Practice-related research

Impact of Non-SMC Condensin I Complex Subunit D2 Upregulation on Oral Squamous Cell Carcinoma Prognosis

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ABSTRACT

Objective: To explore the influence of non-SMC condensin I complex subunit D2 (NCAPD2) on the prognosis of oral squamous cell carcinoma (OSCC) and the correlation between NCAPD2 and OSCC.

Methods: In this study, NCAPD2 gene expression profiles of OSCC and normal tissues were collected from the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). The real-time quantitative polymerase chain reaction (RT-qPCR) was employed to preliminarily validate OSCC cell strains and normal epithelial cell strains. Besides EdU, cell scratch, and transwell assays were performed to assess the proliferation, migration, and invasion of OSCC cell strains with the silence of NCAPD2. Moreover, immunohistochemistry (IHC) staining was utilised to measure the expression of NCAPD2 and tumour-related markers in 74 OSCC specimens. Finally, the Kaplan-Meier analysis was performed to evaluate the influence of NCAPD2 in the prognosis of OSCC.

Results: The expression of NCAPD2 in OSCC tissues was higher than that in normal tissues. Inhibiting NCAPD2 can reduce the proliferation and migration of OSCC cell lines and inhibit the invasion of these cells. The IHC staining results indicated that the high expression of NCAPD2 in OSCC tissues was positively correlated with T stages, Ki67 expression, and affected sites. The Kaplan-Meier analysis results validated that the up-regulated expression of NCAPD2 was significantly correlated with the poor overall survival (OS) of OSCC patients.

Conclusion: NCAPD2 is a potential molecular marker for the poor prognosis of OSCC, and it is expected to become a target for the treatment of this carcinoma.

Introduction

As the most common histologic type of oral cancer, oral squamous cell carcinoma (OSCC) has a proportion of 90% among all oral cancer.[1] The number of patients newly diagnosed with this carcinoma ranks eighth among all new cancer patients. Since 1991, the risk of cancer-induced death has decreased by 32% owing to the advances in early detection, surgical techniques, and targeted therapy.[2] Although certain achievements have been made in the surgical therapy and adjuvant radiotherapy and chemotherapy of OSCC, the survival of this carcinoma is unsatisfactory, with a 5-year survival rate of about 50% to 60%.[3,4] From 2010 to 2019, the mortality of oral cancer and pharyngeal cancer increased by 0.4% per year.[2] As reported in a previous study, TNM staging

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has been regarded as a routine diagnostic and prognostic marker, but this method is not always effective.[5] The early diagnosis and molecular targeted therapy of OSCC are considered to be the key to improving the survival of patients.[6-8] Therefore, it is necessary to investigate the biomarkers for making a diagnosis of OSCC or assessing the prognosis of this carcinoma and identify effective cancer treatment targets, which are of great significance for improving the OS of these patients.

Condensin plays a central role in mitotic chromosome condensation, non-SMC condensin I complex subunit D2 (NCAPD2) is one of the 3 non-SMC subunits in condensin I that is located on chromosome 12p13.3.[9] As a key participant in cell cycles, NCAPD2 is usually related to the abnormal development of the central nervous system (CNS) and tumorigenesis. It has been demonstrated that the abnormality of NCAPD2 is significantly related to Alzheimer's disease (AD),[10] autism,[11] and Parkinson's disease (PD).[12] NCAPD2 is overexpressed in triple-negative breast cancer (TNBC),[13] lung adenocarcinoma,[14] and colorectal cancer.[15] In addition, NCAPD2 is closely related to the prognosis of these cancers and can be considered a risk factor.[13-15] These findings suggest that NCAPD2, as a potential prognostic factor, is involved in the progression and metastasis of tumours. However, it remains unclear about the clinicopathologic significance, prognostic value, and acting mechanism of NCAPD2 in OSCC.

In this study, the expression of NCAPD2 in OSCC tissues and cell lines was measured. Additionally, the correlation between NCAPD2 expression and clinicopathologic parameters in OSCC patients was explored, and the prognostic value of NCAPD2 was also evaluated. Furthermore, the proliferation, migration, and invasion of OSCC cell strains with the silence of NCAPD2 were also assessed, in an attempt to clarify the function of NCAPD2 and relevant molecular mechanisms.

Materials and methods

Data downloading and processing

The transcriptome data (count format) of 546 patients with head and neck squamous cell carcinomas (HNSCCs) were downloaded from UCSC Xena (https://xenabrowser.net/datapages/).[16] These data were converted into TPM format files with the assistance of the IOBR package.[17] Besides, the clinical and survival data of 528 patients with HNSCCs from a study of Liu[18] were also included in this study.

To eliminate the influence of other factors, the inclusion criteria were formulated as follows: (1) patients with primary OSCC; (2) patients with complete data on age, gender, and survival; and (3) the primary site was oral cancer, which was divided into tongue (C01) and other unspecified parts of tongue (C02) and gum (C03), floor of mouth (C04), palate (C05), and other unspecified parts of oral cavity (C06) according to the International Classification of Diseases (ICD-10). The exclusion criteria included: (1) patients with the survival time shorter than 30 days; (2) patients who had received neoadjuvant therapy; (3) patients whose pathological type cannot be classified into OSCC; and (4) patients succumbed to other comorbidities. Based on the inclusion and exclusion criteria, the complete clinical data of 386 patients with OSCC in the TCGA-HNSCC dataset were screened in this study.

To verify the molecular diagnostic efficacy, the transcriptome chip and clinical information dataset (GSE6631)[19] of patients with HNSCC were downloaded from the Gene Expression Omnibus (GEO) database.[20] In this dataset, the Affymetrix Human Genome U95 Version 2 Array chip was used to perform sequencing, including the tumour tissues and para-carcinoma tissues of 22 patients with HNSCC, all of which were included in the verification set for diagnostic efficacy.

