

Original Article

High expression of heat shock protein 10 (Hsp10) is associated with poor prognosis in oral squamous cell carcinoma

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Abstract: Heat shock protein 10 (Hsp10), located in mitochondria, is a co-chaperone involved in the protein folding and aggregation with Hsp60. Besides, a wide range of other extramitochondrial and extracellular activities, such as mammalian mitochondrial chaperonin including modulation of apoptosis, inflammation, and carcinogenesis, have been reported. Expression of Hsp10 protein in oral squamous cell carcinomas (OSCC) and the non-cancerous squamous epithelium was detected using immunohistochemistry we retrospectively evaluated the correlations between Hsp10 expression and the clinicopathological characteristics of OSCC. Our results showed that percentage of high expression of Hsp10 in the OSCC was statistically higher than that in the non-cancerous squamous epithelium ($P = 0.006$). What is more, high Hsp10 expression was significantly associated with shorter overall survival in patients with OSCC ($P < 0.001$). In addition, our results identified that the high expression of Hsp10 was significantly correlated with OSCC patients age, the history of chewing betel nut, pathological grade, lymph node metastasis and radiotherapy after operation ($P = 0.008$, $P = 0.021$, $P = 0.026$, $P = 0.008$, $P = 0.049$ respectively). Multivariate Cox regression analysis further identified that high expression of Hsp10 protein was an independent poor prognostic factor for OSCC ($P < 0.001$). High Hsp10 expression might play important roles in the progression of OSCC, and it might act as a novel valuable independent biomarker to predict poor prognosis in patients with OSCC.

Keywords: Oral squamous cell carcinoma, heat shock protein 10 (Hsp10), lymph node metastasis, biomarker, prognosis

Introduction

About 90% of oral cancer is oral squamous cell carcinoma (OSCC) [1], which is the sixth most common cancer in the world [2]. The clarified predisposing factors of OSCC mainly include tobacco consumption, abusing of alcohol, and chewing betel nut [3, 5]. Other important risk factors also cannot be ignored, including infection with human papilloma virus (HPV) [6] or human immunodeficiency virus (HIV) [6, 7] and so on. So far, the mainstream treatment for OSCC patients is surgery, combining with radiotherapy or chemotherapy. In spite of the increase of alternative approaches for the treatment of OSCC, the overall 5-year survival rate for the patients with OSCC is still disappointed [8]. In this situation, further elucidation

of the molecular mechanism underlying OSCC is vital for the development of new effective therapeutic agents and novel prognostic markers.

Heat shock proteins (Hsps), widely expressed and highly conserved in prokaryotes and eukaryotes, are molecular chaperones playing an important role in various routine biological processes, such as transcription, translation and posttranslational modifications, protein folding, and aggregation and disaggregation of proteins [9]. However, most Hsps are related to carcinogenesis. As to the relationship between Hsps and tumor progression, some Hsps can serve as biomarkers [10]. Hsp10, located in mitochondria, is a co-chaperone involved in the protein folding and aggregation with Hsp60.

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Besides, a wide range of other extramitochondrial and extracellular activities, such as mammalian mitochondrial chaperonin including modulation of apoptosis, inflammation, and carcinogenesis, have been reported [11]. The overexpression of Hsp10 correlated with carcinogenesis and tumor malignancy such as uterine, large bowel and ovarian cancer has already been reported [12, 13]. However, up to now there is not any literature about whether high Hsp10 expression is associated with clinicopathological characteristics and had prognostic implications in OSCC. In this study, we detected the expression of Hsp10 protein by immunohistochemistry (IHC) in 110 cases of OSCC and 32 cases of the non-cancerous control squamous epithelium tissues. Also, we retrospectively evaluated the correlations between Hsp10 expression and the clinicopathological characteristics of OSCC by univariate and multivariate analyses.

