Published in final edited form as:

Med Mycol. 2009 March ; 47(2): 157–167. doi:10.1080/13693780802165797.

A novel immunocompetent murine model for *Candida albicans*-promoted oral epithelial dysplasia

P. P. DWIVEDI^{*}, S. MALLYA[†], and A. DONGARI-BAGTZOGLOU^{*}

^{*}Division of Periodontology, University of Connecticut Health Center, Farmington, Connecticut, USA

[†]Division of Oral and Maxillofacial Diagnostic Sciences, University of Connecticut Health Center, Farmington, Connecticut, USA

Abstract

Candida albicans is a common opportunistic pathogen found in the oral mucosa. Clinical observations indicate a significant positive association between oral *Candida* carriage or infection and oral epithelial dysplasia/neoplasia. The aim of this study was to test whether *C. albicans* is able to promote epithelial dysplasia or carcinoma in a mouse model of infection where a carcinogen (4 Nitroquinoline 1-oxide [4NQO]) was used as initiator of neoplasia. Mice were divided into four groups: group 1 received 4NQO alone; group 2 received 4NQO followed by *C. albicans* (ATCC 90234); group 3 received vehicle dimethyl sulfoxide (DMSO) followed by *C. albicans* and group 4 was untreated. Although 4NQO treated mice did not develop oral lesions, mice exposed to both 4NQO and *C. albicans* developed oral dysplastic lesions 19 weeks after exposure to 4NQO. Mice challenged with *C. albicans* only developed hyperplastic lesions. The expression of Ki-67 and p16, two cell-cycle associated proteins that are frequently deregulated in oral dysplasia/neoplasia, was also tested in these lesions. Ki-67 and p16 expression increased from normal to hyperplastic to dysplastic mucosa and was highest in the group exposed to both 4NQO and *C. albicans*. In conclusion, we showed that *C. albicans* plays a role in the promotion of oral dysplasia in a mouse model of infection when 4NQO was used as initiator of oral neoplasia.

Keywords

Candida albicans; 4NQO; oral epithelial dysplasia; murine model

Introduction

Oral candidiasis is a collective name given to a group of diseases caused by *Candida* spp. in the oral cavity [1]. The major *Candida* species responsible for oral infection is *C. albicans*, a polymorphic organism that resides as a commensal in the human gastrointestinal tract [2]. Asymptomatic carrier rates in healthy individuals range from 3–48% [3] and are even higher

© 2008 ISHAM

Correspondence: Anna Dongari-Bagtzoglou, Division of Periodontology, University of Connecticut Health Center, School of Dental Medicine, 263 Farmington Ave, Farmington, CT 06030–1710, USA. Tel: +1 860 679 4543; Fax: +1 860 679 1673; E-mail: adongari@uchc.edu.

Publisher's Disclaimer: The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

in children, the elderly and immunocompromised individuals (up to 85%) [4]. High carrier loads increase the risk for oral infection [5]. Morbidity from oral infection has increased in recent years due to higher prevalence of immunosuppression attributed to steroids, anti-rejection medications [6,7], myeloablative radiation therapy [8-10] and antineoplastics [11-13].

Squamous cell carcinoma (SCC) of the oral cavity is one of the most common neoplasms with 350,000 new cases diagnosed annually worldwide [14]. A possible association between oral *Candida* colonization and oral neoplasia was first reported in the 1960s [15]. *Candida albicans* was much more frequently detected in the biofilm of oral squamous cell carcinoma tumor sites than control sites [16]. Earlier human studies also showed a statistically significant association between fungal invasion and epithelial dysplasia [17-19]. Other investigations found that there is a positive relationship between oral carriage of yeast and oral epithelial dysplasia. However it remains unclear how oral yeast carriage or infection influences the development and progression of dysplasia, or vice versa [20].

The two major risk factors for oral cancer are tobacco and alcohol in most western populations [21-23]. Tobacco contains many carcinogens that cause carcinogenesis through interaction of reactive intermediates with DNA to form DNA adducts [24]. 4 Nitroquinoline 1- oxide (4NQO) is a water soluble quinoline derivative that can also cause DNA adduct formation, resulting in adenosine substitution for guanosine [25-27]. Thus, 4NQO has a similar mode of activity as tobacco byproducts and has been shown to promote oral SCC in rats [28,29] and mice [30-32]. Application of 4NQO in a mouse model triggers multiple dysplastic, preneoplastic and neoplastic types of lesions after long-term treatment, which mimic oral epithelial neoplastic transformation [30].

The ability of *C. albicans* to colonize, penetrate and damage host tissues depends on an imbalance between *Candida* virulence and specific defects in host immune defenses [33]. A number of murine models have been developed to study virulence properties of *C. albicans* in the oral mucosa over the past half a century [34-40]. However, all of these mouse models are based on pharmacologically or genetically immunocompromised animals, and thus are of limited value in the study of specific host immune factors involved in the pathogenesis of oral infection. Furthermore, none of these mouse models has addressed the role of *C. albicans* as initiator or promoter of oral epithelial dysplasia/neoplasia.

