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***Candida* virulence and ethanol-derived acetaldehyde production in oral cancer and non-cancer subjects**

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Running head: *Candida* virulence and oral cancer

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Key words: Oral cancer, *Candida albicans*, Microbial-induced carcinogenesis, Carcinogens, Acetaldehyde

Abstract

Objectives: To compare biofilm-forming ability; hydrolytic enzymes and ethanol-derived acetaldehyde production of oral *Candida* isolated from the oral cancer and matched non-oral cancer patients.

Material and Methods: Fungal biofilms were grown in RPMI-1640 medium and biofilm-mass and -activity were assessed using crystal-violet staining and XTT-salt reduction assays, respectively. Phospholipase, proteinase and esterase production was measured using agar plate method, while fungal acetaldehyde production was assessed via gas-chromatography.

Results: *Candida* isolated from oral cancer patients demonstrated significantly higher biofilm mass ($p=0.031$), biofilm metabolic activity ($p<0.001$), phospholipase ($p=0.002$) and

proteinas (p=0.0159) activity than isolates from non-oral cancer patients. High ethanol-derived acetaldehyde-producing *Candida* were more prevalent in oral cancer than non-oral cancer patients (p=0.01). In univariate regression analysis high biofilm mass (p=0.03) and metabolic activity (p<0.001), high phospholipase (p=0.003) and acetaldehyde production ability (0.01) were significant risk factors for oral cancer; while in the multivariate regression analysis high biofilm activity (0.01) and phospholipase (p=0.01) were the only significantly positive influencing factors on oral cancer.

Conclusion: These data suggest a significant positive association between the ability of *Candida* isolates to form biofilms, to produce hydrolytic enzymes and to metabolize alcohol to acetaldehyde with their ability to promote oral cancer development.

Introduction

There has been cumulative clinical and laboratory evidence to support a putative role for oral yeasts in oral and oesophageal carcinoma (Field *et al.*, 1989; Hooper *et al.*, 2009). However, the underlying mechanisms linking *Candida* colonization/infection with oral squamous cell carcinoma remains contentious (Mohd Bakri *et al.*, 2010). The apparent epidemiological links between microbial colonization/infection and cancer presence have indirectly implied an aetiological role for these microorganisms (McCullough *et al.*, 2002) which has prompted the study of probable mechanisms by which microbial species may initiate or promote oral carcinogenesis. Apart from *Candida*'s ability to produce direct carcinogens such as

nitrosamine (Hooper *et al.*, 2009), other mechanisms such as the induction of chronic inflammatory cytokines and the metabolism of ethanol to the carcinogenic acetaldehyde, may be involved in *Candida*-related oral carcinogenesis.

Evidence is building that chronic exposure to microorganisms and/or their products, such as endotoxins (lipopolysaccharides) and enzymes (e.g. proteases, collagenases, fibrinolysin, phospholipase), are toxic to host cells and may indirectly induce mutations or change signaling pathways that influence cell proliferation and/or survival of epithelial cells (Lax, 2005; Kurago *et al.*, 2008). *Candida* biofilm forming ability and hydrolytic enzyme production are essential virulence factors that enable this fungus to survive many challenging oral conditions and to adhere to, or invade the underlying host tissues (Calderone and Fonzi, 2001; Ramage *et al.*, 2012). Hence, the chronic mucosal/epithelial colonization by *Candida* with enhanced virulence properties may have a role in oral carcinogenesis.

Acetaldehyde (ACH), the first metabolite of ethanol metabolism, has been found to facilitate the formation of mutagenic acetaldehyde DNA-adducts in clinically relevant concentrations (40- 100 μ M) (Brooks and Theruvathu, 2005; Seitz and Stickel, 2007; Nieminen *et al.*, 2009). *Candida* has been postulated to have a direct carcinogenic role through its ability to convert ethanol into carcinogenic acetaldehyde, which may explain the highly significant epidemiological association between oral *Candida* carriage, alcohol drinking and oral cancer presence reported in our previous investigation (Alnuaimi *et al.*, 2015).

To date, no study has simultaneously examined the association between *Candida* virulence traits, its ability to metabolize alcohol to the carcinogen acetaldehyde and the presence of oral cancer. Therefore, this study mainly aimed to compare the *in vitro* biofilm forming ability, major hydrolytic enzyme (proteinase, phospholipase and esterase) and ethanol-derived acetaldehyde production of *Candida* yeasts previously isolated from oral cavities of oral cancer and matched non-oral cancer patients. The association of these fungal virulence factors with other patients' factors was also examined. Further, this study aimed to compare these virulence attributes in different *Candida* species and *C. albicans* genotypes. This might help to explore additional mechanisms through which this yeast may initiate/promote oral mucosal carcinogenesis.

Methods

Organisms

The organisms used in the present study were obtained from a larger case-control survey investigating oral *Candida* colonization in oral cancer patients and its association with other risk factors of oral cancer (Alnuaimi *et al.*, 2015). Ethical approval was granted, as previously reported, and all patients gave informed consent prior to being enrolled in the present study (Alnuaimi *et al.*, 2015). Of the 156 patients [52 oral cancer patients and 104 age ($\pm$ three years), gender and denture status (with or without denture) matched non-oral cancer patients], 80 patients had *Candida* growth in their oral cavity. Thirty six (69.2%) of oral cancer patients and 44 (42.3%) of non-oral cancer patients were found to be carriers for this yeast (Alnuaimi *et al.*, 2015). CHROMagar *Candida* medium and real time PCR-high resolution melting curve analysis were used to characterize these isolates at the species and sub-species level (Alnuaimi *et al.*, 2014). According to these methods, a total of eight *Candida* species and 90 strains were identified from both patients groups. The distribution of these isolates is presented in Table 1. At the subspecies level, only the species *C. albicans* was broadly genotyped via the ABC genotypic method based on the size and presence of transposable intron in the 25S of ribosomal DNA into *C. albicans* genotype A, genotype B and genotype C (Table 1) (McCullough *et al.*, 1999; Alnuaimi *et al.*, 2014; Alnuaimi *et al.*, 2015).