Materials and methods

Tissue samples and clinical data

All patients with OSCC were submitted to surgical treatment at the Department of Stomatology, the Second Xiangya Hospital of Central South University (Changsha, China) from April 2007 to March 2012. All OSCC samples and non-cancerous squamous epithelium were obtained from Department of Pathology, the Second Xiangya Hospital of Central South University. This study protocol, specimen usage, and data retrieval were approved by the Ethics Review Board of the Second Xiangya Hospital of Central South University (Scientific and Research Ethics Committee). Written informed consent was obtained from all patients. If the patients were minors/children, written informed consent was obtained from the next of kin, caretakers, or guardians on the behalf of the minors/children participants involved in your study. Complete clinical record and follow-up data were available for all patients. Also, written informed consent was obtained from all patients.

These patients had a confirmed histological diagnosis of OSCC according to WHO histological classification of oral tumors. 110 patients (90 males, 20 females), had not been previously treated with chemotherapy and radiotherapy at the time of original operation. Complete

clinical record and follow-up data were available for all patients. Overall survival time was calculated from the date of diagnosis to the date of death or the date of last follow-up if the patient was still surviving. At the time of analysis, 80 patients (72.7%) were still alive with a median follow-up of 33.2 months (range, 10 to 60 months).

Tissue microarrays (TMA) construction

In this study, we used the TMA technology to construct high-throughput OSCC TMAs according to protocol previously described [14]. Representative areas of OSCC and non-cancerous squamous epithelium were marked on each hematoxylin and eosin (H&E) stained slide and tissue paraffin block. The marked areas of tissue paraffin blocks were sampled for the TMAs. Two 0.6-mm-diameter tissue cores were taken from each paraffin block of donor OSCC samples.

IHC and scores

The IHC staining of Hsp10 protein in tissue array of OSCC was carried out using ready-to-use Envision TM + Dual Link System-HRP methods (Dako; Carpinteria, CA). The staining of Hsp10 protein was performed according to the manufacturer's instructions and the staining conditions of antibody were adjusted according to our laboratory experience. Heat based antigen retrieval was accomplished by incubating TMAs with 0.01 M citrate buffer (pH 7.0) in a pressure cooker for 7 minutes, then the samples were immersed into 0.3% H₂O₂ to inactivate endogenous peroxidase at 37°C for 30 minutes. To eliminate nonspecific staining, the slides were incubated with appropriate preimmune serum for 30 minutes at room temperature. After incubation with a 1:1000 dilution of primary antibody to Hsp10 protein (Mouse monoclonal antibody, Catalog: sc-376313, Santa Cruz Biotechnology, Inc.) at 4°C overnight, slides washed with physiological phosphate buffered saline (PBS) three times for 10 minutes each and second antibody conjugated with a labeled polymer-HRP was added in accordance with the manufacturer's instructions and incubated at 37°C for 30 minutes. Staining was developed using 3,3'-diaminobenzidine tetrachloride (DAB) as a chromogen substrate. All slides were counterstained with Mayer's hematoxylin after rinsing with distilled H₂O.

