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Original Article

NEK2 is up-regulated in oral squamous cell carcinoma and correlates with patients' disease severity

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Abstract: Background: Nek2 is a centrosome kinase and an important regulator in mitosis. Nek2 overexpression has been observed in many cancers including breast cancer, colorectal cancer and pancreatic cancer. However, Nek2 expression has not been examined in oral squamous cell carcinoma (OSCC). Objective: Our study aims to examine the expression of Nek2 in OSCC and explore its correlation with patients' clinical features and disease severity. Methods: Sixty tissue specimens were collected from 60 OSCC patients who underwent surgical treatment at the Stomatological hospital affiliated to Guizhou Medical University, China. Sixty normal oral tissues were collected from 60 age- and sex-matched healthy controls. Expression of Nek2 was assessed by Reverse Transcription-Polymerase Chain Reaction assay and Immunohistochemistry. Results: Nek2 was up-regulated in OSCC tissues at both the mRNA and protein level compared to control group ($P<0.05$). Expression of Nek2 in patients with lowly differentiated OSCC was higher than that of patients with highly differentiated OSCC. Expression of Nek2 was higher in patients with stage III and IV than patients with stage I and II OSCC. Higher Nek2 expression was also associated with the presence of vascular and nerve invasion, lymph node and distant metastasis. Conclusions: Expression of Nek2 in OSCC is markedly increased at both the mRNA and protein level; the increased expression of Nek2 correlates positively with clinical stages and tumor differentiation.

Keywords: Oral squamous cell carcinoma, Nek2, clinical features

Introduction

Oral cancer ranks the eighth in total cancer incidence worldwide and is the third most common malignancy in south-central Asia [1, 2]. Oral squamous cell carcinoma (OSCC) accounts for more than 80% of all malignant oral and maxillofacial tumors. According to the latest data, the mortality of oral carcinoma accounts for 0.88% of all cancer deaths in China [3]. In the US, 2-4% of annually diagnosed malignancies are OSCC, resulting in 8,000 deaths each year [1]. In addition, OSCC causes significant morbidity to the patients as they suffer difficulty in daily activities such as feeding, communications and other social activities.

Previous researches suggest that multiple genes are involved in tumorigenesis. These genes play prominent roles in one or more of the following processes: cell proliferation, differentiation and genome stability [4]. Nek2 belongs to the Never In Mitosis A (NIMA) -related kinase family [5]. There are three isoforms of activated Nek2: Nek2A, Nek2B and Nek2C [5]. Nek2, together with Nek2-associated protein 1, Rootletin and β-catenin are paired with centrosome through the cell cycle in alternative splicing events [5, 6]. These proteins, upon phosphorylation, separate from centrosome to facilitate the rapid division of centrosome [5, 6]. Dysregulated expression of Nek2 affects the maturity of centrosome and spindle formation, which is believed to be one of the causes for tumorigenesis.

The expression of Nek2 has been found to increase markedly in breast cancer tissue [7, 8], colorectal cancer tissue [9, 10], pancreatic ductal adenocarcinoma [11], hepatocellular carcinoma [12] and others [13]. However, there are no reports on the expressing of Nek2 in oral neoplasm. In this study, we examined the expression of Nek2 in OSCC tissue by real time-qPCR and Immunohistochemistry. The differences of Nek2 expression between OSCC and

Table 1. NEK2 positive staining in OSCC and normal tissue by IHC analysis

Groups	NEK2 staining					X^2	*P*
	N	-	+	++	Positive rate%		
Normal control	60	42	14	4	30.0	17.38	<0.01
OSCC	60	10	30	20	83.3		

normal tissue, and the correlation between its expression and patients' clinical features were also analyzed.

