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**Apoptin gene therapy in head and neck cancer**  
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## **A mouse model for oral squamous cell carcinoma**

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## **Abstract**

Despite recent advances, the prognosis of oral squamous cell carcinoma is still poor. Therapeutic options such as radiotherapy, chemotherapy, surgery and the novel treatment option gene therapy are being investigated in animal models. Diverse models have been studied to induce oral squamous cell carcinomas. The carcinogenic 4-nitroquinoline 1-oxide (4NQO) model has proven to be successful although until now it is unknown at what time point the established tumor is a representative squamous cell carcinoma and has a suitable volume for scientific treatment. For this end we applied 4NQO 3 times a week during 16 weeks and measured the volume of tumor tissue each week until the end of the experiment at 40 weeks. Concurrent histopathology at different time points up to the end of the experiment revealed that all mice bearing oral tumors were diagnosed with squamous cell carcinoma. Immunohistochemistry with markers cyclin D1 and E-cadherin revealed that the generated mouse oral tumors showed strong similarities with the described immunopathology in human oral tumors. The 4NQO model is a suitable alternative for preclinical gene therapy experiments with primary oral tumors. Future survey of therapeutic options in the carcinogenic 4NQO model should be conducted around 40 weeks after the start of the treatment.

## Introduction

Oral cancer accounts for almost 275,000 new cases worldwide each year<sup>1</sup>. The main risk factors for developing oral cancer are tobacco smoking and consumption of alcohol<sup>2</sup>. Continuation of smoking during treatment can lead to increased morbidity and mortality<sup>3</sup>. Primary treatment is associated with high morbidity and function loss of the organ, while up to 50% die due to recurrence<sup>4</sup>. Present-day treatment, surgery, radiotherapy or chemotherapy or a combination of these three, has had notwithstanding the improvements, no significant improvement in survival<sup>5</sup>. For that reason new treatments, such as (targeted) gene therapy are being developed<sup>6-10</sup>.

New therapies can be investigated both *in-vitro* and *in-vivo*. The drawback of *in-vitro* laboratory study is the dissimilarity between the cell culture and the physiological processes giving misleading results. Several animal models for oral squamous cell carcinoma are used including hamster, rat and mouse models, with each model having its own advantages and disadvantages. Earlier experiments were set to induce tumors by mechanical damaging the jaw in mice<sup>11</sup>. Nowadays xenograft models are widely used. In this model human cells are injected and grow in immunodeficient mice. Advantages are the speed and certainty of tumor development. The premier limitation is the lack of functional T lymphocytes in nude mice resulting in a non physiological tumor response.

The widely used hamster cheek pouch model in which 7,12-dimethylbenz(a)anthracene is used to induce tumors has a disadvantage because the tumors are unlike the human counterparts<sup>12</sup>. After the introduction of 4-Nitroquinoline-1-oxide (4NQO) for inducing tumors in rats<sup>13</sup>, it has been extensively used in both rats and mouse and can be useful for analyzing the effects of anti-tumor agents<sup>14</sup>. The 4NQO oral squamous cell carcinoma model is a lengthy multi-step process in which eventual invasive squamous cell carcinoma is attained after several months.

Once established the tumors are suitable for studying the therapeutic

effect of various therapies including gene therapy. Intratumoral injection of recombinant adeno-viral vectors is mainly used in preclinical models<sup>15</sup>. Therefore at the time of injection a minimum tumor volume must be attained and the realization of squamous cell carcinoma must be assured. Up till now, in the 4NQO mouse model, this time point is unknown.

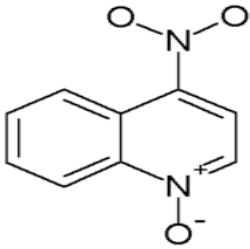
The present study was set up to investigate the time needed for sufficient tumor volume to be developed enabling intra-tumoral injection of anticancer agents such as gene therapy. As important, we have identified that the generated mouse oral tumors resembles human oral tumor formation.

## **Material and Methods**

### *Animals and treatment with carcinogen.*

Thirty-two 7 and 8 weeks old male CBA mice weighing between 23 and 27 g were purchased from Harlan in Zeist, The Netherlands. The experiments were approved in advance by the Leiden University Animal Welfare Committee. All animals were kept at standard laboratory conditions of alternating 12-h periods of light and darkness and were fed sterilized laboratory chow and water ad libitum. The carcinogen 4-Nitroquinoline-1-oxide (Figure 1) was dissolved in propylene glycol to a final concentration of 5 mg/ml. For each session a fresh aliquot was used. Anesthesia was achieved by inhalation of halothane vapour and the tongue was stroked once with a no 3 camel hair brush<sup>16</sup>, which had been dipped in the 4NQO solution. The mice were restrained from drinking the first hour after 4NQO application. The mice were treated with 4NQO during 16 weeks with a frequency of 3 weekly treatments. All mice were carefully inspected daily and weighed weekly.

