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Original Article

Expression of beta adrenergic receptor in oral squamous cell carcinoma and its significance to the prognosis

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Abstract: The aim of this study was to detect the expression of β-AR (Beta Adregenic Receptor) in Oral squamous cell carcinoma (OSCC), para-cancerous and normal oral mucosa and to investigate the relationship between the expression intensity and the characteristics and prognosis of oral cancer. 100 cases of OSCC were collected; 20 cases of paraneoplastic tissues and 10 cases of normal oral mucosa were taken as control. The expression of β-AR was detected by immunohistochemical method and the average optical density determination using Image J software. Finally, the difference of β-AR expression and the correlation with the clinicopathological factors were analyzed statistically. The expression of β-AR in OSCC was higher than that in paracarcinoma and normal mucosa ($P<0.01$). The expression intensity of β1, β2-AR in preoperative lymph node metastasis group was higher than that in patients without lymph node metastasis ($P<0.01$). The expression intensity of β3-AR was not related to pathological grade and tumor size ($P>0.05$). β1 and β2-AR in early stage of OSCC were higher than those in early stage ($P<0.05$). Lymph node metastasis, recurrence, TNM clinical stage, and the expression intensity of β1-AR all had an effect on the cumulative survival rate. All the β1, 2, 3-AR were expressed in OSCC. β1 and β2-AR were involved in lymphatic metastasis and had influence on clinical staging. Metastasis, recurrence, TNM stage and expression of β1-AR had an effect on the cumulative survival rate of tumor. The expression of β3-AR in OSCC was not associated with the pathological grades and tumor growth.

Keywords: Oral squamous cell carcinoma, β-adrenergic receptor, lymph node, metastasis, prognosis

Introduction

Head and neck cancer is the sixth most common types of cancer in the world, this heavy disease ranked third in the developing world, ranked eighth in the developed countries. According to Ferlayand's observation and statistics, the incidence of oral cancer is 263/100000 annually, and the overall mortality rate is 127/100000 [1]. Oral squamous cell carcinoma (OSCC) is the main kind of oral cancer that occurs in head and neck. Although most patients received surgery, postoperative recurrence is still relatively common. The effects of surgery, chemotherapy and radiotherapy for advanced OSCC are not satisfactory. Due to the morbidity of OSCC is lower than lung cancer, liver cancer and other cancers, the basic research of OSCC is not common compared with others. We need a new clinical diagnostic indicator for early warning and auxiliary diagnosis, thus providing the most effective treatment for clinic and improving the effect of the prognosis of patients with oral squamous cell carcinoma.

In recent years, many malignant cells with abnormal expression of neurotransmitter receptors that can be activated by catecholamine hormone (such as epinephrine, norepinephrine, etc.) and regulate proliferation, metastasis, apoptosis, angiogenesis and biological behavior of tumor cells were found by some researches. β adrenergic receptor (β-AR) could be found in many cancer cells, such as lung cancer [2], pancreatic cancer [3], melanoma [4], colon cancer [5], breast cancer [6], ovarian cancer [7] and

prostate cancer [8]. And the expression of the most common is β1, β2 receptor. These studies have demonstrated that the expression of β-AR in tumors is related to the occurrence, development and prognosis of these tumors.

A growing number of clinical and animal experiments have verified that the increase levels of hormone can promote the development of tumors, for example, under stimulation of constant stress factors, the adrenaline (norepinephrine) lead to cancer cell proliferation through the β-AR signaling pathway [9]. It has been found that isoprenaline can regulate cell proliferation and promote the development of pancreatic cancer through β-AR signaling pathway in the study of pancreatic cancer [10]; adrenaline (norepinephrine) can also increase the proliferation of colon cancer cells [11] and regulate the development of lung cancer [5] through β-AR signaling pathway. Through the in-vivo animal trial of breast cancer, it has been verified that β-AR signal expression exists in pathology mechanism of tumor metastasis [12].