Preparation of yeast suspension and biofilm growth

Biofilms were prepared as previously described (Silva *et al.*, 2010; Alnuaimi *et al.*, 2013), except that yeasts cells were suspended in RPMI 1640 medium (pH=7 and 0.2% glucose) supplemented with L-glutamine and buffered with MOPS [3-(N-morpholino)-propanesulfonic acid]; (Department of Microbiology and Immunology, Media preparation unit, The University of Melbourne, Australia). Experiments were carried out in triplicate on two separate occasions for each strain with uninoculated controls. Biofilm mass and metabolic activity was assessed using crystal violet and tetrazolium salt reduction assays, respectively, and as previously described (Silva *et al.*, 2010; Alnuaimi *et al.*, 2013).

Hydrolytic enzymes production assay

Enzymatic activities of all *Candida* isolates were tested in triplicate using the agar plate method (Price *et al.*, 1982; Treviño-Rangel Rde *et al.*, 2013). All test media were prepared at the Department of Microbiology and Immunology, Media preparation unit, The University of Melbourne, Australia.

Aspartyl proteinase activity was assayed using YCB-BSA test medium which was prepared according to previously published protocol (Chakrabarti *et al.*, 1991). Phospholipase activity was evaluated using the egg yolk agar plate (Ghannoum, 2000); while esterase activity was determined using the tween 80 opacity test medium (Slifkin, 2000; Kumar *et al.*, 2006).

A single drop (5 μ L) of un-inoculated RPMI medium and three separate drops (5 μ L each) of RPMI 1640 medium containing overnight *Candida* growth at concentration of 1×10^7 cfu/mL were spotted carefully to outer quadrants of plate containing the specific agar medium. The plates were incubated at 37°C for 5 days for aspartyl proteinase and phospholipase assays and for 10 days for esterase assay. After incubation period, the plates were inspected for zones of degradation and/or precipitation around the yeast colonies. Results were expressed as: (colony diameter plus the precipitation halo/the colony diameter)-1. According to this formula, a zero value indicates no activity (no increase in the total diameter of colony and precipitation zone), while a value above zero means a positive enzymatic activity.

Ethanol-derived acetaldehyde production assay

The methods used herein for ethanol-derived acetaldehyde production are similar to those reported earlier by Nieminen *et al.* (2009) and Gainza-Cirauqui *et al.* (2013) (Nieminen *et al.*, 2009; Gainza-Cirauqui *et al.*, 2013). Yeasts cells were suspended in phosphate buffered saline (PBS) at a final density of 1×10^7 cells/mL via a haemocytometer. Five hundred microliters of this cells suspension was transferred into gas chromatography vials (Grace, Victoria, Australia) containing 500 μ L of ethanol at final concentration of 11 mM. The vials were immediately closed with screw caps (Grace, Victoria, Australia) and incubated, aerobically, at 37°C for 30 minutes. Subsequently, the reaction was stopped by injecting 100 μ L of Perchloric acid (Sigma-Aldrich, NSW, Australia) at final concentration of (0.6 M) through the silicone septum of the test vials. Every isolate was tested in triplicate on two separate occasions and the mean was used for analysis. To measure the baseline ACH, 100 μ L of Perchloric acid was first added to the yeast suspension (500 μ L) and ethanol (500 μ L) was injected afterward. Subsequently, the suspension was incubated at 37°C for 30 minutes. The ACH formed was measured by a gas chromatograph (GC; GC-2010, Shimadzu Co., Kyoto, Japan) as previously described (Ramdzan *et al.*, 2013). Acetaldehyde concentration

was calculated by subtracting values of control samples from that of actual yeast samples. The concentration of ethanol used in the present study is considered clinically pertinent, as it can be detected in blood and saliva following moderate alcohol consumption (Nieminen *et al.*, 2009).

Statistical analysis

Data were analysed by using SPSS (PASW Statistics GradPack 18 Inc., USA) and GraphPad Prism 6 softwares (GraphPad software Inc., USA). The distribution of data for biofilm formation, hydrolytic enzymes and acetaldehyde for these isolates failed the Shapiro-Wilks test of normal distribution ($p < 0.05$). Therefore, data were analysed using Mann-Whitney test and the association between *Candida* virulence attributes and different patients' factors (oral cancer presence, medical problems, gender, smoking, alcohol drinking, alcohol-containing mouthwash use, level of *Candida* colonization, presence of periodontal disease and denture use) was evaluated using Chi square or Fisher's exact tests. Detailed patients' factors such as smoking, drinking habits, tumor site and others can be found in our recent investigation (Alnuaimi *et al.*, 2015) and the association between these patients' factors and *Candida* virulence attributes can be found in table 2. Logistic Regression model for oral cancer presence, with *Candida* virulence factors and colonization, smoking and alcohol drinking as explanatory variables, was performed as well. For biofilm formation and hydrolytic enzyme, the median was used to divide the isolates into high and low biofilm or hydrolytic enzyme producers. For the acetaldehyde production, a cut-off point of 40 μM (Brooks and Theruvathu, 2005; Seitz and Stickel, 2007; Nieminen *et al.*, 2009) was chosen to divide the isolates into high or low acetaldehyde producers. There was a mean of 567 cfu/mL obtained from all *Candida* isolates carriers and this was used as "internal cut-off" limit to assess the degree of candidal colonization amongst different patients (Alnuaimi *et al.*, 2015). In patients colonized by more than one *Candida* species or strain, only the isolates with the highest virulence value was considered as a measure of yeast virulence in those patients. Two-sided P less than 0.05 was considered statistically significant.

