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ANATOMICAL PATHOLOGY

Overexpression of CDK7 is associated with unfavourable prognosis in oral squamous cell carcinoma

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Summary

Increased expression of cyclin-dependent kinase 7 (CDK7), an ubiquitous kinase associated with cell cycle and transcription, contributes to human tumourigenesis and associates with patients' prognosis. In the present study, we sought to investigate the expression pattern of CDK7 and its clinicopathological significance in primary oral squamous cell carcinoma (OSCC). The expression of CDK7 mRNA in OSCC was determined by data mining and interrogation using the Oncomine database. Its protein expression was measured by immunohistochemistry in clinical samples from a retrospective cohort of 113 patients with primary OSCC which were treated at our institution from January 2006 to December 2016. The potential associations between CDK7 abundance and multiple clinicopathological parameters as well as patients' survival were assessed. The 4-nitroquinoline 1-oxide (4NQO)-induced OSCC mouse model was developed to monitor CDK7 expression during cancer initiation and progression. The bioinformatics analyses revealed higher CDK7 mRNA in OSCC samples compared to normal counterparts. Our immunohistochemical staining data revealed significant aberrant overexpression of CDK7 in a large subset of OSCC. Elevated CDK7 expression was found to be significantly associated with higher T-stage ($p = 0.009$) and reduced overall and disease-free survival (Log-rank test, $p = 0.022$, 0.010, respectively). Both univariate and multivariate Cox regression analysis identified the expression status of CDK7 as an important independent prognostic factor for patients' survival. Increased CDK7 expression was parallel with OSCC development in the 4NQO-induced animal model. Our findings indicate that aberrant CDK7 overexpression associates with T-stage and reduced survival in OSCC, thus suggesting that it might play critical roles underlying oral tumourigenesis and also serve as a novel biomarker with diagnostic and prognostic significance.

Key words: CDK7; cell cycle regulation; oral squamous cell carcinoma; prognosis.

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BACKGROUND

Oral squamous cell carcinoma (OSCC) is commonly defined as squamous cell carcinoma originating from the lip, tongue, gingiva, cheek, floor of mouth, and palate. It is the most common head and neck malignancy worldwide with relatively high incidence and mortality.[1] Well-known aetiological factors for OSCC include smoking, alcohol consumption and betel quid chewing.[2] The past decades have witnessed tremendous progress in combined and sequential treatment regimes against OSCC.[3,4] However, the long-term survival for patients with OSCC remains dismal, especially for those with advanced disease. To date, few reliable biomarkers have been equivocally established for diagnostic and prognostic management of oral cancer.[5] Hence, the identification and validation of novel biomarkers and potential therapeutic targets for OSCC are urgent for clinicians to stratify patients and improve their prognosis.

Considerable evidence has established that dysregulation of transcription and cell cycle are hallmarks of cancer.[6] Importantly, therapeutic inhibition of aberrant transcription or targeting regulators of cell cycle in cancer has shown promising outcomes in preclinical animal models.[7,8] Cyclin-dependent kinase 7 (CDK7) is the main enzyme responsible for activating and stabilising RNA Polymerase II (Pol II) during the pre-initiation phase of transcription.[9] Beyond this, CDK7 contributes to cell cycle progression as part of the trimeric CAK complex that phosphorylates and activates CDK2/Cyclin A to ensure the G1/S transition.[10] In addition, CDK7 also modulates gene expression by phosphorylating several key transcription factors including nuclear receptors RARα, AR and ER as well as p53.[11–13] Consistent with the notion that CDK7 is an ubiquitous kinase involved in cell cycle progression and transcription, its aberrant overexpression is commonly observed in a broad spectrum of human cancers including breast, gastric and ovarian cancer, and also significantly associates with aggressive clinicopathological features and unfavourable prognosis.[14–17] Noticeably, several pioneering studies have identified THZ1 as a potent chemical inhibitor of CDK7 with selectivity and revealed significant therapeutic effects of THZ1 against human cancer.[18–20] Collectively, the abovementioned findings offer strong evidence to support that CDK7 serves as a putative oncogene driving tumourigenesis

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and a novel prognostic biomarker as well as a therapeutic target with translational significance.[20]

However, to the best of our knowledge, the expression pattern of CDK7 and its clinicopathological significance in OSCC have not been established yet. In the present study, we sought to investigate the expression of CDK7 protein in primary human OSCC specimens and identify the potential relationship between its abundance and clinicopathological features as well as patients' survival.