Thus it can be seen that expression of β-AR in malignant tumor cells seems to be significantly correlated with the development and prognosis of tumors. In turn, the expression of beta adrenergic receptor in OSCC, the most common oral malignant tumor, is whether or not related to the occurrence, development and prognosis are rarely reported. In this study, in order to examine the expression of β-AR (β1, β2, β3) in OSCC, the relevant para-carcinoma tissues and normal oral mucosa tissues, immunohistochemistry was used to analyze and explore the relationship among the expression intensity of β-AR and the clinical factors of OSCC as well as prognosis.

Materials and methods

Patients, tumor samples and antibodies

From 2004 to 2012, 100 newly diagnosed OSCC patients who underwent cervical lymph node dissection (functional or radical) in the same period in Guangxi Zhuang Autonomous Region People's Hospital were collected. Inclusion criteria were: patients who did not receive preoperative radiotherapy, chemotherapy, or immunotherapy; patients without any serious cardiovascular and cerebrovascular diseases, diabetes and other systemic diseases impacting prognosis and survival; surgery to take the cut edge to send the pathological examination and confirmed no tumor; complete clinical data collection. 50 cases with lymph node metastasis and 50 cases without lymph node metastasis were randomly selected from these cases. 20 cases of non-cancerous tissue were randomly selected from these 100 cases. 10 cases of normal oral mucosa tissue were randomly selected as a control. The follow-up period for patients (from the date of surgery to the date of death, lost or last follow-up) ranged from 1 to 96 months, with 13 patients lost to follow-up, 39 with recurrence and 29 patients died due to oral cancer.

β-AR expression in OSCC, paracancerous tissues and normal mucosa tissues

Sections were deparaffinized in xylene and hydrated using graded alcohol/water baths. Antigen retrieval was performed using 10 mmol/L citrate buffer (pH 6.0) in a Microwave ovens (SUPOR, China) for 7 min, and then the endogenous peroxidase activity was blocked by incubation in endogenous hydrogen peroxide blockers (Maixin Biotechnology, SPKIT-A3, Fuzhou, China) for 10 min. Sections were added primary antibodies (Abcam, ab3442, SC-569, ab140713, USA) and then put in wet boxes, and stored at room temperature for 3 hours. Sections were washed three times with PBS (5 min each time). Sections were added second antibodies (Maixin Biotechnology, MaxVision TM HRP-Polymer anti-Mouse/Rabbit IHC Kit, Fuzhou, China) and then put in wet boxes, and stored at room temperature for 20 min. For negative controls, primary antibodies ware replaced with PBS. Normal myocardium tissue was used in β1-AR positive control. Normal skeletal muscle tissue was used in β2-AR positive control. Normal adipose tissue was used in β3-AR positive control.

Five typical fields of view (400×) (OSCC, adjacent tissue, normal mucosa) were taken randomly from each section. The average optical density (MOD) of each image was measured with Image J software (Java-based image processing and analysis program of public domain developed by Wayne Rasband NIH, Bethesda, MD, USA), and the average value of the MOD values obtained from the five images of each

