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Oral Cavity Carcinogenesis Modeled in Carcinogen-Treated Mice

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Abstract: In the present study, oral squamous cell carcinoma (OSCC) was induced in a mouse model with 20 μg/ml 4-Nitroquinoline-1-oxide (4NQO) solution in drinking water. 120 six-week-old male Balb/C mice were randomly divided into an experimental group (n=110) and a control group (n=10). They were sacrificed after 16 to 48 weeks of exposure to allow for histopathological and immunohistochemical examinations. Gross changes could be observed, including white changes, leukoplakia, erythroplakia, ulceration and papillary tumor appearance on the mucosa of the tongue dorsum of the experimental group mice during the carcinogenesis period. At the same time, no visible and histopathological changes in tongue epithelium were observed in the control group. Survivin expression was positive in dysplasia and OSCC groups but not in normal mucosa, and correlated positively with PCNA expression. Also, survivin protein staining showed statistical significance in the dysplasia group (p<0.05) but not in the OSCC group (p<0.05). Furthermore, PCNA Labelling Index (PI) in survivin positive specimens were found significantly higher than it in survivin negative specimens (p<0.01). These results showed that survivin might be closely related to cell proliferation, differentiation and carcinogenesis. It also showed that survivin might promote unrestricted multiplication and dedifferentiation of cells, making the tumor taking a malignant behavior through promoting cell mitosis, cell apoptosis, and enhancing cell proliferative activity. Therefore, the detection of expression of survivin and PCNA is helpful for early diagnosis of OSCC.

Keywords: Carcinogenesis, Oral cancer, Squamous cell carcinoma, Mouse

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common neoplasms in the world1. The 5-year survival rate of patients diagnosed with OSCC is still less than 30%, even though strong efforts have been devoted to treat it2. Considering that carcinogenesis is a multiple process, which required the accumulation of genetic alterations, it could be desirable to establish an ideal animal model for examining the precise pathobiological mechanisms involved in oral tumorigenesis will be a good way to identify reliable biomarkers for early diagnosis and prevention of OSCC.

Previously, several animal models for OSCC development were established, including hamster, rat, and mouse models3-11. In the past, the most commonly used model was the carcinogen 7,12-dimethylbenzanthracene (DMBA) induced hamster cheek pouch tumor model. However, DMBA or its solvent vehicle is a significant local irritant that causes inflammatory response, necrosis, and sloughing. It is difficult to study early squamous lesions3. Tumors caused by DMBA in hamster cheek pouch exhibit many differences in the histological features of human OSCC4.

4-Nitroquinoline-1-oxide (4NQO), a synthetic water soluble carcinogen, had been confirmed can induce OSCCs in rats5 and mice6,7. Generally, there were two different methods of 4NQO treatment. One was to paint 4NQO on tongues8,9, the other one was to dissolve it in the drinking water for rats10,11. And the results showed 4NQO was a better carcinogen for the production of oral carcinogenesis compared to DMBA in rat model12. It was widely used as a carcinogen in murine models, to study various stages of oral carcinogenesis13,14. Additionally, it produces similar histological results as well as molecular changes as seen in human oral carcinogenesis15.

However, the 4NQO tongue painting method was not convenient and the incidence of tumors was relatively low16. Recently, a genetic mouse model of human oral-esophageal cancer was generated by over-expressing cyclin D1 in p53-null mice17. This model may not completely reflect the multiple genetic changes that occurs during the process of the oral cavity carcinogenesis in humans17,18. Tang et al. induced a mouse model of OSCC with
different dose of 4NQO solution\(^9\). However, it lacked the process of the preneoplastic lesions transforming to the neoplastic lesions in the carcinogenesis which is the key point time to prevent OSCC.

The aim of the present study was to investigate the propercencentration and timepoint to induce the preneoplastic and the neoplastic lesions in mouse model with 4NQO carcinogen in drinking water. Moreover, the expression of survivin and proliferating cell nuclear antigen (PCNA) was assessed by immunohistochemistry in this model. To the best of our knowledge, it was the first study which focuses on the concomitant expression of survivin and PCNA in experimental oral carcinogenesis.