MATERIALS AND METHODS

Data mining and analysis of CDK7 mRNA in OSCC via publicly available database

The original data concerning abundance of CDK7 mRNA in OSCC were retrieved from the publicly available database Oncomine.[21–23] Briefly, the mRNA expression of CDK7 in human cancer was searched on the Oncomine database (https://www.oncomine.org) and only data on squamous cell carcinoma arising from the oral cavity and healthy oral mucosa were included. These original data of CDK7 mRNA in OSCC and healthy counterparts were retrieved and statistically compared.

Patients and tissue specimens

A retrospective cohort of 113 patients with primary OSCC treated at our institution between January 2006 and December 2016 were enrolled. Written informed consent was obtained from these patients. Patient inclusion criteria were described as follows: (1) primary OSCC without any prior history of surgery, chemotherapy or radiotherapy; (2) patients who underwent radical tumour resection and neck dissection (elective or therapeutic neck dissection as required); (3) detailed information available including epidemiological, clinical, pathological and follow-up data. The archived tissue samples were retrieved and haematoxylin and eosin stained slides for each patient were further analysed to confirm the previous diagnosis based on the established histopathological criteria. Sixteen samples of healthy oral mucosa without evidence of epithelial dysplasia, atypia or neoplastic proliferation were obtained from non-cancer surgeries and histologically confirmed by senior pathologists. This study protocol was reviewed and approved by the Research Ethic Committee of Nanjing Medical University.

Clinicopathological categorisation and immunohistochemical staining of CDK7

All relevant clinicopathological information for each case including histological grade, TNM classification and clinical stage was collected. Histological grade for each sample was determined under microscope according to the World Health Organization (WHO) classification of tumours[24] by senior pathologists. TNM staging for each patient was performed based on American Joint Committee on Cancer (AJCC)/International Union for Cancer Control (UICC) TNM classification (7th edition).[25] Immunohistochemical staining for CDK7 was performed on 4-μm formalin fixed, paraffin embedded specimens using routine procedure as previously reported.[26,27] Briefly, tissue sections from representative paraffin blocks were deparaffinised in xylene and rehydrated through graded alcohols. Tissue slides were then processed by microwave heating in 10 mM citrate buffer (pH 6.0) for 15 min for antigen retrieval and 3% hydrogen peroxide for endogenous peroxidase inactivation. These sections were further incubated with primary antibody (anti-CDK7, 1:100 dilution/2 μg/mL; sc-7344; Santa Cruz Biotechnology, USA) at 4°C overnight and developed with 3.3′-diaminobenzidine and counterstained with haematoxylin. The immunoreactivity in each slide was assessed independently by two senior oral pathologists without knowledge about the relevant clinical and pathological data. Negative controls (without primary antibody incubation) were included in each staining run.

Immunoreactivity was semi-quantitatively evaluated according to staining intensity and distribution using the immunoreactive score which was calculated as intensity score × proportion score as previously reported.[28] Intensity score was defined as 0, negative; 1, weak; 2, moderate; 3, strong. The proportion score was defined as 0, negative; 1, <10%; 2, 11–50%; 3, 51–80%; 4, >80% positive cells. Therefore, the total score ranged from 0 to 12. Similar to our previous reports, the immunoreactivity of each slide was categorised into three subgroups based on the final score: 0, negative; 1–4, low expression; 4–12.[26,27,29]