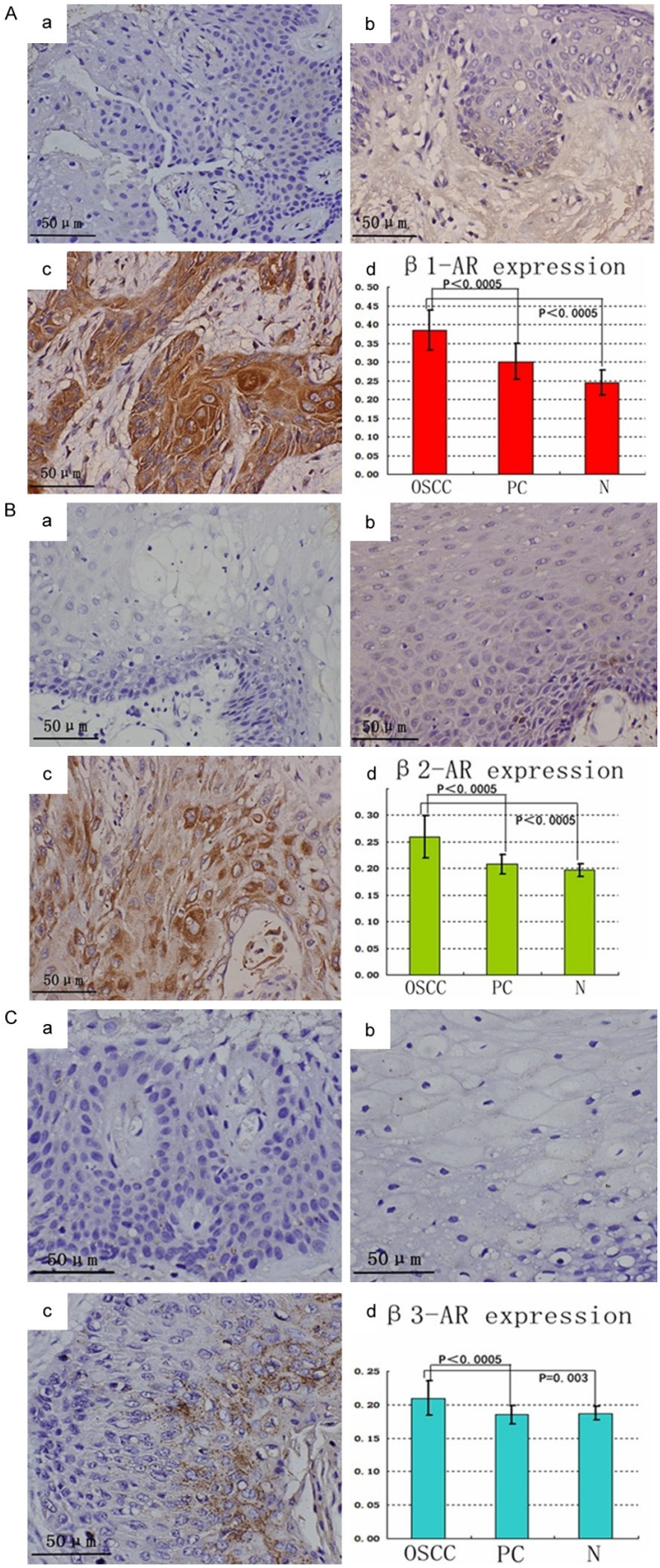

Figure 1. Expressions of β-adrenergic receptor in normal oral mucosa tissues, OSCC tissues and the relevant para-carcinoma tissues (Immunohistochemical method, 400×). The expression of β1-AR, β2-AR and β3-AR in normal mucosa (Aa, Ba and Ca); in paracancerous tissues (Ab, Bb and Cb); in OSCC (Ac, Bc and Cc); the comparison of three groups of average optical density values, β1-AR, Ad; β2-AR, Bd; β3-AR, Cd.

pathological section was used to represent the mean optical density values of the specimens. The median values of the mean optical density (MOD) expressed in the OSCC were used as the cutoff values. The data of each group were divided into two groups: low expression group and high expression group. The expression intensity of the antibody was expressed as the mean ± standard deviation of the mean optical density (x±s). The results were determined by two pathologists who were not related to this study.