Materials and methods

Patients' specimen and clinical data

From September, 2013 to December, 2014, 60 OSCC patients who underwent surgical operation at the Stomatological Hospital Affiliated to Guizhou Medical University, China were enrolled into this study. Sixty tissue specimen were collected from those 60 patients which include tongue cancer tissues, gingiva and mouth floor carcinoma tissues. Patients' age, gender and clinicopathological data were collected (**Table 2**). Sixty normal tissue samples include oral mucosa tissues, gingiva and vermilion tissues and soft palate tissues were collected from 60 age- and sex-matched controls during their dental visits. All OSCC patients underwent surgical operation and received no other treatment such as chemotherapy, radiation therapy or immunotherapy before or after the surgical procedures. Diagnosis was based on histological test and confirmed by pathological examination. Exclusion criteria include: heart disease, high blood pressure, diabetes, other systematic disease or any other genetic defects.

This study is approved by the Research Ethics Committee of the Guizhou Medical University. Informed consent was obtained from each patient.

Real-time qPCR

mRNA was extracted using the paramagnetic particle method according to the manufacturer's instructions (Dynabeads® mRNA DIRECTTM, USA). Briefly, about 1-2 mm^3 segment of tissue were harvested and immediately stored in the RNA*Later* reagent (-80°C). The tissue was then put into lysis buffer with proteolysis enzyme K. The samples were incubated at 55°C until complete digestion. Subsequently, 40 µl Dynabeads dT25 was added to each sample and the samples was rotated at room temperature for 10 min. Samples were then placed on Magnetic rack immediately. Wash Buffer A was then added into each tube and the supernatant was removed without disturbing the Dynabeads pellet. The beads were then washed with Buffer B and the supernatant was removed as much as possible. The samples were then removed from the magnet rack and 13 µl Tris-HCl was added to the pellet, and the Dynabeads was thoroughly resuspended with a pipette tip. Finally, the suspended Dynabeads was heated in a heat block for 2 mins at 80°C to elute mRNA from the Dynabeads. Afterwards, the tubes were placed immediately on the magnetic rack to concentrate the beads. The supernatant (13 µl in Tris-HCl) which contains mRNA was then collected and was added directly into 7.5 µl cDNA Synthesis Master Mix (Transcriptor First Strand cDNA Synthesis Kit, Roche). These samples were placed in a thermal cycler at 42°C for 30 mins and then at 85°C for 5 mins, followed by 4°C for 5 mins. 1 µl of the synthesized cDNA was used in the subsequent PCR reaction with 10 µl reaction system: 5 µl SYBR Green (Light Cycler® 480 SYBR Green I Master, Roche), 4.0 µl primers. PCR reaction was conducted with CFX connect Real Time System (Bio-Rad, USA). The following reaction conditions were used: pre-denaturation for 5 mins at 95°C, denaturation for 10 s 95°C, annealing at 65°C 30 s and a total of 64 cycles. Every sample had three replicates. The mean CT value was analyzed by relative quantitative method with *POLR2A* as internal control. The following primers were used: *NEK2*, forward: 5'-gggcagccctctccttta-3', Reverse: 5'-attgacagacgacgatgatg-3'; *POLR2A* (control) forward: 5'-gcaaattcaccaagagagac-3', reverse: 5'-cacgtcgacaggaacatcag-3'.

Immunohistochemistry and quantification

Immunohistochemistry was conducted according to standard procedures. Briefly, tissue samples were harvested, rinsed with saline and fixed in formalin. The tissue samples were then embedded with paraffin before sectioned to 4 µm thickness. Sections were stained with hematoxylin-eosin (H&E) to confirm the diagnosis. Sections were then deparaffinized in xylene and rehydrated in a series of graded

Table 2. NEK2 positive staining in OSCC by IHC and its relationship with patients' clinical pathological parameters