We set out to develop an immunocompetent mouse model of *Candida* infection, which would establish a role of this organism in the development or progression of oral dysplasia/neoplasia and provide a useful tool for future studies to identify host factors possibly contributing to this process. Using this model we hypothesized that *C. albicans* infection is capable of promoting epithelial dysplasia/neoplasia triggered by 4NQO. To support our histopathologic findings we also examined the expression of two commonly used biomarkers in the histologic assessment of oral epithelial neoplastic transformation (Ki-67 and p16 proteins) [41,42]. We found that long-term exposure to the microorganism alone can trigger oral hyperplasia but not dysplasia in immunocompetent mice. However, in a two-stage initiator (4NQO)-promoter (*C. albicans*) protocol the fungus promoted the development of oral dysplastic lesions.

Materials and methods

Experimental design

The experimental design is an extensive modification of the rat protocol described by O'Grady [29]. Mice ($n=42$) were divided into four experimental groups and received treatments indicated in Fig. 1a. Group1 ($n=12$) received daily 4NQO (Sigma, USA) dissolved in DMSO (Fisher Scientific, USA) vehicle for eight weeks. The second group ($n=12$) received daily

4NQO for the first eight weeks, followed by *C. albicans* challenge. The third group ($n=12$) received DMSO control for eight weeks, followed by *C. albicans*, while the 4th group ($n=6$) was untreated. 4NQO was stored at -20°C and a fresh stock solution was prepared in DMSO as needed. Drinking water containing a fresh 4NQO solution (20 parts per million; ppm) was added on a weekly basis.

To monitor infection and neoplastic changes in the oroesophageal mucosa, subgroups of mice ($n=2-3$) were sacrificed at certain time intervals between 9 and 19 weeks after initiation of 4NQO or DMSO treatment (Fig. 1b). Tissue samples were collected at each time point and were processed for microbial cultures, histology and immunohistochemistry.

Animals

Immunocompetent C57BL/6 female mice, 3 to 5 weeks old, were purchased from Jackson Laboratory, USA. Throughout the experiment they were kept in an isolated environment according to guidelines established by the Animal Care Facilities of the University of Connecticut Health Center.

Microorganism and intraoral challenge

Candida albicans ATCC 90234, which triggers a reproducible infection of the gastrointestinal tract [39], was used in this study. Prior to microbial challenge, Groups 2 and 3 were treated with 0.1% (w/v) tetracycline hydrochloride (TCN; Sigma, USA) for 1 week to suppress the endogenous microflora [43]. The concentration of TCN was reduced to a maintenance dose of 0.01% when the infection process started, and continued as such throughout the 19-week experiment. To deliver the microbial challenge, mice were anaesthetized by an intramuscular injection of ketamine (Fort Dodge Animal Health, USA): xylazine (VEDCO, USA), at 90–100 mg/kg and 10 mg/kg of body weight, respectively. A small cotton pad was soaked with 100 μl of *C. albicans* cell suspension (6×10^8 yeast/ml) and was used to swab the entire oral cavity. Subsequently the swab was left for 2 h under the tongue and was removed before the animals awoke. This procedure was repeated two times in the first week only. This method of oral inoculation has been shown to enhance the severity and reproducibility of oral infection in mice [44]. Subsequently, Groups 2 and 3 were given drinking water containing 6×10^4 organisms/ml to maintain persistent infection throughout the experimental period [45]. A freshly grown suspension of yeast was added to the water daily.

Evaluation of oral *C. albicans* infection

The presence of hyphal organisms invading the oroesophageal mucosa has been traditionally used in humans and animals to distinguish infection from colonization [38] and was used in this study as a histologic criterion for infection. To determine the number of viable organisms in oral tissues, the tongue was longitudinally dissected in three equal pieces and the central portion was processed for colony forming unit (CFU) quantification, by the same examiner, at the time of sacrifice (Fig. 1b) [35]. The tissue was weighed, rinsed with phosphate buffered saline and then homogenized using a tissue homogenizer. Serial dilutions were plated onto Sabouraud dextrose agar plates containing 10 $\mu\text{g/ml}$ chloramphenicol (Sigma, USA). Plates were incubated at 30°C for 48 h and colonies were counted. Results were expressed as log CFU counts/g of tissue.

Histology

Longitudinally dissected tongue and esophageal tissues were fixed with 10% buffered formalin and embedded in paraffin. Five μm thick sections were prepared and stained with hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) stains. The entire length of the tissues was examined for the presence of lesions. Hematoxylin and eosin stained slides were examined for

the presence of hyperplasia or dysplasia by two histopathologists (TVR and EE), blinded to sample type. Periodic Acid Schiff stained slides were used to identify tissue-associated *C. albicans* organisms. Epithelial dysplasia was histologically evaluated based on the World Health Organization criteria for human tissue [46].

