Abstract. It has previously been demonstrated that autophagy and inflammation act synergistically to promote carcinogenesis. However, the precise roles of autophagy in multistep oral carcinogenesis are still unclear, particularly regarding its association with tumor inflammation. The present study established a 4NQO-induced oral cancer mouse model and investigated autophagy status in the multistep process of oral carcinogenesis using immunohistochemistry, western blotting and immunofluorescence staining. Furthermore, the number of Gr-1+CD11b+ myeloid derived suppressor cells (MDSCs) and CD4+Foxp3+ regulatory T cells (Tregs) during oral carcinogenesis and the association with autophagy status was also examined. The results revealed that the expression of autophagy biomarkers, including dihydrosphingosine 1-phosphate phosphatase LCB3 (LC3B), p62/SQSTM1 (p62) and Beclin 1 increased during 4NQO-induced carcinogenesis and in human oral cancer. The number of MDSCs and Tregs also increased during oral carcinogenesis. Furthermore, the expression of LC3B and p62 significantly correlated with the accumulation of MDSCs and the expression of Beclin 1 correlated with the increase of Tregs. These data indicated that autophagy may be activated by the tumor inflammation microenvironment during oral carcinogenesis.

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most commonly occurring cancer in the world and a lethal disease, with a 5-year survival rate of ~50% (1). Despite continuing improvements in therapy, the poor prognosis of OSCC patients remains unsolved around the world. Carcinogenesis is a multistep process including initiation, promotion and progression, and oral cancer usually develops from premalignant lesions of oral mucosa into OSCC (2). It has been revealed that risk factors for human oral carcinogenesis include alcohol consumption, tobacco and human papillomavirus infection (3). However, the underlying molecular mechanism of dynamic oral carcinogenesis has not been completely elucidated.

Macroautophagy (autophagy) is an evolutionarily conserved and self-consumption process involved in preserving organelle function, removing cellular waste products and providing metabolic substrates (4,5). It has been reported that autophagy is an evolving and multifaceted process during cancer initiation and progression (6). In normal cells, autophagy prevents excess reactive oxygen species, DNA damage and genome instability, known causes of cancer initiation and progression (7). In these contexts, autophagy likely serves as a tumor suppressor in the tumor initiation stages (8). However, in the late stages of tumorigenesis, autophagic responses maintain...
tumor metabolism and promote tumor cell survival. In this sense, autophagy exhibits a pro-oncogenic role (6,8).

In addition to a cellular mechanistic role, autophagy has been demonstrated to orchestrate the tumor microenvironment by regulating the inflammation response (9-11). Autophagy enhances the processing and presentation of tumor antigens and thereby activates antitumor immunity (9). Martinez-Outschoorn et al (10) demonstrated that in a co-culture system, cancer cells produced numerous inflammatory mediators in a tumor microenvironment and further induced autophagy in adjacent fibroblasts via oxidative stress and nuclear factor (NF) κB-activation (10). This indicated that inflammation mediators in the tumor microenvironment contribute to tumor progression by activating the autophagy response (10,11).

Hence, the present study established an oral cancer mouse model with 4-nitroquinoline-1-oxide (4NQO) and assessed the expression of autophagy markers dihydrophosphoglycine 1-phosphate phosphatase LC3B (LC3B), p62/SQSTM1 (p62) and Beclin 1 at various stages of tongue lesions of these mice. Furthermore, the number of Gr-1+CD11b+ myeloid derived suppressor cells (MDSCs) and CD4+ Foxp3+ regulatory T cells (Tregs) during oral carcinogenesis and the association with autophagy status was also examined. The data will help to address the dynamic change of autophagy activity during multistage oral carcinogenesis and its association with inflammation.

Materials and methods

Ethics statement. All procedures involving mice were approved by the Subcommittee on Research and Animal Care of Sichuan University (WCHSIRB-D-2016-149, Chengdu, China). The written informed consents were obtained from participants through their signatures. The use of human tissue samples and clinical data was approved by the Institutional Ethics Committee of the West China Medical Center, Sichuan University (WCHSIRB-D-2012-097).