Variables		N	Negative	Positive	Positive rate%	*Z*	*P*
Gender	Male	44	8	36	81.8%	-0.576	0.156
	Female	16	2	14	87.5%		
Age	>45	36	8	28	77.8%	-1.837	0.031
	<45	24	8	16	66.7%		
Tumor size	>2 cm	23	4	19	82.6%	-1.563	0.024
	<2 cm	37	13	24	64.9%		
Tumor differentiation	I	44	10	34	77.3%	-1.954	0.013
	II	16	0	16	100.0%		
Vascular invasion	Y	40	7	33	82.5%	-0.893	0.026
	N	20	6	14	70.0%		
Nerve invasion	Y	48	7	41	85.4%	-1.262	0.002
	N	12	3	9	75.0%		
Lymph node metastasis	Y	22	2	20	90.9%	-0.756	0.032
	N	38	8	30	78.9%		
Distant metastasis	Y	6	1	5	83.3%	-1.235	0.042
	N	54	9	45	83.3%		

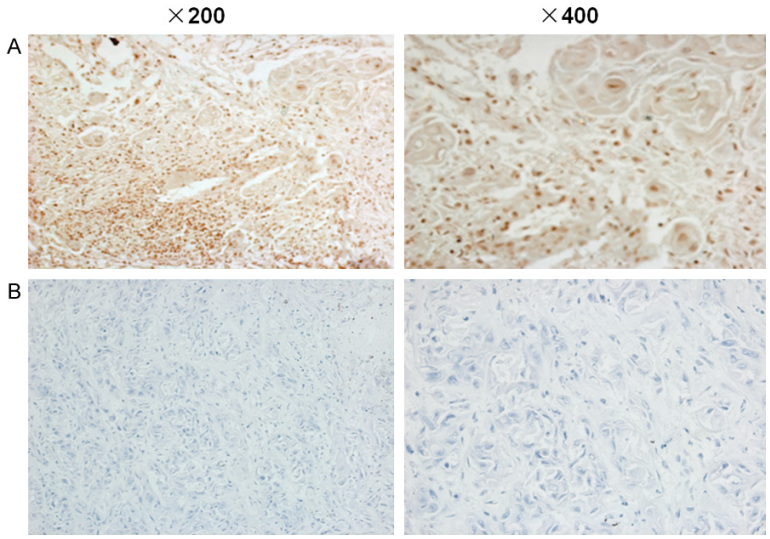

Figure 1. Representative images of the immunohistochemistry (IHC) staining of OSCC and normal tissue. A: NEK2 staining in OSCC tissue; B: IHC staining in normal oral tissue, showing no NEK2 signal.

Table 3. Expression of NEK2 in OSCC and normal tissue measured by optical density analysis

Groups	Cases (n)	MOD (X±S)	t	*P*
Normal	60	0.051±0.015	-24.049	<0.001
OSCC	60	0.448±0.127		

MOD: Mean integrated optical density.

alcohol. Then, sections were immersed in 3.0% hydrogen peroxide for 10 min at room temperature followed by blocking in serum for 1 hour. Sections were then incubated with monoclonal primary antibody against Nek2 (Abcam, UK) and HOXA7 (Abnova, Taiwan) as control at 4°C overnight. Sections were then incubated with secondary biotinylated antibody followed by brief incubation with streptavidin-biotinylated horseradish peroxidase (HRP).

For scoring Nek2 expression, 10 (×400) fields from each section were randomly selected under the microscope. Nek2 positive staining, shown as yellow or brown staining in the nuclei was semi-quantified. First, cells with positive staining were counted and the ratio of positive cells was calculated and scored as follow: score 0 (<10%), 1 (10-30%), 2 (>30%). The positive cells were then further quantified by staining intensity: score 0 (weak or no staining), 1 (light yellow staining), 2 (brownish yellow staining). The two scores were then added together and total score of 0-1 was considered as negative (-), 2-3 as positive (+) and 4 as strongly positive (++).