4-nitroquinoline 1-oxide (4NQO)-induced OSCC animal model

The 4NQO (CAS no. 56-57-5; Cat no. N8141; Sigma, USA) induced OSCC animal model in which squamous cell carcinoma was initiated and maintained in the tongue was performed as per our previous reports with minor modifications.[26,30] For 4NQO-induced tongue SCC in C57BL/6 mice, 6-week-old mice ($n = 32$) were treated with drinking water containing 50 μg/mL 4NQO for consecutive 16 weeks and then given normal drinking water for another 8 weeks. Another group of animals ($n = 8$) with normal drinking water were used as negative control. The lesions in the tongue were visually inspected every week. Samples ($n = 8$) were harvested at 16, 20 and 24 weeks after chemical administration and subjected to further histopathological analyses. The expression of CDK7 in samples from the 4NQO-induced OSCC mouse model was evaluated by immunoreactive score similarly to immunohistochemical scoring of human samples.

Statistical analyses

The associations between CDK7 expression and various clinicopathological parameters of patients were evaluated using the Chi-square test. Survival rates of patients were estimated using the Kaplan–Meier method and compared with the Log-rank test. The prognostic analyses were performed by univariate and multivariate Cox regression models to determine the individual clinicopathological variables with patients' overall survival. p values <0.05 (two-sided) were considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad, USA) or SPSS 18.0 (IBM, USA).

RESULTS

CDK7 mRNA is frequently overexpressed in OSCC

Previous studies have indicated that CDK7 is aberrantly overexpressed in several human cancers and commonly

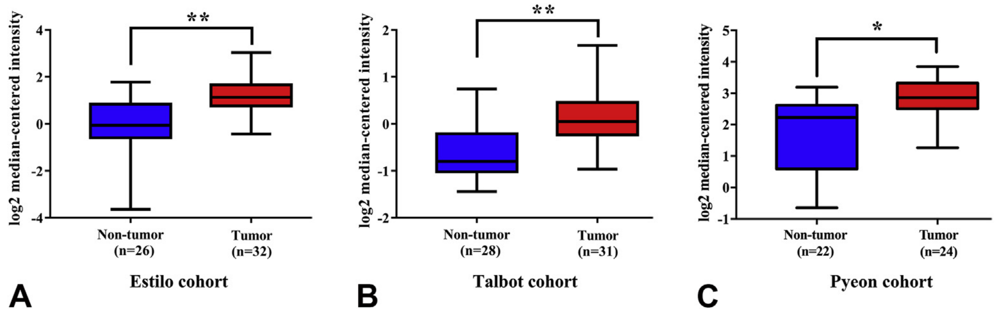

Fig. 1 Overexpression of CDK7 mRNA in three OSCC cohorts. The mRNA levels of CDK7 were compared between OSCC samples and non-tumour counterparts in the cohorts of (A) Estilo *et al.*,[21] (B) Talbot *et al.*[22] and (C) Pyeon *et al.*[23] The original data were retrieved from the Oncomine database, then plotted using GraphPad Prism 7.0 software. Y-axis represents the median intensity, 25th and 75th percentile data.

associates with poor prognosis.[31–33] To initially explore the expression of CDK7 in primary OSCC, we utilised Oncomine, a publicly available database, to interrogate the CDK7 mRNA expression. Only squamous cell carcinoma arising from the oral mucosa, tongue and mouth floor were included. As shown in Fig. 1A–C, our data mining and analyses from the cohorts of Estilo *et al.*,[21] Talbot *et al.*[22] and Pyeon *et al.*[23] indicated significant overexpression of CDK7 mRNA in OSCC samples relative to non-tumour samples.

