Oral Oncology 51 (2015) 139–145

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Oral *Candida* colonization in oral cancer patients and its relationship with traditional risk factors of oral cancer: A matched case-control study

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ARTICLE INFO

Article history:
Received 21 June 2014
Received in revised form 2 November 2014
Accepted 12 November 2014
Available online 9 December 2014

Keywords:
Oral cancer
Candida albicans
Acetaldehyde
Risk factors
Case-controlled study
Squamous cell carcinoma

SUMMARY

Objectives: Candida, an opportunistic fungal pathogen, has been implicated in oral and oesophageal cancers. This study aimed to examine oral *Candida* carriage in 52 oral cancer patients and 104 age-, gender- and denture status-matched oral cancer-free subjects.
Material and methods: We assessed general health, smoking and alcohol drinking habits, use of alcohol-containing mouthwash and periodontal status (community periodontal index of treatment needs). Yeasts were isolated using oral rinse technique and genetically identified via Real-Time PCR-High resolution melting curve analysis of conserved ribosomal DNA. Conditional and binary logistic regressions were used to identify explanatory variables that are risk factors for oral cancer.
Results and conclusion: The frequencies of oral yeasts' presence and high oral colonization were significantly higher in oral cancer than non-oral cancer patients ($p = 001$; $p = 0.033$, respectively). No significant difference in the isolation profile of *Candida* species was found between the two groups, except *C. parapsilosis* was more frequent in non-oral cancer group. Differences were noticed in the incidence of *C. albicans* strains where significantly more *C. albicans* genotype-A was isolated from cancer patients and significantly more *C. albicans* genotype-B isolated from non-cancer patients. Multiple regression analyses showed significant association with cancer observed for alcohol drinking (OR = 4.253; 95% CI = 1.351, 13.386), *Candida* presence (OR = 3.242; 95% CI = 1.505, 6.984) and high oral colonization (OR = 3.587; 95% CI = 1.153, 11.162). These results indicate that there is a significant association between oral cancer occurrence and *Candida* oral colonization and that the observed genotypic diversity of *C. albicans* strains may play a role in oral carcinogenesis.

Introduction

Oral cancer, is the eighth most diagnosed cancer in Victorians in 2009 [1] and globally it ranks the 8th and the 13th most common malignancies for males and females, respectively [2]. The five-year survival rates are poor with 50% achieved only with optimal care [3]. Although the life quality for patients has been improved by surgical advances, overall mortality has not changed in the last 50 years [4]. Tobacco use and heavy alcohol consumption has been strongly associated with oral cancer [5]. Other postulated risk factors include poor oral hygiene [6], viral infections [7] and *Candida albicans* infections. *Candida albicans* has been associated with leukoplakic lesions and the presence of *Candida* species has been recognized as an independent risk factor for oral carcinoma [8].

Candida can cause a spectrum of oral mucosal lesions including chronic hyperplastic candidosis, (candidal leukoplakia). It has been postulated that this variant of oral candidosis carries a significant risk of malignant transformation [9,10]. During the last four decades, increasing clinical and experimental evidence suggests a putative role for *Candida* in the multi-steps process of oral mucosal carcinogenesis. An aetiological role of *Candida* in causing progression to carcinoma was first suggested by Cawson in 1966 [11] with later reports suggesting a link between presence of *C. albicans* and oral squamous cell carcinoma [12–15]. The most significant evidence is the increase in the frequency of *C. albicans* in biofilms from oral squamous cell carcinoma tumor sites compared to control areas [16] and the correlation between oral yeast carriage and the presence and degree of oral epithelial dysplastic and neoplastic changes [17].

To date there has not been a matched case-control study assessing all potential variables confounding oral cancer risk particularly

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http://dx.doi.org/10.1016/j.oraloncology.2014.11.008
1368-8375/

regarding oral *Candida* colonization in oral cancer patients. Thus, this study aimed to firstly: isolate, identify and genotype oral yeasts from oral cancer patients and matched oral cancer-free subjects. Second, this study aimed to elucidate the association, if any, of oral *Candida* colonization with other known risk factors for oral cancer. We hypothesized that certain species, or strains, of *Candida* are associated with the presence of oral cancer and that *Candida* oral carriage and high level of colonization are risk factors in oral cancer.

Materials and methods

Patient samples

Ethical approval was granted by the Human Research Ethics Committees of Melbourne Health (approval number 2012.133), Dental Health Services of Victoria (approval number 254) and Melbourne University (ethics ID 1238737). All patients gave informed consent prior to being enrolled in the present study. A total of 52 consecutive oral cancer patients and 104 age (±three years), gender and denture status (with or without denture) matched non-oral cancer control subjects were enrolled from October 2012 to September 2013. Oral cancer subjects were attending the Head and Neck Oncology Tumour Stream at the Royal Melbourne Hospital; whereas non-oral cancer subjects were recruited from the Oral Medicine, Periodontal, Endodontic, and Prosthodontic clinics at the Royal Dental Hospital of Melbourne.