Statistical analysis

All statistical analysis was performed using the SPSS 19.0 for windows software (SPSS Inc., Chicago, IL, USA). The differences were analyzed respectively of the expression intensity of β1-AR, β2-AR and β3-AR in OSCC, paracarcinoma and normal mucosa; the statistical significance was analyzed of the expression of β1, β2, β3-AR in the clinical features of OSCC, such as tumor size, pathological grade, clinical stage (TNM) and lymph node metastasis. The Kaplan-Meier method was used to analyze the clinicopathological factors and the expression of β-AR in univariate survival. Log-rank test was used to compare and the survival curves of high and low expression of β-AR were constructed. And then the risk

Table 1. Intensity comparison of β-AR expression between OSCC, adjacent and normal tissues

	β1-AR		β2-AR		β3-AR	
	MOD	P	MOD	P	MOD	P
OSCC	0.3843±0.0539	<0.0005	0.2592±0.0404	<0.0005	0.2097±0.0257	<0.0005
PC	0.3008±0.0472		0.2077±0.0186		0.1850±0.0136	
N	0.2444±0.0332	<0.0005*	0.1965±0.0114	<0.0005	0.1871±0.0105	0.003
OSCC	0.3843±0.0539		0.2592±0.0404		0.2097±0.0257	

Note: *for the T test results.

Table 2. Relationship between expression of β-AR and clinicopathological features

	β1-AR		β2-AR		β3-AR	
	MOD	P	MOD	p	MOD	P
Tumor Size						
≤4 cm	0.3820±0.0535	0.585*	0.2604±0.0419	0.840	0.2135±0.0259	0.056*
>4 cm	0.3859±0.0550		0.2572±0.0384		0.2035±0.0245	
Metastasis						
LM	0.4034±0.0483	<0.0005*	0.2726±0.0389	0.001	0.2128±0.0263	0.235*
NLM	0.3651±0.0528		0.2457±0.0377		0.2066±0.0251	
Pathological Grading						
High	0.3809±0.0564	0.383#	0.2576±0.0434	0.076▲	0.2093±0.0234	0.872▲
Moderately	0.3982±0.0422		0.2522±0.0256		0.2105±0.0327	
Poorly	0.3767±0.0591		0.2856±0.0387		0.2108±0.0271	
Clinical Stages						
I and II	0.3679±0.0561	0.019*	0.2477±0.0361	0.04	0.2092±0.0266	0.878*
III and IV	0.3939±0.0505		0.2659±0.0416		0.2100±0.0255	

Note: *for the T test, #for single factor analysis of variance, ▲for the H test.

factors into the Cox proportional hazards model for assessment. All test criteria were set at P<0.05 as statistically significant, P<0.01 as a significant difference.

Results

The expression of β1, β2, β3 adrenergic receptors in OSCC, para-cancerous tissues (PC) and normal mucosa tissues (N) was displayed by immunohistochemistry.

β-AR expression in oral squamous cell carcinoma, paracancerous tissues and normal mucosa tissues

In normal mucosa, there was no significant expression of β1-AR and β2-AR, and was weakly positive in the paracancerous tissues, while the strong positive expression was found in the cancer tissues (**Figure 1A**, **1B**).

There was no obvious expression of β3-AR in normal mucosa tissues and paracancerous tissues, while the positive expression was found in the cancer tissues (**Figure 1C**).

β-AR expression intensity in oral squamous cell carcinoma, paracancerous tissues and normal mucosa tissues

The expression of β1-AR, β2-AR and β3-AR in OSCC were significantly higher than those in normal mucosa tissues and paracancerous tissues (P<0.01) through the comparison of the β-AR expression intensity (**Table 1**). (Histogram comparison was seen in **Figure 1Ad**, **1Bd**, **1Cd**).

Relationship between the expression of β-AR and clinicopathological features

The expression intensity of β-AR in the two groups with different tumor diameters was compared; there were no significant differences in the expression intensity of β-AR between the two groups (P>0.05). So the expression of β-AR intensity was not related with tumor size (**Table 2**).