Immunohistochemistry

Ki-67 and p16, two cell-cycle associated proteins that may be aberrantly expressed in the early stages of oral neoplasia were tested [47]. The following antibodies and dilutions were used: rat monoclonal anti-mouse Ki-67 (1:50 of 7.7 g/l stock; Dako Cytomation, Denmark) and mouse monoclonal anti-p16 (1:100 of 200 µg/ml stock; Santa Cruz Biotechnology, USA). Paraffin embedded tissue sections were deparaffinized in xylene and rehydrated with a series of ethanol and distilled water. Antigen retrieval was performed with 0.01 M citrate buffer (pH 6.0) at 95°C for 20–30 min in a water bath. Sections were incubated with primary antibodies or appropriate isotype controls, for 30 min at room temperature. To detect Ki-67, sections were incubated with secondary antibody for 30 min at 1:200 dilution (goat anti-rat, 1.1 mg/ml; Jakson Immunoresearch, USA), followed by streptavidin-biotin peroxidase complex (ABC Kit, Vector Lab, USA), according to manufacturer protocols. For immunohistochemical detection of p16, we used the mouse on mouse immunohistochemistry kit (Vector Lab, USA) following the manufacturer's instructions. Reactions were developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Lab, USA) and slides were counter-stained with hematoxylin. Appropriate positive (mouse oral carcinoma tissues) and negative (isotype control antibodies, rat IgG2a for Ki-67 and mouse IgG2a for p16; Jakson Immunoresearch, USA) controls were run with each stain. The number of positive cells was calculated using the NIH Image J software (US National Institutes of Health, Bethesda, Maryland, USA; <http://rsb.info.nih.gov/ij/>) and expressed as positive cells per unit area.

Statistical analyses

Mean CFU/gm of tissue and mean Ki-67 and p16 positive cells per unit area among groups were compared by the Student's *t*-test. $P \leq 0.05$ was considered statistically significant.

Results

Infection outcomes

Because even immunosuppressed mice may be able to clear oral *Candida* infection within 7–10 days [48], we examined tongue and esophageal tissues during the first week of infection (2 days [$n=2$] and 7 days [$n=3$] post-*Candida* inoculation) in groups 2 and 3 to obtain histologic evidence of successful infection. Histologic examination of tissues collected during this time period revealed the presence of a large number of microorganisms invading the oroesophageal mucosa in both groups (Fig. 2b, 2c). Organisms formed micro-colonies embedded in a mucoid substance within the esophageal lumen triggering partial obstruction (Fig. 2b, 2c). All mice in group 2 ($n=7$) exhibited tongue lesions throughout the experimental period resulting in 100% incidence of infection (Table 1, Fig. 3d–f). In group 3 mice the infection rate was the same as group 2 at 10–12 weeks (100%, or 6/6 mice), but dropped to 2 out of 4 mice (50%) during the later stages of the experiment (Table 1, 14–19 weeks). In general, fewer intraepithelial organisms were observed in group 3 compared to group 2 animals (Fig. 3d–i) throughout the experiment.

Infectious loads measured in log CFU/g of tongue paralleled histologic findings (Table 2). The number of microorganisms progressively decreased after 12 weeks of infection in both groups 2 and 3, with a more dramatic decrease in group 3. The CFU load was higher in group 2 at 12 and 19 weeks after infection ($P=0.04$ and $P=0.02$, respectively) as compared with group 3 (Table 2). In summary, these results indicate that although *C. albicans* was able to trigger oral

infection alone or in combination with 4NQO in this model, pretreatment with 4NQO increased the severity and duration of oral infection in immunocompetent mice.

Epithelial histopathologic changes in oroesophageal tissues

The oroesophageal mucosa of all mice ($n=12$) treated with 4 NQO only showed essentially normal epithelium with minimal infiltration of inflammatory cells at 12, 14 and 19 weeks (Fig. 3a–c). Our results also showed that this concentration of 4NQO did not cause any dysplastic/neoplastic lesions over the 19 week time period, which is consistent with other reports [14, 40]. Infiltration of inflammatory cells, loss of epithelial maturation and invasion of *Candida* hyphae were found at 12, 14 and 19 weeks in group 2 mice (Fig. 3d–f). Two out of three mice developed hyperplasia after 12 weeks in group 2 (Table 3). Mice sacrificed after 14 weeks in the same group ($n=2$) developed severe hyperplasia with loss of the cornified layer and acute inflammation accompanied by hyphal invasion. Mice sacrificed five weeks later (19 weeks, $n=2$) in the same group had developed moderate dysplasia and exhibited an increase in proliferative changes in the oral mucosa with loss of epithelial maturation, loss of the anucleated keratin layer and few polymorphonuclear cells (Table 3, and Fig. 3f). The degree of inflammatory cell infiltration and loss of maturation was higher in mice that developed dysplasia, when compared with mice that developed hyperplasia only. Mice challenged with *C. albicans* alone, developed hyperplasia with fewer inflammatory cell infiltrates when compared with mice challenged with a combination of *C. albicans* and 4NQO (Fig. 3g–i). The incidence of hyperplasia in this group at 12 weeks was 2/3 mice (66%), at 14 weeks was 1/2 mice (50%) and at 19 weeks was 1/2 mice (50%). These results indicate that *C. albicans* alone can initiate the development of oral hyperplasia. However, histologically detectable dysplasia developed only after prolonged exposure to *C. albicans* following pre-treatment with 4NQO as initiator.

Esophageal tissues were also examined for evidence of candidiasis or preneoplastic changes. In group 2 mice, *C. albicans* was observed intraepithelially in 5/7 (71%) mice at 12, 14 and 19 weeks (Table 1), while in group 3, intraepithelial presence of *C. albicans* was observed in 4/7 mice (57%) (Table 1). None of the mice developed esophageal dysplasia. However, 1/2 mice sacrificed at week 14 and 1/2 mice sacrificed at week 19 in group 2 had developed epithelial hyperplasia in esophageal tissues (Table 3).

