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Original Article

Correlation between autophagy related genes expression and clinical features in carcinogenesis of oral squamous cell carcinoma

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Abstract: Autophagy is considered to be a double-edged sword to participate in the occurrence and development of the malignant tumor, which plays different roles in various types of tumors or pathological stages. The role in carcinogenesis of oral squamous cell carcinoma (OSCC) remains poorly understood. This study aims to investigate the temporal and spatial expression of autophagy related genes mTOR, Beclin1 and LC3B by immuno histochemistry in sixty cases of OSCC and paired dysplasia and normal oral mucosa, and also to analyze the correlation between the expression and clinical features in oral cancer. The positive expression rate of mTOR in OSCC and dysplasia was 75% and 66.7%, respectively, which was significantly higher than that in normal mucosa ($P < 0.05$). Beclin1 and LC3B expression in OSCC (65%, 58.3%) were lower than in normal mucosa (75%, 78.3%), and in dysplasia the expression rate decreased (54.2%, 27.1%, respectively) which was significantly lower than that in normal mucosa ($P < 0.05$). The integral optical density (IOD) of expression of three proteins was significantly higher in the center than in peripheral of tumor ($P < 0.05$). mTOR, Beclin1, LC3B expression was closely related to lymph node metastasis and TNM stage of oral cancer ($P < 0.05$), and the expression of LC3B, Beclin1 was closely related to smoking. Autophagy is involved in the carcinogenesis of OSCC. Direct damage of smoking may be an activator of autophagy. Autophagy may inhibit tumorigenesis in dysplasia, while in formed cancer upregulated autophagy may try to maintain permanent survival and promote the invasion and metastasis.

Keywords: Oral squamous cell carcinoma, autophagy, mTOR, Beclin1, LC3, dysplasia

Introduction

Oral cancer as the most common malignant tumor in head and neck is a serious and growing problem in many parts of the globe. The annual estimated incidence is around 275,000, two-thirds of these cases occurring in developing countries, especially in the South and Southeast Asia, parts of Europe, parts of Latin America and Pacific regions [1]. At present, surgical treatment is a major means in comprehensive therapy for oral cancer, which may result in the head and face tissues defects and seriously affect the facial aesthetics and oral physical function. Though the advances in the treatment of cancer therapy the 5-year survival rate has remained at approximately 50% in the last few decades [2]. Most oral cancer had a longer period of precancerous lesions or dysplasia to develop into invasive cancer under long-term chronic stimulation. Despite of lots of research about oral cancer, which exhibits multiple genetic and epigenetic mutations, the molecular basis of oral cancer is very complicated. In recent years, autophagy is believed to be involved in the tumorigenesis of breast cancer, colorectal cancer, hepatic cancer and gastric cancer [3-6]. Previous studies have indicated that autophagy is related to the growth and invasion of oral cancer [7, 8], and it is expected to be a new target for molecular therapy, which becomes a hot topic in recent researches.

Autophagy is a process of intracellular degradation in which cytoplasmic constituents are enveloped in double-membrane vesicles, ter-