Table 3. Clinicopathological features and univariate survival analysis

Group	Analysis factor	Number of cases	Overall survival rate		X^2	P
			3 years	5 years		
Gender	Male	70	0.711	0.624	0.034	0.855
	Female	30	0.724	0.636		
Age	≤60	62	0.694	0.612	0.205	0.651
	>60	38	0.750	0.656		
Tumor diameter size	≤4 cm	62	0.724	0.699	1.298	0.255
	>4 cm	38	0.701	0.534		
Lymph node metastasis* (Including preoperative and follow-up)	Yes	60	0.625	0.483	9.618	0.002
	No	40	0.864	0.864		
Recurrence	Yes	39	0.424	0.283	41.771	<0.001
	No	61	0.977	0.977		
TNM stage	I and II	36	0.843	0.843	5.854	0.016
	III and IV	64	0.653	0.528		
Pathological grade	High	68	0.744	0.621	1.923	0.382
	Moderately	22	0.674	0.674		
	Poorly	10	0.583	0.583		
β1-AR	High expression	50	0.640	0.494	6.056	0.014
	Low expression	50	0.785	0.744		
β2-AR	High expression	50	0.655	0.546	2.286	0.131
	Low expression	50	0.770	0.702		
β3-AR	High expression	50	0.694	0.594	0.385	0.535
	Low expression	50	0.735	0.658		

*Lymph node metastasis refers to the case of lymph node metastasis before and after the operation.

The differences in expression intensity of β-AR between the preoperative lymph node metastasis group and the preoperative lymph node non-metastasis group were analyzed ,the expression intensity of β1-AR and β2-AR in the patients with lymph node metastasis was higher than that in the group without lymph node metastasis (P<0.01). There was no significant difference in the expression of β3-AR between the two groups (P>0.05) (**Table 2**).

According to **Table 2**, the expression of β-AR (β1, β2, β3) was not associated with the pathological grade of OSCC (P>0.05).

The expression intensity (MOD value) of β-AR in different clinical stages of OSCC was compared. The expression of β1, β2-AR in stage III and stage IV of OSCC was higher than that in stage I and II (P<0.05). But there was no difference between the expressions of β3-AR in stage I, and II of OSCC and that in stage III, IV (P>0.05). Therefore, the expression of β1, β2-AR was correlated with TNM staging of OSCC, the later the clinical stage was, the higher the expression of β1, 2-AR was. And the expression of β3-AR was not correlated with TNM staging of OSCC (**Table 2**).

Survival analysis

The relationship between clinical pathological factors and prognosis (survival rate) was analyzed by Kaplan-Meier method. Univariate survival analysis and log-rank test on the differences between groups tested in **Table 3**.

The overall survival rate was higher in patients without lymph node metastasis than in patients with lymph node metastasis (P<0.01), indicating that the prognosis of patients without lymph node metastasis was significantly better than that of patients with lymph node metastasis. The overall survival rate in the non-recurrence group was significantly higher than that in the postoperative recurrence group (P<0.01), and most of the patients without recurrence survived, showing that recurrence was an extremely serious risk factor; The overall survival rate was higher in patients in TNM clinical stage I and II group than in patients in III and IV group (P<0.05), indicating that the earlier the clinical stage was, the better the prognosis after surgery was; Moreover, the overall survival rate of low expression group of β1 and β2-AR was higher than that of high expression group (P<0.05), which indicated that the prognosis of patients

