLRs in different tumors may be different. Furthermore, the function of TLRs expressed in tumors is still unclear.

Our previous study confirmed the expression of TLR4 in OSCC [18]. Our recent research revealed a relationship between TLRs/NF-κB

and HIF-1 pathway in OSCC [19]. In this study, we focused on the expression of HIF-1 and TLR3 in OSCC patients and its relationship with patient clinical features and prognosis. We also analyzed whether HIF-1 and TLR3/NF-κB could be a suitable target for the treatment of OSCC.

Materials and methods

Patients and tumor tissues

The study group consisted of 90 patients diagnosed with OSCC (45 men and 45 women) who were treated at Department of Oral and Maxillofacial Surgery, Nanjing Stomatological Hospital, Medical School of Nanjing University, between January 2005 and December 2008. All medical records were reviewed retrospectively according to the inclusion and exclusion criteria. Clinical data (sex, age, TNM stage and grade of differentiation) was collected. The follow-up period was calculated from the date of surgery to the date of death, or May 2015, whichever came first. This study was approved by the Ethics Committee of the Stomatological Hospital Affiliated Medical School, Nanjing University.

ShRNA transfections and viral transductions

Recombinant lentivirus pLvx-Luciferase-puro-HIF1a-1+2+3+4 was generated by transfecting HEK293T cells. After 48 h, medium containing viral particles was harvested and passed through a 0.45-μm filter (Millipore New Bedford, MA). For transduction, 10 μl of supernatant containing lentivirus was added to the HSC-3 cells, which were maintained in 2 ml of complete culture medium. In addition, 8 μg/ml polybrene (Sigma-Aldrich) was present to aid transduction. Twenty-four hours later, the media were replaced by fresh complement with 10% FBS containing 0.5 μg/ml puromycin (Sigma-Aldrich). Cells were maintained in puromycin-containing medium for selection of stable transfectants.

Human OSCC transplantation model in nude mice

All mice were maintained in SPF conditions and the animal experiment protocols were approved by the Ethics Committee of Nanjing Stomatological Hospital (approval no. 2017-NKRL013). Immunoincompetent nude mice were injected subcutaneously in the flank with 100 μl of HSC3 or sh-HIF1α HSC3 (1×10^7/ml; 20 mice each group). After 10 days, tumors formed. Each group was divided into two groups (control group and PDTC group). The NF-κB inhibitor PDTC (Sigma-Aldrich) was injected every 2 days (50 mg/kg) from abdomen. Mice in control group were injected with physiological saline (0.2 ml/each time), for a total of 8 times. Tumor tissue was collected and tumor weight was calculated 3 days after the last injection.

Immunohistochemistry

Tumor tissue and xenograft tumor were fixed with 10% formalin, and then paraffin embedded; 5-μm sections were prepared, dewaxed with xylene, and hydrated with graded ethanol. DAKO was used with HIF-1α antibody (1:200, Abcam), TLR3 (1:100, Abcam), Ki67 (1:300, Abcam), VEGF (1:250, Abcam), NF-κB p65 (1:150, Cell Signaling Technology). Sections were counterstained with Mayer's hematoxylin, dehydrated through graded ethanol into xylene, and mounted.

Five fields from each case were selected at a magnification of ×400. Expression of TLR3 or HIF-1α was assessed by semiquantitative analysis. The percentage of immunopositive tumor cells was scored as 0, 0%; 1, positive cells (PC) <25%; 2, 25%<PC<50%; 3, 50%<PC<75%; and 4, 75%<PC<100%. The staining intensity was scored as 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. The immunoscore was calculated by multiplying the percentage score. The mean immunoscore of 90 patients was calculated, and each patient was classified as low or high expression.

Statistical analysis

Statistical analysis was performed with SPSS 18.0 (SPSS Inc, Chicago, IL) and Prism statistical software (version 5.0, GraphPad Software Inc.). All data are presented as the mean±SD or standard error (SE) of the mean. Statistical differences between groups were evaluated using paired Student's t-tests or one-way analysis of variance followed by a Student's t-test with Bonferroni correction as indicated. TNM stage, histologic differentiation status and expression of HIF-1α and TLR3 were correlated with the duration of progression free