Clinicopathological characteristics and CDK7 expression in primary OSCC

To further determine CDK7 expression in OSCC, we next measured the expression of CDK7 protein by immunohistochemical staining in a retrospective cohort of 113 primary OSCC samples. The epidemiological information and relevant clinicopathological features (age, gender, smoking, alcohol use, pathological grade, tumour site, T-stage, clinical stage, and cervical node status) of the included patients are summarised in Table 1. In brief, 67 male and 46 female patients were enrolled with a mean age of 64.5 years (range 34–87 years). The follow-up period ranged from 4 to 96 months with an average of 43.4 months. Based on our immunohistochemistry scoring regime, CDK7 protein abundance in these primary OSCC and non-tumour oral epithelial samples (n = 16) were categorised. As shown in Table 2, CDK7 expression patterns in OSCC samples were graded as negative (0), low (62) and high (51) expression. In parallel, CDK7 expression in normal oral epithelial samples was divided into negative (5), low (8) and high (3). These data showed a significant difference in CDK7 expression pattern between OSCC and non-tumour oral mucosa, and also clearly indicated that CDK7 was aberrantly overexpressed in a significant fraction of primary OSCC. The representative immunohistochemical staining of CDK7 in OSCC and non-tumour oral mucosa is shown in Fig. 2. High CDK7 expression was identified primarily in the nucleus in cancerous cells, whereas weak staining was observed in healthy oral epithelial cells. The detailed associations between CDK7 expression and various clinicopathological variables were further analysed and are shown in Table 1. There were no significant associations between CDK7 expression and patients' gender, age, smoking, alcohol use, tumour site, pathological grade, cervical node metastasis and clinical stage. However, significant association between CDK7 abundance and T-stage was found (p = 0.0090, Chi-square analysis).

Table 2 CDK7 expression patterns in OSCC and normal oral mucosa

	CDK7 expression			p value
	Negative	Low	High	
Normal oral mucosa	5	8	3	<0.0001
OSCC	0	62	51	

Table 1 The associations between CDK7 expression and multiple clinicopathological parameters in primary OSCC

Clinicopathological parameters	Cases	CDK7		p values
		Low	High	
Gender	113	62	51	
Male	67	36	31	0.8482
Female	46	26	20	
Age				
≤60	41	24	17	0.6945
>60	72	38	34	
Smoking				
No	75	43	32	0.5493
Yes	38	19	19	
Alcohol use				
No	90	52	38	0.2470
Yes	23	10	13	
Tumour site				
Lip	5	2	3	0.3966
Buccal	30	17	13	
Mouth floor	8	2	6	
Maxilla	3	1	2	
Tongue	46	26	20	
Gingiva	21	14	7	
T-stage				
T1–T2	75	48	27	**0.0090**
T3–T4	38	14	24	
Pathological grade				
I	67	38	29	0.7020
II–III	46	24	22	
Cervical node metastasis				
N (0)	88	50	38	0.4980
N (+)	25	12	13	
Clinical stage				
I–II	70	37	33	0.6975
III–IV	43	25	18	

Bold indicates statistical significance, with p values less than 0.05.

High CDK7 expression associates with reduced survival in patients with OSCC

To reveal the potential prognostic value of CDK7 expression in OSCC, we attempted to evaluate the association between CDK7 expression and patients' survival. At the last follow-up, 75 of 113 (66.4%) patients were alive and disease-free, 10 (8.8%) patients were alive but with recurrence and/or cervical nodal metastases, and 28 (24.8%) patients died due to local recurrence, metastases, or other unrelated diseases. The results from Kaplan–Meier survival analyses indicated that high CDK7 expression had an adverse prognostic impact on patients' outcomes. In detail, as shown in Fig. 3, patients with high CDK7 abundance were significantly associated with reduced overall survival and disease-free survival (Log-rank, p = 0.022, 0.010). To further assess the clinical significance of CDK7 expression as a prognostic predictor for patients with OSCC, both univariate and multivariate survival analyses (Cox proportional hazards regression model) were performed. As indicated in Table 3, the univariate survival analysis revealed that CDK7 expression status and T-stage were significantly associated with overall survival [hazard ratio (HR) 0.425, 95% confidence interval (95% CI) 0.199–0.906, p = 0.027 for CDK7; HR 0.440, 95% CI 0.209–0.927, p = 0.031 for T-stage, respectively], while other clinicopathological variables didn't reach the statistical significance. To rule out confounding factors, we performed multivariate survival analysis and found that only CDK7 expression status was identified as an independent prognostic marker for overall survival of patients (HR 0.438, 95% CI 0.195–0.986; p = 0.046).