Results

Biofilm formation

Analysis of all 90 *Candida* isolates showed that the median biofilm mass and biofilm metabolic activity/biofilm was 1.86 and 0.38, respectively. The frequency of high biofilm mass (CV absorbance > 1.86) and high biofilm metabolic activity (XTT absorbance > 0.38) was significantly ($p=0.031$, $p=0.0001$, respectively) higher amongst isolates from oral cancer patients [$n=25/36$, (69%), $n=28/36$, (77.7%), respectively] than non-oral cancer patients' isolates [$n=20/44$, (45.4%), $n=14/44$, (31.8%), respectively] (Table 2). However, Mann-Whitney test analysis showed that only biofilm metabolic activity was significantly ($p<0.0001$) higher in the oral cancer than the non-oral cancer patients' isolates (Figure 1B) and the difference was not significant in biofilm mass (Figure 1A).

Significant inter-species variation was observed in biofilm mass and metabolic activity with significantly higher frequency of high biofilm mass and metabolic activity ($p<0.0001$, $p=0.002$, respectively) seen in isolates from *C. albicans* species [$n=42/63$, (66.6%), $n=39/63$, (61.9%), respectively] versus non-*albicans Candida* species [$n=4/27$, (14.8%), $n=7/27$, (25.9%), respectively] (Table 2). Absence of denture was significantly ($p=0.048$) associated with high biofilm mass; whereas smoking and the absence of periodontal disease were significantly ($p=0.019$, $P=0.044$, respectively) associated with high biofilm metabolic activity (Table 2).

At *C. albicans* species level, comparing biofilm formation of *C. albicans* isolates from the two patient's cohorts indicated significantly ($p<0.0001$) higher biofilm metabolic activity in the oral cancer than non-oral cancer *C. albicans* isolates (Figure 2B). No significant difference was found in biofilm mass of this species in both patients groups (Figure 2A).

At the subspecies level, the median biofilm mass and metabolic activity/biofilm for *C. albicans* isolates was 2 and 0.4, respectively. Dividing *C. albicans* isolates into high and low biofilm mass and metabolic activity producers revealed no significant association between biofilm formation and *C. albicans* genotype (Table 2). However, analysis by Mann-Whitney test showed a significantly ($p=0.014$) higher biofilm mass for *C. albicans* genotype A than *C. albicans* genotype B, with no significant difference seen in biofilm metabolic activity between the two genotypes (Figure 3A and 3B).

Hydrolytic enzyme production

In a similar categorization fashion to that of biofilm formation, all 90 *Candida* isolates were divided into high and low hydrolytic enzyme producers according to the median proteinase activity, median phospholipase activity and median esterase activity (1.4, 0.8 and 0.88, respectively). The frequency of high phospholipase producers was significantly ($p=0.002$) higher in the oral cancer [$n=27/36$, (75%)] than the non-oral cancer isolates [$n=18/44$, (40%)] (Table 2). This was supported by Mann-Whitney analysis where *Candida* isolates from oral cancer patients expressed higher ($p=0.005$) phospholipase level than those from non-oral cancer patients (Figure 1D). Whereas no significant difference was revealed by Mann-Whitney analysis in the expression of proteinase and esterase enzymes between oral cancer and non-oral cancer patients' isolates (Figure 1C and 1E, respectively). There was a significant ($p=0.001$) association between high esterase activity and the absence of periodontal disease. No other differences ($p>0.05$) were observed in hydrolytic enzyme production of isolates from any of the 80 *Candida* carriers evaluated in this study (Table 2).

Significant inter- and intra-species differences were also observed in the level of hydrolytic enzyme production with significantly higher number of high phospholipase and esterase enzyme producers ($p<0.0001$, $p=0.011$, respectively) seen in *C. albicans* isolates [$n=46/63$, (73%), $n=37/63$, (58.7%), respectively] versus non-*albicans Candida* isolates [$n=8/27$, (29.6%), $n=8/27$, (42.1%), respectively] and significantly ($P=0.038$) higher number of high proteinase producers was observed in the non-*albicans Candida* isolates [$n=18/27$, (66.6%)] versus *C. albicans* isolates [$n=27/63$, (42.8%)] (Table 2).

At *C. albicans* species level, Mann-Whitney analysis showed significantly ($p=0.015$) higher proteinase activity in *C. albicans* isolates from oral cancer patients than those from non-oral cancer patients and significantly ($p=0.042$) higher esterase activity in isolates from non-oral cancer patients than those from oral cancer patients (Figures 2C and 2E). No difference was observed in phospholipase enzyme production by *C. albicans* isolates from the two patient groups (Figure 2D). At the sub-species level, the median proteinase, phospholipase and esterase activity for *C. albicans* isolates was 1.2, 0.91 and 1, respectively. According to these median values, there was no significant difference in the *in vitro*

expression of these enzymes between *C. albicans* genotypes by chi square or Mann-Whitney analysis (Table 2 and figures 3C, 3D and 3E).

