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# VX2-induced rabbit buccal carcinoma: A potential cancer model for human buccal mucosa squamous cell carcinoma

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## SUMMARY

The buccal mucosa is the site at highest risk of contracting malignancy in habitual betel-quid chewers who expose the buccal mucosa to high doses of carcinogens. Of all oral squamous cell carcinomas (SCCs), those of the buccal mucosa are most associated with the poorest prognoses. Therefore, an animal model would be helpful to evaluate new treatment modalities for buccal SCC. We evaluated whether the VX2 rabbit cancer model could be employed as a cancer model for human buccal SCC. Ten adult male, New Zealand White outbred rabbits were randomly divided into two groups A (n = 2) and B (n = 8). A 0.5 ml VX2 tumor cell suspension containing approximately  $40 \times 10^6$  vital cells was injected intramuscularly into the right hind paw of the two rabbits of group A. Four weeks later, moderately to poorly differentiated hind paw SCCs were apparent in both rabbits of group A. No abdominal organ metastases, but multiple pulmonary metastases, were found in both animals. Fresh solid tumor pieces (about  $5 \times 5$  mm) obtained from group A animals were subsequently inserted into the surgically created spaces of the left cheeks of the eight rabbits of group B. Ulcerated buccal tumors (moderately to poorly differentiated SCCs) were found in all eight animals 6 weeks later. No internal organ metastases were noted in any of the eight rabbits, but a total of 11 with an average of 2.75 cervical lymph node metastases were found in four of the eight animals. Mandibular bone and tooth pulp invasion by cancer cells was also noted in one animal. In conclusion, our findings indicated that VX2-induced rabbit buccal carcinomas could be a potential cancer model for human buccal mucosa squamous cell carcinoma.

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## Introduction

Squamous cell carcinoma (SCC) of the oral cavity is a serious health problem, as indicated by its high incidence in many parts of the world. Oral SCC ranked the 12th most common cancer in the world<sup>1</sup> and the 8th most predominant in males.<sup>2</sup> In central to Southeast Asian countries, oral SCC is the most frequent cancer and constitutes about a third of all cancers due to the practice of chewing betel-quid or tobacco or both.<sup>3</sup> For countries, including Taiwan, with a high prevalence of chewing betel-quid and tobacco,<sup>4-13</sup> buccal SCC is the most common oral cancer. In contrast, in North America and Western Europe, buccal SCC accounts for

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only 10% of all oral carcinomas.<sup>12</sup> Hence, the buccal mucosa appears to be the site at greatest risk of contracting malignancy among habitual betel-quid and/or tobacco chewers who expose the buccal mucosa to high doses of carcinogens.<sup>14</sup> In Taiwan, buccal SCC ranges from about 27% to 37% of all intraoral cancers, as reported in different studies.<sup>4,5,15</sup>

The 3- and 5-year disease-specific survival rates for buccal SCC were 62–91% and 37–76% respectively.<sup>6,12,13,16–23</sup> Locoregional recurrence rates of buccal SCC reported in the literature range from 26% to 80% and is the main cause of death for those patients after surgery or after combined surgery and radiotherapy.<sup>12,13,17–23</sup> A high locoregional failure rate is noted even for  $T_{1-2}N_0$  buccal SCC cancer patients.<sup>13,24</sup> These data suggest that buccal SCCs appear to be more aggressive than those arising from other subsites in the oral cavity, possibly due to a limited anatomic barrier within the buccal space, with essentially no resistance to tumor spread.<sup>25</sup>

In 1936, Kidd et al.<sup>26</sup> successfully induced cutaneous carcinomas after inoculation with extracts containing Shope-papilloma virus<sup>27</sup> and they designated the first successful transplantation of

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carcinoma as 'V1'. Subsequent trials resulted the development of the 'V2' carcinoma cell line, in which successful transplantation was initially low (only 5%) among the inoculated Dutch belted rabbits, but the success rate increased to 21% in the third tumor generation.<sup>28</sup> By then, the V2 tumor became increasingly anaplastic histologically.<sup>29</sup> Because existing different stocks of cell lines worldwide comprised different transplant generations, the carcinoma became designated as 'VX2' tumor. We induced SCC using VX2 tumor cells in the buccal mucosa of rabbits and evaluated whether this animal model could be employed as a cancer model for human buccal SCC.