Immunohistochemical analyses of Ki-67 and p16 expression

The Ki-67 staining pattern in mice treated with 4NQO only was restricted to the basal layer of epithelium (Fig. 4a–c) and was similar to untreated control mice throughout the experimental period (data not shown). In mice treated with 4NQO followed with *C. albicans* challenge, hyperplastic regions showed Ki-67 to be continuously expressed from basal to parabasal layers of epithelium (Fig. 4e). In dysplastic regions, Ki-67 positive cells were seen throughout the entire width of the epithelium from basal to keratin layers (Fig. 4f). At the end of the experimental period, Ki-67 expression in this group (mean $\pm$ SD=63 $\pm$ 5) was significantly higher than expression in mice treated with 4 NQO only (mean $\pm$ SD=24 $\pm$ 3, $P=0.007$) or mice receiving vehicle plus *C. albicans* (mean $\pm$ SD=42 $\pm$ 4, $P=0.02$) (Fig. 4g–i and Fig. 6). Moreover, mice challenged with *C. albicans* only showed increased Ki-67 expression compared to uninfected control groups (mean $\pm$ SD=22 $\pm$ 2) (groups 1 and 4, $P<0.03$).

The p16 staining pattern in mice treated with 4NQO only was restricted to the basal or parabasal layers of epithelium (Fig. 5a–c) and was similar to untreated control mice throughout the experimental period (data not shown). At the end of the experimental period, p16 expression was highest in mice challenged with 4NQO plus *C. albicans* (mean $\pm$ SD=69 $\pm$ 5), compared to *C. albicans* (mean $\pm$ SD=43 $\pm$ 3, $P=0.03$) or 4NQO alone (mean $\pm$ SD=27 $\pm$ 4, $P=0.02$) (Fig. 5d–f and Fig. 6). In addition, mice receiving vehicle followed by *C. albicans*, showed increased p16

expression compared to 4NQO alone group ($P=0.01$) (Fig. 5g–i, Fig. 6). Regardless of hyperplasia or dysplasia, a scattered pattern of p16 staining throughout epithelial layers was observed in groups 2 and 3 (Fig. 5d–i). In summary, at the end of the experimental period, Ki-67 and p16 expression was significantly higher in mice challenged with *C. albicans* when 4NQO used as initiator, when compared to all other groups. This was consistent with the appearance of dysplastic changes in this group.

Discussion and conclusion

It has been shown that when 4NQO is applied to the oral mucosa for a brief time period it functions as an initiator, which suggests that if followed by a promoter (e.g., a phorbol ester) it will lead to neoplastic changes [49]. We found that 4NQO delivered in this manner and followed by a sustained exposure to *C. albicans*, leads to the development of oral dysplastic lesions in immunocompetent mice. Although there are no established biomarkers that reliably predict the transition from oral dysplasia to carcinoma in humans, the combination of histologic findings of dysplasia and higher biomarker scores are generally accepted as accurate prognosticators of malignancy in 4NQO-treated animals [50]. Thus, our histologic observations coupled by increased Ki-67 and p16 immunostaining intensity in dysplastic lesions, provided a clear link to malignant transformation of the tongue mucosa in animals exposed to both 4NQO and *C. albicans*.

In the rat study by O'Grady and Reade [29], where 4NQO was used as initiator and *C. albicans* was used as promoter of oral neoplasia, this organism promoted oral mucosal squamous cell carcinoma, in a similar manner to a known chemical tumor promoter [29]. Although we observed pre-cancerous lesions in our model, we were not able to detect carcinomas, i.e., invasion of atypical cells into the submucosa. These discrepant findings between the two studies could be attributed to several differences in the experimental design. First, we used a much lower 4NQO concentration (20 vs. 5,000 ppm). Second, our total experimental period was much shorter in duration (19 vs. 52 weeks), which may not have provided adequate time for progression of the dysplastic lesions to overt neoplasia. Thirdly, a clinical strain of *C. albicans* was used by O'Grady and Reade, which was different from our ATCC-catalogued strain. Tremendous strain to strain variability exists in the ability of *C. albicans* to establish oral infection in immunocompetent mice [51–53]. In addition, in a study where the ability of *C. albicans* to produce nitrosamines from precursors was tested (postulated to be an indicator of their ability to promote carcinogenesis), wide strain variability was observed [54].

The fact that a commercially available strain (ATCC 90234) was successful in both triggering oral infection and promoting dysplastic changes in our model makes it a valuable tool readily accessible to all investigators. However, based on reports of other oral microorganisms influencing the ability of *C. albicans* to trigger oral infection [55–58], it is possible that polymicrobial interactions similarly influence its ability to promote dysplastic changes. One of the limitations of this model is that these interactions cannot possibly be accounted for since TCN is used to suppress oral flora.

The *in vitro* demonstration of nitrosamine compounds generated by this organism from precursor molecules led some investigators to suggest that *C. albicans* has a direct carcinogenic role, since these compounds have been shown to be carcinogenic in the mouth [54,59]. It is unlikely that a direct, *Candida*-driven, carcinogenesis effect plays a role in the development of dysplasia in our model, since no dysplastic changes were detected in the animals infected with *C. albicans* alone. Rather, we hypothesize that *C. albicans* creates a cell proliferation-conducive environment, leading to clonal expansion of genetically altered cells into histologically identifiable dysplastic foci. The potential role of infection-driven epithelial cell

proliferation is indirectly supported by the finding that 4NQO at this concentration was unable to trigger epithelial cell proliferation, as confirmed by the Ki-67 expression pattern, whereas *C. albicans* alone triggered a significant hyperplastic response in oral epithelium.