In contrast, the transcriptome chip and clinical information dataset (GSE41613)[21] of patients with OSCC were downloaded from the GEO database.[20] In this dataset, the Affymetrix Human Genome U133 Plus 2.0 Array chip was used to perform sequencing, including the tumour tissue chips of 97 OSCC patients with complete survival information, all of which were included in the verification set for prognostic efficacy.

Clinical patient samples

A total of 74 patients who were diagnosed with OSCC and received corresponding surgical treatment in the oral and maxillofacial surgery department of the First Affiliated Hospital of Kunming Medical University from August 2014 to December 2020 and had complete clinical data and survival and prognosis information were included in this study. The IHC staining was performed on the formalin-fixed OSCC tissue specimens from 74 patients and the corresponding paraffin-embedded para-carcinoma tissues (≥2.0 cm away from the tumour edge) from 12 patients. Tumour grading was performed according to the tumour classification standards of the World Health Organization (WHO),[22] and tumour staging was performed according to the tumour-nodes-metastasis (TNM) system recommended by the American Joint Committee of Cancer (AJCC).[23] This study was approved by the Ethics Review Committee of Kunming Medical University and Hospital of Stomatology (No.KYKQ2021MEC073), and all patients signed the written informed consent.

Explorations into the influence of NCPD2 on the diagnosis and prognosis of OSCC based on the TCGA-OSCC dataset

To further explore the influence of NCPD2 on the diagnosis and prognosis of OSCC, we analysed the influence of different characteristics of NCPD2 transcriptome levels on the diagnosis and prognosis of OSCC based on the TCGA-OSCC dataset. The efficacy of NCAPD2 in the diagnosis and prognosis of OSCC at the transcriptome level was verified in different datasets. Meanwhile, we also analysed the correlation between the transcriptional expression level of NCAPD2 and clinical features. The surv_cutpoint function of the survminer package was employed to identify the cut-off value of NCAPD2 that exerted the most significant influence on the survival of OSCC patients in the dataset. This cut-off value was used as a threshold to divide the dataset into the high-score and low-score groups.

Identification of the differential expression genes and function analysis of NCAPD2

The 'Limma' package[24] of R language was adopted to analyse tumour samples obtained from TCGA. The DEmRNA with $P < .05$ was obtained, and the absolute value of log-fold change (|logFC|) was >1.0. The volcano plot was drawn with the 'ggplot2' package.[25]

Immunohistochemistry

All specimens were fully fixed with 10% formalin. After routine dehydration, transparency, wax impregnation, paraffin embedding, and continuous sectioning (4 μm) treatment, these tissue sections were dewaxed, repaired, and sealed with citric acid. Subsequently, these specimens were incubated with the NCAPD2 antibody at 4°C overnight. After these specimens were washed with the phosphate buffer solution (PBS) 5 times, the general secondary antibody IgG was added. Then, these specimens were incubated at 25°C for 30 minutes. Next, the tissue sections were re-stained with 3,3′-diaminobenzidine and haematoxylin and sealed with neutral balsam. The PBS was used to replace the primary antibody as a negative control. A microscope (Olympus) was used to obtain relevant images.

Paraffin embedding and tissue sectioning, dewaxing, and hydration were performed for 74 OSCC tissue specimens and 12 para-carcinoma tissue specimens. After EDTA (pH = 8.0) was added to the boiling water, thermal repair was performed with a pressure cooker for 25 minutes. Then, these specimens were incubated with 3% H_2O_2 at room temperature for 10 minutes. The primary antibody working solution NCAPD2 (diluted by1:200, Proteintech), Ki-67 antibody (at a dilution of 1:200, ZSGB-BIO), and mouse anti-human papillomavirus (HPV) (broad spectrum) monoclonal antibody (clone No.: BPV-1/1H8 + CAMVIR, Sunbio) were incubated at 25°C for 1 hour. The slide was washed with PBS 3 times at room temperature, with each for 5 minutes. Subsequently, these specimens were incubated with the secondary antibody (Batch No.: Z5007; DAKO) at room temperature for 40 minutes. Then, the tissue sections were stained with 3,3′-diaminobenzidine and haematoxylin. Two senior pathologists who were blinded to the clinicopathologic data of these patients were responsible for performing analyses and scoring independently. The images were obtained with the aid of a microscope (Nikon). Image-pro Plus (Media Cybernetics) was used to analyse the image measurement data. According to Beesley classification, the presence of brownish-yellow granules in the nucleus and cytoplasm was rated as positive IHC staining reactions for the expression of NCAPD2 and HPV; the presence of brownish-yellow granules in the nucleus was rated as positive IHC staining reactions for the expression of Ki-67. The staining intensity was graded as 0, 1, 2, and 3, which corresponded to no staining, mild yellow, brownish-yellow, and strong brown, respectively. Five high-power fields (×200) were randomly observed in each section under an optical microscope, and 200 cells were counted in each field. The percentage of positive cells was calculated. The positive cells were scored as 1, 2, 3, and 4, with the corresponding positive cell percentages being 0% to 25%, 26% to 50%, 51% to 75%, and 76% to 100%, respectively. In terms of the expression intensity of NCAPD2, the IHC staining results were assessed by positive cell score × staining colour intensity score. The higher the score, the higher the antibody expression. Specifically, the score ≤4 represented the low expression of NCAPD2. The score >4 represented the high expression of NCAPD2. The presence of brownish-yellow granules in the nucleus and the staining in both the cytoplasm and nucleus ≥10% can be rated as positive IHC staining reactions for HPV. The staining in the cytoplasm or the absence of staining in both the cytoplasm and nucleus was rated as negative IHC staining reactions for HPV, which was scored as 0. In addition, >30% of cells with the staining in the nucleus indicated the high expression of Ki-67, while ≤30% of cells with the staining in the nucleus indicated the low expression of Ki-67.