Experimental model. A total of 38 female wild-type C57BL/6 mice, eight weeks old, weighing 20-25 g were purchased from the Chengdu Dashuo Biological Technology Co., Ltd. (Sichuan, China). The mice were housed in State Key Laboratory of Oral Diseases West China Hospital of Stomatology (Sichuan University), five per cage, maintained at 22±1˚C and within the range of 30-70% relative humidity with a 12-h light/dark cycle. The animals were provided free access to a normal chow (LabDiet with constant nutrition, Dashuo Co. Ltd., Chengdu, China) and drinking water provided in water bottles. A stock solution of 4-nitroquinoline-1-oxide (4NQO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was prepared at 0.5 mg/ml and added to the drinking water to prepare at 0.5 mg/ml 4NQO (low-4NQO group) or 200 µg/ml 4NQO (high-4NQO group) in drinking water. Then, mice in low-4NQO and high-4NQO group (n=18), high‑4NQO group (n=20). In the experimental groups, the mice were given 100 µg/ml 4NQO (low-4NQO group) or 200 µg/ml 4NQO (high-4NQO group) in drinking water. At the end of the experimental period, mice were anesthetized using isoflurane, and blood was collected by vacutainer vials containing heparin. Tongue and spleen tissues were collected in each group and tongue tissues were then longitudinally bisected. One part of each tongue tissue was fixed in 10% buffered formalin at room temperature for 24 h and then embedded with paraffin. The other part was immediately snap-frozen and stored at -80˚C for western blotting or frozen with OCT for immunofluorescence staining.

Histopathological analysis. Sections (4-µm) of tongue tissue from different groups were processed for hematoxylin and eosin (H&E) staining. After deparaffinization and rehydration, the sections were stained with hematoxylin (OriGene Technologies, Inc., Rockville, MD, USA) at room temperature for 5 min. Then the sections were differentiated in 1% hydrochloric acid alcohol for 2 sec followed by incubation in ammonia water for 2 min, and stained with eosin (OriGene Technologies, Inc.) at room temperature for 1 min. Histopathological diagnosis was performed by an experienced oral pathologist in a blind manner, and the samples were classified into the following four types: Normal epithelium, mild-moderate dysplasia, severe dysplasia, and carcinoma, according to the criteria described by the World Health Organization (12). All histopathological examination was conducted using a light microscope (Olympus BX46; Olympus Corporation, Tokyo, Japan).

Tissue microarray (TMA) construction. The tissue microarray used for the present study included 52 oral squamous cell carcinoma (OSCC), 5 squamous cell papilloma and 5 normal mucosa specimens from the Department of Oral Pathology, West China Hospital of Stomatology, Sichuan University between 2013 and 2014. The OSCC samples used in the study were typical keratinizing-type, not verrucous or other variant types. There were 32 male and 20 female patients, and their ages ranged from 27 to 91 years, with a median age of 62 years. The diagnosis of these specimens was confirmed by pathologic examination. The tissue microarray slide was constructed as previously described (13). Briefly, based on the results of HE-stained tissue slides, formalin-fixed, paraffin-embedded tissue blocks were punched to obtain tissue cylinders with a 3 mm diameter containing representative tissue areas. Then the punched tissue cores were placed on a recipient block and arranged with an array pattern. Four-µm-thick sections of TMA were produced for H&E and immunohistochemistry staining.