Ethanol-derived acetaldehyde production capability

A total of 34/80 (42.5%) patients were colonized by *Candida* isolates that were able to produce a mutagenic level of ACH from yeast incubation with 11 mM of ethanol (Table 2). The percentage of *Candida* isolates with high acetaldehyde producing ability ($>40\ \mu\text{M}$) was significantly ($p=0.01$) higher amongst oral cancer [$n=21/36$, (58%)] than non-oral cancer patients [$n=13/44$, (29.5%)] (Table 2). Mann-Whitney analysis also indicated significantly ($p=0.032$) higher amount of acetaldehyde produced by isolates from oral cancer patients compared to those from non-oral cancer patients (Figure 1F). The frequency of *Candida* isolates that produced high levels of ACH was higher ($p=0.037$) in patients with high *Candida* colonization level ($> 567\ \text{CFU}$) than those with low colonization level ($< 567\ \text{CFU}$) (Table 2). No significant association was found between the ability of *Candida* to produce acetaldehyde and all other patients' factors.

Differences amongst *Candida* species and strains were also observed in the amount of ACH produced. Although the frequency of high ACH production was higher in *C. albicans* isolates than the non-*albicans* *Candida* isolates, this did not reach the level of statistical significance ($p=0.088$) (Table 2).

Comparing ACH production of *C. albicans* isolates only, isolates from oral cancer patients produced significantly ($p=0.039$) higher amount of ACH than those from non-oral cancer patients (Figure 2F). No significant association was seen between ACH production and *C. albicans* genotype and there was no difference in ACH production between *C. albicans* genotype A and genotype B (Table 2) (Figure 3F).

The risk effect of *Candida* virulence and ethanol-derived ACH on oral cancer

The univariate and multivariate logistic regression analyses are presented in Table 3. In the univariate analysis high *Candida* biofilm mass and metabolic activity, high phospholipase enzyme activity, high ethanol-derived ACH production, current/daily alcohol drinking and high *Candida* colonization were significant risk factors for oral cancer. However, in the multivariate analysis, high biofilm metabolic activity and phospholipase enzyme production, and alcohol drinking were the only significantly positive influencing factors on oral cancer

risk. Although *Candida* yeasts with high biofilm mass, proteinase and high acetaldehyde production capability did not show a significant risk in the multivariate analysis; the likelihood of cancer presence was higher (odds ratio higher than 1) in patients colonized by these isolates than those colonized by isolates with low virulence (Table 3).

Discussion

The present study is the first to demonstrate a clear association between virulence attributes and ethanol-derived acetaldehyde production capability of *Candida* yeasts and oral cancer. Isolates from oral cancer patients exhibited a higher capacity to form biofilms, to produce phospholipase and proteinase enzymes and to metabolize alcohol to acetaldehyde. Further, the multivariate logistic regression analysis showed that *Candida* high biofilm metabolic activity, high phospholipase enzyme activity and high oral *Candida* colonization level were significant risk factors for oral cancer. While the present study has supplied much useful information about virulence properties of yeasts isolated from oral cancer patients, it has some limitations that must be acknowledged. Firstly, it is important to emphasize the fact that due to the design of the present study (case-controls), the results only give information regarding associations, without a cause-effect relationship. Nonetheless, these data may have clinical and pathological significance as individuals may have chronic oral colonization by yeast isolates that simultaneously have enhanced pathogenic properties, such as biofilm formation, hydrolytic enzyme and ethanol-derived acetaldehyde production capacity. Such chronic colonization could play a role in the promotion of oral premalignant and malignant lesions. High biofilm forming ability is likely to secure a prolonged oral colonization and chronic exposure of host tissues to fungal carcinogens, such as acetaldehyde; while enhanced hydrolytic enzyme production may allow for progressive tissue destruction promoting a chronic inflammatory response.

The chronic microbial irritation may result in a chronic inflammatory response and subsequent release of cascade of cytokines and chemokines that infiltrate disrupted and damaged tissues. Studies have shown a significant positive correlation between virulence potential of pathogenic fungi, such as secreted aspartyl proteinases, and the expression of inflammatory cytokines and chemokines (Beausejour *et al.*, 1998; Schaller *et al.*, 2002; Schaller *et al.*, 2005). It has been postulated that chronic inflammation caused by microbial infection is a major driving force in tumour development and indispensable participant in the neoplastic process (Karin and Greten, 2005). Given the current findings of enhanced

virulence potential of *Candida* isolated from oral cancer patients and the previously reported positive correlation between *Candida* virulence and cytokine expression (Beausejour *et al.*, 1998; Schaller *et al.*, 2002; Schaller *et al.*, 2005), it could be surmised that pro-carcinogenesis inflammation is a working hypotheses behind the strong epidemiological association between oral *Candida* colonization and oral cancer presence (Alnuaimi *et al.*, 2015). However, another limitation of our study which could be an objective of further research is that we did not investigate the correlation between the severity of inflammatory changes at cancerous sites and virulence of *Candida* isolated from these sites.

Previous studies (Ghannoum, 2000; Parahitiyawa *et al.*, 2006; Silva *et al.*, 2010; Sardi *et al.*, 2013) showed that significant variation exists in biofilm mass, biofilm metabolic activity, phospholipase activity, proteinase activity and esterase activity between different *Candida* species. In the present study, *C. albicans* isolates produced higher levels of these virulence-associated traits, confirming the high virulence potential of this species when compared to other *Candida* species. Interestingly, in this study, non-*albicans* *Candida* species had significantly greater proteinase activity.