Cell culture

The human OSCC cell line (CAL27), human tongue squamous cell carcinoma (TSCC) cell line (HSC-3), and immortal human keratinocyte cell strain (HaCaT) were purchased from the American Type Culture Collection (ATCC). CAL27 and HaCaT cells were cultured in the Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum (FBS; GIBCO) and 1% penicillin/streptomycin solutions (GIBCO). HSC-3 cells were cultured in the MEM supplemented with 10% FBS and 1% penicillin/streptomycin solutions.

Quantitative RT-PCR

The total RNA was extracted with the TRIzol reagent (Invitrogen). The first strand cDNA was synthesised from the total RNA with the PrimeScript RT reagent (TaKaRa). The RT-qPCR was performed with the ABI Step One Plus real-time PCR system (Applied Biosystems), and the SYBR Premix Ex Taq II (TaKaRa) reaction system was also adopted. The cycling conditions included the initial denaturation at 95°C for 10 minutes, followed by 40 denaturation cycles at 95°C for 10 seconds, annealing at 58°C for 15 seconds, extension at 72°C for 1 minute, and finally extension at 72°C for 5 minutes. The primers included forward (NCAPD2) 5′-TGGAGGGGTGAATCAGTATGT-3′ and reverse (NCAPD2) 5′-T GCGGGATACCACTTTTATCAGG-3′; forward (GAPDH) 5′-AAAGGGTCATCATCTCTG-3′ and reverse (GAPDH) 5′-GCTGTTGTCATACTTCTC-3′.

Small interfering RNA transfection

Small interfering RNAs (siRNAs) against NCAPD2 were designed and synthesised by OBiO. The NCAPD2 siRNA sequence was presented as follows: NCAPD2-231-Human (sense strand: 5′-CAAAGAAGAUACUCUGCAAUUTT-3′; antisense strand: 5′-AAUUGCAGAGUAUCUUCUUUGTT-3′); NCAPD2-561-Human (sense strand: 5′-CUCAAUAAUUGAAGAAGAAUUTT-3′; antisense strand: 5′-AAUUCUUCUUCAAUUAUUGAGTT-3′); NCAPD2-1239-Human (sense strand: 5′-GGCAGACAAGUCAGUGCUAGUTT-3′; antisense strand: 5′-ACUAGCACUGACUUGUCUGCCTT-3′); sicontrol (sense strand: 5′-UUCUCCGAACGUGUCACGUTT-3′; antisense strand: 5′-ACGUGACACGUUCGGAGAATT-3′). These cells after inoculation were transfected with a mixture of siRNAs and

lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

Western blot

The radioimmunoprecipitation assay lysis buffer was used for cell lysis (Beyotime) to obtain proteins. The protein concentration was measured with the BCA kit (Beyotime). After 25 μg of protein was isolated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis, it was transferred to the polyvinylidene fluoride membrane. Subsequently, the specimens were incubated with a 5% skim milk sealing membrane at room temperature for 1 hour and then incubated with the primary antibody GAPDH (ab8245, 1:3,000, Abcam) and NCAPD2 (13382-1-AP, 1:3,000, Proteintech) at 4°C overnight. The membrane was treated with secondary antibodies at room temperature for 1 hour. After the developing agent was added to the membrane, the detection was performed by a chemiluminescence imaging system (Bio-rad).

Transwell assay

The transwell chamber (Corning) with an aperture of 8 μm was used to assess the migration and invasion of OSCC cells. In cell invasion experiments, the equivalent transfected cells were re-suspended and placed on the top of a membrane precoated with 1 μg/μL matrix (BD Biosciences). After 24 hours of incubation, the cells invading the underside of the membrane were fixed with 4% paraformaldehyde and stained with 10% crystal violet at 37°C according to the manufacturer's instructions. The number of cells migrating and invading in three randomly selected regions was calculated under the IX71 inverted fluorescence phase contrast microscope (Olympus).

Wound-healing assays

After these cells were inoculated in a 6-well plate and reached almost 100% fusion, the cell scratch assay was performed. Then, the cells were washed twice with sterile PBS and cultured in the serum-free medium. The images of scratched areas were obtained at 0, 24, 48, and 72 hours, respectively.

Cell viability assay

The viability of human OSCC cells was determined by the CCK-8 (Dojindo). Specifically, these cells (3000-5000 cells/well) were inoculated in a 96-well plate. Then, 100 μL of culture medium was added to these cells containing 10 μL of CCK8 solutions, followed by the incubation at 37°C for 3 hours. The absorbance was measured at 450 nm.

EdU incorporation assay

The BeyoClick EdU Cell Proliferation Kit was adopted for EdU staining according to the manufacturer's instructions. Specifically, a total of 4×10^5 CAL27 and HSC-3 cells were inoculated in a 6-well plate containing a cover glass. After 15 hours of incubation, the EdU reagent was added to each well and incubated at 37°C for 4 to 6 hours. These cells were washed with PBS, fixed with 4% paraformaldehyde, and then incubated with 0.3% Triton X-100. Finally, these cells were stained with 4′,6-diamidino-2-phenylindole.

Statistical analysis

In this study, all data were calculated and statistically analysed with the aid of R software (https://www.r-project.org/, version 4.2.2). If not specifically indicated, spearman correlation analysis was performed based on the cor function of R software, the KM survival analysis was conducted by the log-rank test, and the independent factors of NCAPD2 were identified by the COX analysis. All P values in the statistical analysis were bilateral. The adjusted $P < .05$ indicated that there was a statistically significant difference for the screening of differential expression genes ($P > .05$, ns; $P < .05$, *; $P < .01$, **; $P < .001$, ***).

Results

High expression of NCAPD2 in OSCC tissues and cell lines

To explore the potential influence of NCAPD2 on OSCC, we conducted a comprehensive analysis of NCAPD2 expression at the tissue and cellular levels. First, the RNA-seq data from TCGA indicated that the NCAPD2 mRNA expression level was significantly up-regulated in OSCC compared with normal tissues (Figure 1A,B). We further performed IHC staining analyses for OSCC tissues from 74 patients and para-carcinoma tissues from 12 patients. Based on the IHC score, the expression of NCAPD2 in OSCC tissues was significantly higher than that in para-carcinoma tissues (Figure 1C-E). Consistent with the IHC analysis results, the NCAPD2 mRNA and protein levels were higher in various OSCC cell strains than in HaCAT cell strains (Figure 1F-H). In addition, the expression of NCAPD2 was positively correlated with T stages and Ki67 expression (Table 1). These results suggested that the amplification and high expression of NCAPD2 were related to the progression of OSCC.