# Materials and methods

# Animals

Adult male, specific-pathogen free, New Zealand White outbred rabbits weighing 2.5–3.5 kg (10 animals; purchased from the Livestock Research Institute, Council of Agriculture, Executive Yuan, Taiwan) were randomly divided into two groups: A (containing two rabbits) and B (containing eight rabbits). Approval of the experimental protocol was obtained from the Animal Experiments Committee of the institution to ensure humane treatment of the test animals; all procedures were conducted in accordance with the NIH Guide for the care and use of animals. The animals were allowed to acclimatize for at least 5 days before the experiments and were kept in a closed system without isolation. The rabbits were housed in individual steel cages under constant conditions: 12-h light-dark cycle with an environmental temperature maintained at 18–20 °C and non-regulated relative air humidity of approximately 60%. The rabbits were fed acidified (hydrochloric acid, pH 2.7) tap water and approximately 100 g of 'complete diet' pellets for rabbits per day ad libitum. All experiments were done during day-time hours.

#### Analgesia, sedation, sacrifice

Analgesia and sedation during interventions were achieved with intramuscular injection of 0.45 ml/kg ketamine hydrochloride (Merial, Lyon, France), 0.3 ml/kg atropine (Bayer, Leverknsen, Germany), and 0.5 ml chanazine (Bayer, Leverknsen, Germany). Rabbits were sacrificed by intravenous injection of pentobarbital 0.3 ml/kg (Bayer, Leverknsen, Germany).

### VX2 cancer cell propagation in rabbit hind paws (group A)

The VX2 cancer cells were kindly donated by Dr. Robert J.J. van Es, Department of Oral and Maxillofacial Surgery, University Medical Centre Utrecht, Utrecht, The Netherlands. VX2 cancer cells were propagated by intramuscular passage in the hind paws of rabbits as described below.<sup>30</sup>

The VX2 cancer cells were treated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Hyclone, Logan, UT) for 24 h. Then, the resulting cell suspension was centrifuged for 5 min at 3000 rpm and used within 2 h. The number of cells in the suspension was counted using a hemacytometer. Viability of the cells was estimated by trypan blue staining. Upon anesthesia and sedation of the two group A rabbits, all of the hair



Figure 1 (A) Intramuscular injection of VX2 cancer cells into the right hind paw of the rabbit; (B) a palpable tumor mass was found at the inoculation site of the right hind paw of the rabbit; (C) gross photograph of the dissected tumor in (B).

on the right hind paws was removed by shaving the skin with a pair of clippers. In order to induce tumors, approximately 0.5 ml of a suspension containing approximately  $40 \times 10^6$  vital cells was injected with an 18-gauge needle intramuscularly in the right hind paws of the two rabbits (Fig. 1A). The rabbits were then observed daily for the development of tumors in their hind paws. The body weights of the rabbits were recorded weekly. Weight loss at sacrifice was defined as a percentage of original weight:  $100 \times (original)$ weight - weight at sacrifice)/original weight. At autopsy, the dimensions of the induced hind paw tumors were measured. Tumor pieces were then taken from non-necrotic, well-vascularized portions of an aseptically excised hind paw tumor from each group A rabbit<sup>31,32</sup> and were cut into approximately  $5 \times 5$ -mm pieces in ice-cold DMEM (Hyclone) and used within 2 h for tumor implantation in the buccal mucosa of group B rabbits. Moreover, pulmonary tissues and abdominal organs were inspected for the presence of metastases.<sup>32,33</sup> All tissues were fixed in 10% buffered formalin for hematoxylin-eosin staining and immunohistochemical analysis.