**Materials and Methods**

**Ethic Statement**
Ethical approval was approved by the ethical commission of the School of Stomatologist, Dalian Medical University and mice used in this study were cared for in accordance with institutional guidelines for animal care.

**Animals and experimental design**
120 six-week-old male Balb/C mice, weighing approximately 250 g, (Shanghai Experimental Animals Institution Shangai, China) were randomly divided into experimental group (110 mice) and control group (10 mice). Mice were allowed to access the drinking water at anytime during the treatment and were fed with a regular diet (Shandong animal food Co. Jinan, China) under room temperature. The experimental group was divided into 2 subgroups. Samples in subgroup A were treated with 20 \(\mu\)g/ml 4NQO (Sigma Aldrich, St. Louis, USA) solution in the drinking water and sacrificed at 16, 20, 24 and 28 weeks for histopathological and immunohistochemical examinations respectively. Samples in subgroup B were treated with 20 \(\mu\)g/ml 4NQO solution in the drinking water for 16, 20, 24 and 28 weeks then observed until 48 weeks, then they were also sacrificed for further examinations. Samples in the control group were treated with water only. Body weight was determined once a week for all samples. At the end of the experiment, gross lesions were identified and photographed. Specimens were taken from palate, tongue, stomach, and liver. These were fixed in freshly prepared 4% paraformaldehyde overnight at 4 °C, then embedded in paraffin, and sectioned into 4\(\mu\)m sections.

**Histopathological examination**
The histological determination of lesions was done by a pathologist without knowledge of the duration of treatment. The lesions observed were classified in six types: epithelial hyperplasia, mild epithelial dysplasia, moderate epithelial dysplasia, severe epithelial dysplasia, carcinoma in situ and invasive epithelial carcinoma according to the report of Kramer et al\(^9\).

**Immunohistochemistry**

The expression of survivin and PCNA was investigated by immunohistochemistry. Serial longitudinal tongue sections of 4 \(\mu\)m were deparaffinized in xylene and rehydrated in graded ethanol then pretreated in a microwave with citric acid buffer (pH=6) at a temperature of 95 °C for 15 minutes for antigen retrieval. They were pre-incubated with 3% hydrogen peroxide then in Phosphate Buffered Saline (PBS) for 10 minutes for inactivation of endogenous peroxidase. The specimens were then incubated with both the anti-survivin polyclonal antibody (1:100) and anti-PCNA monoclonal antibody (1:100) (Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China). Incubation was carried out overnight at 4 °C. This was followed by three washes in PBS for 10 minutes at room temperature. The sections were then incubated with biotin-conjugated secondary antibody anti-rabbit IgG in PBS for 30 minutes. After washes with PBS 3 times, the immunoreactivity was visualized by development for 2 minutes with 0.1% 3,3′-diaminobenzidine and 0.02% hydrogen peroxide (DAB substrate kit, Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China). The bound complexes were visualized by the application of a 0.05% solution of 3,3′-diaminobenzidine solution, and counterstained with Harris hematoxylin. For control studies of the antibodies, the serial sections were treated with the rabbit IgG (Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing) at a concentration of 1:200 instead of the primary antibody.

**Quantification of immunohistochemistry**

Tongue sections stained by immunohistochemistry were analyzed for immunopositive cells under optical microscope at \(\times 400\) magnification per sample. Survivin-positive cells were identified as yellow or brown stained in cytoplasm. A total of 1000 PCNA-positive cells were evaluated as established by Matsumoto et al.\(^{21}\). These values were expressed in percentage (%) as PCNA Labelling Index (PI).

**Statistical analysis**
Statistical analysis for immunohistochemistry data were assessed by \(\chi^2\) test using SPSS software pack (version 18.0 SPSS Inc. Chicago, Illinois, USA). The relationships between survivin and PCNA expression during the development of OSCC were evaluated with Spearman’s correlation coefficient. A \(p\) value<0.05 was considered statistically significant.