**Figure 1**



Chemical structure of 4-nitroquinoline 1-oxide (4NQO).

*Pathological Examination and Immunohistochemistry.*

After the final 4NQO application the oral mucosa of each individual mouse was examined under inhalation anesthesia every week. The volume of exophytic tumors was calculated each week by measuring three dimensions (length, height and width). After 24 weeks following the start of the experiment 6 mice were sacrificed and tongue tissue was removed and fixed in freshly made 4% buffered formalin. Subsequently there were groups of 6 mice sacrificed after 28, 32, 36 and 40 weeks.

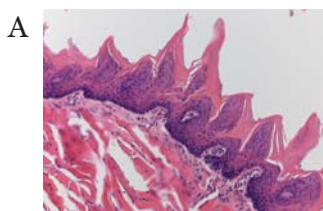
After paraffin-embedding, serial sections of 4  $\mu\text{m}$  width were made and one in five subsequent sections were stained with hematoxylin-eosin (HE). The epithelia was examined by a pathologist in our institution and classified as either normal epithelia, mild dysplasia, moderate dysplasia, sever dysplasia or invasive squamous cell carcinoma. Dysplastic epithelia and invasive squamous cell carcinoma were subsequently investigated by immunohistochemistry with cyclin D1 and E-cadherin. The following antibodies were used from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): the rabbit polyclonal antibody against cyclin D1 (H-295, dilution 1:100) and a rabbit polyclonal antibody against E-cadherin (H-108, dilution 1:50). After the slides were deparaffinized and rehydrated, antibodies were applied according to the manufacture's protocol.

## Results

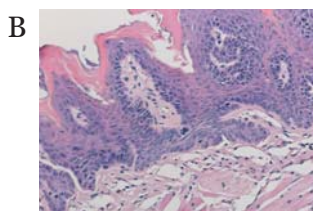
### *Histological findings.*

The first macroscopic changes were seen 19 weeks after starting the experiment. After 24 weeks some tongues did not show any pathological changes (Figure 2A) although at the same phase hyperplasia without atypia and mild dysplastic changes were detected (Figure 2B). Four weeks further on, advance in atypia was found and moderate (Figure 2C) and severe dysplasia (Figure 2D) could be detected. The first evidence of squamous cell carcinoma was identified after 32 weeks (Figure 2E). At the time point of 36 weeks 4 out of 6 animals that were sacrificed showed squamous cell carcinoma at histopathological examination. At 40 weeks, the end of our experiment, all 6 tumors were squamous cell carcinomas (data not shown).