Ki-67 is a cell cycle associated nuclear protein, used as proliferation marker and linked with malignancies [41]. Previous studies showed an increase in Ki-67 expression according to the severity of epithelial dysplasia in the upper and lower gastrointestinal tract [60-62]. Our data showed an increase in Ki-67 expression in mice challenged with *C. albicans* alone or in combination with 4NQO, as well as a gradual increase from normal to hyperplastic to dysplastic epithelium. Taken together these data confirm the role of *C. albicans* in the transition of normal to dysplastic epithelium.

Protein p16, is the product of the cyclin dependent kinase inhibitor 2A (*CDKN2A*) gene located at chromosome 9p21 [63,64] and plays a crucial role in the negative regulation of the cell cycle [42,64-66]. Inactivation of p16 by genetic deletion, point mutation or promoter methylation is a common finding in oral epithelial dysplastic and neoplastic lesions, and p16 is thought to play an important role in the early process of neoplastic development [42]. In the present study we found an increase in the expression of p16 in the hyperplastic and dysplastic epithelium. We speculate that this increase in p16 immunoreactivity is secondary to increased cell proliferation. One possibility is that the molecular pathways of *Candida albicans* plus 4NQO-induced dysplasia represent a discrete set of events that does not require p16 inactivation. Indeed HPV-induced oral and cervical neoplastic lesions are characterized by an increase in p16 expression, which occurs via a negative feedback due to HPV-induced phosphorylation of the retinoblastoma protein [67,68]. However, it is also important to consider that the hyperplastic and dysplastic lesions that we observed in this study represent very early events in the neoplastic process, and perhaps if these lesions were allowed to progress to severe dysplastic/invasive neoplasms, we would have observed loss of p16 expression.

In summary we have developed a novel animal model, which can be used to provide critical new insights into the complex role of *C. albicans* infection in the development or progression of oral dysplasia/neoplasia. It is known that tumor suppressor genes, oncogenes, apoptosis genes and inflammatory pathway genes are involved in the development of cancer, but to date no single gene directly related to the oral dysplasia/neoplasia has been identified. This mouse model and its variants with host response abnormalities are undoubtedly well suited to evaluate the role of cell cycle regulatory genes as well as inflammatory, humoral and cellular immune responses in the development of oral dysplasia/neoplasia.

Acknowledgements

We thank Dr T. V. Rajan and Dr Ellen Eisenberg for expert histopathologic advice. This work was supported by USPHS Research Grant R01 DE 13986 to A.D.B. from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892.

Abbreviations

4NQO, 4 Nitroquinoline 1-oxide; DMSO, Dimethyl sulfoxide; ppm, parts per million; H&E, Hematoxylin and Eosin; PAS, Periodic Acid Schiff; TCN, Tetracycline hydrochloride; CFU, Colony Forming Unit; PDD, phorbol-12, 13-didecanoate; SCC, Squamous cell carcinoma.