Correlation between the transcriptional expression level of NCAPD2 and the diagnostic prognosis and clinical features of OSCC

To further explore the correlation between NCAPD2 and OSCC progression, we conducted multivariate analyses on clinicopathologic variables and NCAPD2 expression based on TCGA-OSCC, GSE6631, and OSCC patient sample data, respectively. The Kaplan-Meier curve and ROC curve were also plotted.

Based on TCGA-OSCC and GSE6631, the diagnostic efficacy of the transcriptional expression level of NCAPD2 was analysed. The AUC of the transcriptional expression level of NCAPD2 was 0.896 and 0.787 based on TCGA and GSE6631, respectively, with favourable diagnostic efficacy in both groups of data (Figure S1A). Besides, the influence of the transcriptional expression level of NCAPD2 on survival was further analysed based on TCGA-OSCC. It was found that the transcriptional expression level of NCAPD2 exerted an impact on survival based on both datasets. The higher the expression

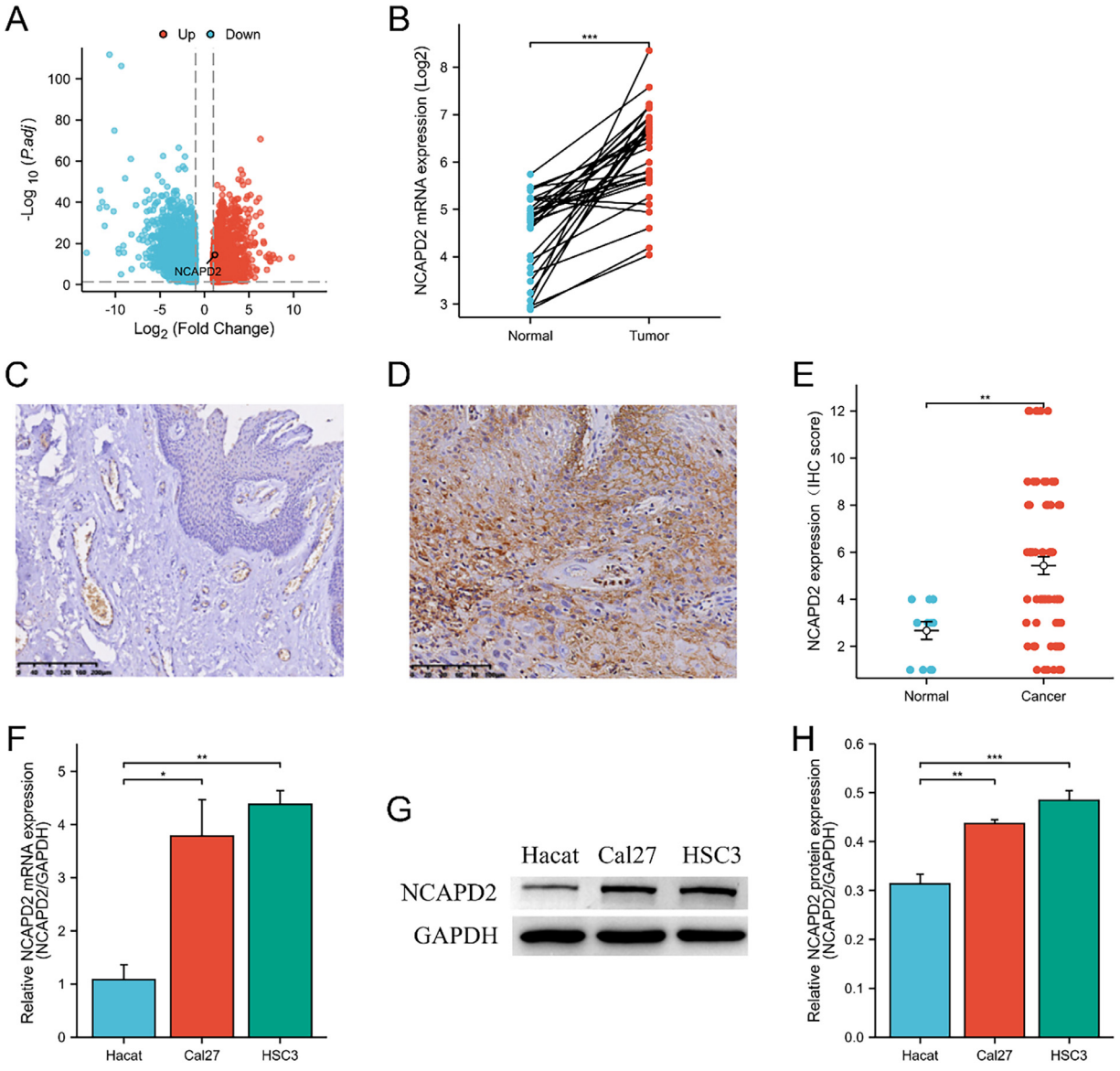

Fig. 1 – NCAPD2 is frequently upregulated in OSCC. (A,B) NCAPD2 mRNA expression in OSCC from TCGA database. (A) Volcano plot depicting significantly differentially expressed genes, upregulated and downregulated genes were represented red and blue, respectively. NCAPD2 log2 (fold change) 1.1632, −log10 (P-value) 14.4657. (B) NCAPD2 mRNA levels were significantly upregulated in OSCC patient, compared with paired normal tissue. In B, each line indicated one pair of matched adjacent normal-tumour samples. (C,D) Representative pictures of the expression levels of NCAPD2 in normal tissues and tumour tissues detected by immunohistochemistry (IHC) (200× magnification). (E) Plots indicate the NCAPD2 levels in OSCC patients. (F-H) NCAPD2 mRNA and protein were expressed across Cal27 cells and HSC3 cells, compared with Hacat cells. *P < .05, *P < .05, **P < .01, ***P < .001.