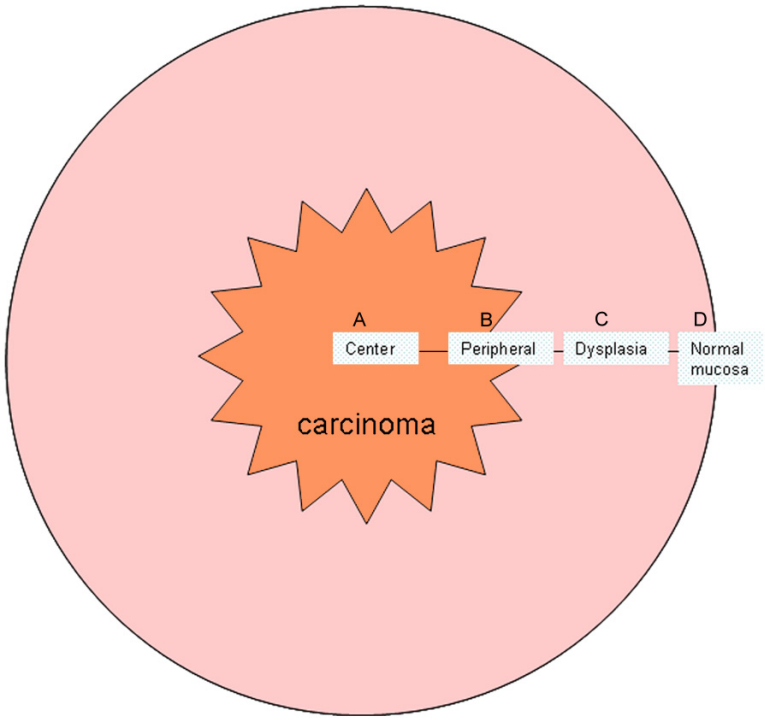

Figure 1. Sketch of pathologic specimen cutting. All samples of every patient include the center of tumor (A), peripheral of tumor that is invasive tumor front (B), precancerous dysplasia tissues (C) and paired normal mucosa (D) that was more than 15 mm away from the edge of tumor.

med autophagosomes, that deliver the contents to the lysosome for degradation [9, 10]. In the past, autophagy was thought to be a programmed cell death, but at present autophagy is considered to be a pathway to keep cells survival, and to maintain the balance of protein metabolism and cell environment stability [11, 12]. The remarkable characteristic of the malignant tumor is that the tumor cells can protect themselves, generate stress tolerance, and maintain the stable living environment to keep unlimited proliferation [13, 14]. So, the effect of autophagy is beneficial to the survival and growth of tumor cells in the environment of hypoxia, lack of nutrition, pathological stimulation. A variety of autophagy related genes (Atg) are involved in the autophagy process, such as Atg1 compound, Atg12-Atg5 compound, Beclin1 and so on. Microtubule associated protein 1 light chain 3-(LC3) as a specific substrate of autophagy is essential for autophagosome formation, and recognized as a marker of autophagy activity [15]. In different types of tumor or different pathological stages, autophagy is considered to be a double-edged sword which may promote or inhibit the occurrence and development of tumor. Autophagy is strictly regulated by different signal transduction pathways including mammalian target of rapamycin (mTOR), Beclin1/VPS34 complex. The interaction and mechanism in tumor is not yet clear. There has been no reports about the correlation expression of mTOR , Beclin1 and LC3 in oral dysplasia and invasive cancer according to the document available. This study aimed to investigate autophagy activity and its regulation pathway by detecting the expression of mTOR, Beclin1 and LC3 in oral carcinogenesis and correlated the results with clinical and pathological characteristics of patients.

Materials and methods

Patients and samples

Sixty patients with OSCC were obtained from the archives of the Department of Pathology who had undergone surgical resection with cervical lymph node dissection, Binzhou Medical University Hospital, China. And both clinical and pathological data were collected for the study. None of the patients had received radiotherapy, chemotherapy or any other treatment before surgery. The patients were ranged from 29 to 75 years old (average age 56.3; 37 males and 23 females). The locations of cancer include tongue (29), gingival (13), floor of mouth (8), lip (6), buccal division (2) and soft palate (2). The TNM Classification of Malignant Tumors (TNM) was evaluated according to American Joint Committee on Cancer 7th edition criteria [16] (I + II stage 20 cases; III + IV stage 40 cases). The longest diameter of the tumor represents tumor size, which was ranged from 10 mm to 40 mm (average 25 mm, < 25 mm 14 cases, ≥ 25 mm 46 cases).

All specimens were cut including the center (A) and peripheral (B) of primary carcinoma, precancerous dysplasia (C) and oral normal mucosa (D) (more than 15 mm away from the edge of cancer), of which there were only 48 cases

Table 1. Expression of mTOR, Beclin1 and LC3B in OSCC, dysplasia and normal mucosa

Group	n	mTOR expression		Beclin1 expression		LC3B expression	
		+	-	+	-	+	-
Carcinoma	60	45 (75%)	15*	39 (65%)	21	35 (58.3%)	25*,#
Dysplasia	48	32 (66.7%)	16*	26 (54.2%)	22*	13 (27.1%)	35*
Normal mucosa	60	16 (26.7%)	44	45 (75%)	15	47 (78.3%)	13

There was statistical significance of the positive expression rate between this group and normal mucosa (*, $P < 0.05$). The positive expression rate of tumors was higher than that in dysplasia (#, $P < 0.01$).

associated with dysplasia. Sketch of pathologic specimen cutting was shown in **Figure 1**. All of the samples were fixed in 4% paraformaldehyde for 24 hours, followed by gradient dehydration and transparency, then embedded in paraffin, 4 μm slice were cut for the following studies. The study was approved by the Ethics Committee of Binzhou Medical University Hospital as required by the Declaration of Helsinki. Before sample collection, written consent was obtained from all patients.

