NIT2 overexpression predicts poor prognosis in tongue squamous cell carcinoma patients

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Abstract

There is disputable on the role of nitrilase-like 2 (NIT2) in cancer. Its expression and its relationship with clinicopathological features in tongue squamous cell carcinoma (TSCC) are not yet clear. The purpose of this study is to investigate the expression of NIT2 in TSCC and its correlation with clinicopathological characteristics in TSCC patients. Through proteomic identification, we found that the protein NIT2 was related to the development of TSCC. q-PCR, western blot and immunohistochemistry techniques were applied to detect the expression of NIT2 in TSCC. The relationship between the expression of NIT2 and clinicopathological features was analyzed by Chi square tests. The results showed the expression of NIT2 in TSCC was significantly higher than that in normal tongue tissues ($p < 0.05$). Univariate and multivariate analysis showed that the positive expression of NIT2 and N classification were associated with decreased disease-free survival rate (DFS) and overall survival (OS) ($p < 0.05$). The results suggested that NIT2 is overexpressed in TSCC and NIT2 may be a potential therapeutic target for TSCC.

Keywords Tongue squamous cell carcinoma (TSCC) · Prognosis · Survival · Proteome · Nitrilase-like 2

Background

The most common malignant oral and maxillofacial tumor is tongue squamous cell carcinoma (TSCC) with an increasing incidence year by year [1, 2]. So far, the prognosis and

treatment strategies of tongue cancer still rely on some traditional indicators, such as the classification of clinical tumor node metastasis (TNM), local lymph node metastasis, and the surgical margin [3–5]. However, these clinical prognostic factors still fail to predict prognosis in many cases of TSCC. Although progress has been made in research and treatment, the 5-year survival rate of TSCC patients is still low. Nowadays, biomarkers have become a research hotspot in tumor treatment and prognosis [6]. However, there is still no specific biomarker for recurrence and metastasis of tongue cancer.

Nitrilase 2 (NIT2), a member of the nitrilase superfamily, is a homologous protein of the mammalian tumor suppressor NIT1 with high sequence homology [7]; however, the function of NIT2 is not clear. Meister et al. found that there is an enzyme in the liver that can hydrolyze α -ketoxipamide and α -ketoglutarate monoamide. This enzyme can further deaminate the toxic substances produced by transamination of glutamine and asparagine to ammonia, and decompose them into reusable oxaloacetic acid and α -ketoglutarate for the maintenance of health [8, 9]. In 1972, Hersh proposed the enzyme catalytic reaction of NIT2 classified as ω -amidase [10]. In 2009, Krasnikov et al. identified ω -amidase that Meister found in the liver as the protein NIT2 [11, 12].

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Recent studies have suggested that NIT2 is a candidate tumor suppressor protein [13] and that NIT2 overexpression appears to arrest the cell cycle in G2/M phase without inducing apoptosis [14]. However, another study has reported that NIT2 may be an oncogene in human colon cancer [15].

To date, the role of NIT2 in cancer is unclear, and thus the present study investigated the expression of NIT2 and its correlation with clinicopathological features in TSCC patients.

Methods

Patients and tissue samples

Three independent cohorts of 112 TSCC patients participated in our study. In cohort 1 and 2, 19 fresh TSCC samples and corresponding adjacent tissues (1.5 cm away from the tumor resection margin, confirmed as tumor-free by intraoperative frozen biopsy) were collected from 19 patients who initially underwent malignant neoplasm resection at the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) between June 2014 and July 2015. Three TSCC tissues and corresponding adjacent tissues from cohort 1 were used for proteome analysis. Another 16 TSCC tissues and corresponding adjacent tissues in cohort 2 were used for western blot and quantitative reverse transcription-polymerase chain reaction (q-PCR). In cohort 3, 93 TSCC tissues from patients who were treated with malignant neoplasm resection between January 1999 and December 2012 at the same hospital were used for the survival analysis. The use of tissue samples in this study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University.