References

1. Arendorf TM, Walker DM. Oral candidal populations in health and disease. Br Dent J 1979;147:267–272. [PubMed: 393283]

2. Walsh TJ, Merz WG. Pathologic features in the human alimentary tract associated with invasiveness of *Candida tropicalis*. *Am J Clin Pathol* 1986;85:498–502. [PubMed: 3953503]
3. Saltarelli CG, Gentile KA, Mancuso SC. Lethality of *Candida* strains as influenced by the host. *Can J Microbiol* 1975;21:648–654. [PubMed: 1092440]
4. Arendorf TM, Walker DM. The prevalence and Intraoral distribution of *Candida albicans* in man. *Arch Oral Biol* 1980;25:507–538.
5. Samaranayake LP. Oral mycoses in HIV infection. *Oral Surg Oral Med Oral Pathol* 1992;73:171–180. [PubMed: 1549312]
6. Cumming CL, White C, Blackwell CL, Wray D. Denture stomatitis in the elderly. *Oral Microbiol Immunol* 1990;5:82–85. [PubMed: 2087353]
7. Taschdjian CL, Seelig MS, Kozinn PJ. Serological diagnosis of candidal infections. *CRC Crit Rev Clin Lab Sci* 1973;4:19–59. [PubMed: 4614938]
8. Heimdahl A, Nord CE. Oral yeast infections in immunocompromised and seriously diseased patients. *Acta Odontol Scand* 1990;48:77–84. [PubMed: 2181814]
9. Horn R, Wong B, Kiehn TE, Armstrong D. Fungemia in a cancer hospital: changing frequency, earlier onset, and results of therapy. *Rev Infect Dis* 1985;7:646–655. [PubMed: 4059756]
10. Wingard JR, Merz WG, Rinaldi MG, et al. Increase in *Candida krusei* infection among patients with bone marrow transplantation and neutropenia treated prophylactically with fluconazole. *N Engl J Med* 1991;325:1274–1277. [PubMed: 1669837]
11. Bodey GP. Fungal infection and fever of unknown origin in neutropenic patients. *Am J Med* 1986;80:112–119. [PubMed: 3717185]
12. Gold JW. Opportunistic fungal infections in patients with neoplastic disease. *Am J Med* 1984;76:458–463. [PubMed: 6367457]
13. Maksymiuk AW, Thongprasert S, Hopfer R, et al. Systemic candidiasis in cancer patients. *Am J Med* 1984;77:20–27. [PubMed: 6093530]
14. Tang XN, Knudsen B, Bemis D, Tickoo S, Gudas LJ. Oral cavity and esophageal carcinogenesis modeled in carcinogen-treated mice. *Clin Can Res* 2004;10:301–313.
15. Cawson RA. Leukoplakia and oral cancer. *Proc R Soc Med* 1969;62:610–614. [PubMed: 4895896]
16. Nagy KN, Sonkodi I, Szoke I, Nagy E, Newman HN. The microflora associated with human oral carcinomas. *Oral Oncol* 1998;34:304–308. [PubMed: 9813727]
17. Roed-Petersen B, Renstrup G, Pindborg JJ. *Candida* in oral leukoplakias. A histologic and exfoliative cytologic study. *Scand J Dent Res* 1970;78:323–328. [PubMed: 4097885]
18. Renstrup G. Occurrence of *Candida* in oral leukoplakias. *Acta Pathol Microbiol Scand [B] Microbiol Immunol* 1970;78:421–424.
19. Daftary DK, Mehta FS, Gupta PC, Pindborg JJ. The presence of *Candida* in 723 oral leukoplakias among Indian villagers. *Scand J Dent Res* 1972;80:75–79. [PubMed: 4502584]
20. McCullough M, Jaber M, Barrett AW, et al. Oral yeast carriage correlates with presence of oral epithelial dysplasia. *Oral Oncol* 2002;38:391–393. [PubMed: 12076705]
21. Tobacco smoking. Vol. 38. International Agency for Research on Cancer; Lyons, France: 1986. IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans.
22. Alcohol drinking. Vol. 44. International Agency for Research on Cancer; Lyons, France: 1988. IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans.
23. Blot WJ. Invited commentary: more evidence of increased risks of cancer among alcohol drinkers. *Am J Epidemiol* 1999;150:1138–1140. [PubMed: 10588074]
24. Phillips DH. Smoking-related DNA and protein adducts in human tissues. *Carcinogenesis* 2002;23:1979–2004. [PubMed: 12507921]
25. Panigrahi GB, Walker IG. The N2-guanine adduct but not the C8-guanine or N6-adenine adducts formed by 4-nitroquinoline 1-oxide blocks the 3'–5' exonuclease action of T4 DNA polymerase. *Biochemistry* 1990;29:2122–2126. [PubMed: 2109634]
26. Thomas DC, Husain I, Chaney SG, Panigrahi GB, Walker IG. Sequence effect on incision by (A)BC excinuclease of 4NQO adducts and UV photoproducts. *Nucleic Acids Res* 1991;19:365–370. [PubMed: 1901645]