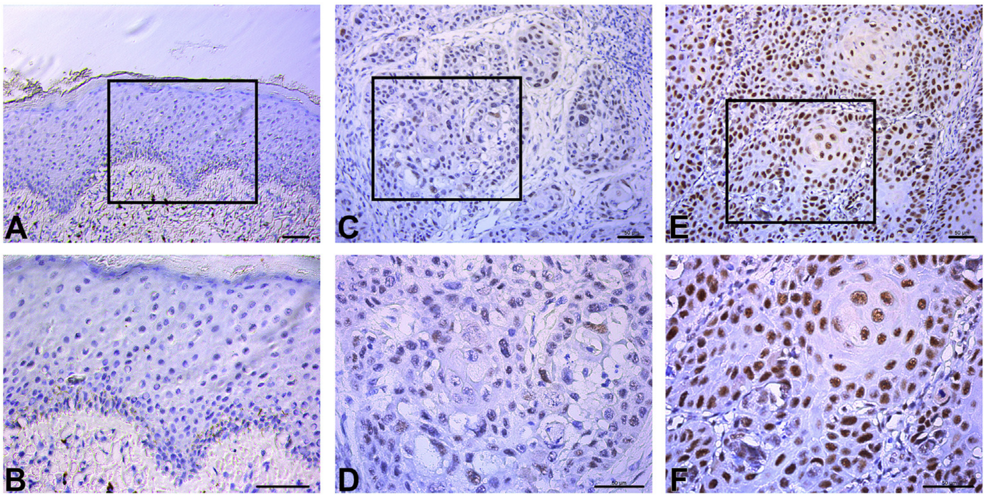

Fig. 2 CDK7 protein expression in healthy oral mucosa and OSCC samples by immunohistochemical staining. (A,B) Representative low expression of CDK7 in healthy oral mucosa; (C,D) representative low expression of CDK7 in primary human OSCC sample. (E,F) Representative high expression of CDK7 in primary human OSCC sample. Nuclei are counterstained with haematoxylin. Scale bar = 50 µm. The areas marked by the black box in A, C, E (upper panel) are shown in larger magnification in B, D, F (lower panel), respectively.

CDK7 is involved in chemical-induced OSCC tumourigenesis

Having revealed aberrantly high expression of CDK7 and prognostic significance in human OSCC samples, we next developed a well-established chemical-induced animal model to characterise the expression pattern of CDK7 during OSCC initiation and progression. Pathological lesions were primarily identified in the tongue following 4NQO treatment and displayed typical changes from epithelial hyperplasia, dysplasia, carcinoma *in situ* and invasive SCC, thus largely recapitulating the multiple-staged tumourigenic process in human OSCC. Furthermore, as shown in Fig. 4A–D, no evident lesions of the tongue were observed in mice with normal water drinking (Fig. 4A), while small, white lesions and discolouration were seen at the 16th week (Fig. 4B). Lesions grew to small berry-like elevations at 20th week (Fig. 4C) and to ill-defined indurated white or congestive areas, often ulcerated at the 24th week (Fig. 4D). Immunohistochemical staining of CDK7 in these samples indicated negative or low staining in normal tongue mucosa (Fig. 4E,I) and epithelial hyperplasia (Fig. 4F,J), while prominent strong nuclear staining was present in dysplasia/carcinoma *in situ* (Fig. 4G,K) and invasive carcinoma (Fig. 4H,L). Data from immunohistochemical staining revealed that significant CDK7 overexpression was observed in carcinoma (87.5%, 7/8), whereas much less was detected in healthy mucosa (16.7%, 1/8), samples with hyperplasia (25.0%, 2/8) or dysplasia/carcinoma *in situ* (50.0%, 4/8). Together, our findings from the chemical-induced animal model provide support to the notion that CDK7 might be critically involved in OSCC development by serving as a putative oncogene.