Proteome analysis

Proteins were extracted and purified from TSCC tissues and adjacent tissues and then isoelectric focusing (IEF) was performed. In detail, protein samples were dissolved in 200 µl rehydration buffer, then loaded onto DryStrips (pH 4–7, 24 cm; BIORAD, USA). The rehydration buffer contained 7 M urea (Amersham, Sweden), 2 M thiourea (Amersham, Sweden), and 4% CHAPS detergent (Usb, USA). IEF was performed using a Protean IEF Cell with an immobilized pH gradient (BIORAD, USA) at 20 °C. After IEF, the gel strips were equilibrated with equilibration buffer (6 M urea, 2% SDS, 50 mM Tris–HCl pH 8.8, 30% glycerol, 0.002% Bromophenol blue, 100 mM DTT) for 15 min, then placed into another equilibration buffer containing 2.5% iodoacetamide for 15 min. SDS-PAGE (12.5%) was performed for two-dimensional gel electrophoresis (2-DE). After 2-DE, the gels were fixed for 30 min in fixative solution containing

100 ml ethanol, 25 ml acetic acid, and 0.17 g anhydrous sodium acetate (plus distilled water to 250 ml), washed four times with distilled water, and stained (0.3 g Coomassie brilliant blue G-250, 25 g ammonium sulfate, 50 ml 20% methanol, 25 ml 10% phosphoric acid, and distilled water to 250 ml). The 2-DE gels were scanned and analyzed using the Image Master 2D Platinum 5.0 (GE, USA) software. The spots where the gray value ratio was 1.5 times more between the two groups were screened as the differential protein spots. 2-DE gels were washed in water/acetonitrile 1:1 and then dehydrated in acetonitrile. The gel pieces were rehydrated in 50 µl 100 mM NH_4HCO_3 and 100 µl 50% acetonitrile. Then the gel pieces were air-dried, rehydrated in 50 mM NH_4HCO_3 and 20 ng/µl trypsin, and incubated at 37 °C for 12 h. The supernatant was collected and peptides were extracted from the gel and the combined extracts were evaporated to dryness in a vacuum centrifuge. Mass analyses were performed with an ABI4800 mass spectrometer (MALDI-TOF/TOF, USA) and survey MS spectra from 700 to 3500 Da were acquired. Proteins were identified by automated database spectra search (MASCOT, matrix-science) using the NCBI databases.

Western blot analysis

Total tissue protein was extracted, separated by 10% SDS-PAGE, and transferred to the PVDF membrane (Amersham, Freiberg, Germany). The PVDF membrane was blocked for 1 h and then incubated with rabbit antibody against NIT2 (Proteintech, Chicago, IL, USA) or GAPDH antibody (Proteintech, Chicago, IL, USA) overnight at 4 °C. The membranes were washed for 5–10 min three times with washing buffer and then second antibody was added at room temperature for 1 h, which was followed by another three washes. The membranes were observed with chemiluminescence.

Quantitative RT-PCR (q-PCR)

Trizol (Thermo Fisher Scientific, USA) was used to extract total RNA from tissues. The specific primer sequences of NIT2 for RT-PCR were as follows: 5'-TGGAGGCTCTATCCCTGAAGA-3', 5'-ACTATCACCCGGACTCAATGTT-3' (Invitrogen). GAPDH (5'-TCCTCTGACTTCAACAGC GACAC-3', 5'-CACCTGTGTGCTGTAGCCAAATTC-3') was used as an endogenous control. Relative gene expressions of NIT2 were calculated as fold-change values ($\text{fold} = 2^{-\Delta\Delta C_t}$) by DataAssist v3.0 (Applied Biosystems).

Tissue microarray

Hematoxylin and eosin staining of sections of selected tumors were performed to pre-label representative tumor regions. A tissue core sample with a diameter of 2 mm

was produced from each donor block using tissue microarray (Quick Ray) and placed in an empty paraffin wax with a maximum of 48 cores (UNITMA, Korea). Therefore, two kinds of tissue microarray blocks were constructed. Four-micron-thick sections were cut from the receptor block and transferred to the slide for hematoxylin and eosin staining to confirm the tumor tissue. It was found that two cases were adenocarcinoma, one case was lost during the experimental procedure, four cases exhibited serious defects, and three cases could not be tracked, as the complete follow-up data was not available. In the end, 93 samples were obtained from the two different tissue microarray blocks of TSCC (Fig. 1a).