## VX2 cancer cell implantation to rabbit mucosa (group B)

VX2 cancer cells were implanted into the left buccal mucosae of the eight group B rabbits via an extra-oral approach (Fig. 2A and B). After shaving the hair of the left cheek under anesthesia and sedation, a small space was aseptically created about 1.5–2 cm in depth using blunt dissection (Fig. 2A). A solid tumor piece was subsequently inserted into the surgically created space of the left cheek of each rabbit (Fig. 2B). Finally, the surgical space was closed with sutures. The rabbits were then monitored for recovery from sedation and observed daily for the development of tumors in the buccal mucosae. The body weight of the rabbits was recorded weekly. Weight loss at sacrifice was defined as a percentage of original weight:  $100 \times (\text{original weight} - \text{weight at sacrifice})/\text{original weight}$ .

Implantation success was denoted by the tumor uptake rate. Under anesthesia and sedation, the induced tumor dimensions in the buccal mucosa were recorded. Enlarged cervical lymph nodes were dissected bilaterally. The lungs as well as the abdominal organs were inspected for the presence of metastases.<sup>32,33</sup> All tissues were fixed in 10% buffered formalin for microscopic examination. The resected induced buccal tumor was fixed in 10% neutral buffered formalin solution, and paraffin sections (5 µm) were prepared for hematoxylin–eosin staining and immunohistochemical analysis.

## Immunohistochemistry

Following tissue sectioning of paraffin-embedded samples, immunohistochemical staining was performed using a standard avidin-biotin peroxidase complex (ABC) method.<sup>34</sup> Mouse monoclonal antibodies against pan cytokeratin (Abchem Corporation, Cambridge, UK; Cat. No. ab6401) and against vimentin (Abchem, Cat. No. ab28028) were used; their specificity was established in previous studies.<sup>35,36</sup>

Tissue sections were mounted on gelatin-chrome alum-coated slides. Following deparaffinization in xylene (twice) and rehydration in decreasing concentrations of ethanol (absolute, 95%, 70%, and 30% ethanol, and then water), the tissue sections were microwave-treated three times (5 min each time) in citrate buffer (10 mM; pH 6.0) to recover antigenicity. Endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 60 min. Prior to immunohistochemical staining, the specimen was incubated with 10% solution of normal goat serum for 60 min at room temperature to inhibit non-specific staining. The sections were subsequently incubated with antibodies against keratin (1:100) and vimentin (1:100), respectively, overnight at 4 °C. Following subsequent rinsing with Tris buffered saline (TBS) (three times, 10 min each), all tissue sections were incubated for 60 min at room temperature with biotin-conjugated goat anti-mouse IgG (Vector, Burlingame, CA, USA; 1:100). Following this, all sections were again washed with TBS (three times, 10 min each) and then incubated with an avidin-biotin complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA, USA) for another 60 min. After washing with TBS (three times, 10 min each), peroxidase binding was visualized as brown reaction products via a benzidine reaction. The sections were then counterstained with Mayer's hematoxylin. Each set of experiments included a specimen known to express keratin and vimentin, respectively, which served as positive controls and ensured the reproducibility of the staining process. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments.

## Results

Four weeks after the VX2 tumor cells were injected into the right hind paws of both rabbits in group A, two grossly palpable tu-



Figure 2 (A) Surgically created space about 1.5–2 cm in depth in the left cheek of the rabbit; (B) implantation of the fresh VX2 tumor mass into the surgically created space shown in (A).

mor masses, measuring 5 cm and 3 cm in maximum diameter, respectively, were found at each of the inoculation sites of both hind paws (Fig. 1B); a representative dissected tumor is shown in Figure 1C. The percentage of body weight loss of both animals of group A were 12% and 11%, respectively, at the time of sacrifice.

