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# Antioxidant enzymes in oral verrucous carcinoma

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**BACKGROUND: Verrucous carcinoma is a non-metastasizing variant of welldifferentiated squamous cell carcinoma, which has been associated with reactive oxygen species generated by betel quid chewing. Salivary antioxidant systems have been suggested to play a protective role in reducing the oxidative damage. Herein, we investigated the difference of the enzymatic antioxidant system expressions in oral verrucous carcinoma and oral squamous cell carcinoma.**

**METHODS: The enzymatic antioxidant system expressions, including manganese superoxide dismutase, glutathione peroxidase, and catalase were evaluated by immunohistochemistry in a series of 202 surgically resected oral squamous cell carcinoma and 20 oral verrucous carcinoma specimens, using tissue microarray slides.**

**RESULTS: The immuno-staining intensities of superoxide dismutase and glutathione peroxidase were strongest in the oral squamous cell carcinoma group than in verrucous carcinoma. The catalase expression showed no difference between different pathological groups.**

**CONCLUSIONS: The different degrees of superoxide dismutase and glutathione expressions in verrucous carcinoma and squamous cell carcinoma may be helpful for pathologists to differentiate these two entities, especially between oral verrucous carcinoma and well differentiated oral squamous cell carcinoma.**

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**Keywords:** antioxidant enzyme; glutathione peroxidase; manganese superoxide dismutase; oral cancer; squamous cell carcinoma; verrucous carcinoma

## Introduction

Oral verrucous carcinoma (VC) is a rare variant of oral squamous cell carcinoma (SCC) with unique histopathological features (1, 2). In 1948, Ackermann first described this oral mucous membrane neoplasm, which is also known as 'Verrucous carcinoma of Ackermann' or 'Ackermann's tumor' (3). Oral VC is predominantly seen in males aged over sixth decade and occurred most commonly in oral cavity (4). In terms of tumor biology, oral VC is distinct in its slow growth and ability to become locally aggressive if not treated appropriately. However, even with local tumor progression, it is intriguing that regional or distant metastasis is still rare.

Tobacco and alcohol use are considered the major risk factors for buccal carcinoma in the United States, while betel quid (BQ) chewing is the main risk factor in Taiwan (5–8). BQ chewing produces reactive oxygen species (ROS), resulting in carcinogenesis of the oral mucosa. The increase in ROS may have been the event that led to the consumption and reduction of salivary antioxidant systems, thus explaining the oxidative damage to the DNA and proteins, and possibly the promotion of oral cancer (9). The pathogenesis of oral VC is unclear; however, studies have shown strong association with tobacco use, including both inhaled and smokeless tobacco, alcohol, and human papillomavirus (10–14).

The enzymatic antioxidant system includes manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPx), and catalase. The function of SOD is to catalyze the dismutation of two molecules of superoxide anion into molecular oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$) (15). Yokoe et al. reported that MnSOD was highly expressed at the transcriptional level in oral SCC tissue compared with normal tissue counterparts, whereas no significant changes in Cu/Zn-SOD were detected. Therefore, MnSOD may be a more important physiologic factor in oral tumors (16). A few studies exist in oral SCC (17–23), but the expressions of antioxidant enzymes in oral VC have not previously been studied.

In this study, we have investigated the expression status of MnSOD, GPx, and catalase in normal oral epithelium, VC, and SCC tissues, to compare in both of these carcinomas, and to investigate the possible correlation of antioxidant enzyme expression in different histological grades of oral SCC.