Table 4. Relationship between the expression of NEK2 in OSCC as measured by optical density analysis and patients' clinical features

Variables		N	MOD	t	*P*
Gender	Male	44	0.45±0.13	0.222	0.825
	Female	16	0.44±0.13		
Age	>45	36	0.42±0.12	-1.941	0.057
	<45	24	0.49±0.14		
Tumor size	>2 cm	37	0.49±0.11	3.232	0.002
	<2 cm	23	0.39±0.12		
Tumor differentiation	I	44	0.43±0.14	-2.825	0.007
	II	16	0.50±0.07		
Vascular invasion	Y	40	0.47±0.12	2.355	0.022
	N	20	0.40±0.13		
Nerve invasion	Y	48	0.48±0.11	5.007	<0.001
	N	12	0.31±0.08		
Lymph node metastasis	Y	22	0.48±0.08	2.033	0.047
	N	38	0.43±0.14		
Distant metastasis	Y	6	0.54±0.05	3.884	0.001
	N	54	0.44±0.13		

Table 5. Relative expression of NEK2 at the mRNA level in normal oral tissues and OSCC

Groups	Cases (n)	NEK2 (X±S)	t	*P*
Normal	60	0.038±0.020	-15.466	<0.001
OSCC	60	1.125±0.544		

Note: Oral squamous cell carcinoma compared to normal tissue, P<0.05.

Optical density (OD) analysis was based on photograph of 10 fields (×200, 768×556 pixel) from each slide. Mean integrated optical density (IOD) was analyzed by Image pro-plus (Media Cybernetics, Inc., USA). MOD was calculated as: MOD=IOD/area (Sum), of which area (Sum) was the total area with positive staining.

Statistical analysis

Statistical analyses were conducted using SPSS statistical software, version 10.0. Chi-square analysis was used in comparison between two groups. Student *t* test was used to compare between multiple groups. P<0.05 was considered statistically significant.

Results

Increased Nek2 expression in OSCC by IHC staining

Expression of Nek2 was assessed by IHC in 60 OSCC and 60 normal tissue samples. Increased Nek2 staining was observed in the nuclei of cancer cells as yellow or brown staining (**Figure 1A**), compared to very weak or no staining in normal tissue (**Figure 1B**). As shown in **Table 1**, the ratio of cells with positive Nek2 staining (+ or ++) was 83.3% in the 60 tumor specimen compared to 30.0% in the normal group. The difference is statistically significant by chi-square analysis (χ^2=17.38, P<0.01).

We then analyzed the positive staining rate in the 60 OSCC patients and its correlation with their clinicopathological parameters. Our result shows that the number of individuals with positive Nek2 expression in moderately and poorly differentiated OSCC group (100%) was higher than that in highly differentiated group (77.3%) (z=-1.954, P=0.013) (**Table 2**). Similarly, the number of patients with positive Nek2 staining increased significantly with the progression of the clinical stages and disease severity, such as tumor size (P=0.024), vascular invasion (P=0.026), nerve invasion (P=0.002), lymph node metastasis (P=0.032) and distal metastasis (P=0.042). Nek2 expression also correlated with patients age, and patients age >45 had higher Nek2 positive ratio (P=0.031). However, no correlation between Nek2 expression and gender was observed (P=0.156).

To further quantify the IHC staining, we measured the median optical density (MOD) value of Nek2 in OSCC and normal tissue (**Table 3**). Result shows that the MOD value of Nek2 in OSCC was higher than that in normal tissue analyzed by two independent samples t-test (t=-24.049, P<0.001). This result is in consistent with earlier counting result. Therefore, we conclude that the expression of Nek2 protein in OSCC was higher than in normal tissues.

We further examined the correlation between the MOD value and patients' clinical pathological parameters (**Table 4**). The MOD values of Nek2 in the poor and moderate differentiation group were higher than that in the well differentiation group (0.43±0.14 vs. 0.50±0.07, P=0.007). MOD value increased significantly with the increase of tumor size (P=0.002) the

Table 6. Correlation between *NEK2* expression at mRNA level and the clinical features of OSCC