Immunohistochemistry

NIT2 primary antibody was diluted (1:100) and secondary antibody was labeled with peroxidase and stained with 3,3'-diaminobenzidine (DAB). Sample tissues were sliced, routinely dewaxed, treated with hydrogen peroxide, antigen repaired, and washed with phosphate buffer (PBS). Five visual fields with $\times 400$ magnification were selected for each sample under a microscope and the results were assessed by the double-blind method. According to the staining intensity of cells and the proportion of positive cells, the expression of NIT2 was evaluated by multiplying the results of the two scores. Immunohistochemical staining was ranked into four grades: (0) colorless, (1) yellow, (2) yellow–brown, and (3) brown. The corresponding positive rates were ranked into four grades: (0) 0–25%, (1) 26–50%, (2) 51–75%, and (3) 75–100%. For statistical purposes, the specimens were divided into negative (–) expression (0–2 points) and positive (+) expression (3–9 points).

Ethics approval and consent to participate

Use of tissue for this study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University [(2011) 69]. Written consent was obtained with all participants.

Statistical analysis

The experimental data were processed and analyzed by SPSS 18 and represented as mean $\pm$ standard deviation (SD). The χ^2 test was used to compare the correlation between NIT2 expression and clinicopathological features and the Wilcoxon test was used to detect the expression of NIT2 mRNA by qPCR and Western blot. Disease-free survival (DFS) and overall survival (OS) were calculated from operation date to recurrence date and death date, respectively. The survival curves were drawn by the Kaplan–Meier method

Fig. 1 Tissue microarray. **a** One hundred and three specimens were detected using tissue chip technique and 93 cases were finally available. Two cases were adenocarcinoma, one case was lost during the experimental procedure, four cases exhibited serious defects, and three cases could not be tracked, as the complete follow-up data was not available. In the end, 93 samples were obtained from the two different tissue microarray blocks of TSCC. **b** NIT2 was highly expressed in tongue cancer tissues by immunohistochemical staining, mainly in cytoplasm

and compared using the log-rank test. The Cox regression model was used for multivariate analysis. $p < 0.05$ indicates that the difference is statistically significant.

Results

Proteomic analyses identified altered protein expression in TSCC

Representative gel images of the protein in TSCC and adjacent tissue are shown in Fig. 2. A comparison of the 2-DE images revealed that six protein spots were

up-regulated in TSCC ($T/N > 1.5$) and 11 protein spots were down-regulated in TSCC ($N/T > 1.5$). Finally, 13 proteins were identified from 17 spots by mass spectrometry (Table 1). As the role of NIT2 in cancer is unclear, we selected this protein for further study.

Western blot analysis and q-PCR were performed on 16 pairs of TSCC and adjacent tissues to explore the expression of NIT2. Western blotting demonstrated that NIT2 protein was overexpressed in twelve TSCC tissues

Fig. 2 Representative 2-DE images of differentially expressed proteins isolated from TSCC and adjacent tissue. **a** Representative 2-DE images of differentially expressed proteins isolated from adjacent non-tumor tissue. Significantly up-regulated spots ($N/T > 1.5$) in para-

carcinoma tissues of tongue are marked with numbers. **b** Representative 2-DE images of differentially expressed proteins isolated from TSCC samples. Significantly up-regulated spots ($T/N > 1.5$) in TSCC are marked with numbers

Table 1 Identified different protein expressions between TSCC and adjacent nontumor tissues