27. Venkat JA, Shami S, Davis K, et al. Relative genotoxic activities of pesticides evaluated by a modified SOS microplate assay. *Environ Mol Mutagen* 1995;25:67–76. [PubMed: 7875128]
28. Tanuma J, Shisa H, Hiai H, et al. Quantitative trait loci affecting 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in the rat. *Cancer Res* 1998;58:1660–1664. [PubMed: 9563479]
29. Grady JF, Reade PC. *Candida albicans* as a promoter of oral mucosal neoplasia. *Carcinogenesis* 1992;13:783–786. [PubMed: 1586990]
30. Hawkins BL, Heniford BW, Ackermann DM, et al. 4NQO carcinogenesis: a mouse model of oral cavity squamous cell carcinoma. *Head Neck* 1994;16:424–432. [PubMed: 7960739]
31. Steidler NE, Reade PC. Experimental induction of oral squamous cell carcinomas in mice with 4-nitroquinolone-1-oxide. *Oral Surg Oral Med Oral Pathol* 1984;57:524–531. [PubMed: 6427721]
32. Yuan B, Heniford BW, Ackermann DM, Hawkins BL, Hendler FJ. Harvey ras (H-ras) point mutations are induced by 4-nitroquinoline-1-oxide in murine oral squamous epithelia, while squamous cell carcinomas and loss of heterozygosity occur without additional exposure. *Cancer Res* 1994;54:5310–5317. [PubMed: 7923158]
33. de Repentigny L. Animal models in the analysis of *Candida* host-pathogen interactions. *Curr Opin Microbiol* 2004;7:324–329. [PubMed: 15288620]
34. Cantorna MT, Balish E. Mucosal and systemic candidiasis in congenitally immunodeficient mice. *Infect Immun* 1990;58:1093–1100. [PubMed: 2180820]
35. Sundstrom P, Balish E, Allen CM. Essential role of the *Candida albicans* transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice. *J Infect Dis* 2002;185:521–530. [PubMed: 11865405]
36. Deslauriers N, Coulombe C, Carre B, Goulet JP. Topical application of a corticosteroid destabilizes the host-parasite relationship in an experimental model of the oral carrier state of *Candida albicans*. *FEMS Immunol Med Microbiol* 1995;11:45–56. [PubMed: 7599602]
37. Balish E, Balish MJ, Salkowski CA, Lee KW, Bartizal KF. Colonization of congenitally athymic, gnotobiotic mice by *Candida albicans*. *Appl Environ Microbiol* 1984;47:647–652. [PubMed: 6372689]
38. Rahman D, Mistry M, Thavaraj S, Challacombe S, Naglik J. Murine model of concurrent oral and vaginal *Candida albicans* colonization to study epithelial host-pathogen interactions. *Microbes Infect* 2007;9:615–622. [PubMed: 17383212]
39. Turner JR, Butler TF, Johnson ME, Gordee RS. Colonization of the intestinal tract of conventional mice with *Candida albicans* and treatment with antifungal agents. *Antimicrob Agents Chemother* 1976;9:787–792.
40. Ide F, Oda H, Nakatsuru Y, et al. Xeroderma pigmentosum group A gene action as a protection factor against 4-nitroquinoline 1-oxide-induced tongue carcinogenesis. *Carcinogenesis* 2001;22:567–572. [PubMed: 11285190]
41. Schlifter C, Duchrow M, Wohlenberg C, et al. The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. *J Cell Biol* 1993;123:513–522. [PubMed: 8227122]
42. Goodger NM, Gannon J, Hunt T, Morgan PR. Cell cycle regulatory proteins – an overview with relevance to oral cancer. *Oral Oncol* 1997;33:61–73. [PubMed: 9231162]
43. Jones JH, Russell C, Young C, Owen D. Tetracycline and the colonization and infection of the mouths of germ-free and conventionalized rats with *Candida albicans*. *J Antimicrob Chemother* 1976;2:247–253.
44. Kamai Y, Kubota M, Kamai Y, et al. New model of oropharyngeal candidiasis in mice. *Antimicrob Agents Chemother* 2001;45:3195–3197. [PubMed: 11600377]
45. Clemons KV, Gonzalez GM, Singh G, et al. Development of an orogastrointestinal mucosal model of candidiasis with dissemination to visceral organs. *Antimicrob Agents Chemother* 2006;50:2650–2657. [PubMed: 16870754]
46. Bouquot JE, Speight PM, Farthing PM. Epithelial dysplasia of the oral mucosa-Diagnostic problems and prognostic features. *Current Diagnostic Pathology* 2006;12:11–21.
47. Vairaktaris E, Spyridonidou S, Papakosta V, et al. The hamster model of sequential oral oncogenesis. *Oral Oncol* 2008;44:315–24. [PubMed: 18061531]

48. Louis DR, Martine P, Leo-Gilles M. Gastrointestinal colonization and systemic dissemination by *Candida albicans* and *Candida tropicalis* in intact and immunocompromised mice. *Infect Immun* 1992;60:4907–4914. [PubMed: 1399001]
49. Steidler NE, Reade PC. Initiation and promotion of experimental oral mucosal carcinogenesis in mice. *J Oral Pathol* 1986;15:43–47. [PubMed: 3080566]
50. Marilena V, Noam Y, Dan D. 4NQO oral carcinogenesis: animal models, molecular markers and future expectations. *Oral Oncol* 2005;41:337–339. [PubMed: 15792604]
51. Allen CM, Saffer A, Meister RK, Beck FM, Bradway S. Comparison of a lesion-inducing isolate and a non-lesional isolate of *Candida albicans* in an immunosuppressed rat model of oral candidiasis. *J Oral Pathol Med* 1994;23:133–139. [PubMed: 7517452]
52. Balish E, Filutowicz J, Oberley TD. Correlates of cell-mediated immunity in *C. albicans* colonized gnotobiotic mice. *Infect Immun* 1990;58:107–113. [PubMed: 2403528]
53. Deslauriers N, Cote L, Montplaisir S, de Repentigny L. Oral carriage of *Candida albicans* in murine AIDS. *Infect Immun* 1997;65:661–667. [PubMed: 9009328]
54. Krogh P, Holmstrup P, Thorn JJ, Vedtofte P, Pindborg JJ. Yeast species and biotypes associated with oral leukoplakia and lichen planus. *Oral Surg Oral Med Oral Pathol* 1987;63:48–54. [PubMed: 3543797]
55. Bunetel L, Bonnaure-Mallet M. Oral pathoses caused by *Candida albicans* during chemotherapy: update on development mechanisms. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1996;82:161–165. [PubMed: 8863305]
56. Nseir S, Jozefowicz E, Cavestri B, et al. Impact of antifungal treatment on *Candida-Pseudomonas* interaction: a preliminary retrospective case-control study. *Intensive Care Med* 2007;33:137–142. [PubMed: 17115135]
57. Hogan DA, Kolter R. *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* 2002;296:2229–2232. [PubMed: 12077418]
58. Thein ZM, Samaranayake YH, Samaranayake LP. Effect of oral bacteria on growth and survival of *Candida albicans* biofilms. *Arch Oral Biol* 2006;51:672–680. [PubMed: 16620775]
59. Farrelly JG, Stewart ML, Saavedra JE, Lijinsky W. Relationship between carcinogenicity and *in vitro* metabolism of nitrosomethyl-ethylamine, nitrosomethyl-N-butylamine, and nitrosomethyl-(2-phenylethyl)amine labeled with deuterium in the methyl and α -methylene positions. *Cancer Res* 1982;42:2105–2109. [PubMed: 7074592]
60. Jordan RC, Bradley G, Slingerland J. Reduced levels of the cell-cycle inhibitor p27Kip1 in epithelial dysplasia and carcinoma of the oral cavity. *Am J Pathol* 1998;152:585–590. [PubMed: 9466585]
61. Radović S, Vukobrat-Bijedić Z, Selak I, Babić M. Expression of p53, bcl-2, and Ki-67 proteins in the inflammatory regenerative and dysplastic epithelial lesions of flat colonic mucosa. *Bosn J Basic Med Sci* 2006;6:39–45. [PubMed: 16533178]
62. Zhao J, Guo B, Ma SC, Zhou XD. Expression of p53 and Ki-67 genes in epithelial dysplasia from old oral mucosa and clinical significance. *Sichuan Da Xue Xue Bao Yi Xue Ban* 2005;36:689–691. [PubMed: 16235539]
63. Kamb A, Gruis NA, Weaver-Feldhaus J, et al. A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994;264:436–440. [PubMed: 8153634]
64. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993;366:704–707. [PubMed: 8259215]
65. Yeudall WA, Jakus J. Cyclin kinase inhibitors add a new dimension to cell cycle control. *Eur J Cancer B Oral Oncol* 1995;31:291–298. [PubMed: 8704644]
66. Sugerman PB, Joseph BK, Savage NW. The role of oncogenes, tumour suppressor genes and growth factors in oral squamous cell carcinoma: a case of apoptosis versus proliferation. *Oral Dis* 1995;1:172–188. [PubMed: 8705824]
67. Klaes R, Friedrich T, Spitkovsky D, et al. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer* 2001;92:276–84. [PubMed: 11291057]
68. Cunningham LL Jr, Pagano GM, Li M, et al. Overexpression of p16INK4 is a reliable marker of human papillomavirus-induced oral high-grade squamous dysplasia. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;102:77–81. [PubMed: 16831676]