Immunohistochemistry

After deparaffinization and hydration, the slice was incubated with 0.01 mol/L citrate buffer (PH 6.0) in microwave oven at 98°C for 10 minutes for antigen retrieval. Endogeneous peroxidase was blocked by treatment with 3% H_2O_2 for 20 minutes at room temperature. After pretreatment with normal goat serum for 30 minutes to block nonspecific binding, the slice was incubated with rabbit anti-mTOR polyclonal antibody (Abcam, ab32028, Cambridge, UK), rabbit anti-Beclin1 polyclonal antibody (Abcam, ab55878, Cambridge, UK), rabbit anti-LC3B polyclonal antibody (Abcam, ab63817, Cambridge, UK) at dilutions of 1:800, 1:300, and 1:60 respectively, overnight at 4°C. Human colon carcinoma tissue was used as a positive control. Negative control was obtained by replacing the primary antibody with phosphate buffer saline (PBS). Biotinylated goat antirabbit immunoglobulin G was applied as a secondary antibody for 15 minutes at 37°C. The sections were exposed to streptavidin-peroxidase conjugate for 10 minutes at 37°C. Then, the peroxidase reactivity was visualized by the application of 3,3'-diaminobenzidine solution (Zhongshan, Beijing, China) for 5 minutes. Finally, the sections were counterstained with hematoxylin and then mounted.

The immunoreactivity of mTOR, Beclin1 and LC3B was evaluated for percentage of positive cells and intensity of reactivity at high magnification (40 ×). The proportion of tumor, dysplasia or normal mucosa cytoplasm and/or cytomembrane showing positive staining was evaluated as follows: 0, staining in < 1% of cells; 1, staining in 1% to 10%; 2, staining in 11% to 50% and 3, staining in > 50% of cells. The intensity was also recorded as 0, 1, 2 and 3 for negative (no staining), weak (faint yellow), moderate (yellow) and strong (brown) respectively [17]. The total score, ranging from 0 to 9 was obtained by multiplying the proportion and intensity scores. The total score of equal or less than 3 was recorded as negative (-), whereas score of greater than 3 was recorded positive (+). Image pro plus 6.0 software was used to analyze the integral optical density (IOD) results.

Statistical analysis

The data were analyzed with SPSS19.0 software (SPSS Inc, Chicago, IL). Chi-square test was applied to compare the positive rate among different groups, and Non-parametric test (Manny-Whitney Test) was used to analyze ranked data. Spearman rank test was used to verify the correlations. $P < 0.05$ was considered to indicated statistical significance.

Results

The expression of mTOR, Beclin1 and LC3B in OSCC, dysplasia and normal mucosa

Sixty patients' tumors were tested for mTOR, Beclin1 and LC3B immunoreactivity. The expression of the three proteins in carcinoma, paired dysplasia and normal mucosa were summarized in **Table 1**. Forty-five patients (75%) of tumors and thirty-two patients (66.7%) of dysplasia showed positive mTOR expression located in cytoplasm (**Figure 2A-C**), and the positive rates were higher than that in normal mucosa (16, 26.7%) ($\chi^2 = 28.04$, $P < 0.01$; $\chi^2 = 17.28$, $P < 0.01$). The positive expression of