No.	Proteins name	Protein AC	pI	MASS	MASCOT score	Sequence coverage (%)
G08	Endoplasmin, HSP90B1	ENPL	4.76	92,696	189	15
G09	Endoplasmin, HSP90B1	ENPL	4.76	92,696	173	15
G13	Ezrin	EZRI	5.94	69,484	74	17
G14	Keratin, type II cytoskeletal	K2C6A	8.09	60,293	193	26
G16	Keratin, type II cytoskeletal	K2C6A	8.09	60,293	222	28
G20	Actin, alpha skeletal muscle	ACTS	5.23	42,366	252	44
G22	Keratin, type I cytoskeletal 10	K1C10	5.13	59,020	143	20
G28	Clathrin light chain A	CLCA	4.43	27,174	154	20
G30	Keratin, type I cytoskeletal 14	K1C14	5.09	51,872	186	34
G33	Omega-amidase NIT2	NIT2	6.82	30,988	251	34
G34	Inosinetriphosphate pyrophosphatase	ITPA	5.5	21,831	150	43
H04	Actin, alpha skeletal muscle	ACTS	5.23	42,366	411	50
H12	Alpha-actinin-2	F6THM6	5.41	80,081	221	27
H14	Keratin, type I cytoskeletal 13	K7ERE3	4.76	45,574	294	38
H16	Suppressor of G2 allele of SKP1	SUGT1	5.07	41,284	124	17
H18	Actin, alpha skeletal muscle	ACTS	5.23	42,366	341	52
H19	Keratin, type I cytoskeletal 16	K1C16	4.99	51,578	334	28

Fig. 3 Expression of NIT2 in TSCC by Western Blot and qPCR. N, adjacent normal tissues of tongue; T, TSCC tissue. ($p=0.026$ (a), $p<0.001$ (b))

compared with their adjacent tissues, whereas in four samples NIT2 protein expression was higher in adjacent tissues compared to TSCC tissues (Fig. 3b, $p=0.008$). q-PCR also showed similar results. NIT2 mRNA in twelve TSCC tissues was higher than that in adjacent tissues, whereas in four cases NIT2 mRNA in adjacent tissues was higher than that in TSCC tissues (Fig. 3a, $p=0.01$). The difference between TSCC and adjacent tissues was statistically significant. These results demonstrate that NIT2 is highly expressed in TSCC tissues.

NIT2 expression was detected by immunohistochemistry

Immunohistochemistry on paraffin sections showed that the localization of NIT2 protein was mainly in the cytoplasm. NIT2 staining was strong in TSCC tissues and weak in adjacent tissues (Fig. 1a). The positive expression rate of NIT2 in 93 cases of TSCC was 71.0%.

The relationship between NIT2 overexpression and clinicopathological parameters in TSCC patients

As summarized in Table 2, analysis of 93 TSCC samples indicated that NIT2 overexpression was significantly associated with classification of lymph node metastasis (N classification) ($p=0.008$). In contrast, there was no significant relationship between NIT2 protein level and variables including age, gender, smoking history, and tumor grade ($p>0.05$).

Table 2 Correlation between NIT2 and clinicopathologic variables of TSCC patients

Variables	Cases	NIT2		<i>p</i>
		Negative (%)	Positive (%)	
Age (years)				
≤60	67	21 (31.3)	46 (68.7)	0.783
> 60	26	9 (34.6)	17 (65.4)	
Gender				
Male	57	18 (31.6)	39 (68.4)	0.594
Female	36	14 (38.9)	22 (61.1)	
Smoking history				
Yes	26	8 (30.8)	18 (69.2)	0.552
No	67	25 (37.3)	42 (62.7)	
N classification				
Negative	42	21(50.0)	21 (50.0)	0.008
Positive	51	12 (23.5)	39 (76.5)	
T classification				
T1–T2	82	28 (34.1)	54 (65.9)	0.492
T3–T4	11	5 (45.5)	6 (54.5)	
Clinic stage (TNM)				
I–II	46	15 (32.6)	31 (67.4)	0.572
III–IV	47	19 (40.4)	28 (59.6)	

TSCC tongue squamous cell carcinoma

The relationship between NIT2 protein expression and DFS and OS in TSCC patients

Patients with TSCC were followed up for 2–163 months (median: 70 months). At the end of the follow-up, 50 patients survived without local recurrence and 38 patients developed cervical lymph node metastasis 2–154 months after surgery (median: 37 months). The 1-, 3-, and 5-year DFS rates were 78.4%, 74.3%, and 72.0% in the NIT2-positive group and 96.7%, 91.7%, and 89.2% in the negative group, respectively ($p=0.001$, Table 3, Fig. 4a). The 1-, 3-, and 5-year OS rates were 80.3%, 60.6%, and 52.9% in the NIT2-positive group and 88.9%, 77.8%, and 74.1% in the negative group, respectively ($p=0.005$, Table 3, Fig. 4b). The DFS and OS rate of NIT2-positive patients was significantly shorter than that of NIT2-negative patients.