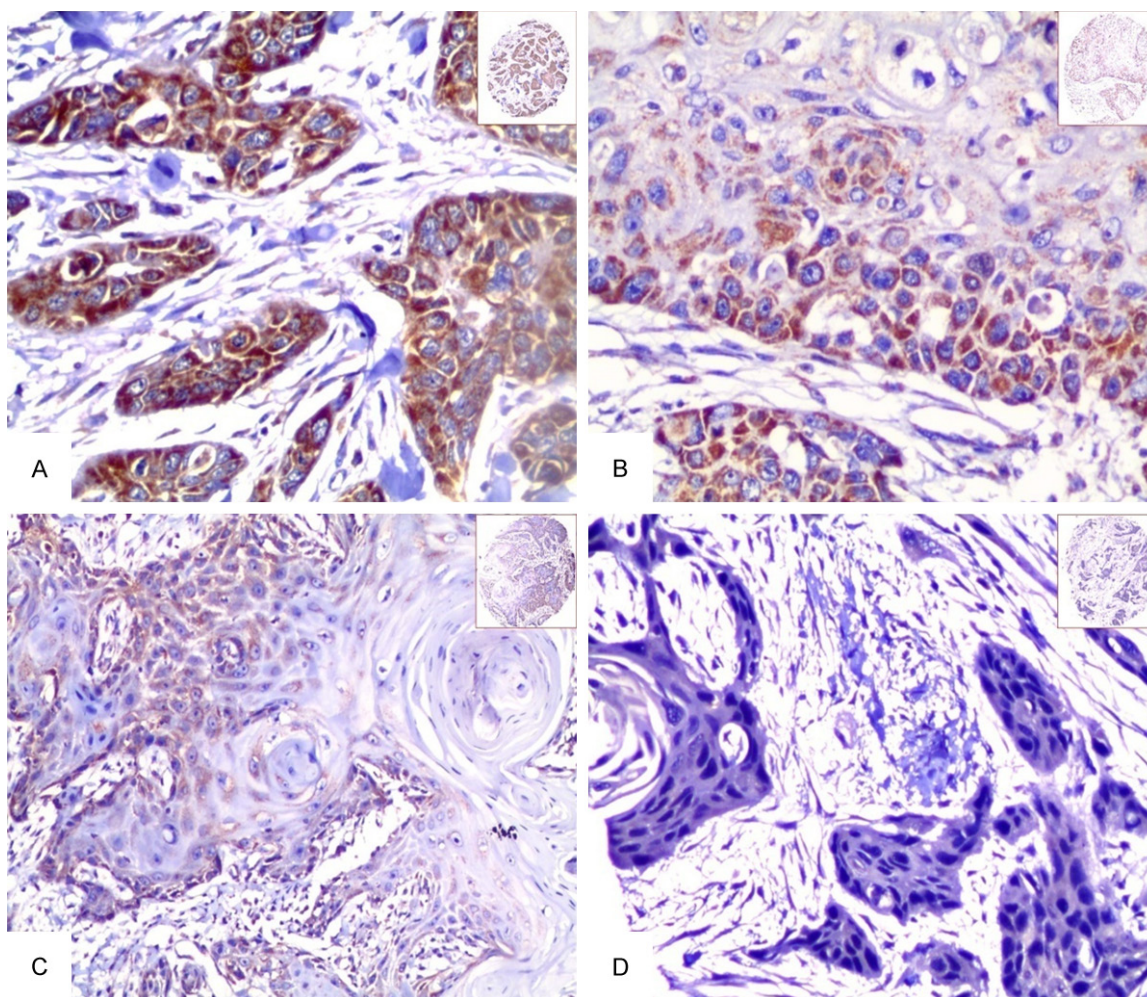


Figure 1. Expression of Hsp10 in OSCC tissue was detected by IHC. The expression of Hsp10 was detected by IHC using specific antibodies as described in the section of materials and methods. Strong positive staining of Hsp10 was identified in the cytoplasm of OSCC (A, 20 \times , IHC, DAB staining). Moderate positive staining of Hsp10 was showed in the cytoplasm of OSCC cells (B, 20 \times , IHC, DAB staining). Weak positive staining of Hsp10 was localized in the cytoplasm of OSCC cells (C, 20 \times , IHC, DAB staining). Negative staining of Hsp10 was in the OSCC cells (D, 20 \times , IHC, DAB staining).

Immunohistochemically staining of TMA sections of OSCC was evaluated independently by JF and SF, who were blinded to the clinicopathological data, at 200 \times magnification light microscopy. The positive staining of Hsp10 was located in the cytoplasm of cancer cells. Representative micrographs in the **Figures 1** and **2** showed that strong positive staining of Hsp10 was found only in OSCC, while only moderate and weak positive staining of Hsp10 presented in the non-cancerous control squamous epithelium tissues.

A semiquantitative evaluation of Hsp10 was performed using a method described in the literature [15] as follows: staining intensity for

Hsp10 was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Staining extent was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%), depending on the percentage of positive-stained cells. Staining positivity was determined by the formula: overall score = positive percentage score \times intensity score. The total score ranged from 0 to 12, scores of 0, 1, 2, 3, 4 and 6 (0-6) were considered low expression and scores of 8, 9 and 12 (8-12) were defined as high expression. Agreement between the two evaluators was 98%, and all scoring discrepancies were resolved through discussion between the two evaluators.