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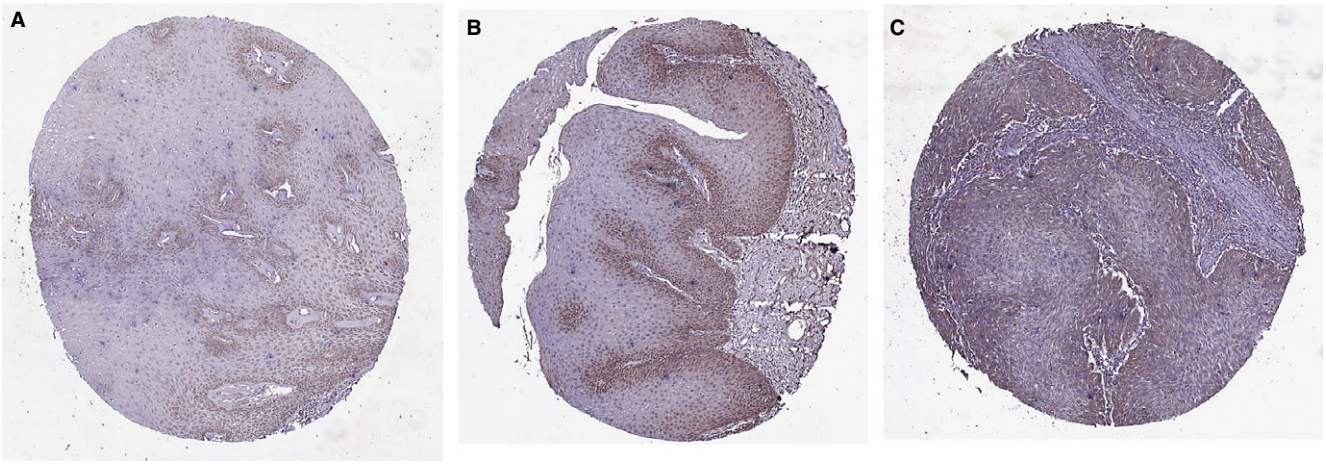


**Figure 1** (A) Immunoreactivity of MnSOD in verrucous with mild intensity; (B) Immunoreactivity of MnSOD in verrucous with moderate intensity; (C) Immunoreactivity of MnSOD in well-differentiated SCC with strong intensity 428 × 150 mm (120 × 120 DPI).

## Materials and methods

*Tissue specimens*

Two hundred and two specimens of primary oral SCC and 20 specimens of oral VC were collected at Kaohsiung Veterans General Hospital over 13-year period (between 1993 and 2006). Moreover, 27 specimens of normal squamous epithelium from patients who received uvulopalatopharyngoplasty for sleep apnea during the same period were used as controls.

The pathological staging was classified at the time of their initial resection of tumor according to the guidelines of 2002 American Joint Committee on Cancer system. The study was approved by the Institutional Review Board of Kaohsiung Veterans General Hospital (IRB number: VGHKS97-CT2-08).

*Tissue microarray (TMA) construction*

Tissue microarray was performed as described in our previous study (23, 24). Pathologist selected two tumor tissues and its adjacent tissues on the paraffin blocks. Another five cores of normal uvula epithelium were included. Totally, six TMA blocks were constructed and sliced in 4-μm paraffin sections using standard techniques.

*Immunohistochemistry*

We used polyclonal anti-MnSOD antibodies (Stressgen Biotechnologies, Victoria, BC, Canada), monoclonal anti-GPx antibodies (Stressgen Biotechnologies, Victoria, BC, Canada), and monoclonal anti-catalase antibodies (clone CAT-505; Sigma, Saint Louis, Missouri, USA) for immunohistochemistry study. TMA blocks were sliced into 4-μm serial sections, deparaffinized in xylene, rehydrated in graded ethanol, and washed for 5 min with phosphate-buffered saline. The sections were boiled and immersed in a high pH solution (DAKO, S3308) for antigen retrieval. Endogenous peroxidase was blocked by incubation of the slides for 10 min with 3% hydrogen peroxide in methanol. After blocking, primary antibodies were immediately applied and incubated for 1 h at room temperature (MnSOD 1:1000, GPx 1:1000, catalase 1:1000) and were detected