Based on a previous observational study [17], the expected proportions of oral cancer and oral cancer-free individuals with *Candida* were 0.7 and 0.3, respectively. Sample size calculation based on matched pairs and the methods described in Case-Control Studies: Design, Conduct, Analysis, [18] showed that the sample size was more than adequate to detect this size of effect.

A standardised questionnaire was used for general health (diabetes, hypertension, rheumatic fever, prosthetic heart valve, epilepsy, psychiatric treatment, tuberculosis, asthma, hepatitis, kidney disease, HIV infection, HPV infection, bleeding disorders and joint problems), smoking and alcohol drinking habits, and the use of alcohol-containing mouthwash. Periodontal status was assessed using community periodontal index of treatment needs according to the WHO [19].

Inclusion criteria for the study were newly diagnosed oral squamous cell carcinoma patients who had not undergone any treatment (i.e. surgery, radiotherapy and chemotherapy) and non-oral cancer patients with no oral mucosal abnormalities. The exclusion criteria were patients with recent use (less than 2 weeks) of antifungal containing mouthwashes or medications.

Each patient received a detailed oral examination that also included recording of the presence or absence of dentures, clinical evidence of oral candidosis or any other mucosal abnormalities. Oral rinses were obtained to determine the presence and degree of oral *Candida* colonization.

Oral rinses and determination of colony forming unit (cfu)/ml

The oral rinse technique [20] with patients rinsing with 10 ml of sterile saline for at least one min was used. Each mouth rinse was vortexed for 30 s and 100 μl spread evenly on Sabouraud Dextrose Agar (SDA; Department of Microbiology and Immunology, Media preparation unit, The University of Melbourne, Melbourne, Vic., Australia) plate containing chloramphenicol and incubated for 48 h at 37 °C. Two tenfold serial dilutions (in sterile normal saline) were plated onto SDA/chloramphenicol plates and incubated for 48 h at 37 °C to assess the degree of colonization. Non-identical, single colonies were isolated and re-cultured to confirm their identity.

Identification of Candida species

All isolates were phenotypically classified into different *Candida* species using CHROMagar *Candida* medium (Department of Microbiology and Immunology, Media preparation unit, The University of Melbourne, Melbourne, Vic., Australia). One hundred microliters of undiluted oral rinse was inoculated onto CHROMagar plate, incubated for 48 h at 37 °C and then presumptively identified by colony color evaluation. Further, all clinical isolates were identified at species and subspecies level using the high resolution melting curve assay (HRMA) described in our previous study [21]. Briefly, type *Candida* species and strains previously assessed [22] were used as reference strains and genomic DNA was extracted using Master Pure Yeast DNA Purification kit (Epicentre, NSW, Australia). Subsequently, real time polymerase chain reaction (PCR)-HRMA analysis using primers spanning internal transcribed spacer regions (ITS1 and ITS2) in ribosomal DNA (rDNA) was conducted to characterize *Candida* isolates at the species level [21]. At the sub-species level, primers spanning the regions that include the site of the group 1 transposable intron of the 25S rDNA were used to study the genetic diversity of *C. albicans* as previously described [23]. According to this method, *C. albicans* can be classified into four distinct genotypes based on the PCR amplification product, namely, genotype A (450 bp), B (840 bp), C (450 and 840 bp) and D (1080 bp; *C. dubliniensis*) [23].

Statistical analysis

Results were analysed using SPSS software (PASW Statistics GradPack 18 Inc., USA). Kruskal Wallis test was used to assess intra-group variation in colony forming units and nonparametric chi-square statistics and Fisher's exact test were initially used for testing differences between patients and controls. Differences among or between groups were assumed to be significant when the probability (P) was less than or equal to 0.05.

As this study was a matched case-control study where each oral cancer patients was age-, gender- and denture status-matched with two controls, conditional logistic regression using Cox regression models were used to identify explanatory variables that were risk factors for oral cancer. The primary explanatory variables of interest were those related to the presence of *Candida*. The presence of *Candida* was measured in two different ways with binary variables indicating *Candida* presence or absence, then, separately, the presence or absence of *C. albicans*. These two measures of oral yeast are related, and so could not be included in the same Cox regression model. Thus, two separate models were conducted, one for the presence/absence of all *Candida* species and another for the presence/absence of *C. albicans*.

To assess the independent risk effect of the level of oral colonization, a further binary logistic regression analysis was conducted. The mean cfu/ml of mouth swill obtained from all patients was used as a cut-off point for the level of colonization with below the mean cfu/ml indicating low colonization and above the mean was considered as high colonization.

In addition to the above oral yeast factors, the effect of the following explanatory variables was assessed: the presence of any medical problem; smoking; alcohol drinking; alcohol containing mouthwash use; the presence of periodontal disease. Patients who are fully edentulous (with or without full denture) were classed as having "no periodontal disease".

Smoking, alcohol drinking and use of alcohol containing mouthwash behaviors were defined for statistical analyses. Firstly, a "current smoker" were those who reported smoking on a daily basis within the previous 12 months; a "past smoker" an individual who had discontinued smoking for more than 12 months prior to the interview; "non-smokers" those who had never smoked. As