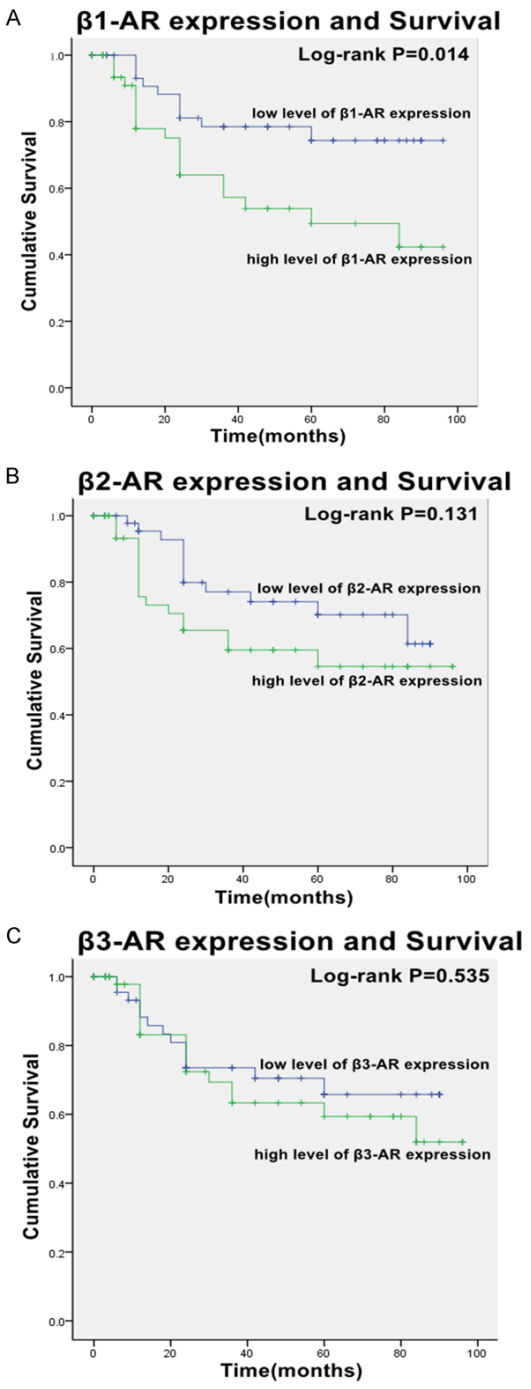

Figure 2. β-AR expression and CS. Blue solid line indicates the β-AR low expression group; solid green line, the β-AR high expression group.

with low expression of β1, β2-AR was better than that of high expression group. There was no statistically significant difference among the other groups (P>0.05). Therefore, it could be concluded that lymph node metastasis, recurrence, β1-AR, β2-AR expression intensity and TNM staging were closely related to the situation of prognosis (survival rate).

According to **Figure 2A**, the survival rate of the low expression group of β1-AR was higher than that of the high expression group (P<0.05), indicating that the lower expression of β1-AR, the better prognosis. The survival rate of β2-AR low expression group was higher than that of high expression group (P<0.05), as shown in **Figure 2B**, indicating that the lower expression of β2-AR related with the better prognosis. According to **Figure 2C**, there was no significant difference in the survival rate of high and low expression of β3-AR (P>0.05), indicating that the expression intensity of β3-AR was not associated with prognosis.

In the light of the univariate analysis, the four factors of metastasis, recurrence, TNM stage, and β1, β2-AR expression were correlated with tumor prognosis. So metastasis, recrudescence, TNM staging and β1 and β2-AR were involved in multivariate regression analysis. The forward stepwise conditional method was selected, finally, only the relapse was included in the independent variable.

Among the many risk factors associated with survival, only relapse was an independent risk factor, P<0.001 (**Table 4**), and the expression of β1, β2-AR could not be considered an independent impact on survival.

Discussion

The sympathetic nervous system “responds to or evades” stress response to release epinephrine or norepinephrine into the circulatory system, as well as releasing norepinephrine into the tumor microenvironment through local sympathetic nerve fibers. These two catecholamines bind to β-AR on the surface of tumor cells and regulate the pathways of β-AR signaling [13, 21]. β-AR is a class of tissue receptors that mediate catecholamine action and is one of the G protein-coupled receptors. Three subtypes of β-AR have been identified: β1, β2, and β3. β1-AR is mainly distributed in the myocardium, β2-AR is mainly distributed in skeletal muscle, bronchus (vascular) smooth muscle tissue, and β3-AR is mainly distributed in adipose tissue. They have a similar spatial structure, the amino-side in the cell side, the carboxyl terminal in the cell side, in the middle of the