**Table 1** Demographic clinicopathological data of oral squamous cell carcinoma and oral verrucous carcinoma patients

| *Variables* | *Verrucous carcinoma Number (%)* | *Squamous cell carcinoma Number (%)* |
|---|---|---|
| Age (years) | | |
| ≤40 | 3 (15.0) | 31 (15.3) |
| >40 | 17 (85.0) | 171 (84.7) |
| Cell Differentiation | | |
| Well (G1) | 20 (100.0) | 60 (29.7) |
| Moderate (G2) | 0 (0.0) | 130 (64.4) |
| Poor (G3) | 0 (0.0) | 12 (5.9) |
| Pathological Stage | | |
| I | 7 (35.0) | 71 (35.1) |
| II | 9 (45.0) | 48 (23.8) |
| III | 2 (10.0) | 25 (12.4) |
| IV | 2 (10.0) | 58 (28.7) |
| T classification | | |
| $T_1$ | 7 (35.0) | 76 (37.6) |
| $T_2$ | 9 (45.0) | 71 (35.1) |
| $T_3$ | 2 (10.0) | 14 (6.9) |
| $T_4$ | 2 (10.0) | 41 (20.3) |
| N classification | | |
| $N_0$ | 20 (100.0) | 152 (75.2) |
| $N_1$ | 0 (0.0) | 27 (13.4) |
| $N_2$ | 0 (0.0) | 23 (11.4) |
| Postoperative radiotherapy | | |
| No | 20 (100.0) | 144 (71.3) |
| Yes | 0 (0.0) | 58 (28.7) |
| Preoperative chemotherapy | | |
| No | 20 (100.0) | 202 (100.0) |
| Yes | 0 (0.0) | 0 (0.0) |
| Postoperative chemotherapy | | |
| No | 20 (100.0) | 196 (97.0) |
| Yes | 0 (0.0) | 6 (3.0) |
| Margin free | | |
| No | 1 (5.0) | 21 (10.4) |
| Yes | 19 (95.0) | 181 (89.6) |
| Betel nut chewing | | |
| No | 5 (25.0) | 37 (18.3) |
| Yes | 15 (75.0) | 163 (81.7) |
| Multiple tumors[a] | | |
| No | 10 (50.0) | 161 (79.7) |
| Yes | 10 (50.0) | 41 (20.3) |

[a]Multiple tumors means second primary tumor, including synchronous with either the index tumor or the metachronous tumor occurring after an interval of longer than 6 months (27).

using secondary antibodies (rabbit anti-mouse antibody; VECTASTAIN® ABC kits, Vector Laboratories, Burlingame, CA, USA). The color development used 3, 3-diaminobenzidine tetrahydrochloride as chromogen (DAB substrate kit, VECTASTAIN® ABC kits, Vector Laboratories) after reaction for 2.5 min. Inflammatory cells adjacent to the tumor tissue were used as intrinsic positive controls.

*Immunohistochemical analysis*

Two pathologists reviewed the slides independently, blinded to the information of clinical outcomes. For discrepancies, two pathologists reviewed the slides together to reach consensus. We graded the immunoreactivity by a semi-quantitative approach. Cytoplasmic staining was calculated based on staining intensity (0, no signal; 1, mild; 2, moderate; and 3, strong; Fig. 1) and percentage of cells staining at each intensity level (0–100%). The intensity score and percentage of the positive cells were then multiplied to produce the final scores as used in previous studies (16, 23).

*Statistical analysis*

One-way analysis of variance (ANOVA) and Kruskal–Wallis test were used to compare antioxidant enzyme expressions in the oral VC, oral SCC, and normal oral mucosa. All statistics were performed using IBM SPSS software (version 20; IBM Corp., Armonk, NY, USA). A value of $P < 0.05$ (two-sided) was considered significant.

## Results

*Clinical and pathological data*

The clinicopathological characteristics of the patients are summarized in Table 1. All 202 oral SCC cases were male with a mean age at 51.9 years (age range, 24–82 years), while the mean age of 20 male oral VC was 52.3 years (age range, 37–70 years). Seven (35%) patients with oral VC were classified as stage I, nine (45%) stage II, two (10%) stage III, and two (10%) stage IV. None of the patients with oral VC received postoperative radiotherapy nor adjuvant chemotherapy.

*The relationship of antioxidant enzymes with normal epithelium, oral VC, and oral SCC*

The immunostaining intensity of MnSOD was strongest in the oral SCC group than in the VC group (Table 2). The normal mucosal tissue stained only mildly for MnSOD. The staining of GPx was weaker than that of MnSOD, but both two stains had the similar results. The immunostaining intensity of GPx was strongest in the oral SCC group than in the VC group. The normal mucosal tissue stained only mildly for GPx. In contrast, the immunoreactivities for VC and SCC showed no difference for catalase.