Immunohistochemical analysis. Paraffin-embedded sections at 4-µm thickness were deparaffinized and rehydrated in graded ethanol series and distilled water. For antigen retrieval, slides were immersed in 0.01 M sodium citrate buffer, pH 6.0 in a water bath at 95˚C for 30 min. Endogenous peroxidases were inhibited by treatment with 3% hydrogen peroxide for 20 min. After blocking with goat serum albumin (OriGene...
Table I. Incidence of tongue lesions in various 4-NQO-treated mice groups.

| Group            | No. of mice | Lesion number* | Lesion size (mm)* | Mild-moderate dysplasia (%) | Severe dysplasia (%) | Total (%) | Carcinoma (%) |
|------------------|-------------|----------------|-------------------|-----------------------------|----------------------|-----------|---------------|
| 14 weeks         | 11          | 0.7            | 1.3               | 2/11 (18)                   | 4/11 (36.4)          | 6/11 (54.4)| 5/11 (45.5)   |
| Low-dose 4NQO    | 5           | 0.8            | 1.0               | 1/5 (20)                    | 1/5 (20)             | 2/5 (40)  | 3/5 (60)      |
| High-dose 4NQO   | 6           | 0.7            | 1.6               | 1/6 (17.7)                  | 3/6 (50)             | 4/6 (67.7)| 2/6 (33.3)    |
| 18 weeks         | 8           | 1.6            | 1.3               | 4/8 (50)                    | 2/8 (25)             | 6/8 (75)  | 2/8 (25)      |
| Low-dose 4NQO    | 4           | 1.7            | 1.5               | 1/4 (25)                    | 1/4 (25)             | 2/4 (50)  | 2/4 (50)      |
| High-dose 4NQO   | 4           | 1.5            | 1.1               | 3/4 (75)                    | 1/4 (25)             | 4/4 (100)| 0/4 (0)       |
| 22 weeks         | 9           | 2.0            | 1.6               | 1/9 (11.1)                  | 2/9 (22.2)           | 3/9 (33.3)| 6/9 (66.6)    |
| Low-dose 4NQO    | 4           | 1.8            | 1.4               | 1/4 (25)                    | 1/4 (25)             | 2/4 (50)  | 2/4 (50)      |
| High-dose 4NQO   | 5           | 2.2            | 1.7               | 0/5 (0)                     | 1/5 (20)             | 1/5 (20)  | 4/5 (80)      |
| 26 weeks         | 10          | 1.9            | 2.1               | 0/10 (0)                    | 2/10 (20)            | 2/10 (20)| 8/10 (80)     |
| Low-dose 4NQO    | 5           | 1.6            | 1.8               | 0/5 (0)                     | 1/5 (20)             | 1/5 (20)  | 4/5 (80)      |
| High-dose 4NQO   | 5           | 2.2            | 2.4               | 0/5 (0)                     | 1/5 (20)             | 1/5 (20)  | 4/5 (80)      |
| Total            | 38          | 1.5            | 1.9               | 7/38 (18.4)                 | 10/38 (26.3)         | 17/38 (44.7)| 21/38 (55.3) |

*The lesion number of each mouse was obtained and recorded, and then the sum of lesion number in each group was calculated. Average number of lesion was assessed by the formula: The sum of lesion number/mice number of each group. The mice were divided into four experimental groups, 14w, 18w, 22w, 26w groups, and the mice of each group were subdivided into low-4NQO group and high-4NQO group. *The size of all lesions in each mouse was obtained and recorded, and then the sum of lesion size in each group was calculated. Average number of lesion was assessed by the formula: The sum of lesion size/mice number of each group. The mice were divided into four experimental groups, 14w, 18w, 22w, 26w group, and the mice of each group were subdivided into low-4NQO group and high-4NQO group.
in 1% BSA in PBS and incubated with the sample for 1 h at 37°C in the dark, followed by counterstaining with DAPI to visualize the nuclei. The slides were observed under laser scanning confocal microscope (Olympus FluoView FV1000; Olympus Corporation) at x400 magnification, and excitation wavelength was 559 nm for detection of Rhodamine.