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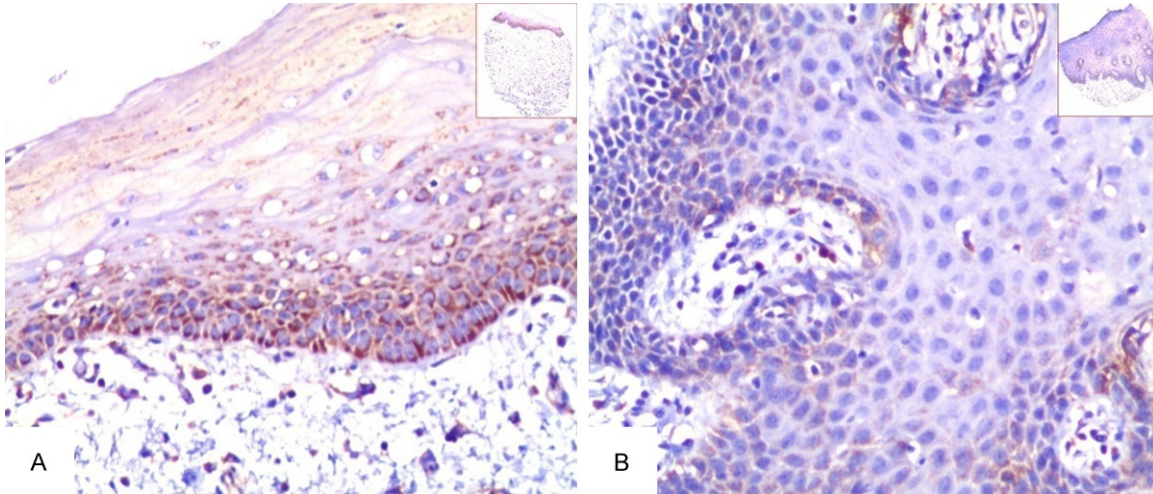


Figure 2. Expression of Hsp10 in the non-cancerous squamous epithelium was detected by IHC. The expression of Hsp10 was detected by IHC using specific antibodies as described in the section of materials and methods. Moderate positive staining of Hsp10 was showed in the cytoplasm of the non-cancerous squamous epithelium (A, 20 \times , IHC, DAB staining). Weak positive staining of Hsp10 was displayed in the cytoplasm of the non-cancerous squamous epithelium (B, 20 \times , IHC, DAB staining).

Table 1. Association between expression of Hsp10 and OSCC clinical pathological feature (n = 110)

Clinical pathological characteristics (n)	Expression of Hsp10		P-value
	High	Low	
Age (yr)			
<45 (n = 27)	20 (74.1)	7 (25.9)	0.008*
\geq 45 (n = 83)	37 (44.6)	46 (55.4)	
Gender			
Female (n = 20)	7 (35.0)	13 (65.0)	0.096
Male (n = 90)	50 (55.6)	40 (44.4)	
Chewing betel nut			
Yes (n = 74)	44 (59.5)	30 (40.5)	0.021*
No (n = 36)	13 (36.1)	23 (63.9)	
Clinical stages			
Stages I and II (n = 71)	35 (49.3)	36 (50.7)	0.475
Stages III and IV (n = 39)	22 (56.4)	17 (43.6)	
Pathological grades			
Well (n = 55)	24 (43.6)	31 (56.4)	0.026*
Moderate (n = 43)	29 (67.4)	14 (32.6)	
Poor (n = 12)	4 (33.3)	8 (66.7)	
LNM status			
LNM (n = 41)	28 (68.3)	13 (31.7)	0.008*
No LNM (n = 69)	29 (42.0)	40 (58.0)	
Radiotherapy after operation			
Yes (n = 48)	30 (62.5)	18 (37.5)	0.049*
No (n = 62)	27 (43.5)	35 (56.5)	
Survival status			
Alive (n = 80)	31 (38.8)	49 (61.3)	0.000*
Dead (n = 30)	26 (86.7)	4 (13.3)	

Abbreviation: LNM: lymph node metastasis. *The data were tested using the two-tailed chi-square test, statistically significant difference ($P < 0.05$).