**Results**
The mice showed no ill effects from the carcinogen applications, the body masses of both control and experimental mice remained similar. The 4NQO treated mice tended to be lighter than the controls at the end of experiment. In total of 110 mice given 4NQO solution, 9 mice died during the administration period. Autopsies were not performed because of advanced autolysis.
Figure 1 shows the gross changes of the mouse tongue during carcinoma-esis. (A) There was no change in normal control; (B) a focal loss of the lingual papillae and a micronodular surface texture formation (mild epithelial dysplasia); (C) thickening of the mouse tongue epithelium with a whitened appearance (moderate epithelial dysplasia); (D) leukoplakia (severe epithelial dysplasia); (E) red areas in association with the white patches (carcinoma in situ); (F) red areas in association with the white patches (carcinoma in situ); (G) papillary appearance (carcinoma in situ); (H) papillary appearance (early invasive carcinoma); (I) ulcer with a raised margin or a granular appearance (early invasive carcinoma).

Figure 2 illustrates the multi-step process of mouse tongue carcinogenesis. (A) No histopathological change (control); (B) mild epithelial dysplasia; (C) moderate epithelial dysplasia; (D) severe epithelial dysplasia; (E), (F), (G) carcinoma in situ of mouse tongue epithelium; (H), (I) early invasive carcinoma of the mouse tongue of well differentiated type; (J) carcinoma cell nest (Hematoxylin and Eosin stain).

Gross and histopathological evaluation following 4NQO treatment

No gross and histopathological changes in tongue epithelium were observed in the control group (Fig. 1A and Fig. 2A).

To the gross lesions on the dorsum of the tongue, the alteration in the lingual epithelium was a focal loss of the lingual papillae and a micronodular surface texture at the end of 14 weeks (Fig. 1B). Microscopically, there was mild epithelial dysplasia with hyperkeratosis, acanthosis, and basal cell hyperplasia (Fig. 2B). This was followed by a whitened appearance of all the dorsum of the tongue at 18-20 weeks (Fig. 1C). Moderate epithelial dysplasia was also found in 18 weeks (Fig. 2C). With 20-24 weeks of carcinogen treatment, thickening of the mouse tongue epithelium with a whitened appearance (Fig. 1D), severe epithelial dysplasia were found with grossly disturbed stratification, loss of polarity of cells, nuclear and cellular pleomorphism, individual cell keratinization, hyperchromatism, and the appearance of many mitotic figures, some of which were morphologically bizarre (Fig. 2D). Lesions with such changes involving the entire thickness of epithelium and the basal membrane remaining intact were considered as carcinoma in situ (Fig. 2E,F). Carcinoma in situ was grossly observed as red areas developed in association with
the white patches (Fig. 1E) and small erosions (Fig. 1F), when the mice received 4NQO solution for 28 weeks. Some carcinoma in situ was papillary in nature (Fig. 1G and Fig. 2G). Two growth patterns of invasive carcinoma were observed at 28-48 weeks. Exophytic type lesions were broadbased and occasionally pedunculated, with a cauliflower or coarse to pebbly surface (Fig. 1H). Endophytic type lesions showed superficially ulcerated surface with a raised margin or a granular appearance (Fig. 1I). Invasive lesions were identified by the invasion of neoplastic epithelial cells into the subepithelial tissues, forming small nests with typical keratin pearl formation (Fig. 2H,I). Nuclear pleomorphism was marked. Mitoses were frequent (Fig. 2J).

The severity of the lesions corresponded with the duration of the treatment and the length of the observation. Metastasis were not detected. The histopathological findings were summarized in Table 1. The epithelial dysplasia or carcinoma was not found in the mucosa of the palate, the cheek, or the floor of the mouth. Neither were molecular markers examined in these regions. No gross and microscopic lesions were observed in esophageal, forestomachs, lungs, liver and colons.

Immunohistochemistry

Survivin expression was detected predominantly in the cytoplasm of lingual epithelium cell. Expression of survivin was absent in control group and mild epithelial dysplasia (Fig. 3A). Overall, the rate of survivin expression was considered negative in these tissues. On the other hand, survivin positive cells were found in the superficial layers of epithelium after 12 weeks of carcinogen treatment, although no histopathological changes were induced during this period (Fig. 3B). In the following sequence of 4NQO treatment, mouse tongues started to show early proliferative changes of epithelium characterized by moderate and severe dysplasia. These preneoplastic lesions contained survivin positive cells (Fig. 3C). The rate of survivin positive expression was 53.9% in carcinomas in situ, and 62.5% in invasive carcinomas. Invasive carcinomas displayed survivin positive cells adjacent to keratin pears (Fig. 2D). Data analysis showed statistically significant (p<0.05) findings in all type of lesions compared to the controls (Fig. 3A). The survivin expression results are summarized in Table 2.