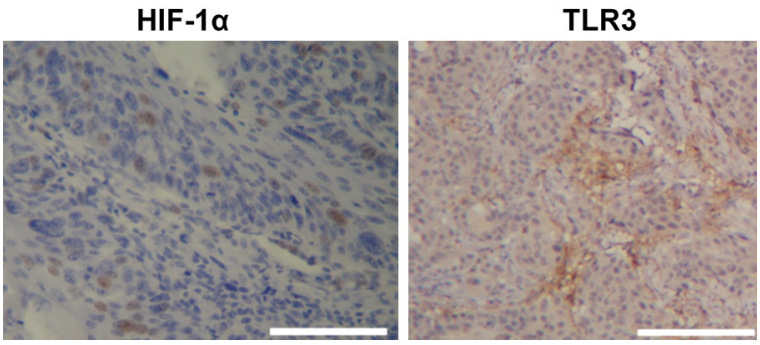

Figure 1. Expression of HIF-1α and TLR3 was detected in 90 OSCC specimens (×200) (n=90).

Table 1. TLR3 expression with regard to OSCC clinical features

Clinical features	Total No.		high		low		X^2	*P* value
	n	%	n	%	n	%		
Sex								
male	45	50%	24	55.8	21	44.7	1.13	*0.291*
female	45	50%	19	44.2	26	55.3		
age								
≤60	35	38.9	13	30.2	22	46.8	2.596	*0.107*
>60	55	61.1	30	69.8	25	53.2		
T								
1	23	25.6	13	30.2	10	21.3		
2	42	46.6	16	37.2	26	55.3	3.633	*0.304*
3	7	7.8	3	7.0	4	8.5		
4	18	20.0	11	25.6	7	14.9		
N								
N0	58	64.4	26	60.5	32	68.1	0.569	*0.451*
N+	32	35.6	17	39.5	15	31.9		
Pathologic grade								
I	38	42.2	10	23.3	28	59.6		
I-II	43	47.8	26	60.5	17	36.2	13.036	*0.001*
≥II	9	21.0	7	16.2	2	4.2		

survival. Progression free survival curves were constructed according to Kaplan and Meier. The log-rank test was used and difference between groups was performed with multivariate Cox regression analysis. A value of $P<0.05$ was considered significant.

Results

Clinical and pathological features

The 90 study patients were clinical and histological proven cases of primary OSCC. The patient ages ranged from 32 to 84 years, and the mean age at primary diagnosis was 62.5 years. There were 45 men and 45 women. TNM staging was performed according to the American Joint Committee on Cancer (AJCC): 23 cases were T stage 1, 42 cases were T stage 2, 7 cases were T stage 3 and 18 cases were T stage 4. Fifty-eight cases were lymph node negative and 32 cases were lymph node positive for metastasis. Histological classification results showed that 38 cases were well differentiated, 43 cases were moderately differentiated and nine cases were poorly differentiated.

Association of HIF-1α or TLR3 expression with clinicopathologic findings in OSCC patients

Expression of HIF-1α and TLR3 was detected in all 90 OSCC specimens (**Figure 1**). Both HIF-1α and TLR3 displayed an intracellular localization pattern. We then evaluated the possible relationship between TLR3, HIF-1α expression and clinicopathologic factors of OSCC, including age, sex, TNM stage, and pathologic grade as summarized in **Tables 1** and **2**. Expression of HIF-1α and TLR3 was divided into two groups: high and low. TLR3 expression was significantly correlated withpoorly differentiated status of tumor (**Table 1**) (P=0.001). TLR3 expression was not correlated with other measures, such as sex (P=0.291) and age (P=0.107).

HIF-1α expression was also correlated with pathologic grade (**Table 2**) (P=0.006). Furthermore, our results showed the relationship between expression of HIF-1α and patient age ($P<0.001$). No significant differences were found in any other clinical measure such as sex (P=0.205) and T stage (P=0.270).

Association of HIF-1α or TLR3 expression with clinical outcome in OSCC patients

To confirm whether patients' prognosis could be predicted by gene expression, postoperative survival curves were calculated by HIF-1α or