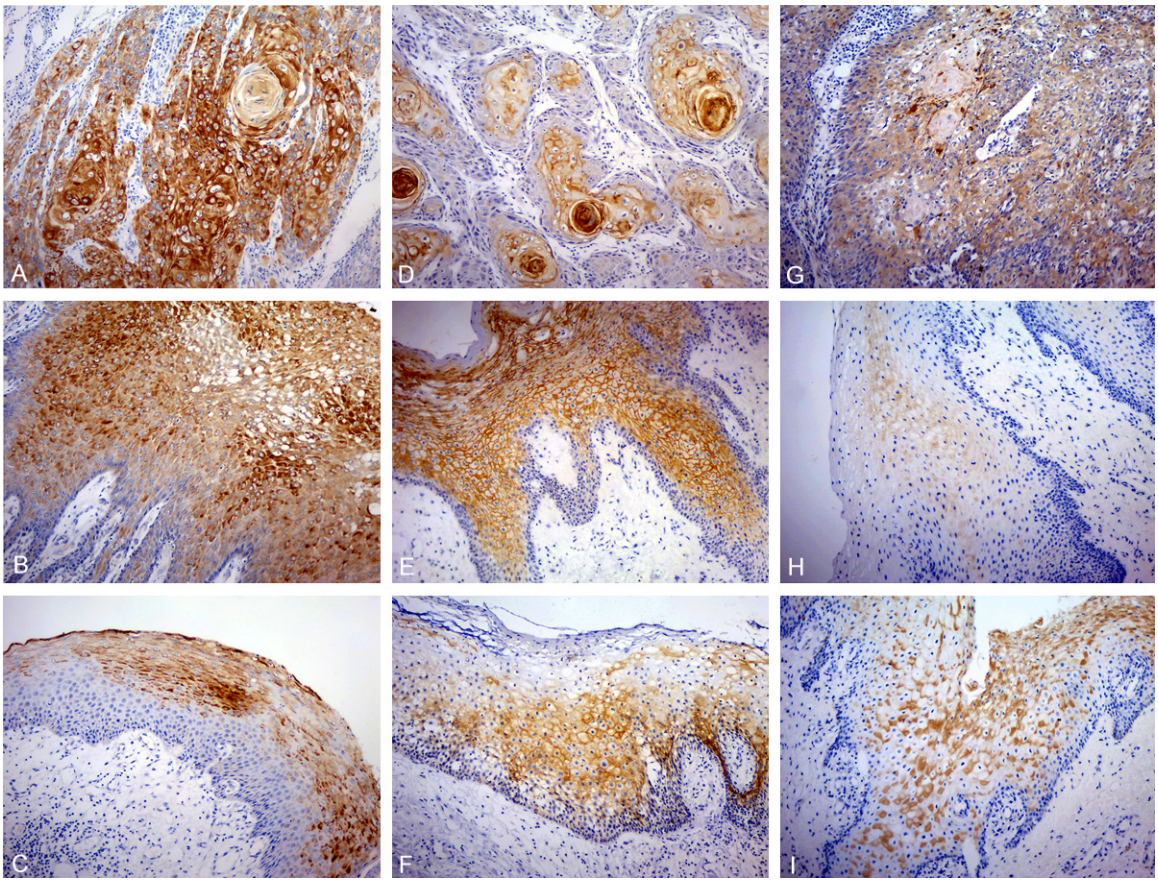

Figure 2. The immunohistochemical expression of mTOR, Beclin1 and LC3B in OSCC, dysplasia and normal mucosa (original magnification × 100). mTOR immunohistochemical staining located in cytoplasm shows strong positive in most OSCC (A), and dysplasia (B). However, the mTOR expression in normal mucosa (C) was lower, and only located the upper of the stratified squamous epithelium. The expression of Beclin1 mainly located in cytomembrane and/or cytoplasm shows positive in OSCC (D), dysplasia (E), and normal mucosa (F). LC3B located in cytoplasm takes on higher positive rate in normal mucosa (I) than that in OSCC (G) and dysplasia (H).