**Western blot analysis.** Total protein lysates were extracted from the mouse tongue from each experimental group by using a Total protein lystate kit (catalog no. KGP250; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer’s protocol. Protein concentration was quantified using the bicinchoninic acid assay (catalog no. KGP902; Nanjing KeyGen Biotech Co., Ltd., and 30 μg of total protein was subjected to 12% SDS-PAGE. The fractionated samples were transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), immersed in 5% blotto at room temperature for 60 min to block non-specific binding and then incubated with anti-GAPDH antibody (1:5,000; catalog no. 10494-1-AP; Proteintech Group), anti-LC3B antibody (1:1,000; catalog no. 14600-1-AP; Proteintech Group), anti-P62 antibody (1:500; catalog no. WL02385; Wanleibio Co., Ltd.) and anti-Beclin 1 antibody (1:500; catalog no. WL02508; Wanleibio Co., Ltd.) overnight at 4°C. The membranes were then incubated with the HRP-conjugated goat anti-rabbit IgG (1:5,000; catalog no. SA00001-2; Proteintech Group) for 1 h at room temperature. The specific bands were detected using the Immobilon Western Chemiluminescent HRP Substrate detection kit (EMD Millipore). The intensities of the protein bands were quantified with Quantity One 4.5.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Data were analyzed using SPSS software, version 22.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). The normally distributed quantitative data were expressed as the mean ± standard deviations and were compared using one-way analysis of variance. The SNK test was performed for multiple testing among all groups. Comparisons of the positive rates of autophagy marker expression in human tissues were performed using Fisher’s exact test and Bonferroni correction was performed for multiple testing (data not shown). P<0.05 was considered to indicate a statistically significant difference. Pearson correlation analysis was performed to evaluate the correlation between autophagy marker expression and the number of MDSCs and Tregs. Immunohistochemical analysis, flow cytometry analysis, immunofluorescence staining and western blot analysis were repeated at least three times.

**Results**

**Characteristics of mouse oral tumor model in macroscopic examinations.** In the present study, mice were exposed to low or high doses of 4NQO for different time-periods and returned to normal drinking water for 8 weeks. Visible and gross lesions of the oral cavity were observed both in low-dose and high-dose 4NQO groups after a period of 6 weeks 4NQO treatment and 8 weeks observation. At 14 weeks, the average lesion number and lesion size of low-dose 4NQO-treated mice were 0.8 and 1 mm respectively, and 0.7 and 1.6 mm in high-dose 4NQO-treated mice (Table I). At 26 weeks, the average lesion number and lesion size of low-dose 4NQO-treated mice were 1.6 and 1.8 mm respectively, and 2.2 and 2.4 mm in high-dose 4NQO-treated mice. This indicated that lesion sizes and number in each mouse increased with the increase of 4NQO exposure duration. However, none of the control mice exhibited visible changes during the period of observation (n=10).

**Histopathological analysis and immunohistochemical staining of PCNA.** Histological examination of 4NQO-induced lesion tissues was conducted by a trained pathologist blinded to the sample identities. As expected, 4NQO-treated mice induced oral carcinogenesis with a well-defined progression from normal epithelia, through dysplasia of different severity to early invasive carcinoma (Fig. 1A). Histopathologically, the tumor lesions were usually squamous cell carcinoma of well-differentiated or moderately-differentiated type with typical keratin pearl formation. A few of the tumor lesions exhibited a papillary surface configuration and spread into the submucosa and underlying muscle layer. At 14 weeks, 40% low-dose 4NQO-treated mice exhibited dysplasia, and 60% exhibited squamous cell carcinoma (SCC), whereas 67.7% high-dose 4NQO-treated mice exhibited dysplasia lesion, and 3.3% showed SCC. At 18 weeks, 50% low-dose 4NQO-treated mice showed squamous cell carcinoma (SCC), whereas no high-dose 4NQO-treated mice showed SCC. At 26 weeks, 80% mice of both the low-dose 4NQO group and high-dose 4NQO group showed SCC. The histopathological analysis was summarized in Table I.

PCNA is a marker of cell proliferation and the PCNA expression in mouse tongues was detected by immunohistochemistry. In the control group, PCNA expression was weakly observed only in the basal layer and suprabasal layers of tongue epithelium. However, PCNA expression was markedly increased in the dysplasia and SCC groups (Fig. 1B).