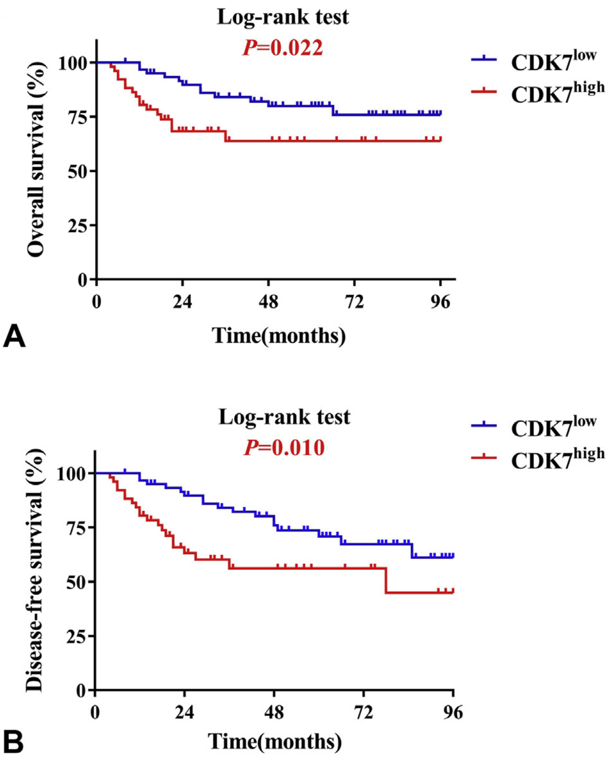

Fig. 3 High CDK7 expression associates with reduced survival in OSCC patients. (A) Overall and (B) disease-free survival analyses of patients with high or low expression of CDK7 were estimated by Kaplan–Meier method and compared with Log-rank test.

DISCUSSION

Deregulated cell cycle and aberrant transcription addition are common features of cancer.[6] The importance of CDK7 in cell-cycle regulation and transcription has highlighted this kinase as a potential diagnostic, prognostic biomarker as well as a potential therapeutic target for cancer.[18,19,34,35] Here, we determined the CDK7 expression pattern in primary OSCC samples and lesions developed from a chemical-induced animal model and found that CDK7 is significantly overexpressed in a large fraction of OSCC and is involved in initiation and progression of OSCC.

Several lines of evidence have revealed that CDK7 is commonly upregulated in various cancers and associates with their aggressive characteristics.[14–16] For example, CDK7 was significantly upregulated and positively associated with tumour grade, infiltration depth, lymph node and Ki-67 in

Table 3 Univariate and multivariate survival analyses (proportional hazards method) for patients with primary OSCC

Variable	Univariate survival analysis			Multivariate survival analysis		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Gender (male, female)	0.819	0.378–1.774	0.612			N/A
Smoking (no, yes)	0.956	0.441–2.071	0.908			N/A
Alcohol (no, yes)	0.504	0.228–1.115	0.091			N/A
Age (≤60, >60)	0.736	0.332–1.629	0.449	0.728	0.323–1.639	0.443
T-stage (T1–T2, T3–T4)	0.440	0.209–0.927	**0.031**	0.727	0.290–1.870	0.521
Pathological grade (I, II–III)	0.566	0.269–1.191	0.134	0.812	0.350–1.884	0.627
Cervical nodal metastasis (N0, N+)	0.515	0.233–1.138	0.101	0.856	0.283–2.588	0.783
Clinical stage (I–II, III–IV)	0.501	0.238–1.054	0.069	0.607	0.184–2.001	0.412
CDK7 expression (low, high)	0.425	0.199–0.906	**0.027**	0.438	0.195–0.986	**0.046**

Bold indicates statistical significance, with *p* values less than 0.05.