*Association of antioxidant enzymes and the different groups of oral SCC*

We further studied the expression of antioxidant enzymes in different groups of oral SCC (Table 3). Poorly differentiated tumors stained more strongly than well-differentiated tumors, but it did not reach statistical significance, while the poorly differentiated tumors stained mildly for catalase. GPx staining was similar in all tumor histopathological groups.

## Discussion

In the present study, we found that MnSOD, GPx, and catalase were commonly expressed in oral SCC and oral VC specimens. The expression levels of MnSOD and GPx in oral VC were significantly higher than in normal oral epithelium but lower than in oral SCC. To the best of our knowledge, this is the first observation of the expressions of antioxidant enzymes in oral VC.

The etiology of oral VC is not completely established, but it was suggested that risk factors such as tobacco use, alcohol, and opportunist viral activity associated with human papillomavirus (HPV) may play important roles in its pathogenesis (12–14). In this study, the positive history of betel nut use was present in 75% of the patients with oral VC. It suggests that betel nut use contributes to the pathogenesis of oral VC in Taiwan, similar to oral SCC.

Betel quid chewing produces ROS. Low levels of ROS are required in various homeostatic mechanisms and are effectively counterbalanced by an array of antioxidant enzymes.

**Table 2** The relationship between expressions of antioxidant enzymes with normal oral epithelium, oral verrucous carcinoma, and oral squamous cell carcinoma

| | *Normal epithelium* | *Verrucous carcinoma* | *Squamous cell carcinoma* | P-*value* |
|---|---|---|---|---|
| number | 27 | 20 | 202 | |
| MnSOD (mean±SD) | 38.61 ± 57.02 | 72.72 ± 36.16 | 104.46 ± 51.62 | <0.001 |
| GPx (mean±SD) | 2.22 ± 5.77 | 5.47 ± 11.71 | 16.48 ± 27.13 | <0.001 |
| Catalase (mean±SD) | 8.33 ± 9.53 | 21.91 ± 31.01 | 20.20 ± 26.63 | 0.055 |

MnSOD, manganese superoxide dismutase; GPx, glutathione peroxidase; SD, standard deviation.

**Table 3** Quantitative antioxidant enzyme immunoreactivities in the different groups of oral squamous cell carcinoma

| *Differentiation of squamous cell carcinoma* | *Well differentiated* | *Moderately differentiated* | *Poorly differentiated* | P-*value* |
|---|---|---|---|---|
| Number | 60 | 130 | 12 | |
| MnSOD (mean±SD) | 93.67 ± 44.99 | 108.03 ± 54.34 | 119.79 ± 46.61 | 0.155 |
| GPx (mean±SD) | 16.22 ± 27.74 | 16.64 ± 26.49 | 16.04 ± 32.97 | 0.509 |
| Catalase (mean±SD) | 18.19 ± 22.17 | 22.12 ± 29.27 | 9.48 ± 8.76 | 0.419 |

MnSOD, manganese superoxide dismutase; GPx, glutathione peroxidase; SD, standard deviation.

When there is an excess of ROS or a state of depleted antioxidant enzymes (known as oxidative stress), lipid peroxidation is initiated and thus can cause DNA damage (25, 26). The elevated expressions of antioxidant enzymes in tumor tissues in our cases probably reflect the adaptive response to the ROS burden in oral mucosa, which is supported by the observation that normal oral epithelium had the lowest antioxidant enzyme activities. Similar results have also been observed in oral SCC. Yokoe et al. reported significant upregulation of MnSOD mRNA expression in oral SCC tissues compared with the normal tissue counterparts (16).

In general, oral VCs are locally aggressive, but have a low propensity for regional as well as distant metastasis (1, 2). This is reconfirmed by the fact that none of 20 patients with our oral VC in the present study developed regional or distant metastasis. The distant metastasis associated with VC can be a consequence of an incorrect diagnosis or of the presence of foci of SCC in lesions with warty features. Significant clinical and biologic differences are found between invasive oral SCC and oral VC. However, the differential diagnosis between well-differentiated SCC and VC can sometimes be less certain. In our study, the different degree of MnSOD and GPx expression in oral VC and oral SCC may be helpful for pathologists to differentiate these two entities, especially between oral VC and well-differentiated oral SCC.

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## Conflict of interest

All authors reported no conflict of interests relevant to this work to declare.