Table 4. COX multivariate regression analysis

Parameter	Regression Coefficient (B)	Standard Error (SE)	Wald	Relative Risk (Exp)	95% Confidence Interval		P
					Upper Limit	Lower Limit	
Relapse	1.867	0.459	16.519	6.466	2.629	15.905	<0.001

formation of seven transmembrane α-helix and three extracellular loops, three intracellular rings. The third ring of the cytoplasmic surface can be combined with G protein, then the extracellular signal into the cell to regulate cell metabolism and cell behavior through the cyclic adenosine monophosphate (cAMP) signaling pathway. CAMP activates both intracellular biochemical effects [14]: (1) cAMP phosphorylates multiple target proteins by activating protein kinase A (PKA), including cAMP response element binding protein (CREB), transcriptional activator of transcription (ATF), beta adrenergic receptor kinase (BARK). The first two are involved in about 20% of human genes. β-AR kinases call β -inhibitory proteins to inhibit β-AR signaling and activate Src kinase, activating transcription factors such as focal adhesion kinase (FAK). Activation of FAK regulates cell migration and migration through cell-scaffold kinetics, as well as cell resistance to apoptosis (such as anoikis); activation of PKA-dependent Bcl-2 family members, such as the proapoptotic protein (BAD), can make cancer cells resistant to chemotherapy-induced apoptosis; (2) cAMP activates the exchange protein (EPAC), resulting in Rap1A regulating the activation of B-Raf/MAP signal pathways and affecting many downstream cellular processes, including transcription of genes regulated by the AP-1 and Ets family transcription factors. Recent studies have shown that β-AR pathways are implicated in various stages of cancer initiation and progression. Many cellular and molecular processes influence tumor progression through modulation of the β-AR pathway [14]; increasing expression of pro-inflammatory cytokines such as IL-6 and IL-8 in tumor cells and immune cells [15, 16]; regulating the increase of VEGF leading to angiogenesis [17], the increase of related matrix metalloproteinases leading to tissue invasion [18] and tumor cell migration; regulating FAK to escape anoikis [19]; regulating proapoptotic proteins (BAD) to escape chemically induced apoptosis [20]. Beta-adrenergic signaling also inhibits p53-mediated DNA repair [21], suppresses cytotoxic T-lymphocyte and NK cell responses [22], inhibits the expression of type I interferon [12], upregulates Her2 signaling pathways [23] and so on are suggested by some evidence. It can be seen that activation of the β-AR pathway can inhibit tumor cell apoptosis, can regulate tumor cell adhesion, migration, invasion and immune response, can promote angiogenesis, and can affect tumor growth and metastasis [24].

A large number of clinical and animal studies have shown that norepinephrine (adrenaline) play a catalytic role in the development of tumors through the β-AR pathways, such as isoproterenol promotes the proliferation, mobility and invasion of pancreatic cancer through the β-AR pathways [25]; Adrenaline (norepinephrine) also promotes the development of acute lymphoblastic leukemia [26] and promotes the proliferation of tumor cells such as prostate cancer [20] through the β-AR pathway. Landen et al hold that β-AR has the ability to regulate tumor cell migration and invasion [18]. The OSCC is a common malignant tumor in the head and neck, while the relationship of the expression of β-AR and the development and metastasis of oral cancer is rarely reported.

We found that β1, β2, β3-AR in OSCC cells were expressed and higher than the adjacent tissue and normal tissue, indicating that the β-AR signal pathways involved in tumor development; More higher the expression of β1, β2-AR was, the more prone to lymph node metastasis, indicating that β1, β2-AR signal pathway involved in tumor lymphatic metastasis process. Tumor metastasis is a complex process of cell biology. It is the behavior of a series of cellular and molecular activities, including local invasion of cancer cells, vascular infiltration, transport, vascular extravasation, micro metastasis and colonization [27, 28]. A number of in vivo studies of breast cancer have shown that beta-AR signals are involved in breast cancer metastasis [6]. In these studies, it was found that tumor metastasis rate (including lymph nodes or lung) could be up-regulated by 30-fold by stress-mediated or drug-stimulated β-AR. The positive rate of β2-adrenergic expression in OSCC with lymph node metastasis was 85.3% was found by Shang et al [29] through 65 cases of oral can-

cer tissue study, implied that β2-AR play a role in the occurrence of oral cancer and lymph node metastasis, which is consistent with our findings.