**Expression of LC3B, p62 and Beclin 1 in different periods of 4NQO-induced oral carcinogenesis.** The present study examined LC3B expression, a common marker of autophagy, in tongue tissue of normal, mild-moderate dysplasia, severe dysplasia, and SCC groups. Immunohistochemical analysis demonstrated that LC3B was mainly expressed in the cytoplasm of the cells and infrequently in the perinuclear membrane and nucleus (Fig. 2A). By semiquantitative assessment of IHC staining, LC3B was highly expressed in dysplasia epithelia and SCC compared to control group, and LC3B levels in SCC were increased compared with dysplasia epithelia (P<0.05; Fig. 2B). Additionally, immunofluorescence assessment revealed that LC3B puncta (representing autophagosomes) formation also increased during oral carcinogenesis (Fig. 2C). These results indicated that increased LC3B levels were associated with tongue carcinogenesis progression. Then, the present study further tested the isoform conversion of LC3, another indicator of autophagosome formation during 4NQO-induced tongue carcinogenesis. The result of the western blot analysis demonstrated that the LC3B-II expression level was upregulated in SCC compared with normal and dysplasia tissues (Fig. 2D). Taken together, the increased LC3B expression...
suggested that autophagosome formation increased during oral carcinogenesis.

p62, a ubiquitin-binding scaffold protein, serves as an autophagy substrate and cargo adapter that can be selectively degraded by autophagy. To identify the level of autophagy flux, p62 expression was assessed by immunohistochemistry and western blot analysis at various stages of 4NQO‑induced tongue carcinogenesis. p62 was primarily expressed in the cytoplasm and infrequently in the nucleus, consistent with the finding that p62 can shuttle between nucleus and cytoplasm (14). Normal tongue epithelium exhibited weak p62 expression, which was predominantly detected in the stratum spinosum of stratified squamous epithelia. In dysplastic epithelium and SCC, increased cytoplasmic expression of p62 was observed. Furthermore, SCC exhibited increased cytoplasmic expression of p62 compared with dysplastic epithelium (Fig. 2A and B). Western blot analysis of tongue tissues from different groups also revealed that the levels of p62 increased during oral carcinogenesis (Fig. 2D).

Furthermore, the present study also evaluated expression of autophagy related 7 (Atg7), a key regulator of autophagosome maturation, in 4NQO‑induced mice tongue carcinogenesis by western blotting. Consistent with other autophagy markers, elevated Atg7 expression was also detected in dysplastic epithelium and SCC (Fig. 2D). Taken together, the autophagy level increased during the progressive stages of 4NQO‑induced tongue carcinogenesis.

Expression of autophagy markers in human oral cancer. The present study investigated the protein level of autophagy-associated genes in resection specimens from 5 normal mucosa, 5 squamous cell papilloma and 52 OSCC patients by a tissue microarray and immunohistochemical staining (Fig. 3A). Compared with negative expression of autophagy markers