Histopathologically, the induced hind paw tumors of both animals were moderately to poorly differentiated SCC (Fig. 3A and B). Immunohistochemically, the tumors stained positively for keratin and negatively for vimentin. On autopsy, no abdominal organ metastases were noted. Multiple pulmonary metastases were



Figure 3 The VX2-induced hind paw tumor was moderately to poorly differentiated squamous cell carcinoma (A × 100; B × 400; hematoxylin and eosin stain).



**Figure 4** (A) Multiple white nodes of lung metastases were noted in the rabbit with a VX2-induced hind paw tumor; the metastatic lung lesion was moderately to poorly differentiated squamous cell carcinoma (B × 100; C × 400; hematoxylin and eosin stain).

found in both animals (Fig. 4A). The histopathologic (Fig. 4B and C) and immunohistochemical staining results of the lung metastatic lesions were similar to those of the primary hind paw lesions.

Six weeks after VX2 tumor tissue implantation into the left cheeks of eight group B rabbits, ulcerated tumor masses (measuring  $4.6 \pm 0.8$  cm in average maximum diameter) were found in the left buccal mucosa of all eight rabbits (Fig. 5A). The average percentage of body weight lost among group B rabbits was  $12 \pm 2\%$  at the time of sacrifice. Histopathologically, the induced buccal tumors of all the animals were moderately to poorly differentiated SCCs (Fig. 5B and C) and were keratin positive and vimentin negative on immunohistochemical staining (Fig. 6A and B). On autopsy, no internal organ metastases were found. Significantly, a total of 11 with an average of 2.75 cervical lymph node metastases were noted in four of the eight rabbits (Fig. 7A–D). Mandibular bone and tooth pulp invasion by cancer cells were also noted in one animal (Fig. 8A–D).

# Discussion

SCCs arising from different types of oral epithelia are reported to possess distinctive characteristics regarding their clinical behavior and responsiveness to different treatment modalities.<sup>37</sup> Of all oral SCCs, those of the buccal mucosa are most often associated with the poorest prognoses.<sup>38</sup> Therefore, an animal cancer model is essential to elucidate the molecular characteristics of buccal SCC or to evaluate the potential of new treatment modalities for SCC originating from the buccal mucosa.

VX2 successfully induces SCCs in rabbits in the oral cavity.<sup>39,40</sup> Jefferis and Berenbaum<sup>39</sup> has demonstrated the rabbit VX2 tumor as a model for carcinomas of the tongue with the tumor uptake rate to be 92.6% (25/27); however, the status of cervical lymphadenopathy has not been reported.<sup>39</sup> Chikit et al.<sup>40</sup> has also been successful to induce SCC of the floor of mouth using the VX2 rabbit cancer model and 20 metastatic cervical lymph nodes have been



Figure 5 (A) The intra-oral photograph of the VX2-induced ulcerated buccal carcinoma (\*) in the rabbit; the ulcerated induced buccal tumor was moderately to poorly differentiated squamous cell carcinoma ( $B \times 40$ ; C  $\times 200$ ; hematoxylin and eosin stain).



Figure 6 Immunohistochemical staining of VX2-incuced buccal squamous cell carcinoma for (A) cytokeratin (positive) and (B) vimentin (negative) (×100; avidin–biotin peroxidase complex staining).

L.-M. Lin et al./Oral Oncology 45 (2009) e196-e203



**Figure 7** Clinical (A) and gross (B) pictures of the cervical lymph node metastases of the rabbit with VX2-induced buccal squamous cell carcinoma; the metastatic cervical lymph node lesions were moderately to poorly differentiated SCC ( $C \times 200$ ;  $D \times 400$ ; hematoxylin–eosin stain).

identified but the tumor uptake rate has not been stated. SCC of the buccal mucosa, induced by VX2, in a rabbit cancer model is not reported. In the current study, a 100% buccal tumor uptake rate and 50% cervical lymph node metastasis rate in a relatively shorter span of tumor induction were observed. Furthermore, as all the previous studies on rabbit VX2 tumor model to induce oral SCCs were via intra-oral injection of the VX2 tumor cell suspension,<sup>39-41</sup> the current study would be the first to demonstrate a detailed extra-oral approach to induce oral SCCs via implantation of fresh solid VX2 tumor mass.