Speaking of the PCNA immunomarker, PCNA-positive nuclei were found in both control and experimental mouse lingual mucosa. Positive nuclei were confined to the cells of the basal cell layer arranging in line and absent in other layers in the control group. The quantity of positive nuclei was small in the control group (Fig. 4A). In epithelium dysplasia group, PI increased with the severity of dysplasia increasing and the positive nuclei were found in the stratum spinosum layer (Fig. 4B). As to carcinoma in situ, positive nuclei were mainly distributed in the active proliferating cells adjaenting to the cancer nests, while PCNA expression of the cells in the central of the cancer nests was negative (Fig. 4C). In contrast, with respect to early invasive carcinomas, PI was increased and PCNA-positive nuclei were detected in the majority of tumor cells. PCNA-positive nuclei were distributed in cancer nests and cancer cells cords while single cancer cells appeared in the normal tissue irregularly (Fig. 4D). Data analysis revealed that PI reached statistically significance for all comparisons, except for the normal oral mucosa and mild...
dysplasia cases (p<0.01). Findings are summarized in Table 3.

The relationship between expression survivin and PCNA is shown in Table 4. All cases were divided into two groups, namely a survivin positive and a survivin negative group. The results showed that PI of the 34 survivin positive cases was significantly higher than that of 75 survivin negative cases (t=53.29, p<0.01).

Discussion

Carcinogenesis is a multi-step process, which is the result of multiple molecular changes22). These changes include involve genetic damage, mutation in critical genes related to the control of cell division, cell death, metastatic potential, and activation of signaling or metabolic pathways which give cells favorable growth and survival characteristics23). In human carcinogenesis, the molecular analysis of these multiple steps was not available, because of the unavailability of biopsies at all the stages of carcinogenesis. Therefore, animal models of carcinogenesis might add evidence by showing all stages under controlled conditions, including normal tissues. These findings were then amenable to pathological, genetic, and biochemical investigations24). Moreover, an ideal chemical carcinogenesis model helped to examine the hazard risk caused by environmental agents as well as to determine the putative precancerous lesions which will progress.

In a previous study that the mice treated with 20, 50, and 100 µg/ml 4NQO in drinking water for 8 weeks and observed for another 16 weeks. 50 and 100 µg/ml 4NQO treatments caused carcinomas in the mouse tongue, 20 µg/ml treatments did not19). Yet, in the present study, OSCC could be induced in mouse tongue with 20 µg/ml 4NQO solution. The different outcome between these studies may be explained by timepoint. The lesions of mouse tongue were mainly epithelium dysplasia after 4NQO treatment for 16-24 weeks. The histological results showed moderate and severe dysplasia after 4NQO treatment for 28 weeks. The carcinoma inducing rates of the mice receiving 4NQO solution for 16, 20, 24, 28 weeks were 0 %, 5 %, 12.5 %, 16 % respectively. In contrast, the carcinoma inducing rates of mice receiving 4NQO solution for 16, 20, 24, 28 weeks, and then the regular water to 48 weeks, were 10 %, 25 %, 37.5 %, 45.4% respectively. Mouse tongues did not exhibit any inflammation and necrosis during the experimental time. The incidence of neoplastic lesions increased over time after 4NQO treatment was completed. These results indicated that in this model, the lesions continue to grow even after removing the carcinogen.

Tobacco is one of the main etiological factors causing human OSCC, because it contains many carcinogens that might cause carcinogenesis through the formation of DNA adducts in many human tissues25,26). 4NQO is a water-soluble quinoline derivative that can cause DNA adduct formation, resulting in adenosine substitution for guanosine26-28). 4NQO may also undergo redox cycling to produce reactive oxygen species that result in mutations and DNA strand breaks29-31). In the present study, the lesions continuously progressed after 4NQO treatment, from hyperplasia, mild and moderate and then severe dysplasia before OSCC, then papilloma, carcinoma in situ and invasive carcinoma. The mouse gross and histological feature showed similarity features with that of humans32). Moreover, the process of carcinogenesis was relative slow with all stages of preneoplastic lesions transforming to neoplastic lesions. The present data demonstrated that the mouse model was useful for the analysis of oral cavity carcinogenesis.