Figure 1. Histopathology of tongue mucosa from the mice treated with 4-nitroquinoline-1-oxide or vehicle. Representative tissue sections of (A) hematoxylin and eosin and (B) immunohistochemical detection of proliferating cell nuclear antigen of normal mucosa (control group), mild-moderate dysplasia, severe dysplasia, and invasive SCC. Scale bars of the top and bottom panels in the images are 100 and 20 µm, respectively. Magnification, x100 and x400. SCC, squamous cell carcinoma.
Figure 2. Expression levels of LC3B, p62, Beclin 1 and Atg7 during 4NQO-induced oral carcinogenesis. (A) Representative immunohistochemical analysis of LC3B, p62 and Beclin 1 expression in sectioned tissue samples from the 4NQO-treated mice. All images were taken at x400 magnification with 20 µm scale bars. (B) Semiquantitative staining analysis showed that the expression of LC3B, p62 and Beclin 1 was gradually upregulated from normal mucosa, mild-moderate dysplasia, severe dysplasia, and SCC. (C) Immunofluorescent staining revealed LC3 puncta in tongue tissue of 4NQO-treated mice accumulated during oral carcinogenesis. (D) Representative western blot images displaying 30 µg of total protein extracted from the tongue tissue of each experimental group showed increased levels of LC3, p62, Beclin 1 and Atg7 in dysplasia and SCC groups, compared with normal mucosa group. Data are expressed as the mean ± standard deviation. *P<0.05. LC3B, dihydrosphingosine 1-phosphate phosphatase LCB3; p62, p62/SQSTM1; Atg7, autophagy related 7; SCC, squamous cell carcinoma; 4NQO, 4-nitroquinoline-1-oxide.
in normal mucosa specimens, OSCC specimens exhibited stronger immunohistochemical staining (Fig. 3B-D). Specifically, 86.3%, 70.6 and 78.4% OSCC specimens showed LC3B, p62 and Beclin 1 positive expression, respectively. Notably, squamous cell papilloma showed a higher positive rate of LC3B, p62 and Beclin 1 (20%, 40%, 20% respectively) compared with normal mucosa, however this was decreased compared with OSCC (data not shown). These results suggested that autophagy was associated with human OSCC progression.
Number of MDSCs and Tregs in peripheral blood and spleen tissue of 4NQO-treated mice at different stages of 4NQO-induced oral carcinogenesis. To examine the number of MDSCs and Tregs in 4NQO-treated mice, peripheral blood and spleen cells were screened by flow cytometry. A substantial accumulation of MDSCs and Tregs was observed in the peripheral blood and spleens during oral carcinogenesis. It was revealed that the number of MDSCs in the peripheral blood and spleens gradually increased in mild-moderate dysplasia, severe dysplasia, and SCC groups compared with control group (Fig. 4A and B). A similar change in the Tregs number was observed in the peripheral blood and spleens of the control, mild-moderate dysplasia, severe dysplasia, and SCC groups (Fig. 4C and D).

Relationship between autophagy-associated proteins and inflammation during oral carcinogenesis. The present study used Pearson correlation analysis to evaluate the correlation between autophagy marker expression and the number of MDSCs and Tregs during oral carcinogenesis (data not shown). The expression levels of LC3B and p62 were positively associated with the number of MDSCs in the peripheral blood and spleen tissues, whereas the expression of Beclin 1 was closely associated with the number of Tregs in the peripheral blood and spleen tissues. These results indicated that the expression of autophagy-associated proteins appeared to be associated with tumor inflammation during oral carcinogenesis.

Discussion

It has been recognized that autophagy is closely associated with carcinogenesis (15). Previous evidence has revealed that inflammation in the tumor microenvironment can induce autophagy, which produces recycled nutrients to 'feed' anabolic cancer cells and maintain tumor cell survival (10). In the present study, the results revealed that the expression levels of LC3B, p62 and Beclin 1 and the number of MDSCs and Tregs increased during oral carcinogenesis. A close association was observed between the overexpression of autophagy biomarkers and the accumulation of myeloid derived suppressor cells. In addition, autophagy activation was also observed in squamous cell papilloma and OSCC patient specimens. These data indicated that autophagy may be activated by tumor inflammation microenvironment during oral carcinogenesis. To the best
oral carcinogenesis (30). Although a previous study reported that normal tongue epithelia exhibited limited cytoplasmic when autophagy is suppressed (32). The results revealed TNM stage and unfavorable outcome (31).

is closely associated with lymph node metastasis, advanced from human normal oral mucosa, verrucous hyperplasia to which LC3B puncta formation exhibited an increasing trend specimens. These were consistent with a previous study in increased LC3B expression was also detected in human OSCC squamous cell carcinoma of 4NQO‑treated mice. Similarly, LC3B expression was increased in premalignant lesion and many cancers (27‑29). The present study demonstrated that elevated and positively associated with poor survival in Previous studies have reported that LC3 expression is and can be degraded by interaction with LC3 (32). However, gliomas (30,33‑35). p62 is considered an autophagy substrate has been found to be upregulated in several human cancers, normal oral mucosa, verrucous hyperplasia, and OSCC, p62 that no differences in p62 expression were observed among.

of the authors’ knowledge, this is the first study to evaluate the role of autophagy in 4NQO‑induced oral carcinogenesis, which was demonstrated to provide a favorable tumor microenvironment for oral carcinogenesis.