173 gastric cancers by immunohistochemical staining assay.[14] In addition, both CDK7 mRNA and protein were markedly increased in breast cancer compared to adjacent normal breast tissue, especially in the triple-negative breast cancer subtype.[18] Consistent with previous findings, our data reveal that CDK7 is aberrantly overexpressed in a significant fraction of OSCC and its overexpression positively associates with higher T-stage. To the best of our knowledge, this is the first study to uncover an abnormal expression pattern of CDK7 in OSCC.

The past decades have witnessed tremendous progress in diagnosis and treatment for OSCC.[4] However, the 5-year survival rate remains disappointing, highlighting that accurate prognostic prediction is a great challenge but highly beneficial in the clinic. Previous studies have revealed that CDK7 overexpression associates with unfavourable prognosis in patients and usually is identified as a key independent prognostic predictor for cancer. For example, overexpression of CDK7 positively correlated with shorter overall survival in patients with triple-negative breast cancer, gastric cancer and ovarian cancer.[14,16,18] In line with these findings, our results indicate that patients with high CDK7 have significantly shorter overall and disease-free survival as compared to those with low CDK7. Furthermore, multivariate regression analyses revealed CDK7 expression as an independent prognostic factor affecting survival of patients with OSCC. However, some conflicting results regarding prognostic roles of CDK7 in diverse cancer contexts have been reported. Elevated expression of CDK7 was significantly associated with better prognosis with improved survival in oestrogen receptor-positive breast cancer but poorer survival in triple-negative breast cancer.[15,18] We reason that this discrepancy might be attributed to sample size, method for patient stratification as well as heterogeneous genetic background. Collectively, our data support the prognostic significance of