Table 2. HIF-1α expression with regard to OSCC clinical features

Clinical features	Total No.		high		low		X^2	*P* value
	n	%	n	%	n	%		
Sex								
male	45	50%	24	57.1	21	43.8	1.607	*0.205*
female	45	50%	18	42.9	27	56.2		
age								
≤60	35	38.9	27	64.3	8	16.7	21.373	*0.001*
>60	55	61.1	15	35.7	40	83.3		
T								
1	23	25.6	8	19.0	15	31.3		
2	42	46.6	21	50.0	21	43.8	3.922	*0.270*
3	7	7.8	2	4.8	5	10.4		
4	18	20.0	11	26.2	7	14.5		
N								
N0	58	64.4	25	59.5	33	68.8	0.832	*0.3622*
N+	32	35.6	17	40.5	15	31.2		
Pathologic grade								
I	38	42.2	12	47.6	26	37.5		
I-II	43	47.8	22	40.5	21	54.2	10.271	*0.006*
≥II	9	21.0	8	11.9	1	8.3		

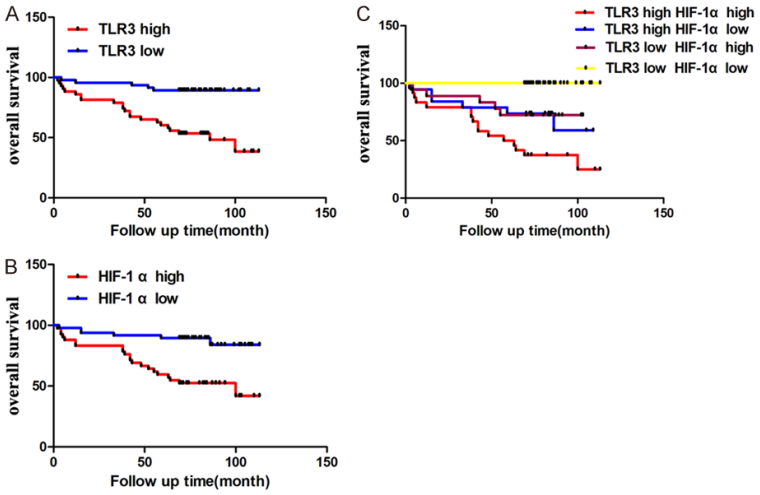

Figure 2. Expression of HIF-1α and TLR3 with respect to the prognosis of OSCC patients. (n=90). A. Expression of TLR3 according to prognosis of OSCC patients. P<0.0001. B. Expression of HIF-1α according to prognosis of OSCC patients. P=0.0001. C. Expression of HIF-1α and TLR3 according to the prognosis of OSCC patients.

Table 3. Expression of TLR3 and HIF-1α

TLR3 expression	HIF-1α expression	
	High (n=42)	Low (n=48)
High (n=43)	24	19
Low (n=47)	18	29

TLR3 expression including high/low expression. Data was available for all 90 patients with follow-up periods ranging from 2 to 113 months (mean). The result showed that HIF-1α or TLR3 expression was associated with a poor prognosis and shorter survival (**Figure 2A**, **2B**; P<0.001).

Next, expression of HIF-1α and TLR3 together was studied (**Table 3**). Kaplan-Meier analysis was performed. Co-detection of HIF-1α and TLR3 was significantly associated with prognosis. Patients with high expression of both markers had poorer prognosis (**Figure 2C**).

Targeting HIF-1 and NF-κB in OSCC xenografts of nude mice

Our previous study revealed the positive relationship between HIF-1 and TLR3/NF-κB. Then we detected whether inhibition of HIF1 and NF-κB could result in a better treatment result in an OSCC nude mice model. Forty nude mice were divided into four groups: control group; inhibition of HIF-1 group; inhibition of NF-κB group, and inhibition of HIF-1 and NF-κB group. After the treatment period, tumor tissue was collected and the weight of each tumor tissue was calculated (**Figure 3**). From the results, we concluded that both size and weight of OSCC tumor tissues were reduced in the last group (inhibition of HIF-1 and NF-κB).

Furthermore, we used IHC to assess the expression of HIF-1, NF-κB (p65), Ki67, and VEGF (**Figure 4**). Our results showed that expression of these markers were lower in the inhibition of HIF-1 and NF-κB group than that in other groups.