The correlation between NIT2 expression and TSCC prognosis

Univariate analysis showed that N classification ($p=0.002$, Table 3, Fig. 4c) and the positive expression of NIT2 ($p=0.001$, Table 3, Fig. 4a) were related to shorter DFS. Multivariate Cox regression analysis indicated that NIT2 positive expression ($p=0.0145$, Table 4), N classification ($p=0.006$, Table 4), and TNM ($p=0.009$, Table 4) predicted shorter DFS and were independent prognostic

Table 3 Prognostic factors for DFS and OS by univariate analysis

Variables	n	DFS				OS			
		1-year (%)	3-year (%)	5-year (%)	p	1-year (%)	3-year (%)	5-years (%)	p
Age (years)									
≤ 60	66	89.0	83.2	81.0		81.8	62.0	56.7	
> 60	27	73.2	68.9	68.9	0.095	85.2	63.0	51.9	0.443
Sex									
Male	57	80.2	76.0	73.6		84.2	61.4	56.1	
Female	36	91.4	84.1	84.1	0.415	80.6	69.4	63.8	0.877
Smoking									
Yes	26	84.4	80.0	80.0		79.1	65.7	61.1	
No	67	84.4	78.8	76.7	0.904	92.3	65.4	53.8	0.822
N classification									
N0	55	92.0	81.2	73.0		95.2	83.3	76.0	
N1–N3	38	61.2	46.8	40.4	0.002	72.0	50.0	44.0	<0.01
T classification									
T1–T2	82	83.7	77.4	75.7		82.9	64.6	58.5	
T3–T4	11	90.9	90.9	90.9	0.283	81.8	72.7	63.5	0.441
Clinic stage (TNM)									
I–II	46	89.1	81.6	78.8		91.3	73.9	67.4	
III–IV	47	79.7	76.7	76.7	0.943	74.5	57.4	51.1	0.112
NIT2									
Negative	27	96.7	91.7	89.2		88.9	77.8	74.1	
Positive	66	78.4	74.3	72.0	0.001	80.3	60.6	52.9	0.005

DFS disease-free survival, OS overall survival

indicators of TSCC. On the other hand, univariate analysis and Kaplan–Meier survival curves revealed that NIT2 positive expression ($p=0.005$, Table 3, Fig. 4b) and lymph node metastasis ($p<0.01$, Table 3, Fig. 4d) were statistically significant. Multivariate Cox regression analysis indicated that positive NIT2 expression ($p=0.015$, Table 4), lymph node metastasis ($p=0.005$, Table 4), and TNM ($p=0.045$, Table 4) were associated with OS and were independent factors for shorter OS of patients with TSCC.

Discussion

To date, the TNM staging system is still the main basis for treatment strategies and prognosis of tongue cancer. Moreover, some studies have reported that clinical indicators such as regional lymph node metastasis status [16] are of significance in the poor prognosis of patients with TSCC. In previous research, we found that lymph node metastasis predicted a poor prognosis for TSCC [17]. Consistent with these findings, TNM and lymph node metastasis are significant for the poor prognosis of TSCC in this study. Even so, the risk classification system of TSCC is still unsatisfactory.