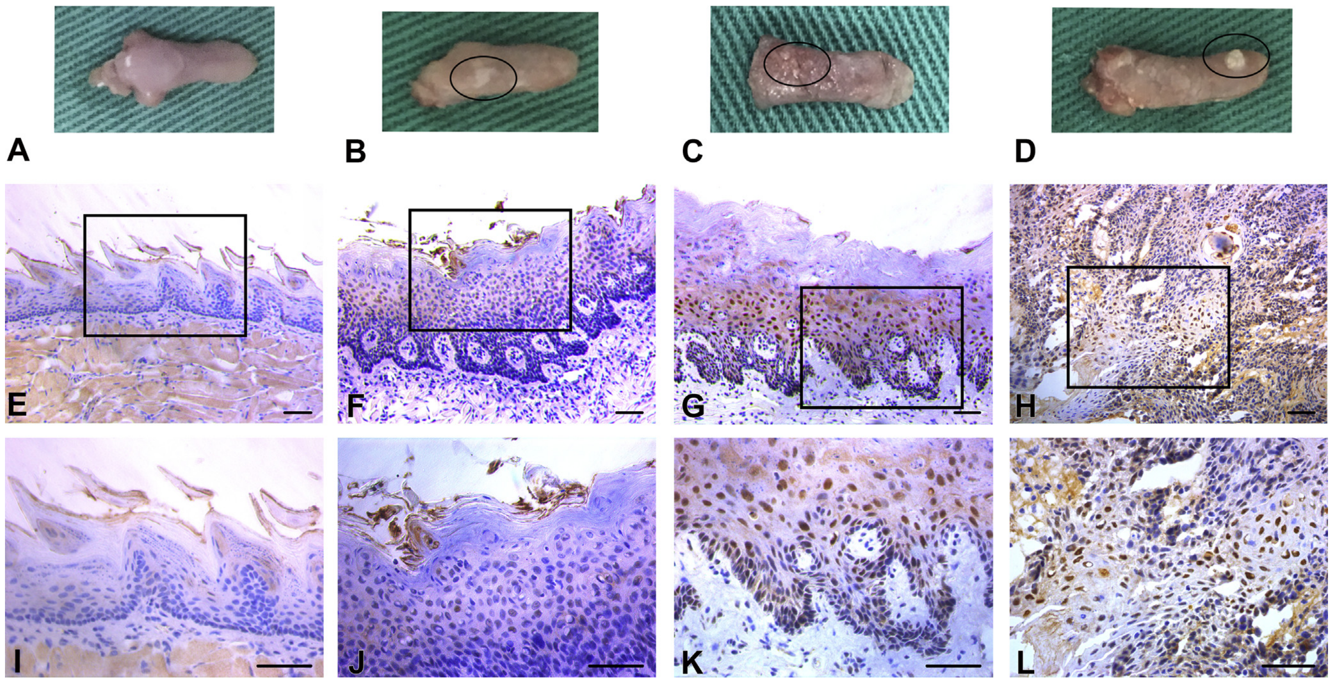

Fig. 4 CDK7 expression during OSCC tumourigenesis in 4NQO-induced animal model. (A–D) Representative images showing lesions in the tongue of mice ($n = 8$ for each subgroup) (A) fed with normal water and (B) 4NQO at the 16th week, (C) 20th week and (D) 24th week. (E–H) Immunohistochemical staining of CDK7 in samples from diverse stages in 4NQO-induced OSCC animal model. Upper panel (E, F, G, H) shows representative staining of CDK7 in normal, epithelial hyperplasia, epithelial severe dysplasia/carcinoma *in situ* and squamous cell carcinoma, respectively. Lower panel (I, J, K, L) are magnified from the black box area in the corresponding images in the upper panel. Scale bar = 50 µm.

CDK7 in OSCC, and suggest that the expression status of CDK7 evaluated by immunohistochemical staining of postoperative samples might offer valuable information about patients' prognosis and indication for effective follow-up management.

The frequent CDK7 overexpression in human cancer and remarkable therapeutic promise by targeting CDK7 strongly underscores its essential tumourigenic roles.[18,20] Our findings derived from a well-established chemical-induced OSCC animal model strongly suggest essential oncogenic functions of CDK7 driving OSCC tumourigenesis. Several lines of evidence have revealed that the pro-oncogenic functions conferred by CDK7 might be associated with its critical roles involved in cell cycle, cell proliferation and transcriptional regulation.[14,18] In particular, CDK7 was critically involved in gene transcription with preferential repression of E2F-regulated genes and transcripts with super-enhancers such as RUNX1, MYC and MYCN which promoted tumourigenesis in different cancer types.[19,20,34,36] However, the detailed biological functions of CDK7 underlying OSCC initiation and progression remain largely unknown, thus necessitating in-depth investigations in the near future.

Several breakthrough studies have identified THZ1 as a selective and potent chemical inhibitor of CDK7 and have shown remarkable therapeutic outcomes against cancer in preclinical settings. THZ1 significantly inhibited cancer cell proliferation and migration while it induced apoptotic cell death both *in vitro* and *in vivo*.[18–20] These previous findings together with ours suggest that CDK7 serves as not only a novel cancer biomarker with diagnostic and prognostic values, but also a viable therapeutic target with tremendous translational potentials. We speculate that a CDK7 inhibitor like THZ1 might have potent anti-oncogenic functions with therapeutic potential, given the overexpression of CDK7 in the large subset of OSCC which might confer sensitivity to CDK7 inhibition. It remains an open and interesting question to develop efficient approaches to therapeutically targeting CDK7 in OSCC.

In conclusion, our data reveal that CDK7 is aberrantly overexpressed in a significant fraction of OSCC and might be a novel biomarker with prognostic significance. More studies are warranted to further unravel the mechanistic insights of CDK7 dysregulation during the initiation and progression of OSCC and develop therapeutic approaches by targeting CDK7.

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