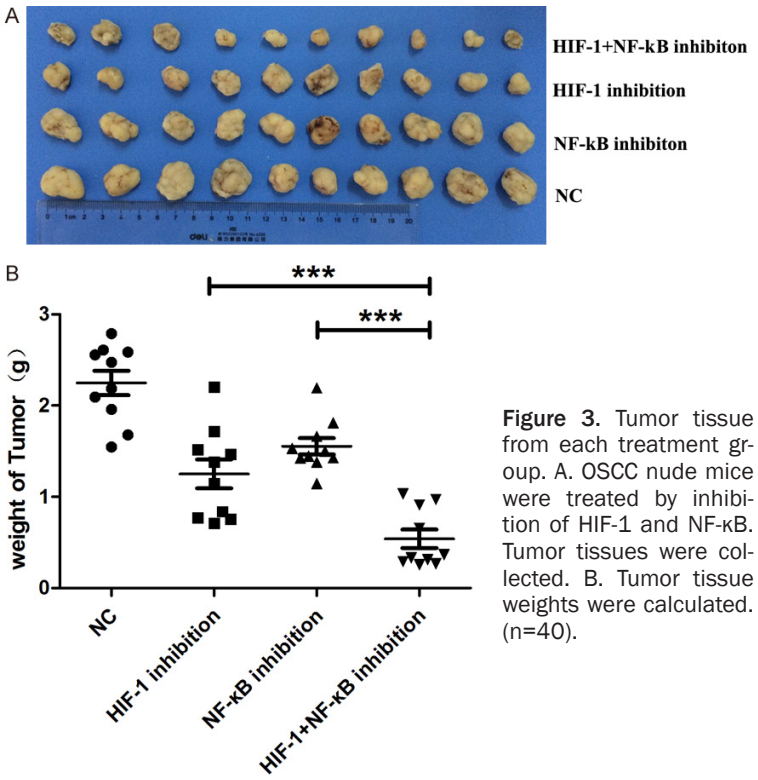

Figure 3. Tumor tissue from each treatment group. A. OSCC nude mice were treated by inhibition of HIF-1 and NF-κB. Tumor tissues were collected. B. Tumor tissue weights were calculated. (n=40).

Discussion

Our previous study revealed the crosstalk between HIF-1 and TLR3/NF-κB in the OSCC microenvironment. In this study, we proved that the expression of HIF-1 and TLR3 was associated with OSCC patients' clinical features and clinical outcomes. In addition, inhibition of HIF-1 and NF-κB in the OSCC xenografts of nude mice showed a better treatment result.

TLR3 was studied first in TLRs, and it is mainly expressed in immune and epithelial cells [20]. The activation of TLR3 facilitates the activation of NF-κB [21]. TLR3 was identified as the signal transducer for poly I:C [22]. Increasing evidence suggests that TLR3 is also expressed in many tumor cell lines or tumor tissues, especially in epithelium-derived cancer [15, 17, 18, 23]. Our previous study revealed that TLR3 was overexpressed in OSCC. Also, activation of the TLR3/NF-κB pathway could help increase the HIF-1 level [19]. Although our previous study revealed the expression of TLR3 in OSCC, its function remains unclear. In this study, we found that overexpression of TLR3 was related to pathologic stage. The Kaplan-Meier analysis showed that the mean survival of patients with low TLR3 expression in tumor tissue was significantly longer than that of patients with high TLR3 expression. These data indicated that TLR3 expression in OSCC may contribute to patient progression.

In solid tumor, rapid proliferation can outpace the oxygen supply, and result in tissue hypoxia. Tumor tissue adaptation to hypoxia is a key step in tumor progression. This adaptation is regulated by HIF-1 which is known to have an essential role in tumor environment [24, 25]. In this study, our results showed that expression of HIF-1 was associated with pathologic stage and patient age. The Kaplan-Meier analysis also showed that the mean survival of patients with low HIF-1 expression in tumor tissues was significantly longer than the survival of patients with high HIF-1 expression. These data indicated that HIF-1 expression in OSCC may contribute to patient progression. These results were consistent with previous studies [8].

Our previous study showed a positive regulatory loop between TLR3/NF-κB and the HIF-1 pathway in OSCC cells. In this study, we found the lowest survival rate in OSCC patients with high expression of HIF-1 and TLR3. Our next endeavors focused on whether HIF-1 and TLR3/NF-κB could be a therapy target for OSCC treatment. Our results showed that weight and size of tumor tissue in the group of inhibition of HIF-1 and NF-κB were smaller than that in other groups. This indicated that HIF-1 and NF-κB could be a novel target for OSCC treatment.

In summary, this study revealed that both TLR3 and HIF-1 are related to the survival of OSCC patients. TLR3/NF-κB and HIF-1 could be a novel target for further treatment.