TSCC is a multifactorial disease caused by multiple genetic changes and environmental factors [1, 2]. With the rapid development of molecular biology, molecular targeted

therapy plays an important part in the treatment of tumors, as it is of great clinical significance to define a target gene for specific and differentiated treatment. By detecting the expression of a particular gene, the tumor can be detected as early as possible and its expression can be regulated by specific activators or blockers that affect its downstream pathway by inhibiting the proliferation, invasion, or migration of tumor cells or by promoting apoptosis of cancer cells [18]. However, the molecular mechanisms of the development and metastasis of TSCC are still unclear, which greatly impedes the application of molecular targeted therapy in the treatment of TSCC. Therefore, the exploration of gene therapy targets and prognostic indicators for effective treatment of TSCC is an important issue that needs to be researched. Using 2-DE combined with MS analysis, we successfully identified NIT2 as a novel biomarker in TSCC tissues.

The nitrilase superfamily is divided into 13 branches and is widely distributed in nature [19–21]. Human nitrilase (NIT) proteins belong to the nitrilase superfamily and contain two proteins, namely NIT1 (nitrilase-like protein 1) and NIT2 (nitrilase-like protein 2) [21, 22]. NIT2 shares 40% sequence identity [22] and 55% homology [14] with NIT1. NIT2, identified as ω -amidase, plays an important metabolic role by catalyzing hydrolysis of α -ketoglutarate and α -ketosuccinamate, yielding α -ketoglutarate and oxaloacetate, respectively [8, 9]. Some studies have speculated that

Fig. 4 Kaplan–Meier survival curves of patients with TSCC after radical tongue cancer resection. The patients were divided into negative group and positive group of NIT2. Disease-free survival ($p=0.001$, log-rank, **a**) and overall survival ($p=0.005$, log-rank, **b**) of patients with positive group was shorter than those with nega-

tive group. **c** and **d** Kaplan–Meier survival curves of N classification for patients with TSCC after radical tongue cancer resection. The patients were divided into N0 group and N1–N3 group. Disease-free survival ($p=0.002$, log-rank) and overall survival ($p<0.01$, log-rank) of patients with N1–N3 group was shorter than those with N0 group

Table 4 Prognostic factors for DFS and OS by multivariate Cox proportional hazards regression model

Variables	DFS			Variables	OS		
	HR	95% CI	<i>p</i>		HR	95% CI	<i>p</i>
NIT2	2.789	1.230–6.326	0.014	NIT2	2.713	1.218–6.047	0.015
N classification	3.131	1.397–7.020	0.006	N classification	2.972	1.379–6.408	0.005
TNM	2.441	1.252–4.758	0.009	TNM	1.96	1.015–3.782	0.045

HR hazard ratio, CI confidence interval. Other abbreviations as in Table

NIT2 is a potential tumor suppressor [23], whereas other studies have suggested that NIT2 is a carcinogenic protein in colon cancer [15]. The role of NIT2 in cancer remains elusive and, to the best of our knowledge, no study to date has reported the role of NIT2 in TSCC.

In this study, we presented the first evidence of NIT2 overexpression in TSCC. Furthermore, we evaluated the possibility of using NIT2 as a potential clinical indicator to identify aggressive TSCC as well as its prognostic value for patient survival in cancer. Immunostaining demonstrated

that the high expression level of NIT2 significantly correlated with reduced DFS time of TSCC patients. Taken together, our present results are consistent with previous studies [15, 23] and suggests that NIT2 might represent a valuable prognostic indicator for differentiating aggressive TSCC. However, the mechanisms of NIT2 tumorigenic effects in TSCC have not been elucidated. NIT2 may be an oncogene in human colon cancer, as its downregulation triggers the apoptosis of colon cancer cells through the caspase-3 and poly (ADP-ribose) polymerase pathways [15]. Activation of caspase-3 has been reported to play a key role in inducing apoptosis [24] and the deregulation of caspase-3 is a frequent event in TSCC [25, 26]. Thus we hypothesize that NIT2 promotes the development of TSCC by activating caspase-3 pathways. This warrants further research to investigate the mechanisms of the tumorigenic effects of NIT2 in TSCC.

In conclusion, our data have demonstrated that NIT2 is highly expressed in TSCC. NIT2 is an independent indicator of prognosis in patients with TSCC after radical resection and may serve as a potential therapeutic target in TSCC.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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