Variables		N	NEK2 mRNA	t	*P*
Gender	Male	44	1.14±0.56	0.329	0.743
	Female	16	1.09±0.52		
Age	>45	36	1.12±0.61	-0.132	0.896
	<45	24	1.14±0.43		
Tumor size	>2 cm	37	1.29±0.59	3.803	<0.001
	<2 cm	23	0.85±0.30		
Tumor differentiation	I	44	0.98±0.51	-3.873	<0.001
	II	16	1.53±0.44		
Vascular invasion	Y	40	1.25±0.60	3.158	0.003
	N	20	0.87±0.36		
Nerve invasion	Y	48	1.23±0.53	3.030	0.004
	N	12	0.73±0.39		
Lymph node metastasis	Y	22	1.54±0.40	5.553	<0.001
	N	38	0.88±0.46		
Distant metastasis	Y	6	1.83±0.22	3.655	0.001
	N	54	1.05±0.51		

presence of vascular invasion (P=0.022), nerve invasion (P<0.001), lymph node metastasis (P=0.047) and distant metastasis (P=0.001). However, no correlation between Nek2 expression and patient's age and gender was observed (P>0.05).

Increase NEK2 expression in OSCC tissue at mRNA level by qRT-PCR analysis

Nek2 was amplified successfully by qRT-PCR with Ct value between 18 and 35. The specificity of Nek2 amplification was confirmed by a single peak melting curve [5]. **Table 5** showed the result of our qRT-PCR analysis. The relative expression of Nek2 was 0.038±0.020 in normal tissue and 1.125±0.544 in OSCC tissue, the difference is statistically significant by t test (t=-15.466, P<0.001) (**Table 5**). Thus, Nek2 expression was up-regulated in OSCC at both the mRNA and protein level.

The correlation between Nek2 expression at mRNA level and patient's clinicopathological features was analyzed (**Table 6**). Expression of Nek2 in moderately and poorly differentiated patients was higher than in well differentiated patients (P<0.001). Similarly, patients with tumor size larger than 2 cm had higher Nek2 expression compared with those with <2 cm (P< 0.001). Nek2 expression also increased with the presence of vascular invasion (P=0.003) and nerve invasion (P=0.004), lymph node metastasis (P<0.001) and distant metastasis (P=0.001). Finally, no correlation between Nek2 mRNA level with age and gender was found (P>0.05).

Discussion

Aberrant Nek2 expression has been associated with many types of cancers and Nek2 has been proposed as an important prognostic biomarker as well as a promising therapeutic target for cancer treatment [5, 7-12, 14]. Overexpression of Nek2 results in centrosomal abnormalities, monopolar spindles and aneuploidy [6, 15]. Down-regulation of Nek2, on the other hand, inhibits cell proliferation and induces apoptosis [13, 16]. Nek2A, a major isoform of Nek2, plays an oncogenic role through such essential biological processes including cell cycle, chromosome instability, senescence, autophagy, as well as drug resistance [17].

In this study, we found that Nek2 was up-regulated at both the mRNA and protein level in OSCC tissue. This result is consistent with earlier studies reporting the high expression of Nek2 in many other cancer types [7-12]. In addition, Nek2 overexpression at the mRNA and protein level correlates with advanced OSCC stages and increased disease severity, such as poor tumor differentiation, vascular and nerve invasion, and increased lymph node and distant metastasis.

Despite the advancement of OSCC diagnosis and surgical treatment, its mortality rate remains unchanged. A better molecular and clinical staging system for OSCC is needed and will allow a more specific and individualized therapy. On the molecular level, increased Nek2 expression activates both AKT and canonical Wnt signaling pathway, resulting in increased cell proliferation and chromosomal instability [5]. The inactivation of tumor suppressors such as P16 and p53, and the overexpression of oncogenes such as EGFR, c-myc, and PRAD-1 are also involved in OSCC develop-

ment [18]. In this study, for the first time, we identified that Nek2 is overexpressed in OSCC and its expression correlates to patients' disease severity, adding it to the molecular network underlying OSCC tumorigenesis.

In conclusion, our study showed that Nek2 expression was increased in OSCC compared to normal tissues. Further study is needed to examine the mechanism of Nek2 induced tumorigenesis and the signaling pathway involved. Our study provides valuable insights for early detection, diagnosis as well as therapeutic treatment of Oral Squamous Cell Carcinoma.

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Disclosure of conflict of interest

None.

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