Statistical analyses

All statistical analyses were performed using SPSS 18.0. The chi-square test was used to analyze the relationship between the expression of Hsp10 protein and clinicopathological characteristics of OSCC. The Spearman's rank correlation coefficient was hired to assess the significance of the expression of Hsp10 in OSCC. Overall survival (OS) rates according to Hsp10 expression status was analyzed using Kaplan-Meier survival analysis, and statistical significance was estimated with the log-rank test. The Cox proportional hazard regression model was performed to identify whether high expression of Hsp10 protein was an independent prognostic factor of overall survival for OSCC. All P -values were based on the two-sided statistical analysis and P -value less than 0.05 was considered to be statistically significant.

Results

Association between expression of Hsp10 and clinicopathological features of OSCC

The positive expression and cellular location of Hsp10 in OSCC and in the non-cancerous squamous epithelium were detected by IHC. Positive staining of Hsp10 was identified in the cytoplasm of OSCC and non-cancerous squamous epithelium (Fig-

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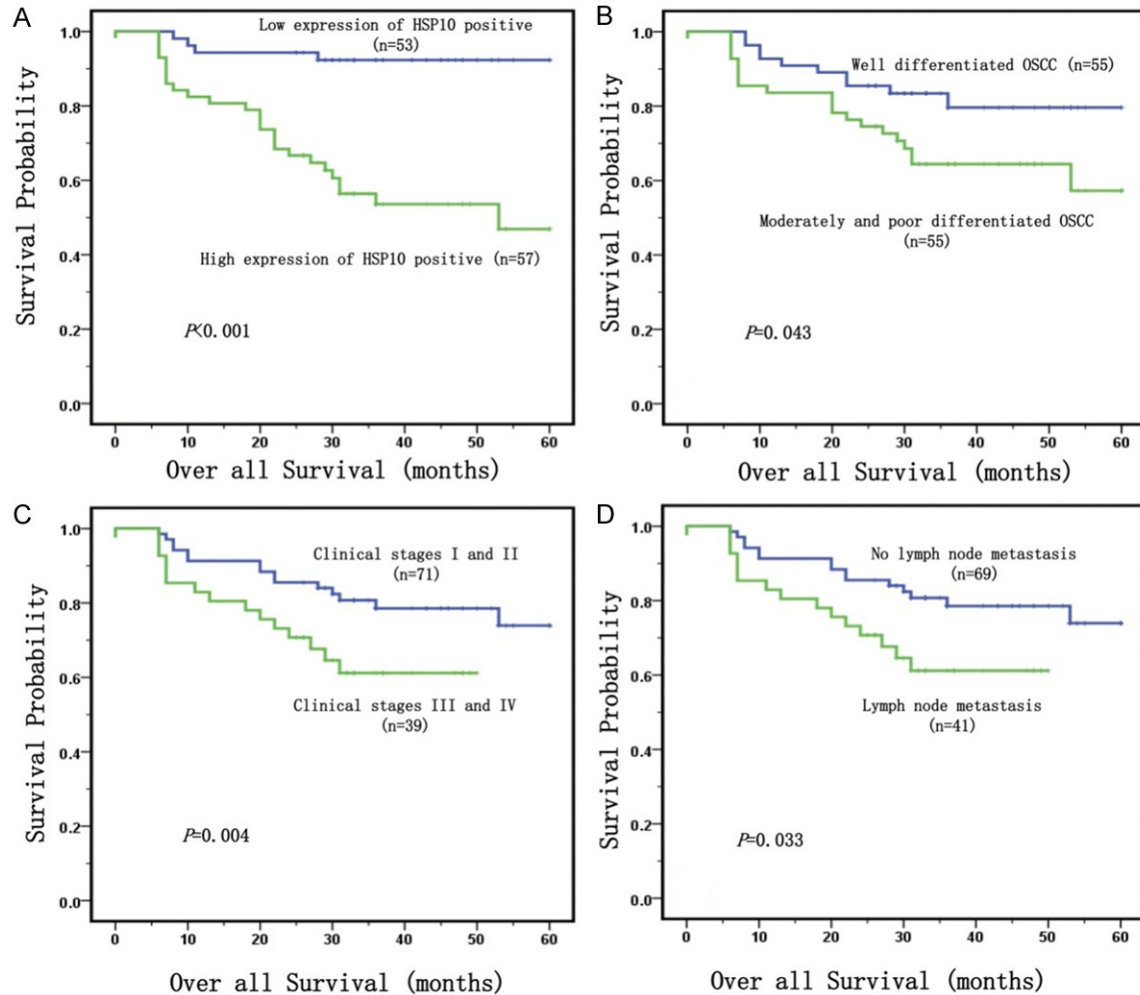