Previous studies have demonstrated a significant association between ABC genotyping and the virulence potential of *C. albicans* strains (Qianming *et al.*, 2004; Zeng *et al.*, 2008; Sardi *et al.*, 2012). In our previous investigation, a significant epidemiological relationship was found between oral cancer presence and *C. albicans* genotype (Alnuaimi *et al.*, 2015). Specifically, a significantly higher frequency of genotype A was observed in *C. albicans* strains isolated from oral cancer patients and significantly higher frequency of genotype B was seen in non-oral cancer patients. This called for investigating whether this genotypic variation would have any pathogenic or carcinogenic implication. However, consistent with other reports (Manfredi *et al.*, 2006; Mane *et al.*, 2012), a relationship could not be established between genotype and any of the investigated virulence factors other than significantly higher biofilm mass was obtained by genotype A than genotype B isolates. This highlights the need for investigating further genetic linkages, and perhaps, using more discriminatory molecular genotyping methods such as multilocus sequence typing (MLST) analysis that could help dissect the molecular basis for this preferential strain selection.

Alcohol, tobacco smoking and poor oral hygiene are the most significant environmental risk factors for upper aerodigestive tract cancers (Boffetta and Hashibe, 2006; Pelucchi *et al.*, 2008). Alcohol itself is not carcinogenic, but the increased risk of oral and upper digestive tract cancers in high alcohol consumers has been linked to the increased local level of ACH, particularly amongst those with poor oral hygiene (Homann *et al.*, 2000a;

Homann *et al.*, 2000b). Acetaldehyde is metabolized from ethanol by alcohol dehydrogenases (ADH) from oral mucosal epithelial cells and from oral microbiota (Hooper *et al.*, 2009). Several oral bacteria such as *Neisseria* and *Streptococci* species have been associated with marked ADH enzyme activity resulting in high acetaldehyde production (Muto *et al.*, 2000; Kurkivuori *et al.*, 2007). The same has also been reported for some yeast species (Tillonen *et al.*, 1999; Salaspuro, 2003). One of the most significant hypotheses on how *Candida* may be associated with oral cancer development relates to its well-documented ability to produce acetaldehyde from ethanol by oxidation. (Mohd Bakri *et al.*, 2010). There are few comparative studies that have been conducted regarding the level of ACH produced by *Candida* isolated from patients with precancerous and cancerous lesions versus isolates from healthy patients (Gainza-Cirauqui *et al.*, 2013; Marttila *et al.*, 2013). However, these studies have not examined the inter- and intra-species variation in ACH production or their data were not derived from matched case-control study design. In addition, researchers have not simultaneously examined ACH production and other *Candida* virulence attributes. One such study showed no significant difference in ACH production between *Candida* isolated from oral cancer and healthy patients (Marttila *et al.*, 2013); while the other, interestingly, showed higher level of ACH in the healthy control patients (Gainza-Cirauqui *et al.*, 2013). In the present study, a significantly higher level of ACH was produced by *Candida* isolated from oral cancer patients compared with matched non-oral cancer patients, suggesting a significant association between the ability of isolates to produce acetaldehyde and to promote cancer development. This may also partly explain the highest cancer risk, observed in our previous investigation, when *Candida* presence was conjugated with alcohol drinking (Alnuaimi *et al.*, 2015).

Conclusions

These data support the concept of preferential selection of *Candida* isolates with enhanced virulence properties and carcinogenic acetaldehyde production in oral cancer-affected individuals. These data also suggest a significant positive association between the ability of *Candida* isolates to form biofilms, to produce hydrolytic enzymes and to metabolize alcohol to acetaldehyde with their ability to promote oral cancer development. Thorough understanding of the biological behaviour of yeasts associated with oral cancer will be useful in assessment of their role in oral mucosal carcinogenesis and may lead to significant

advances in our understanding of oral cancer aetiology, potentially opening up new approaches for therapeutic intervention. Controlled prospective studies are necessary to continue exploring the potential association between *Candida* and oral cancer. Further, in-depth, studies using more complex setting resembling *in vivo* conditions are warranted to elucidate the interaction of *Candida* with oral mucosal cells at a molecular level to broaden our understanding of the relationship of yeasts to oral cancer risk.

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Conflicts of interest: Non to declare.

References

- Alnuaimi AD, O'Brien-Simpson NM, Reynolds EC, McCullough MJ (2013). Clinical isolates and laboratory reference *Candida* species and strains have varying abilities to form biofilms. *FEMS Yeast Res*, 13, 689–699.
- Alnuaimi AD, Wiesenfeld D, O'Brien-Simpson NM, Reynolds EC, Peng B, McCullough MJ (2014). The development and validation of a rapid genetic method for species identification and genotyping of medically important fungal pathogens using high resolution melting curve analysis. *Mol Oral Microbiol*, 29, 117-130.
- Alnuaimi AD, Wiesenfeld D, O'Brien-Simpson NM, Reynolds EC, McCullough MJ (2015). Oral *Candida* colonization in oral cancer patients and its relationship with traditional risk factors of oral cancer: A matched case-control study. *Oral Oncol*, 51, 139-145.
- Beausejour A, Grenier D, Goulet JP, Deslauriers N (1998). Proteolytic activation of the interleukin-1beta precursor by *Candida albicans*. *Infect Immun*, 66, 676-681.
- Boffetta P, Hashibe M (2006). Alcohol and cancer. *Lancet Oncol*, 7, 149-156.
- Brooks PJ, Theruvathu JA (2005). DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis. *Alcohol*, 35, 187-193.
- Calderone RA, Fonzi WA (2001). Virulence factors of *Candida albicans*. *Trends Microbiol*, 9, 327-335.
- Chakrabarti A, Nayak N, Talwar P (1991). In vitro proteinase production by *Candida* species. *Mycopathologia*, 114, 163-168.