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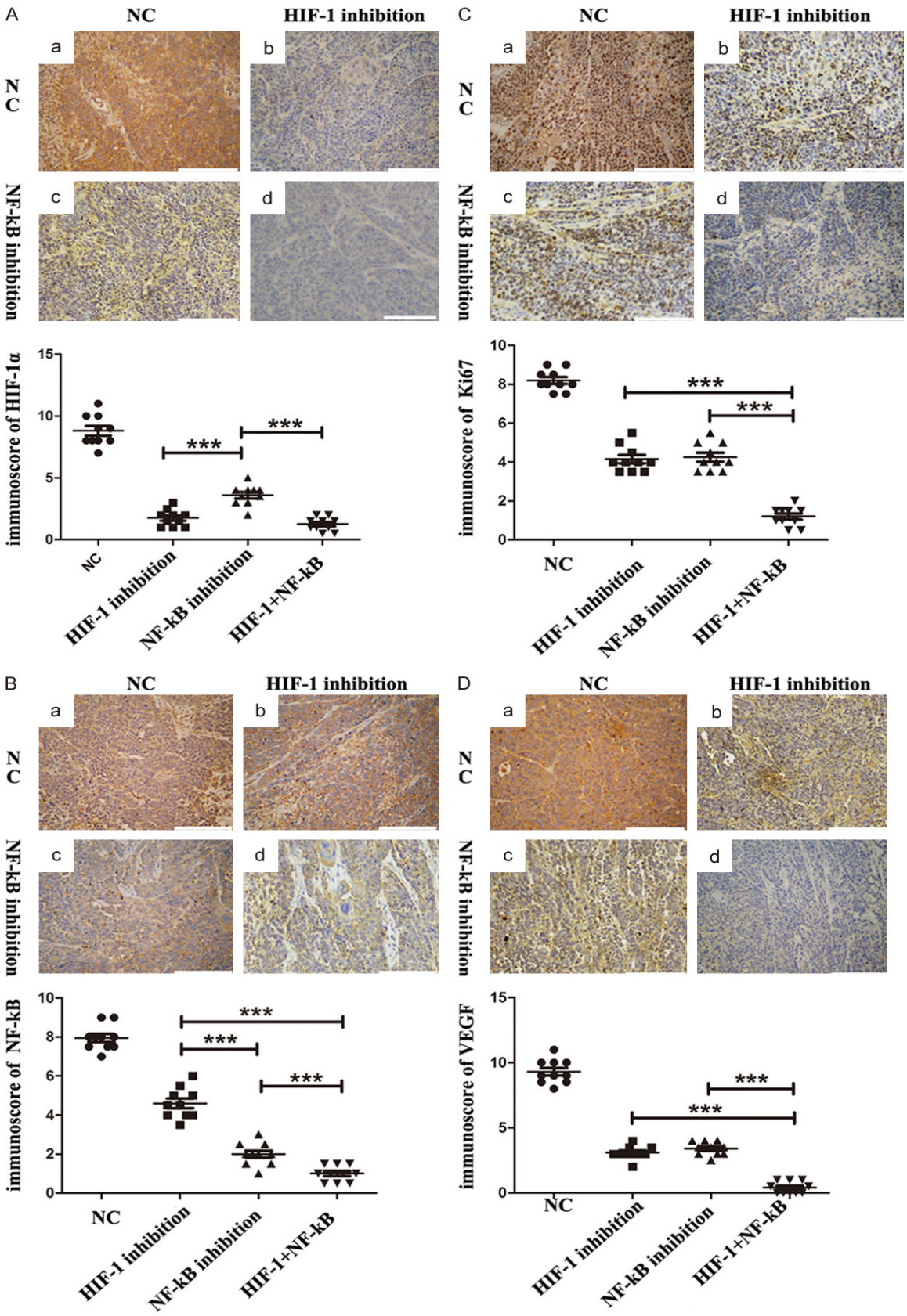

Figure 4. IHC analysis of each tumor tissue collected from the OSCC nude mice model. (n=40) OSCC nude mice were treated by inhibition of HIF-1 or NF-κB. A. HIF-1α expression. B. NF-κB expression. C. Ki67 expression. D. VEGF expression (×200).

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Disclosure of conflict of interest

None.

Address correspondence to: Wei Cheng, Department of Dental Implantology, Nanjing Stomatological Hospital, Medical School of Nanjing University, 30 Zhongyang Road, Nanjing 210008, China. Tel: +86-25-83620140; Fax: +86-25-83620140; E-mail: dentist1992@163.com

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