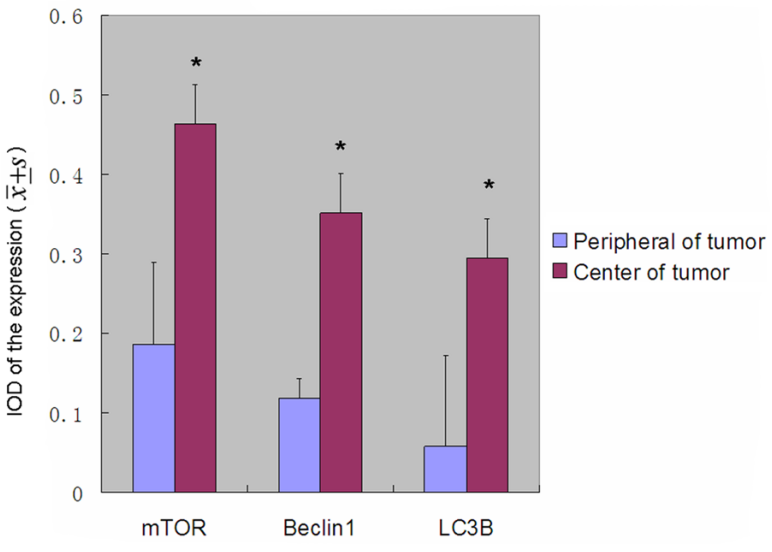

Figure 3. Correlation of the expression of proteins between the center and the peripheral of OSCC. IOD of the mTOR, Beclin1 and LC3B in the tumor center was significantly higher than that in the periphery of tumor.

Beclin1 located in cytomembrane and/or cytoplasm in tumors cells, dysplasia and normal mucosa (**Figure 2D-F**) were thirty-nine (65%), twenty-six (54.2%) and forty-five (75%), respectively. There was a statistically significant difference between the group of dysplasia and normal mucosa ($\chi^2 = 5.14$, $P < 0.05$). No significant relationship was found between the group of tumors and dysplasia, oral normal mucosa ($P > 0.05$). The expression of LC3B located in cytomembrane and/or cytoplasm (**Figure 2G-I**) was significantly higher in normal mucosa (47, 78.3%) than that in carcinoma (35, 58.3%) and

Table 2. Correlation between the expression of mTOR, Beclin1 and LC3B in OSCC and clinical pathological factors

	n	mTOR expression		*P* value	Beclin1 expression		*P* value	LC3B expression		*P* value
		+	-		+	-		+	-	
Age				> 0.05			> 0.05			> 0.05
≤ 56	29	20	9		16	13		17	12	
> 56	31	25	6		23	8		18	13	
Sex				> 0.05			> 0.05			> 0.05
Male	37	27	10		26	11		22	15	
Femal	23	18	5		13	10		13	10	
Smoker				> 0.05			< 0.01			< 0.01
Yes	39	32	7		31	8		30	9	
No	21	13	8		8	13		5	16	
Tumor size				< 0.05			< 0.01			> 0.05
< 25 mm	14	7	7		5	9		6	8	
≥ 25 mm	46	38	8		34	12		29	17	
Tumor grade				< 0.01			> 0.05			> 0.05
I	42	37	5		27	15		22	20	
II/III	18	8	10		12	6		13	5	
Lymph node metastasis				< 0.05			< 0.05			< 0.05
Yes	39	33	6		30	9		27	12	
No	21	12	9		9	12		8	13	
TNM stage				< 0.01			< 0.01			< 0.01
I/II	20	10	10		7	13		6	14	
III/IV	40	35	5		32	8		29	11	

dysplasia (13, 27.1%) (χ^2 = 5.54, P < 0.05; χ^2 = 28.37, P < 0.01), also, the LC3B expression in carcinoma was higher than that in dysplasia (χ^2 = 10.547, P < 0.01).

Expression of mTOR, Beclin1 and LC3B in peripheral and center of tumor

Integral optical density (IOD) was analyzed in peripheral and center of the carcinoma as **Figure 3** shows. The average IOD of mTOR, Beclin1 and LC3B in the center of tumor was significantly higher than that in the peripheral of tumor (P < 0.05).

Analysis of correlation between the expression of mTOR, Beclin1 and LC3B in squamous cell carcinoma tissues and clinical pathological factors

The correlation between the expression of mTOR, Beclin1 and LC3B in OSCC tissues and clinical pathological factors had been summarized in **Table 2**. There was a significant difference between the expression of mTOR and tumor size (P < 0.05), tumor grade (P < 0.01), lymph node involvement (P < 0.05) and TNM stage (P < 0.01). The expression of Beclin1 was significantly correlated with tumor size (P < 0.01), smoking (P < 0.01), lymph node metastasis (P < 0.015) and TNM stage (P < 0.01). The expression of LC3 was closely related with smoking (P < 0.01), lymph node involvement (P < 0.05) and TNM stage (P < 0.01). There was no significant correlation between the expression of these three proteins with age and sex of the patients (P > 0.05).