level of NCAPD2, the worse the survival of OSCC patients (Figure S1B, $P = .010$). The same results were also observed in the validation set GSE41613 (Figure S1C, $P = .044$). Meanwhile, we also analysed the correlation between the transcriptional expression level of NCAPD2 and clinical features. It was found that the sex of OSCC patients can affect the transcriptional expression level of NCAPD2, and the transcriptional expression level of NCAPD2 in male patients is higher than that in female patients (Figure S1E). Additionally, the transcriptional expression level of NCAPD2 can affect the differentiation of OSCC. Specifically, the higher the transcriptional expression level of NCAPD2, the lower the differentiation of OSCC (Figure S1F).

In this study, these OSCC patients were 31 to 78 years old (mean: 54.78 ± 10.03 years old), including 30 males and 44 females. Further, we analysed the correlation between NCAPD2 expression and clinicopathologic features in 74 OSCC patients. Based on the IHC score, higher NCAPD2 expression was observed in the OSCC tissues from 48.6% (36/74) patients. From the localisation of the lesion site, the high expression rate of NCAPD2 in the tongue, gingiva, floor of mouth, palate, and cheek was 73.3% (22/30), 88.8% (16/18),

Table 1 – Association between clinicopathologic parameters and protein expression in patients with oral squamous cell carcinoma.

Characteristics	NCAPD2, n (%)			Ki67, n (%)			HPV, n (%)		
	Low	High	P-value	Low	High	P-value	Negative	Positive	P-value
Age			.821			.139			.234
<55	20 (27%)	18 (24.3%)		18 (24.3%)	20 (27%)		30 (40.5%)	8 (10.8%)	
≥55	18 (24.3%)	18 (24.3%)		11 (14.9%)	25 (33.8%)		24 (32.4%)	12 (16.2%)	
Gender			.219			.714			.001
Female	18 (24.3%)	12 (16.2%)		19 (25.7%)	11 (14.9%)		26 (35.1%)	18 (24.3%)	
Male	20 (27%)	24 (32.4%)		18 (24.3%)	26 (35.1%)		28 (37.8%)	2 (2.7%)	
T. stage			.009			.038			.192
T1+T2	34 (45.9%)	23 (31.1%)		26 (35.1%)	31 (41.9%)		39 (52.7%)	18 (24.3%)	
T3+T4	4 (5.4%)	13 (17.6%)		3 (4.1%)	14 (18.9%)		15 (20.3%)	2 (2.7%)	
N stage			.778			.714			.313
N0	22 (29.7%)	22 (29.7%)		18 (24.3%)	26 (35.1%)		34 (45.9%)	10 (13.5%)	
N1+N2	16 (21.6%)	14 (18.9%)		11 (14.9%)	19 (25.7%)		20 (27%)	10 (13.5%)	
Clinical stage			.253			.459			.732
Stage I/II	24 (32.4%)	18 (24.3%)		18 (24.3%)	24 (32.4%)		30 (40.5%)	12 (16.2%)	
Stage III/IV	14 (18.9%)	18 (24.3%)		11 (14.9%)	21 (28.4%)		24 (32.4%)	8 (10.8%)	.234
Tumour site			<.001			.019			.345
Tongue	8 (10.8%)	22 (29.7%)		16 (21.6%)	14 (18.9%)		20 (27%)	10 (13.5%)	
Gingiva	2 (2.7%)	16 (21.6%)		1 (1.4%)	17 (23%)		16 (21.6%)	2 (2.7%)	
Flour of the mouth	2 (2.7%)	2 (2.7%)		2 (2.7%)	2 (2.7%)		2 (2.7%)	2 (2.7%)	
Palate	8 (10.8%)	4 (5.4%)		6 (8.1%)	6 (8.1%)		8 (10.8%)	4 (5.4%)	
Cheek	4 (5.4%)	6 (8.1%)		4 (5.4%)	6 (8.1%)		8 (10.8%)	2 (2.7%)	
Lymph node metastasis			.856			.989			.815
No	24 (32.4%)	22 (29.7%)		18 (24.3%)	28 (37.8%)		34 (45.9%)	12 (16.2%)	
Yes	14 (18.9%)	14 (18.9%)		11 (14.9%)	17 (23%)		20 (27%)	8 (10.8%)	
Differentiation,			.231			.112			.254
Well	24 (32.4%)	16 (21.6%)		19 (25.7%)	21 (28.4%)		32 (43.2%)	8 (10.8%)	
Moderate	10 (13.5%)	16 (21.6%)		6 (8.1%)	20 (27%)		16 (21.6%)	10 (13.5%)	
Poorly	4 (5.4%)	4 (5.4%)		4 (5.4%)	4 (5.4%)		6 (8.1%)	2 (2.7%)	
Drinking history			.682			.600			<.001
No	28 (37.8%)	28 (37.8%)		21 (28.4%)	35 (47.3%)		48 (64.9%)	8 (10.8%)	
YES	10 (13.5%)	8 (10.8%)		8 (10.8%)	10 (13.5%)		6 (8.1%)	12 (16.2%)	
Smoking history			.236			.877			.094
No	20 (27%)	14 (18.9%)		13 (17.6%)	21 (28.4%)		28 (37.8%)	6 (8.1%)	
YES	18 (24.3%)	22 (29.7%)		16 (21.6%)	24 (32.4%)		26 (35.1%)	14 (18.9%)	
Ki 67			<.001						.090
Low	29 (39.2%)	0 (0%)					36 (48.6%)	9 (12.2%)	
High	9 (12.2%)	36 (48.6%)					18 (24.3%)	11 (14.9%)	
HPV			.051			.090			
Negative	24 (32.4%)	30 (40.5%)		18 (24.3%)	36 (48.6%)				
Positive	14 (18.9%)	6 (8.1%)		11 (14.9%)	9 (12.2%)				
NCAPD2						<.001			.051
Low				29 (39.2%)	9 (12.2%)		24 (32.4%)	14 (18.9%)	
High				0 (0%)	36 (48.6%)		30 (40.5%)	6 (8.1%)	

HPV, human papillomavirus; NCAPD2, non-SMC condensin I complex subunit D2.