One of the most common animal cancer models for studying SCC occurring in the buccal mucosa was the hamster cheek pouch SCC induced by topical application of the carcinogen: 7,12-dimethya[a]nthracene (DMBA).<sup>42</sup> However, induction of cancer using this model was time-consuming, with carcinomas produced after 12-14 weeks of DMBA application. Moreover, in contrast to human oral SCC, the induced hamster buccal SCCs did not often generate infiltrating carcinomas and cervical lymph node metastases were rarely seen.<sup>42</sup> In the current study, both buccal tumors and cervical lymph node metastases in a relatively shorter span of tumor induction were observed. Furthermore, mandibular alveolar bone invasion and tooth pulp involvement by the cancer cells were also found. All of these findings indicated that the pathologic behaviors of VX2-induced rabbit buccal SCCs are superior to those of the hamster buccal pouch model and more likely mimic human buccal SCCs. Therefore, we suggest that VX2-induced rabbit buccal SCC is a potential cancer model for human buccal SCC. On the other hand, chemically-induced carcinoma has an advantage over implanted tumors, as the tumors originate in the epithelium of the test animal, despite that they take considerably longer to develop. There is a limitation to the rabbit cancer model because rabbits do not grow tumors spontaneously in the oral mucosa, and all the tumors had to be implanted beneath the overlying oral epithelium. However, all the induced tumors in the current study had an ulcerated surface and were comparable in epithelial origin to tumors that occur in humans. Finally, the rabbit cancer model has a further merit that the tumors could be implanted at several sites at the same time in the same animal, which could not be done with the hamster buccal pouch model.

VX2 cell transplantation can be performed either by injecting tumor cell suspensions<sup>39–41,43–45</sup> or by implanting fresh solid tumor pieces.<sup>46</sup> Transplantation by injection of cell suspensions is technically easy and allows for determining the ratio of injected vital cells (evaluated by trypan blue staining) and to inject approximately equal amounts of vital cells for individual transplantations. However, the injection pressure can produce a phenomenon called 'seeding at implantation,' causing unintentional inoculation of tumor cells into the blood vessels, with resultant early lung metastases and early nodal metastases.<sup>32,47</sup> Indeed, our experience with injecting tumor cell suspensions showed technical feasibility, and it was easy to cause achieve intramuscular rabbit hind paw tumors. However, for injection of a tumor cell suspension into a site in the

#### L.-M. Lin et al./Oral Oncology 45 (2009) e196-e203



Figure 8 Mandibular alveolar bone (A  $\times$  40; B  $\times$  200; C  $\times$  400; hematoxylin–eosin stain) and tooth pulp (D  $\times$  400 hematoxylin–eosin stain) invasion of cancer cells (\*) was noted for a rabbit with VX2-induced buccal squamous cell carcinoma (T, tooth; TP, tooth pulp).

oral cavity, the exact depth of injection is difficult to locate, and the tumor cells spread out uncontrollably within the area of injection. Furthermore, trismus caused by anesthesia and the limited working field cause additional difficulty for intra-oral tumor cell injection. The dead cells injected inevitably have an immunization effect, stimulating an effective immune-response, which could prevent tumor growth. Thus, we choose to implant fresh solid tumor pieces into the cheeks of the rabbits via an extra-oral approach. This could explain why we achieved a high tumor uptake rate.

In conclusion, we successfully established a rabbit VX2 tumor model for carcinomas of the buccal mucosa. Our results provide useful information for future studies to evaluate new treatments and molecular features for buccal SCCs.

## **Conflicts of Interest Statement**

None declared.

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L.-M. Lin et al./Oral Oncology 45 (2009) e196-e203

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