- Field EA, Field JK, Martin MV (1989). Does *Candida* have a role in oral epithelial neoplasia? *J Med Vet Mycol*, 27, 277-294.
- Gainza-Cirauqui ML, Nieminen MT, Novak Frazer L, Aguirre-Urizar JM, Moragues MD, Rautemaa R (2013). Production of carcinogenic acetaldehyde by *Candida albicans* from patients with potentially malignant oral mucosal disorders. *J Oral Pathol Med*, 42, 243-249.
- Ghannoum MA (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev*, 13, 122-143.
- Homann N, Tillonen J, Meurman JH, Rintamaki H, Lindqvist C, Rautio M, Jousimies-Somer H, Salaspuro M (2000a). Increased salivary acetaldehyde levels in heavy drinkers and smokers: a microbiological approach to oral cavity cancer. *Carcinogenesis*, 21, 663-668.
- Homann N, Tillonen J, Salaspuro M (2000b). Microbially produced acetaldehyde from ethanol may increase the risk of colon cancer via folate deficiency. *Int J Cancer*, 86, 169-173.
- Hooper SJ, Wilson MJ, Crean SJ (2009). Exploring the link between microorganisms and oral cancer: a systematic review of the literature. *Head Neck*, 31, 1228-1239.
- Karin M, Greten FR (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol*, 5, 749-759.
- Kumar CP, Menon T, Sundararajan T, Nalini S, Thirunarayan MA, Rajasekaran S, Venkatadesikal M (2006). Esterase activity of *Candida* species isolated from immunocompromised hosts. *Rev Iberoam Micol*, 23, 101-103.
- Kurago ZB, Lam-ubol A, Stetsenko A, De La Mater C, Chen Y, Dawson DV (2008). Lipopolysaccharide-squamous cell carcinoma-monocyte interactions induce cancer-supporting factors leading to rapid STAT3 activation. *Head Neck Pathol*, 2, 1-12.
- Kurkivuori J, Salaspuro V, Kaihovaara P, Kari K, Rautemaa R, Grönroos L, Meurman JH, Salaspuro M (2007). Acetyldehyde production from ethanol from by oral streptococci. *Oral Oncol*, 43, 181-186.
- Lax AJ (2005). Opinion: Bacterial toxins and cancer--a case to answer? *Nat Rev Microbiol*, 3, 343-349.
- Mane A, Gaikwad S, Bembalkar S, Risbud A (2012). Increased expression of virulence attributes in oral *Candida albicans* isolates from human immunodeficiency virus-positive individuals. *J Med Microbiol*, 61, 285-290.

- Manfredi M, McCullough MJ, Al-Karaawi ZM, Vescovi P, Porter SR (2006). In vitro evaluation of virulence attributes of *Candida* spp. isolated from patients affected by diabetes mellitus. *Oral Microbiol Immunol*, 21, 183-189.
- Marttila E, Uttamo J, Rusanen P, Lindqvist C, Salaspuro M, Rautemaa R (2013). Acetaldehyde production and microbial colonization in oral squamous cell carcinoma and oral lichenoid disease. *Oral Surg Oral Med Oral Pathol Oral Radiol*, 116, 61-68.
- McCullough MJ, Clemons KV, Stevens DA (1999). Molecular and phenotypic characterization of genotypic *Candida albicans* subgroups and comparison with *Candida dubliniensis* and *Candida stellatoidea*. *J Clin Microbiol*, 37, 417-421.
- McCullough M, Jaber M, Barrett AW, Bain L, Speight PM, Porter SR (2002). Oral yeast carriage correlates with presence of oral epithelial dysplasia. *Oral Oncol*, 38, 391-393.
- Mohd Bakri M, Mohd Hussaini H, Rachel Holmes A, David Cannon R, Mary Rich A (2010). Revisiting the association between candidal infection and carcinoma, particularly oral squamous cell carcinoma. *J Oral Microbiol*, 2, doi: 10.3402/jom.v2i0.5780.
- Muto M, Hitomi Y, Ohtsu A, Shimada H, Kashiwase Y, Sasaki H, Yoshida S, Esumi H (2000). Acetaldehyde production by non-pathogenic *Neisseria* in human oral microflora: implications for carcinogenesis in upper aerodigestive tract. *Int J Cancer*, 88, 342-350.
- Nieminen MT, Uttamo J, Salaspuro M, Rautemaa R (2009). Acetaldehyde production from ethanol and glucose by non-*Candida albicans* yeasts in vitro. *Oral Oncol*, 45, e245-248.
- Parahitiyawa NB, Samaranayake YH, Samaranayake LP, Ye J, Tsang PW, Cheung BP, Yau JY, Yeung SK (2006). Interspecies variation in *Candida* biofilm formation studied using the Calgary biofilm device. *APMIS*, 114, 298-306.
- Pelucchi C, Gallus S, Garavello W, Bosetti C, La VC (2008). Alcohol and tobacco use, and cancer risk for upper aerodigestive tract and liver. *Eur J Cancer Prev*, 17, 340-344.
- Price MF, Wilkinson ID, Gentry LO (1982). Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia*, 20, 7-14.
- Qianming C, Xin Z, Li B, Min Z (2004). The genotypic profiles of *Candida albicans* isolates from patients with oral leukoplakia and oral squamous cell carcinoma and the correlation with their virulence attribute. *Cancer Res*, 45, 109.
- Ramage G, Rajendran R, Sherry L, Williams C (2012). Fungal biofilm resistance. *Int J Microbiol*, 2012, 528521.