The 4NQO‑induced oral tumorigenesis mouse model has been proven to provide an ideal model for studying tumor pathobiology during sequential progression of human oral cancer (16). Numerous studies have established the 4NQO‑induced oral cancer mouse model by adding various does of 4NQO in drinking water, but the problems of time‑consuming and slow effect of this mice model remain unsolved (17‑19). In the present study, based on the histological analysis, continuous 4NQO exposure in the mouse model induced the multistage oral carcinogenesis lesions including different grades of dysplasia and squamous cell carcinoma, similar to human oral carcinogenesis. Consistent with previous studies, a long period (26 weeks) of 4NQO exposure increased the mice oral carcinogenesis rate compared with the short period (14 weeks) (18,20,21). However, 200 µg/ml 4NQO exposure does not further accelerate oral carcinogenesis, although a similar lethality rate remains (22,23). It therefore became evident that 100 µg/ml was an appropriate dose with which to induce mice oral cancer in this model and a higher dose cannot accelerate oral carcinogenesis. This is in line with most previous studies, in which the 4NQO dose used to treat mice is usually lower than 100 µg/ml (22,24,25). However, this result may be attributed to the small number of mice in the study, and further studies of larger sample size should be conducted in the future.

LC3 is considered a well‑established marker of autophagy activity for monitoring autophagosome formation (26). Previous studies have reported that LC3 expression is elevated and positively associated with poor survival in many cancers (27‑29). The present study demonstrated that LC3B expression was increased in premalignant lesion and squamous cell carcinoma of 4NQO‑treated mice. Similarly, increased LC3B expression was also detected in human OSCC specimens. These were consistent with a previous study in which LC3B puncta formation exhibited an increasing trend from human normal oral mucosa, verrucous hyperplasia to OSCC (30). Additionally, high LC3 expression status in OSCC is closely associated with lymph node metastasis, advanced TNM stage and unfavorable outcome (31).

p62, as a selective substrate, is routinely used as a biomarker to monitor autophagy flux and p62 accumulates when autophagy is suppressed (32). The results revealed that normal tongue epithelia exhibited limited cytoplasmic p62 expression, whereas both 4NQO‑induced squamous cell carcinoma and human OSCC exhibited increased expression, suggesting that p62 expression was upregulated during oral carcinogenesis (30). Although a previous study reported that no differences in p62 expression were observed among normal oral mucosa, verrucous hyperplasia, and OSCC, p62 has been found to be upregulated in several human cancers, including colorectal carcinoma, epithelial ovarian cancer and gliomas (30,33‑35). p62 is considered an autophagy substrate and can be degraded by interaction with LC3 (32). However, high levels of p62 protein have been revealed to induce onco
genetic transformation independent of its autophagy‑associated functions (36). Valencia et al (37) demonstrated that in the tumor microenvironment, p62 can promote the inflammatory response via NF‑κB activation and upregulation of c‑Myc genes by activating mechanistic target of rapamycin kinase complex 1 (37). Moreover, p62 also activates the nuclear factor, erythroid 2 like 2‑dependent anti‑oxidant response and thereby controls cell death and survival (38). Taken together, p62 may act as a pro‑oncogenic regulator in oral carcinogenesis but the underlying mechanism is not clear.

Beclin 1 has been recognized as a central regulator of autophagy. In the present study, Beclin 1 expression was revealed to be upregulated during 4NQO‑induced carcinogenesis. Similarly, Beclin 1 overexpression was also observed in papilloma and OSCC patient specimens. Consistent with the results of the present study, the accumulation of Beclin 1 has been found in breast, colon and ovarian cancer tissues (29,39,40). Notably, increase of Beclin 1 has been reported to be a marker of poor prognosis in colon cancer (40). However, Hu et al (41) reported that Beclin 1 expression is decreased in oral tongue squamous cell carcinoma tissues relative to the matched non‑cancerous tissues (41). Moreover, Beclin 1−/− mutant mice develop a large number of spontaneous tumors, including lymphomas, lung cancer and hepatocarcinoma (42,43). The reason for these controversial outcomes in a variety of cancers may be that the role of autophagy depends upon intrinsic properties of the tumor type and upon the specific tumor environment.