In the analysis of the clinicopathological features of the tumor, the expression intensity of the three isoforms (β1, β2, β3-AR) was not related to the pathological grade and diameter of the tumor, but to the clinical stage: The higher the expression of β1-AR and β2-AR related with the advanced the tumor, which showed that the β-AR signaling pathway was not involved in the regulation of differentiation of tumor cells. According to UICC 2002 staging criteria for TNM classification of oral cancer, once lymph node metastasis appears, clinical stage is more than III. So the clinical stage was closely related to lymph node metastasis. It has been showed that β2-AR expression is higher, the more prone to lymph node metastasis by the above results. Therefore, it is not difficult to draw the conclusion that β1, β2-AR expression is higher, clinical stage is later.

In the single factor analysis of tumor prognosis, there were five factors influencing the prognosis of metastasis: recurrence, lymph node metastasis, TNM staging, β1 and β2-AR expression. The postoperative survival rate of patients with no lymph node metastasis, no recurrence, early clinical stage, beta 1, beta 2-AR low expression of is higher, and the prognosis is better. Gender, age, tumor diameter, pathological grade and β3-AR were not associated with prognosis. This indicates that once the tumor recurrence or lymph node metastasis happens, the disease will quickly become advanced, the prognosis is poor; and the expression of β1, β2-AR also play a role of risk indicator in the survival rate of postoperative survival. Interestingly, we found that the size of the tumor and the degree of cell differentiation did not seem to affect the prognosis.

Lymph node Metastasis, recurrence, TNM stage, β1 and β2-AR were analyzed by COX multivariate regression model. It was found that only recurrence was an independent risk factor ($P<0.05$). Lymph node metastases had an impact on TNM staging (as described above), and the expression of β1, β2-AR was also associated with lymph node metastasis, then there was a correlation between lymph node metastasis, TNM stage and β-AR. Therefore, lymph node metastasis, TNM stage and β-AR are not independent factors, and only relapse is an independent risk factor for prognosis.

It had been found that the proliferation and metastasis of some tumors were inhibited by the application of adrenergic receptor blockers in some studies. Epidemiological studies in recent decades have shown that the use of nonselective beta-blockers (propranolol) can reduce the progression of cancer, the rate of metastasis, and reduce tumor mortality [30]. For example, the use of β-AR blockers could reduce the rate of proliferation of esophageal cancer cells [31], reduce the incidence of early breast cancer and lymph node metastasis [32], improve relapse-free survival in patients with breast and triple-negative breast cancer [33], reduce the risk of progression of melanoma patients [34], reduce prostate cancer mortality [35].

In conclusion, β1, β2 and β3-AR are positively expressed in OSCC, and the expression of β1 and β2-AR is involved in lymph node metastasis. The expression of β1, β2-AR is related to the clinical stage of tumor. Pathological grade has nothing to do with it. The expression of β1, β2-AR in the survival analysis also has a certain role in the prognosis of patients, indicating that β1, β2-AR plays an important role in the recurrence and development of oral cancer. But the molecular mechanism of its action, such as the related signaling pathways and clinical application prospect of β-blockers, requires further in vitro and in vivo studies to explore. The relationship between the expression of β3-AR and clinicopathological characteristics of the tumor remains to be further studied. Despite small numbers and limited follow-up in our series, further study of impact and mechanism of β-AR blockers in the treatment of OSCC is required.

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Disclosure of conflict of interest

None.

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