- Ramdzan AN, Mornane PJ, McCullough MJ, Mazurek W, Kolev SD (2013). Determination of acetaldehyde in saliva by gas-diffusion flow injection analysis. *Anal Chim Acta*, 786, 70-77.
- Salaspuro MP (2003). Acetyldehyde, microbes and cancer of the digestive tract. *Crit Rev Clin Lab Sci*, 40, 183-208.
- Sardi JC, Duque C, Höfling JF, Gonçalves RB (2012). Genetic and phenotypic evaluation of *Candida albicans* strains isolated from subgingival biofilm of diabetic patients with chronic periodontitis. *Med Mycol.*, 50, 467-475.
- Sardi JC, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJ (2013). *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J Med Microbiol*, 62, 10-24.
- Schaller M, Mailhammer R, Grassl G, Sander CA, Hube B, Korting HC (2002). Infection of human oral epithelia with *Candida* species induces cytokine expression correlated to the degree of virulence. *J Invest Dermatol*, 118, 652-657.
- Schaller M, Korting HC, Borelli C, Hamm G, Hube B (2005). *Candida albicans*-secreted aspartic proteinases modify the epithelial cytokine response in an in vitro model of vaginal candidiasis. *Infect Immun*, 73, 2758-2765.
- Seitz HK, Stickel F (2007). Molecular mechanisms of alcohol-mediated carcinogenesis. *Nat Rev Cancer*, 7, 599-612.
- Silva S, Henriques M, Oliveira R, Williams D, Azeredo J (2010). In vitro biofilm activity of non-*Candida albicans* *Candida* species. *Curr Microbiol*, 61, 534-540.
- Slifkin M (2000). Tween 80 opacity test responses of various *Candida* species. *J Clin Microbiol*, 38, 4626-4628.
- Tillonen J, Homann N, Rautio M, Jousimies-Somer H, Salaspuro M (1999). Role of yeasts in the salivary acetaldehyde production from ethanol among risk groups for ethanol-associated oral cavity cancer. *Alcohol Clin Exp Res*, 23, 1409-1415.
- Treviño-Rangel Rde J, González JG, González GM (2013). Aspartyl proteinase, phospholipase, esterase and hemolysin activities of clinical isolates of the *Candida parapsilosis* species complex. *Med Mycol*, 51, 331-335.
- Zeng X, Xiong C, Wang Z, Jiang L, Hou X, Shen J, Zhou M, Chen Q (2008). Genotypic profiles and virulence attributes of *Candida albicans* isolates from patients with oral lichen planus. *APMIS*, 116, 284-291.

Table 1: Candida species/strain distribution in oral cancer and non-oral cancer patients (Alnuaimi et al., 2015).

Candida species/strains	Patients	
	Oral cancer	Non-oral cancer
<i>C. albicans</i>	31 (79.5%)	32 (62.75%)
Genotype A	27 (69.23%)	15 (29.41%)
Genotype B	4 (10.25%)	16 (31.37%)
Genotype C	0 (0%)	1 (1.96%)
<i>C. dubliniensis</i>	3 (7.7%)	3 (5.88%)
<i>C. glabrata</i>	2 (5.12%)	3 (5.88%)
<i>C. parapsilosis</i>	0 (0%)	9 (17.65%)
<i>C. krusei</i>	1 (2.56%)	0 (0%)
<i>C. tropicalis</i>	0 (0%)	3 (5.88%)
<i>C. lusitaniae</i>	0 (0%)	1 (1.96%)
<i>C. guilliermondii</i>	2 (5.12%)	0 (0%)
Total	39 (100%)	51 (100%)

Patients	Biofilm mass above the median	Biofilm mass below the median	P ^e value	Biofilm activity above the median	Biofilm activity below the median	P ^e value	IP ^a above the median	IP ^a below the median	P ^e value	IPL ^b above the median	IPL ^b below the median	P ^e value	IE ^c above the median	IE ^c below the median	P ^e Value	ACH ^d Above 40 µM	ACH ^d below 40 µM	P ^e value
Cancer	25	11	0.031	28	8	<0.0001	23	13	0.1	27	9	0.002	16	20	0.271	21	15	0.01
No cancer	20	24		14	30		20	24		18	26		25	19		13	31	
Medical problems	29	21	0.684	23	27	0.133	25	25	0.385	26	24	0.323	27	23	0.525	22	28	0.726
No medical problems	16	14		19	11		18	12		19	11		14	16		12	18	
Male	21	16	0.932	22	15	0.248	21	16	0.617	19	18	0.413	18	19	0.666	15	22	0.742
Female	24	19		20	23		22	21		26	17		23	20		19	24	
Smoker	11	14	0.136	18	7	0.019	17	8	0.085	15	10	0.649	15	10	0.291	12	13	0.502
Non smoker	34	21		24	31		26	29		30	25		26	29		22	33	
Alcohol drinker	9	4	0.303	8	5	0.476	8	5	0.538	5	8	0.158	6	7	0.688	7	6	0.366
Non-alcohol drinker	36	31		34	33		35	32		40	27		35	32		27	40	
Alcohol mouthwash	16	9	0.346	12	13	0.587	13	12	0.832	15	10	0.649	15	10	0.291	8	17	0.2
No alcohol mouthwash	29	26		30	25		30	25		30	25		26	29		26	29	
> 567 cfu/ml	14	5	0.0797	13	6	0.112	12	7	0.346	12	7	0.487	8	11	0.361	12	7	0.037
< 567 cfu/ml	31	30		29	32		31	30		33	28		33	28		22	39	
Periodontal disease	37	27	0.573	30	34	0.044	36	28	0.37	34	30	0.260	27	37	0.001	26	38	0.497
No periodontal disease	8	8		12	4		7	9		11	5		14	2		8	8	
Denture	10	15	0.048	12	13	0.587	14	11	0.786	22	13	0.316	15	10	0.291	8	17	0.2
No denture	35	20		30	25		29	26		33	12		26	29		26	29	
C. albicans	42	21	<0.0001	39	24	0.002	27	36	0.038	46	17	<0.0001	37	26	0.011	30	33	0.088
Non-albicans Candida	4	23		7	20		18	9		8	19		8	19		8	19	
C. albicans A	21	21		21	21		25	17		25	17		19	23		21	21	
C. albicans B	8	12	0.436	9	11	0.553	9	11	0.310	7	13	0.116	13	7	0.218	8	12	0.436
C. albicans C	1	0		1	0		0	1		0	1		1	0		1	0	