50% (2/4), 33.3% (4/12), and 60% (6/10), respectively, and the difference was statistically significant (Table 1). The high expression of Ki67 was found in 45.7% (45/74) tumour tissue specimens (Table 1; Figure 2I). The incidence of HPV infection was 27% (20/74) (Table 1). HPV-positive cells were distributed throughout the tumour tissue (Figure 2G). Moreover, it was found that NCAPD2 expression was positively correlated with such adverse clinicopathologic features as T stages, Ki67 expression, and affected sites (Table 1; Figure 2A-E and J). These results indicated that NCAPD2 was related to the proliferation of OSCC cells. However, there was no significant correlation between the expression level of NCAPD2 and the age, gender, pathological grade, smoking history, drinking history, and HPV infection of patients (Table 1). The Kaplan-Meier curve presented that NCAPD2, higher Ki67 expression, T stages, smoking history, and drinking history were associated with the poor OS of OSCC patients ((Figure S2A, B, D-F). These results suggested that the high expression of NCAPD2 in OSCC may be a risk factor for the poor prognosis of this carcinoma. In addition, the univariate Cox regression analysis of OS showed that NCAPD2 expression, KI67 expression, T stages, smoking history, and drinking history were all effective prognostic factors (Table 2, all $P < .05$). The further multivariate Cox regression analysis results confirmed that NCAPD2 expression was an independent prognostic indicator for the OS of OSCC patients (Table 2, $P = .022$). It was verified that NCAPD2 may be a biomarker for the diagnosis and prognosis of OSCC.

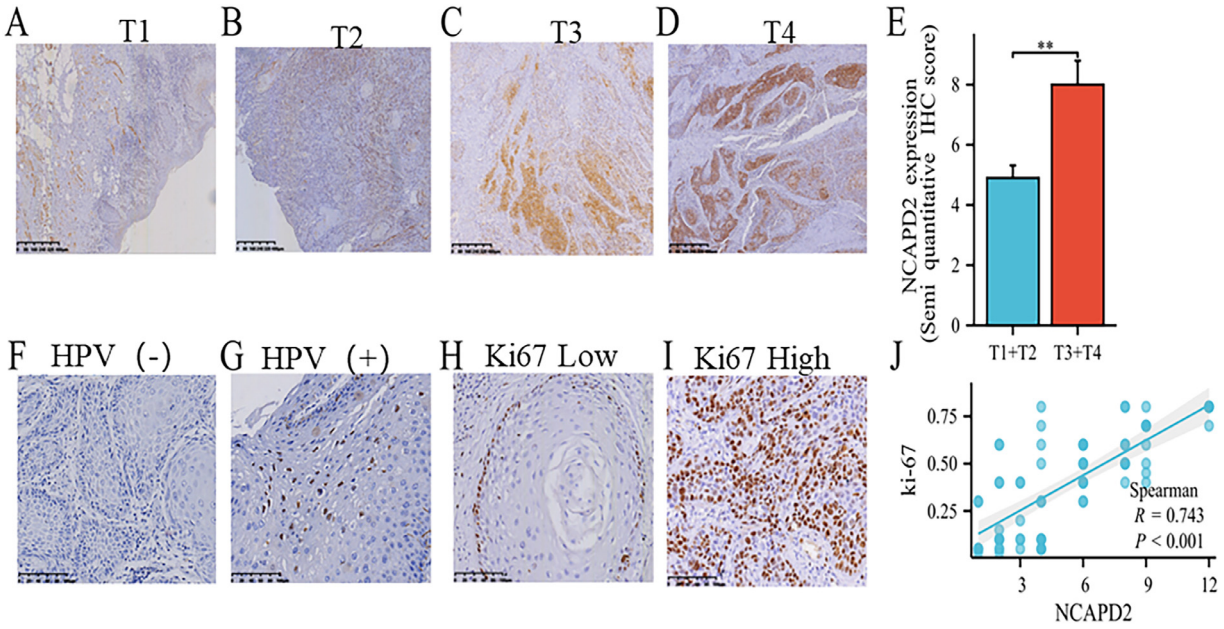

Fig. 2 – NCAPD2 expression is associated with clinicopathologic features in OSCC. (A-E) Images and plots indicate the protein levels of NCAPD2 across different tumour stages of patients with OSCC (magnification: 40 ×; scale: 400 μm). (F-I) Representative images of HPV expression levels in tumour tissues through IHC (magnification: 200 ×; scale: 100 μm). J Graphs show Pearson correlation in IHC scores of NCAPD2 and Ki67 expression levels. **P < .01.

Down-regulation of NCAPD2 decreased the proliferation, migration, and invasion of OSCC cells

As NCAPD2 was overexpressed in OSCC tissues and cells, it can be speculated that NCAPD2 may play a role in the tumorigenesis of OSCC. To further clarify the reasons why the differential expression of NCAPD2 can result in different prognostic states in patients with OSCC, the siRNA interference technology was adopted to knockdown NCAPD2 in HSC3 and CAL27 cell strains. Besides, EdU, cell scratch, and transwell assays were performed. It was verified that knockdown NCAPD2 can inhibit the proliferation, migration, and invasion of OSCC cells (Figure 3). The effect of siRNAs on cell proliferation was then assessed by EdU assay and significantly less of EdU-positive cells with silencing NCAPD2 was detected by comparing with the control cells (Figure 3A). In addition, the transwell assay results suggested that the down-regulation of NCAPD2 led to a significant decrease in the invasion of OSCC cells (Figure 3B). Furthermore, In vitro scratch assays were performed to test the effect of NCAPD2 knockdown on the migratory behaviour of OSCC cells. We found that NCAPD2 depletion significantly impaired migration potential compared to control in both Cal27 and HSC3 cells (Figure 3C).