Discussion

Autophagy plays an important role in the process of cell growth, development and structure reconstruction by degrading cellular damaged organelles and recycling intracellular biological macromolecules [9, 10]. The normal cells have lower level of autophagy, named basic autophagy. Under pressure stimulation, autophagy can control cell quality and maintain homeosta-

sis, which was named induced autophagy [18, 19]. During this process, the level of autophagy is strictly regulated by different signal transduction pathways.

mTOR signaling pathway is currently recognized as a signal transduction pathway involved in the regulation of autophagy, which acts as a "gatekeeper" [20]. The lack of nutrition and energy is the activator of mTOR. When the nutrient is abundant or normal oxygen mTOR signal is activated and autophagy is inhibited, while nutrient deficiency or hypoxia can suppress the mTOR signal and activate the autophagy [21, 22]. There are two complexes of mTOR in the organism, mTOR complex 1 (mTORC1) and mTORC2, of which mTORC1 plays an important role in regulating autophagy. Ras signal pathway and phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) pathways are upstream regulators of mTORC1. Growth factors such as insulin can activate their cognate receptors and subsequently activate the PI3K/AKT signaling axis leading to mTORC1 activation [23-27]. The activation of the PI3K/AKT/mTOR pathway may promote cell survival, proliferation and angiogenesis in most human tumors such as breast cancer, lung cancer, prostate cancer and renal cancer [28-34]. AMP-activated protein kinase (AMPK) pathway is another important upstream regulator of mTORC1, which is a sensor of cellular energy levels and is activated by a high AMP/ATP ratio. Hypoxia or nutrient deficiency can decrease intracellular ATP level resulting in the increase of AMP/ATP ratio and subsequently inhibit mTORC1 [23, 35, 36].

Beclin1 is an important tumor suppressor gene related to autophagy, which can inhibit the tumor cells growth and development by inducing antophagy and apoptosis [37]. LC3 (a mammalian homolog of yeast Atg8) originally identified as a subunit of microtubule-associated protein 1A and 1B, becomes tightly associated with the autophagosomal membranes [38]. During the autophagosome formation, cytosolic LC3 (LC3-I) is transformed into membrane-bound LC3-II by conjugating to phosphatidylethanolamine (PE) [39, 40]. The intra-autophagosomal LC3-I is degraded by lysosomal hydrolases during the fusion of autophagosomes with lysosomes, and LC3-II can be detected with immunohistochemistry or Westren blot. The conversion of LC3-I to LC3-II is associated with autophagic activity. So, detecting the level of LC3-II is generally used as a specific marker for the activity of autophagy [41-44].

Autophagy is considered as a "double-edged sword" in various types of tumor and different pathological stages [45, 46]. Studies have reported that autophagy may inhibit the occurrence of tumor in the early stage of cancer, but it may play a reverse role in the formed tumors [47]. Oral cavity is constantly surrounded by physical (such as hot and cold, sweet and sour, smoking burns, mechanical damage), chemical (food and metabolism decomposition of chemical compounds) and biological (a variety of pathogenic microorganisms) stimulus. So, oral mucosa should have higher damage resistance to maintain the steady state and structure. This study had detected the expression of mTOR, Beclin1 and LC3B proteins in sixty cases of oral carcinogenesis including normal mucosa, dysplasia and invasive squamous cell carcinoma by immunohistochemical staining. In normal oral mucosa, mTOR showed lower positive rate (26.7%), while Beclin1 and LC3B showed a higher level of expression (75%, 78.3%, respectively). From this we can see that the basic level of autophagy in normal mucosa was higher. And low activity of mTOR and high level of Beclin 1 might take synergistic effects to invoke autophagy to maintain homeostasis and metabolic profile in normal mucosa cells.