Apoptosis is a tightly regulated process of genetically programmed cell death by which senescent, damaged, and superfluous cells are eliminated from the body33). Apoptosis is involved in the etiology of many chronic and degenerative diseases, including cancer34-36). In mammals, apoptosis is mainly modulated by two protein families, the bcl-2 and inhibitor of apoptosis proteins (IAP)37). Survivin is a recently characterized IAP protein which has been found to be abundantly expressed in solid and hematologic malignancies, but undetectable in most normal adult tissues38-40). In OSCC, increased survivin expression has been reported in few studies in humans and Syrian golden hamsters38-41). In the present study, the expressivity of survivin following 4NQO administration was evaluated in order to determine its role during oral tumorigenesis, phase by phase. In normal control epithelium, represented by the control group, survivin protein was absent in all specimens. This was consistent with published papers showing the lack of survivin immunoreactivity in normal adult tissues38). The present study found the survivin expression rate was 30 % (24/80) in the oral mucosal dysplasia, and it was 57.1 % (12 / 21) in oral squamous cell carcinoma. Survivin expression could be seen in normal oral mucosa 12 weeks after the start of 4NQO administration. This result was consistent with the study of Ribeiro et al.42) Survivin positive substances were mainly detected in the cytoplasm of cancer cells and the distribution of staining cells showed obvious heterogeneity. These results suggested that the expression of survivin might be an important event in the initiation of oral carcinogenesis.

In humans, only a small portion of oral premalignant lesions develops oral carcinomas43). Therefore, the challenge is to identify which lesions have the malignant potential. Although it was postulated that oral epithelial dysplasia is a pre-neoplastic lesion that occasionally develops into squamous cell carcinomas, the understanding of the biologic basis of oral squamous cell carcinoma development and progression was far from satisfactory. In our study, positive expression of survivin was found in oral premalignant lesions and oral squamous cell carcinomas respectively. The same result was reported by Lo Muzio et al.39,40). An increasing trend in the level of survivin expression between pre-malignant lesions and squamous cell carcinomas 4NQO-induced was also found. The statistical analysis showed that
survivin expression in pre-malignant lesions and squamous cell carcinomas tissues had a significant difference (p<0.05), assuming that survivin protein accumulation was an important event during oral carcinogenesis and progression.

Tumor cell proliferation is an important mechanisms of tumor occurrence and development. Among proliferating cellular markers, PCNA is a DNA polymerase delta auxiliary protein of 36 kDa, which is closely related to the replication of DNA and is indispensable to cell proliferation. PI was negatively correlated to the tumor conversion, stage, transfer and prognosis44,45. In the current study, PI gradually increased from normal mucosa, oral mucosa dysplasia to oral mucosa carcinoma. The statistical analysis showed that there was a significant difference (p<0.01) in PI among these tissues. In general, the proliferating cells located in basal layer in normal oral mucosa. With the degree of differentiation and maturation increasing, cells close to the surface layer were well differentiated but the cell proliferation activity weakened. Because of the disorder of cell differentiation and maturation in oral mucosal dysplasia, cells in the stratum spinosum layer retain higher proliferating activity, and the area of cell proliferation was enlarged in oral mucosa dysplasia46. The present result was consistent with these findings, the tissue of high PI was at high risk of potential malignant transformation, although it showed no morphological changes. It was helpful to apply early diagnosis and therapy to prevent premalignant lesions transforming to malignant lesions. On the basis of the present data, it was assumed that proliferation activity played an important role in precancerous lesions progressing to cancerous lesions.

To our knowledge, the relationship between survivin, PCNA and apoptosis in OSCC had been rarely reported. In the present study, it could be demonstrated that survivin was related to the promotion cell proliferative activity. The mechanism of survivin promoting cell proliferation remains unclear. The further study is needed. It is assumed that, as a powerful inhibitor of apoptosis, survivin may be directly involved in the formation and development of OSCC.

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