Discussion

The survival of OSCC is unsatisfactory, with a 5-year survival rate of about 50% to 60%.[3,4] The early diagnosis and molecular targeted therapy of OSCC are considered to be the key to improving the survival of patients.[6-8] Therefore, it is of great significance to clarify the driving factors for the occurrence and development of OSCC and identify the markers for the prognosis and targeted therapy of patients with this carcinoma. Lectins are a category of multi-subunit protein complexes, and NCAPD2 is one of the non-SMC subunits of condensin I. NCAPD2 plays an important role in chromosome aggregation and segregation during the mitosis of cells.[26] In 2019, Zhang et al[13] first reported the close relationship between NCAPD2 and cancers. They proved that NCAPD2 played an important role in the progression of triple-negative breast cancer. The prognostic value of NCAPD2 in colorectal cancer,[15] breast cancer,[26] and lung adenocarcinoma[14] has been revealed in some recent studies, and it can be used as a potential therapeutic target. In this study, it was found that the expression of NCAPD2 mRNA and protein in OSCC tissues and cell strains was significantly higher than that in healthy oral tissues and epithelial cell strains (Figure 1). Based on TCGA and OSCC patient cohorts, the high expression of NCAPD2 was positively correlated with larger tumour size and reduced OS (Figures 2 and S2), which was consistent with the research results related to colorectal cancer,[15] breast cancer,[26] and lung adenocarcinoma.[14] Based on TCGA-OSCC and GSE6631, the diagnostic efficacy of the transcriptional expression level of NCAPD2 was analysed. The AUC of the transcriptional expression level of NCAPD2 was 0.896 and 0.787 based on TCGA and GSE6631, respectively, with favourable diagnostic efficacy in both groups of data. The COX multivariate analysis results demonstrated that the high expression of NCAPD2 was an independent survival marker in the whole cohort of OSCC patients after the adjustment of other prognostic factors. To our knowledge, it may be the first evidence to prove that the high expression of NCAPD2 was related to the poor prognosis of OSCC. These findings indicated that NCAPD2 exhibited considerable potential and possible clinical effectiveness as a target of OSCC.

Table 2 – Univariate and multivariate Cox regression analysis of overall survival in patients with oral squamous carcinoma.

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
NCAPD2	74		.039		
NCAPD2 High	36	Reference		Reference	
NCAPD2 Low	38	0.605 (0.377-0.973)	.038	2.912 (1.171-7.244)	.022
T.stage	74		<.001		
T1+T2	57	Reference		Reference	
T3+T4	17	3.143 (1.758-5.619)	<.001	7.674 (3.017-19.518)	<.001
N.stage	74		.084		
N1+N2	30	Reference		Reference	
N0	44	0.654 (0.407-1.051)	.079	1.036 (0.476-2.256)	.929
Age	74	1.021 (0.998-1.045)	.068	1.044 (1.002-1.089)	.040
Gender	74		.185		
Male	44	Reference			
Female	30	0.726 (0.449-1.172)	.190		
Tumour site	74		.032		
Gingiva	18	Reference		Reference	
Tongue	30	0.491 (0.268-0.901)	.022	0.688 (0.285-1.660)	.406
Cheek	10	1.056 (0.474-2.352)	.894	1.326 (0.422-4.162)	.629
Palate	12	1.196 (0.558-2.562)	.645	1.776 (0.643-4.903)	.267
Flour of the mouth	4	1.425 (0.471-4.313)	.531	1.212 (0.305-4.814)	.784
Ki 67	74		.012		
High	45	Reference		Reference	
Low	29	0.538 (0.329-0.881)	.014	0.379 (0.157-0.916)	.031
HPV	74		.388		
Positive	20	Reference			
Negative	54	0.792 (0.470-1.333)	.379		
Lymph node metastasis	74		.187		
Yes	28	Reference			
No	46	0.721 (0.447-1.164)	.181		
Differentiation	74		.764		
Moderate	26	Reference			
Well	40	0.891 (0.534-1.488)	.660		
Poorly	8	0.753 (0.339-1.672)	.486		
Drinking history	74		.005		
No	56	Reference		Reference	
YES	18	2.331 (1.339-4.057)	.003	3.054 (1.433-6.506)	.004
Smoking history	74		.010		
No	34	Reference		Reference	
YES	40	1.905 (1.157-3.137)	.011	2.152 (1.151-4.022)	.016

CI, confidence interval; HPV, human papillomavirus; NCAPD2, non-SMC condensin I complex subunit D2.

In this study, we investigated the clinicopathologic correlation between the expression of NCAPD2 and OSCC patients. It was observed that the up-regulated expression of NCAPD2 was positively correlated with larger tumour size at diagnosis and the high expression of Ki67. The immunohistochemical expression of Ki-67 was negatively correlated with the OS and PFS of patients with OSCC. Interestingly, it has also been reported that Ki67 is a biomarker for the unfavourable diagnosis and poor prognosis in OSCC patients.[27,28] Ki67 is a large nonhistone protein in the nucleus and nucleolar region, and it is one of the optimal markers to evaluate the proliferation and local invasion of tumour cells.[29] Given that the expression of NCAPD2 was positively correlated with the expression of KI67, we speculated that the up-regulated expression of NCAPD2 promoted the proliferation, migration, and invasion of OSCC cells. Besides, EdU, cell scratch, and transwell assays were performed. It was verified that the proliferation, migration, and invasion of OSCC cell strains were inhibited after the knockout of NCAPD2, which was consistent with the research results related to breast cancer[26] and lung adenocarcinoma.[14] With the decrease in the incidence of OSCC related to tobacco, alcohol, and betel nut consumption, the number of OSCC patients without typical risk factors is increasing gradually in clinical practice. HPV is the most serious risk factor for the occurrence of OSCC.[30] It has been confirmed in different studies that the infection rate of HPV in OSCC patients varies greatly (6%-84%).[31-33] This can be mainly attributed to regional differences and inconsistent testing methods and inclusion standards. The positive rate of HPV infection in this study was 27%. However, it remains controversial over the etiological role and prognostic significance of HPV in OSCC. It has been reported that HPV infection in OSCC can result in a worse prognosis[34] or has no influence on the prognosis of patients with OSCC.[33] In this study, the presence or absence of HPV infection in patients with OSCC had no influence on their prognosis. Additionally, the correlation between NCAPD2 expression and HPV infection was discussed, and it was validated that there was no statistically significant correlation between both. The infection rate of HPV in OSCC was much lower than that in oropharyngeal squamous cell carcinoma (OPSCC). At least 70% of