Dysplasia is the precancerous lesion of oral cancer, which is due to the excessive proliferation of aberrant cells under pathological stimulation such as smoking, growth factors and stress stimulus. Once the abnormal proliferation cells are involved in the whole epithelial layer, and break through the basement membrane, the invasive cancer is formed. Recent studies suggest the malignant transformation rate of dysplasia is from 8% to nearly 18% [48]. In dysplasia the expression of mTOR was significantly higher (66.7%) than that in normal, while the expression of Beclin1 and LC3B decreased in this study. From the result we inferred that with pathological stimulus intensity increasing and prolonging, the damaged cells will be induced to apoptosis and/or autophagy death to prevent the aberrant cells hyper proliferation once the cells are unable to removal damaged organelle and maintain homeostasis by autoph-

agy survival. So, the down-regulation of autophagy may prevent the transition of dysplasia to SCC, which is realized through the joint regulation of mTOR and Beclin1 [47, 49]. We speculate a hypothesis that in dysplasia stage we could strive to improve the level of autophagy by inhibiting mTOR and promoting Beclin1 to maintain the normal state of the cells and suppress the malignant transformation, which is a promising idea to suppress the carcinogenesis of oral cancer.

As stated earlier, mTOR can be activated through PI3K/AKT and AMPK pathway in tumors. Activated target protein mTOR may accelerate the rapid proliferation and cell cycle to facilitate tumor development rapidly [4]. Autophagy is highly expressed in the multi tumors, which can produce nutrients and energy to maintain the survival of tumor cells, and relieve stress in the external environment. In this study, the expression of mTOR, Beclin1 and LC3B were higher in oral cancer. We further analyzed the expression of mTOR, Beclin1 and LC3B in the center and periphery of carcinoma. The results showed that the expressions of three gene proteins were significantly higher in the central region than those in peripheral cancer. The particular temporal and spatial expression of the phenomenon makes us infer that cancer cells in the center of tumor confronting with more serious hypoxia need to enhance autophagy to maintain permanent survival and proliferation to spread [52, 53]. While in the peripheral of tumor, with angiogenesis lower level of autophagy can maintain the survival. During the process of tumor metastasis, autophagy can protect tumor cells through the vascular system and survival in the distance [50]. Some reports showed that the activation of autophagy in tumor was also an important mechanism of drug resistance [51]. Karantza-Wadsworth V, et al reported that if autophagy related important genes were knocked down, the tumor cells in hypoxia would be necrosis [52]. According to the results of this study, combined with existing research reports, we hypothesized that combined application of targeted mTOR and autophagy inhibitors may be a promising way to overcome cancer. In clinical practice, rapamycin (a mTOR inhibitor) has been successfully used in the treatment of renal cell carcinoma and lymphoma. So, it is valuable to study the level of autophagy in OSCC, and is also the direction of our future study efforts.

It is well known that smoking is the main risk factor for oral cancer [49, 53]. This study also showed that the expression of Beclin1 and LC3B was significantly correlated with smoking, which suggests direct damage of smoking may be an activator of autophagy. So, smoking prohibited is good to the treatment of OSCC. Meanwhile, the expression level of mTOR and Beclin1 was significantly correlated with tumor size. Some reports thought that when mTOR is activated, autophagy should be inhibited [54]. But this study showed autophagy marker protein LC3B was highly expressed in invasive carcinoma, and the positive regulation of autophagy gene Beclin1 was also expressed in high level. These findings suggest that mTOR is not the only regulatory pathway of autophagy, and autophagy is regulated by multiple genes and pathways. Study also found that there was significant correlation between the expression of mTOR, Beclin1, LC3B and lymph node metastasis, TNM staging of oral cancer, indicating that autophagy is not only involved in the carcinogenesis of oral cancer, but also conducive to the invasion and metastasis of tumor.

In summary, autophagy is involved in the carcinogenesis of OSCC. In normal oral mucosa higher level of autophagy maintains homeostasis and structure. In dysplasia autophagy is reduced to remove distorted cells by inducing apoptosis or autophagy death to inhibit tumor. In carcinoma autophagy is enhanced especially in the central region of the cancer. Cancer cells try to maintain permanent survival and proliferation by up regulated autophagy in order to convey enough seed cells to spread. During the process of carcinogenesis, autophagy is regulated by multiple factors such as mTOR, Beclin1 genes and smoking stimuli, which will help to further clarify the mechanism of carcinogenesis of oral cancer, and lay a foundation based on autophagy target for treatment of oral cancer.

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Disclosure of conflict of interest

None.

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