Fig. 1.
(a) Intervention schedule; (b) sacrifice schedule.

Fig. 2. Esophageal mucosa of mice at 10 weeks after initiation of the experiment. (a) untreated mice; (b) mice receiving 4NQO followed by *Candida albicans*; and (c) mice receiving vehicle control plus *C. albicans* (200 × magnification, PAS stain).

Fig. 3. PAS-stained tongue sections of mice treated with 4 NQO (a–c); 4NQO+*C. albicans* (d–f) or vehicle+*Candida albicans* (g–i). Epithelial dysplasia (arrows) was noted after 19 weeks in *C. albicans*-infected animals exposed to 4NQO (200×magnification, PAS stain).

Fig. 4.

Immunohistochemical detection of Ki-67 expression in mouse tongues: (a–c) 4NQO-treated mice showing positive cells restricted to the basal layer of epithelium; (d–f) 4NQO plus *Candida albicans*-treated mice showing increased expression of Ki-67 protein distributed from basal to superficial layers of epithelium (arrows); (g–i) *C. albicans*-infected mice showing increased Ki-67 expression primarily in basal and parabasal layers (200×magnification).

Fig. 5. Immunohistochemical detection of p16 expression in mouse tongues: (a–c) 4NQO-treated mice; (d–f) 4NQO plus *Candida albicans*-treated mice showing increased, scattered expression of P16 throughout the epithelium; (g–i) Similar pattern of p16 expression in mice treated with vehicle plus *C. albicans* (200×magnification).

Fig. 6.
Comparison of Ki-67 and p16 expression in different groups of mice at the end of the experiment (19 weeks).

Table 1
Proportion of animals with histological evidence of oroesophageal candidiasis.

Groups	12 weeks		14 weeks		19 weeks	
	Tongue	Esophagus	Tongue	Esophagus	Tongue	Esophagus
(2) 4NQO+C. albicans	3/3	2/3	2/2	1/2	2/2	2/2
(3) C. albicans	3/3	2/3	1/2	1/2	1/2	1/2

Table 2

Average CFU per gram of tongue tissue in *Candida albicans*-infected groups.

Group number	logCFU/ g of tissue (mean±SD)		
	12 weeks	14 weeks	19 weeks
(2) 4NQO+ <i>C. albicans</i>	6.7±0.2	5.4±0.1	4.7±0.2
(3) <i>C. albicans</i>	6.2±0.1	5.5±0.4	3.2±0.3

Table 3
Proportion of animals with histological evidence of oroesophageal hyperplasia or dysplasia.

Groups	12 weeks				14 weeks				19 weeks			
	Hyperplasia		Dysplasia		Hyperplasia		Dysplasia		Hyperplasia		Dysplasia	
	T*	E**	T	E	T	E	T	E	T	E	T	E
Group 1: 4NQO	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Group 2: 4NQO+C. <i>albicans</i>	2/3	0/3	0/3	0/3	2/2	1/2	0/2	0/2	2/2	1/2	2/2	0/2
Group 3: <i>C. albicans</i>	2/3	0/3	0/3	0/3	1/2	0/2	0/2	0/2	1/2	0/2	0/2	0/2
Group 4: Untreated	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

* T, Tongue
** E, Esophagus.