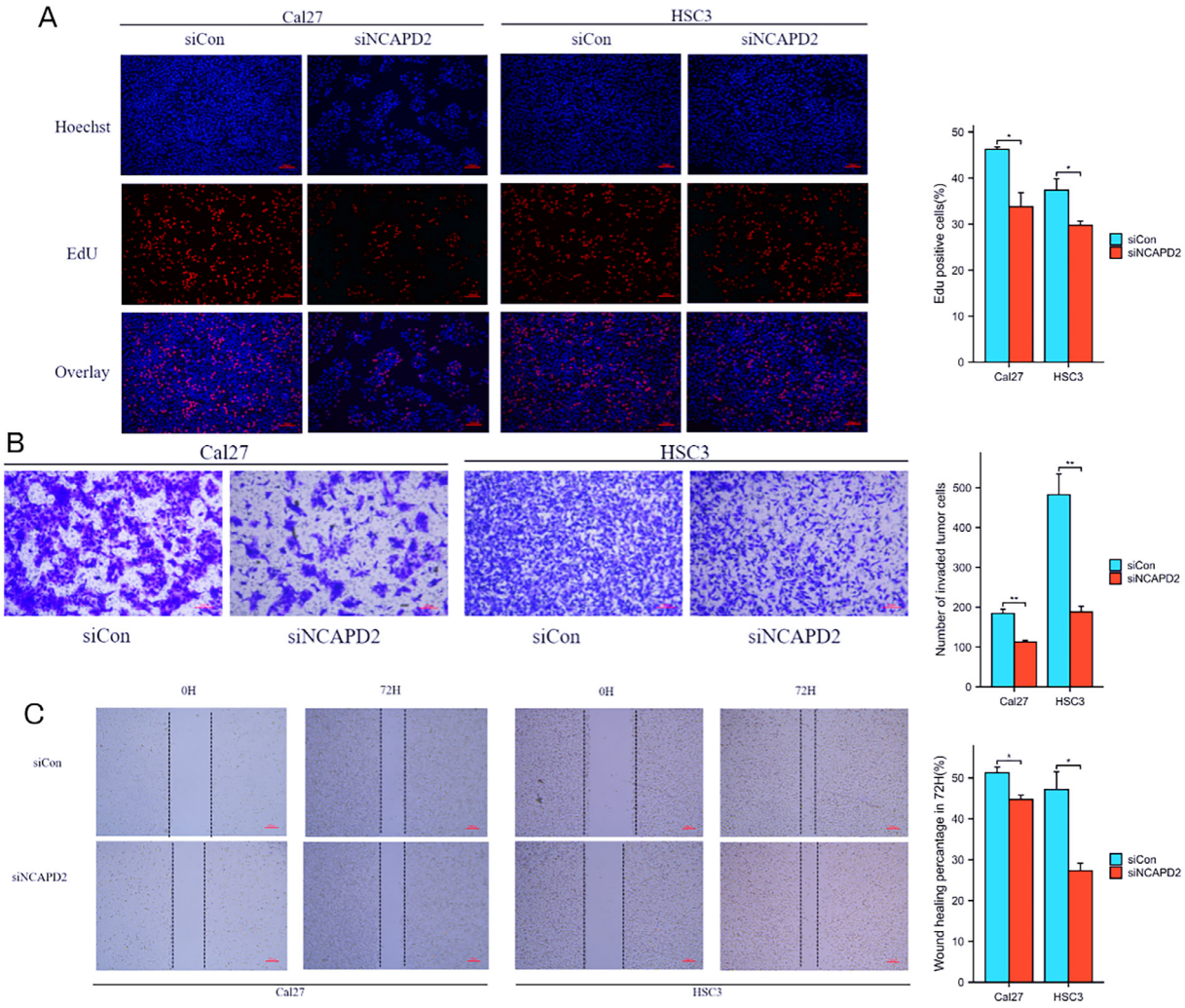

Fig. 3 – Association of NCAPD2 knockdown in OSCC cell lines with oncogenic progression. Knockdown of NCAPD2 reduced OSCC cell proliferation (A, Scale bars indicate 100 μm), invasion (B, Scale bars indicate 100 μm) and migration (C, Scale bars indicate 200 μm) of both Cal27 and HSC3 cells. Statistical significance (t-test): *P < .05, **P < .01.

patients with OPSCC in the United States exhibited positive HPV results.[35] In view of the small sample size, it is necessary to further explore the correlation between NCAPD2 expression and HPV infection based on a larger OSCC sample size or based on OPSCC.

Conclusions

In conclusion, it was found that NCAPD2 was overexpressed in both OSCC cell lines and primary tumours, which promoted the proliferation and migration of OSCC cells and was related to the survival and prognosis of patients with OSCC. However, given the inherent limitations of our study, such as a relatively small sample size and a retrospective single-centre design, it is imperative to expand the sample size in future multicentre large-scale studies. This will enable us to elucidate the underlying mechanism of action of NCAPD2 in the pathogenesis and progression of OSCC, thereby providing novel insights for molecular targeted therapy targeting OSCC.

Conflict of interest

None disclosed.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.identj.2024.03.015.

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