Accumulating evidence has indicated that inflammation contributes to tumor initiation and progression (44). MDSCs and Tregs, two populations of inflammatory cells, notably increase in tumor‑bearing mice and cancer patients and contribute to an immunosuppressive tumor microenvironment (17,23). The present study observed a progressive increase in the proportion of MDSCs and Tregs in the spleens and peripheral blood during oral carcinogenesis. These results are consistent with previous studies (17,23). Tregs are not considered a homogenous population. The multiple subpopulations of Tregs are distinguished by the expression of different cell surface markers, including FoxP3, CD4, CD25 and CD127 (45,46). The present study examined the level of CD4+ Foxp3+ Tregs, which has been widely reported in previous studies (47‑52). The multiple subpopulations of Tregs may play different roles in oral carcinogenesis and the authors hope to examine CD4+ CD25+ CD127 low FoxP3+ Tregs in future studies.

Autophagy has been demonstrated to be activated by inflammation in the tumor microenvironment (10). In the present study, it was demonstrated that the expression of LC3B and p62 was positively associated with MDSCs number, and that the expression of Beclin 1 was closely associated with an increase in Treg number. This indicated that there was a correlation between autophagy and inflammation in oral carcinogenesis. However, the study cannot address how inflammation and autophagy act synergistically in carcinogenesis. One hypothesis is that autophagy can be activated in the inflammation microenvironment via activation of NLR Family Pyrin Domain Containing 3 and inflammasomes or oxidative stress‑induced NF‑κB activation (10). However, given that activated autophagy can suppress excessive inflammation by regulating the secretion of cytokines and chemokines and activate antitumor immunity by presentation of tumor antigens, further study to address this issue is necessary (9).
In conclusion, the study demonstrated that the expression of LC3B, p62 and Beclin 1 was upregulated in 4NQO tongue carcinogenesis, accompanied with MDSCs and Tregs accumulation. The close correlation between autophagy markers and inflammatory cell number suggested that autophagy may be activated by tumor inflammation which provides a favorable tumor microenvironment for cancer development. However, temporal and adjustable autophagy inhibition studies in animal models are required in order to further confirm the roles of autophagy in oral carcinogenesis and its mechanisms in the tumor inflammation environment.

Acknowledgements

Not applicable.

Funding

The present study was supported by National Project on Key Research Project of China (grant no. 2016YFC0902700), National Natural Science Foundation of China grants (grant nos. 81672672, 81572650, 81772891, 81502357 and 81621062), Natural Science Foundation of Zhejiang Province (grant no. Q142114001), Zhoushan Science and Technology Bureau Project (grant no. 2014C3106) and by State Key Laboratory of Oral Diseases Special Funded Projects (2018).

Availability of data and materials

The majority of data generated or analyzed during this study are included in this published article. The data of expression of autophagy markers in human oral cancer and Pearson correlation analysis were not shown.

Authors' contributions

JSW conducted the study, carried out most of experiments and drafted the manuscript. XHL and MZ conceived the study and participated in its design. JSW, LL, SRS and XP assisted in development of methodology and acquisition of data. SSW and JBW performed the analysis or interpretation of data and drafted the manuscript. YJT and YLT participated in scoring of immunohistochemistry, performed data interpretation and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving mice were approved by the Subcommitee on Research and Animal Care (SRAC) of Sichuan University. The written informed consents were obtained from participants through their signatures. The use of human tissue samples and clinical data was approved by the Institutional Ethics Committee of the West China Medical Center, Sichuan University, China (WCHSIRB-D-2012-097).

Patient consent for publication

Informed consent for the publication was obtained from the participants.

Competing interests

The authors declare that they have no competing interests.

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