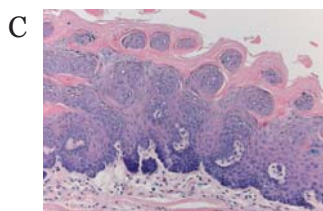
**Figure 2**



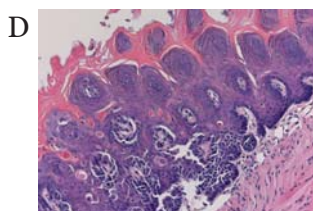
normal; 24 weeks



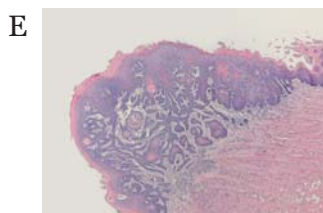
mild dysplasia; 24 weeks



moderate dysplasia; 28 weeks



severe dysplasia; 28 weeks



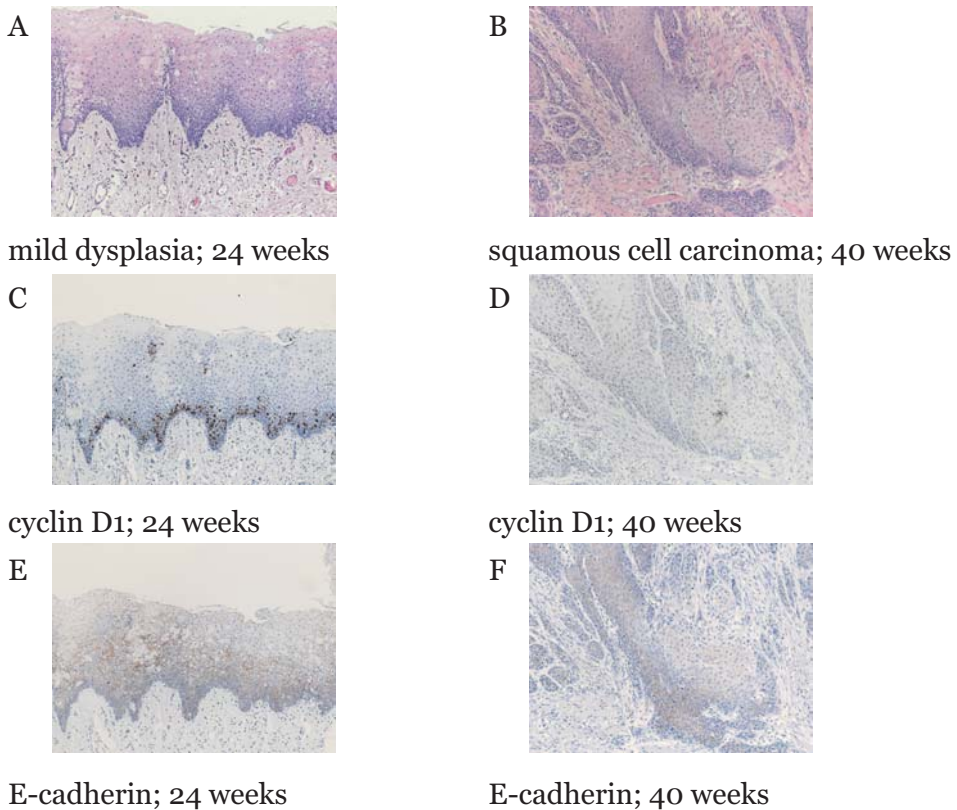
invasive squamous cell carcinoma; 32 weeks

Histological findings of tongue in mice treated with 4NQO during 16 weeks. (A) Normal tongue with no histopathological changes 24 weeks after starting the experiment. (B) Mild dysplasia after 24 weeks. (C) Moderate dysplasia after 28 weeks. (D) Severe dysplasia after 28 weeks. (E) Invasive squamous cell carcinoma after 32 weeks. Original magnification x 10.

*Immunohistological findings.*

Once all 6 mice bearing tumors at the 40 weeks time point were diagnosed with squamous cell carcinoma, immunohistochemistry was performed. A comparison of Cyclin D1 and E-cadherin expression was made in mild dysplasia established after 24 weeks (Figure 3A) and squamous cell carcinoma detected after 40 weeks (Figure 3B). Immunohistological evaluation was done in the tumors of our interest.

**Figure 3**



Histopathology and immunohistochemistry staining with biomarkers cyclin D1 and E-cadherin. The left panel shows mild dysplasia after 24 weeks, the right panel shows squamous cell carcinoma after 40 weeks. Hematoxylin-eosin staining (HE) of mild dysplasia (A) and of squamous cell carcinoma (B) are shown. Cyclin D1 is primarily expressed in basal cells in mild dysplasia (C) and throughout the tumor and in tumor nests in squamous cell carcinoma (D). E-cadherin is expressed in the parabasal cell layer of the epithelium, but not on the surface of basal cells in mild dysplasia (E) and is partly lost in the invasive front of squamous cell carcinoma (F). Original magnification x 40.

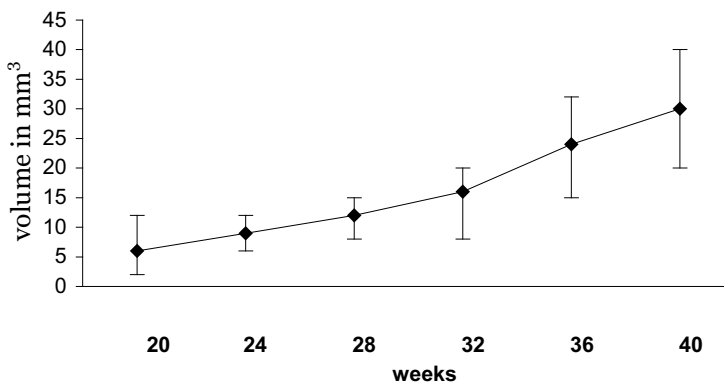


Cyclin D1 expression was investigated by assessing the nuclear staining of the antigen. Only nuclear staining was considered positive, while cytoplasmatic staining was regarded as negative. Mild dysplasia exposed predominantly basal cells expressing cyclin D1 (Figure 3C). The stainability increased with the progression from dysplasia to carcinoma and the positive ratio was the highest in the squamous cell carcinoma (Figure 3D). After 40 weeks, squamous cell carcinoma showed a distribution throughout the tumor (Figure 3D) as well as the basal cell layer and lower spinous cells. Staining of the tongue after 24 weeks showed that E-cadherin was strongly expressed in the parabasal cell layer, but not on the surface of basal cells (Figure 3E). In the oral squamous carcinoma we noticed a loss of E-cadherin expression in the invasive front and positive staining of cancer cell nests (Figure 3F).