Figure 3. Kaplan-Meier curves for overall survival of OSCC were assessed using the log-rank test. A: High expression of Hsp10 had been significantly related to short survival times of OSCC patients ($P < 0.001$, two-sided). B: OSCC patients with well differentiation were significantly related to higher overall survival rates compared to patients with moderate and poor differentiation ($P = 0.043$, two-sided). C: OSCC patients with clinical stage III and IV were significantly related to lower overall survival rates compared to those patients with clinical stage I and II ($P = 0.004$, two-sided). D: OSCC patients with lymph node metastasis were significantly related to poor overall survival rates compared to those patients without lymph node metastasis ($P = 0.033$, two-sided).

ures 1, 2). Strong and moderate positive staining of Hsp10 was found in the OSCC (Figure 1A, 1B). However, strong positive staining of Hsp10 was not found in the non-cancerous squamous epithelium (Figure 2A, 2B). In the 110 cases of OSCC, the percentage of high expression of Hsp10 was 60.0% (66/110) and low expression was 40.0% (44/110). In the 32 cases of the non-cancerous squamous epithelium, the percentage of high expression was 31.3% (10/32) and low expression of Hsp10 was 68.7% (22/32). There was an observably higher percentage of high expression of Hsp10 in OSCC than the non-cancerous squamous epithelium ($P = 0.006$).

Furthermore, we investigated the associations between the expression of Hsp10 and clinico-pathological characteristics of OSCC, containing patient age, gender, status of chewing betel nut, clinical stages, pathological grades, lymph node metastasis, radiotherapy after oral cancer operation and survival status, all data were analyzed by univariate chi-square test. Data in Table 1 showed that the level of Hsp10 protein expression in OSCC was significantly correlated with patient age, history of chewing betel nut, pathological grades, lymph node metastasis status, radiotherapy of operation and survival status ($P = 0.008$, $P = 0.021$, $P = 0.026$, $P = 0.008$, $P = 0.049$, $P < 0.001$ respectively). How-

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Table 2. Summary of multivariate analysis of Cox proportional hazard regression for overall survival in 110 cases of OSCC patients

Parameter	Wald	Sig.	Exp (B)	95.0% CI for Exp (B)	
				Lower	Upper
Gender	0.369	0.543	0.683	0.155	2.668
Age	0.360	0.549	1.302	0.549	3.086
Chewing betel nut	0.354	0.552	0.704	0.221	2.239
Clinical stages	7.231	0.007*	3.448	1.399	8.501
Pathological grades	1.386	0.239	1.439	0.785	2.638
Lymph node metastasis	0.935	0.334	0.565	0.178	1.797
Radiotherapy after operation	1.415	0.234	1.778	0.689	4.587
Expression of Hsp10	14.539	0.000*	8.710	2.863	26.498

95% CI: 95% confidence interval, * $P < 0.05$.

ever, no statistically difference was found in relation to gender and clinical stage ($P > 0.05$, for all).