^a: Proteinase activity index (IP); ^b: Phospholipase activity index (IPL); ^c: Esterase activity index (IE); ^d: Acetaldehyde (ACH).
Table 2: Biofilm forming ability, hydrolytic enzymes and ethanol-derived acetaldehyde production obtained from all the Candida isolates carriers.
 Statistical analysis was performed by sub-classifying the values above and below the median of each assay (biofilm mass: 1.86, biofilm metabolic activity: 0.38, proteinase activity: 1.4, phospholipase activity: 0.8; esterase activity: 0.88 for all isolates) and (biofilm mass: 2, biofilm metabolic activity: 0.4, proteinase activity: 1.2, phospholipase activity: 0.91; esterase activity: 1 for C. albicans isolates). While for ACH, values were sub-classified into above and below 40 µM. Chi square or Fisher's exact test were used to analyse the obtained data.

Table 3: Univariate and logistic regression analysis of possible risk effects of Candida virulence traits and some patients' factors in oral cancer.

Factor	Univariate analysis			Multivariate analysis		
	OR	95% CI	P value	OR	95% CI	P value
Low biofilm mass	1.00			1.00		
High biofilm mass	2.72	1.08-6.87	0.03 [*]	2.37	0.64-8.75	0.19
Low biofilm metabolic activity	1.00			1.00		
High biofilm metabolic activity	7.5	2.73-20.58	P<0.001 [*]	4.81	1.32-17.45	0.01 [*]
Low phospholipase activity	1.00			1.00		
High phospholipase activity	4.33	1.65-11.36	0.003 [*]	5.50	1.34-22.45	0.01 [*]
Low proteinase activity	1.00			1.00		
High proteinase activity	2.12	0.86-5.23	0.1	1.97	0.55-7.07	0.29
Low esterase activity	1.00			1.00		

High esterase activity	0.6	0.25-1.47	0.52	0.52	0.15-1.78	0.3
Low Candida colonization	1.00			1.00		
High Candida colonization	3.58	1.19-10.72	0.02*	2.84	0.54-14.81	0.21
Low Ethanol-derived ACH	1.00			1.00		
High Ethanol-derived ACH	3.33	1.32-8.43	0.01*	1.91	0.57-6.41	0.28
Non-smoking	1.00			1.00		
Smoking	2.42	0.92-6.39	0.07	1.72	0.4-7.31	0.4
No-alcohol drinking	1.00			1.00		
Current/daily alcohol drinking	5.25	1.32-20.9	0.01*	14.97	2.02-110.91	0.008*

OR, odds ratio; CI, confidence interval, ACH, acetaldehyde.

*Statistically significant

Figure 1

Figure 1: Comparison of biofilm forming ability (A and B), hydrolytic enzymes (C, D and E) and ethanol-derived acetaldehyde production capability (F) of all *Candida* isolated from oral cancer and non-oral cancer patients. Each dot represents the mean of measurements performed in triplicate on two separate occasions and the horizontal line represents the median value of each subgroup.

*P value by Mann-Whitney analysis; CV: biofilm mass as assessed by crystal violet assay; XTT: biofilm metabolic activity as assessed by XTT salt reduction assay; ACH: acetylaldehyde produced from incubation of yeast with 11 mM ethanol.

Figure 2

Figure 2: Comparison of biofilm forming ability (A and B), hydrolytic enzymes (C, D and E) and ethanol-derived acetaldehyde production capability (F) of *C. albicans* yeasts isolated from oral cancer and non-oral cancer patients. Each dot represents the mean of measurements performed in triplicate on two separate occasions and the horizontal line represents the median value of each subgroup.

*P value by Mann-Whitney analysis; CV: biofilm mass as assessed by crystal violet assay; XTT: biofilm metabolic activity as assessed by XTT salt reduction assay; ACH: acetylaldehyde produced from incubation of yeast with 11 mM ethanol.

Figure 3

Figure 3: Comparison of biofilm forming ability (A and B), hydrolytic enzymes (C, D and E) and ethanol-derived acetaldehyde production capability (F) of all *C. albicans* genotypes isolated from oral cancer and non-oral cancer patients. Each dot represents the mean of measurements performed in triplicate on two separate occasions and the horizontal line represents the median value of each subgroup.

*P value by Mann-Whitney analysis; CV: biofilm mass as assessed by crystal violet assay; XTT: biofilm metabolic activity as assessed by XTT salt reduction assay; ACH: acteylaldehyde produced from incubation of yeast with 11 mM ethanol.

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