#### *Tumor volume.*

The initial measurement of a volume of exophytic tumor was done after twenty weeks following the start of the experimental procedure. By measuring the size of the tumor in three dimensions the volume was calibrated in mm<sup>3</sup> by using the following formula:  $V = l \times w \times h$ . The volumes and ranges of each time point are shown in Figure 4. After 20 weeks, the average volume was 6 mm<sup>3</sup> with a range of 2 to 12 mm<sup>3</sup>. Gradually with time the volume increased and reached 30 mm<sup>3</sup> at the 40 weeks time point.

**Figure 4**



## Discussion

Oral squamous cell carcinoma is one of the most common cancers in the world. Novel treatment is best studied in animal models that mimic the same clinical features as human squamous cell carcinomas.

In this study we induced oral squamous cell carcinomas by applying 4NQO orally during 16 weeks in mice. Premalignant lesions appeared 3 weeks after the last application of 4NQO. The typical tumor progression for squamous cell carcinoma was seen before invasive squamous cell carcinomas appeared. At 36 weeks two thirds of the tumors were squamous cell carcinomas, 4 weeks further all 6 oral tumors were squamous cell carcinomas. These results are in accordance with earlier observed results in which squamous cell carcinomas were detected between 12 and 33 weeks after applying 4NQO during 16 weeks<sup>16,17</sup>.

Immunohistochemistry was performed on the tissues to assess the similarities with human squamous cell carcinoma. Biomarkers have been studied to predict behavior of tumor cells and are used to characterize tumors<sup>18</sup>. Cyclin D1 plays a pivotal role in transition from G<sub>1</sub> to S phase and cyclin D1 expression pattern is associated tumor characteristics and survival<sup>19</sup>. As is seen in human and other animal models<sup>20,21</sup> cyclin D1 expression showed an increased expression in the oral squamous cell carcinoma in our mice. E-cadherin, a calcium dependent membrane protein that is essential for the formation of adherens junction between cells. There is a connection with changing expression of E-cadherin and the transition from hyperplasia to invasive carcinoma<sup>22</sup>. We observed less expression of E-cadherin in invasive section of the carcinoma and evenly distributed expression in earlier stages, a pattern that has been reported earlier in human oral squamous cell carcinoma<sup>23</sup>.

Average tumor volume advanced from 6 mm<sup>3</sup> after 20 weeks to 30 mm<sup>3</sup> at the 40 weeks time point. For intra-tumor injection with an adenovirus it is desirable that the size of each axis of the tumor is at least 2 mm, though

preferable higher. Eventual tumor size reduction after treatment is then feasible. Assuming that the 4NQO oral tumor model will not have the prospect to develop in to a large tumor volume such as the model in which tumor cells are injected subcutaneously in the flank, tumor volume will not easily exceed 100 mm<sup>3</sup>. Previous experiments with intra-tumor injection of gene therapy showed that the injected volume must not surpass 30% of the volume of the tumor<sup>24</sup>. Taking this in to account and knowing that the volume of an oral squamous cell carcinoma in mice is small we estimate that a tumor volume of at least 15 mm<sup>3</sup> is desirable. After 32 weeks the tumor volume surpassed this point and after 40 weeks the volume averaged 30 mm<sup>3</sup>. We estimate that a desirable tumor volume will be reached after these 40 weeks and the histopathology will reveal a squamous cell carcinoma, without hampering the animal's welfare.

The two most frequent used experimental procedures for investigating therapies are in-vitro cell lines and the nude mouse. The 4NQO model has several advantages and disadvantages over these laboratory experiments. Compared with the cell lines and the nude mouse model, the close similarity of the 4NQO model to the physiological process is a big benefit. The biggest drawback of the nude mouse model is the lack of an immunocompetent component. The weakness of the hamster cheek pouch model is the earlier mentioned dissimilarity of the tumors with the human equivalent. Application of DMBA causes an inflammatory response and necrosis making it hard to study early squamous lesions<sup>25</sup>. No inflammatory reaction or necrosis was seen in our specimens. Other drawbacks of this model are that the cheek pouch has no anatomic counterpart in human, the epithelium of the cheek pouch is significantly thinner than other parts of the oral mucosa of humans and mice and the tumors seem to progress from papillomas<sup>26,27</sup>, which is uncommon in humans and was not seen in our samples. The clear disadvantage of the 4NQO model is that both the cell line and the nude mouse are less time consuming and that cell line experiments are less costly and most of the time readily available

In our conclusion taking both advantages and disadvantages in to account the 4NQO oral tumor model has clear benefits and seems suitable for therapeutic research applications.

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