Impact of high expression of Hsp10 protein on overall survival of patients with OSCC

Since the high expression of Hsp10 was significantly correlated with the survival status of OSCC in univariate such as chi-square tests, Hsp10 expression levels and the clinical follow-up information in all 110 patients with OSCC were further evaluated by Kaplan-Meier analysis and log-rank test. **Figure 3** illustrated the Kaplan-Meier survival plots for OSCC patients with expression of Hsp10 (**Figure 3A**). Univariate survival (long-rank test) analysis showed that the overall survival rate of OSCC patients with high expression of Hsp10 was significantly lower than those with low expression of Hsp10 ($P < 0.001$). We also plotted the survival curves for OSCC patients with conventional prognosis parameters including pathological grades, clinical stages and status of lymph node metastasis. As shown in **Figure 3B-D**, the patients with advanced-stage OSCC (clinical stage III and IV), lymph node metastasis and moderately and poor differentiation had a lower overall survival rate than patients with early stage OSCC (clinical stage I and II) without lymph node metastasis and with well differentiated tumors ($P = 0.004$, **Figure 3C**, and $P = 0.033$, **Figure 3D**, and $P = 0.043$, **Figure 3B**, respectively).

Besides univariate analysis, multivariate Cox proportional hazard regression analysis was also carried out to further investigate whether high expression of Hsp10 protein was an inde-

pendent prognostic factor for OSCC, and these results were revealed in **Table 2**. In multivariate analysis of the features of OSCC patients, which included age, gender, history of chewing betel nut, clinical stages, pathological grades, status of lymph node metastasis, treatment strategy after operation and expression of Hsp10 protein, results showed that high expression of Hsp10 protein was identified as an independent poor prognostic factor for OSCC ($P < 0.001$), so did clinical stages ($P = 0.007$).

Discussion

In the past, TNM-staging system is a mainly determined factor for the prognosis in OSCC [16]. However, more and more biomarkers can not only play an important role in contributing to establish an accurate diagnosis [17, 18], but also provide occurrence, progression and prognosis data [18] for OSCC. This is a kind of trend that propels people to discover new biomarkers for the development of therapeutic strategies to improve outcome and survival for patients with OSCC.

The levels of Hsp10 beyond the normal range could have a pathogenic significance. A past study had shown that Hsp10, single stranded DNA-binding protein (SSBP1) and peptidyl-prolyl cis-trans isomerase A, which was deemed as increasing in the tumor samples. In this study, Hsp10 was emphasized to be closely correlated with protein folding and translation [19]. In addition, the increase of Hsp10 expression in cancers could be involved in regulation of apoptosis of tumor or dysplastic cells [20]. It had been proved that there was a higher expression of Hsp10 in large bowel cancer, and it may be closely related to the presence of lymph node metastases [21]. Furthermore, Hsp10 was overexpressed early in prostate carcinogenesis [22]. In our present study, the expression of Hsp10 in OSCC was significantly higher than that in the non-cancerous squamous epithelium. And high expression of Hsp10 in the poorly differentiated OSCC was significantly higher than that in the well differentiated OSCC. There was significantly negative association between high expression of Hsp10 and overall survival rates of patients with OSCC. The most interesting result in the study is that the expression of

Hsp10 in OSCC patients who received radiation therapy of after oral cancer operation was significantly higher than that of OSCC patients who did not receive radiation therapy. And the expression of Hsp10 in OSCC patients with lymph node metastasis was significantly higher than the patient without lymph node metastasis. It is well known that lymph node metastasis status is a classic predictor of poor prognosis of patients with advanced tumors. Because of these findings, we definitely consider that Hsp10 is a potential novel biomarker for prognosis and represents a therapeutic target for the treatment of OSCC patients.

Moreover, we provided the evidence that high expression of Hsp10 protein might play a major pathogenic role in the development of OSCC. Hobbies of tobacco smoking, alcohol consumption and chewing betel nut were known risk factors for OSCC [3-5]. We found that OSCC patients with high Hsp10 expression whom had the longer history of chewing betel nut than other groups. In additional, expression of Hsp10 in the younger patients was significantly higher than that in the older ones. The hobby of chewing betel nut in young people whether increased the risk of the high expression of Hsp10 in OSCC? The molecular mechanism of Hsp10 about pathogenic factor remained unclear. Further studies are required for us to elucidate the precise mechanism underlying the role of Hsp10 in OSCC.

In summary, this study demonstrated that high expression of Hsp10 was significantly correlated with worse prognosis of patients with OSCC. High expression of Hsp10 protein might act as a valuable independent biomarker to predict poor prognosis of patient with OSCC.

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Disclosure of conflict of interest

None.

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