Analysis of oral yeast microflora in patients with oral squamous cell carcinoma

Csaba Berkovits, Adél Tóth, Judit Szenzenstein, Tünde Deák, Edit Urbán, Attila Gácser and Katalin Nagy

Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most common form of oral cancer, in this study, the association between OSCC and oral yeast carriage was investigated.

Findings: 20 patients having OSCC as well as 40 healthy controls were tested for the presence of yeasts in the oral cavity. Fungal burdens were examined by colony forming unit determinations, while the different yeast genera in patient samples were identified by matrix-associated laser desorption/ionization-time of flight-mass spectrometry. We found that the level of oral yeast carriage was significantly higher in patients with OSCC that was accompanied by a higher diversity of yeasts in the oral cavity of these patients. We also examined the extracellular enzyme production of isolated Candida spp.; however, we found that there was no association between the lipase/protease producing capacity of Candida strains and the higher colonisation rate of neoplastic epithelium.

Conclusions: In conclusion, our results corroborate the findings of previous studies regarding the association between oral yeast carriage and epithelial carcinoma.

Keywords: Oral squamous cell carcinoma, MALDI-TOF, Candida, Yeast

Background

Oral squamous cell carcinoma (OSCC) is the most common form of oral cancer, representing up to 80–90% of all malignancies of the oral cavity (Pires et al. 2013). Among others, risk factors for OSCC include smoking, alcohol consumption, ultraviolet radiation and poor oral hygiene (Mohd et al. 2010). The occurrence of OSCC has also been associated with Candida infections, although the underlying pathogenic mechanisms are poorly understood (Mohd et al. 2010).

Candida species belong to the normal flora and are frequently isolated from various mucosal surfaces in healthy individuals (Sardi et al. 2013). However, they may cause cutaneous or systemic infections when the immunity of the host is compromised. Furthermore, although little is known about the role of fungal infections in cancer, Candida spp. have long been implicated in various epithelial malignancies. There are several reports about chronic mucocutaneous candidiasis (CMC) patients developing oral or esophageal carcinoma (Mohd et al. 2010). Furthermore, chronic hyperplastic candidiasis (CHC, candidal leukoplakia), a rare form of oral candidiasis has been shown to often undergo malignant transformation (Cawson 1966). A recent study has found that Candida species can be isolated with higher frequency from patients with oral epithelial dysplasia compared to healthy subjects (Hebbar and Pai 2013). Candida spp. have several attributes that may promote oral cancer development, such as the ability to produce carcinogens (e.g. nitrosamines), metabolize procarcinogens or induce inflammation (Mohd et al. 2010). However, the etiological relationship between oral cancer and Candida infections is still a matter of debate and needs to be further investigated.

In this study, we assessed the presence of Candida spp. in the oral cavity of patients having keratinizing OSCC as well as healthy controls to investigate the association between Candida infections and oral cancer development.
**Methods**

**Patients**

A total of 60 subjects [20 OSCC patients (14 males, 6 females, median age: 62 (61.95), range 44–86) and 40 controls (22 males, 18 females, median age: 67 (67.62), range 49–82)] were enrolled in this study. The patients and the controls were recruited from among the patients of the Departments of Dentoalveolar Surgery and Maxillofacial Surgery at the Faculties of Dentistry and Medicine at the University of Szeged. OSCC patients were eligible for this study if they had a histologically confirmed diagnosis and if they had not received any treatment for OSCC up to their participation. Controls were recruited from outpatients free of oral mucosal pathology who arrived for routine procedures (e.g. tooth extraction). The study design complied with the tenets of the Declaration of Helsinki in all respects, and it was approved by the Research Ethics Committee for Human Medical Biology at the University of Szeged. The participation was voluntary and it was based on informed consent. Before their enrolment, all subjects received information about the background, aims and procedures of the study, and they had to sign an informed consent form to signify that they opted for participation by their own free will and based on the information they got. Frequency and locations of cancer sites in oral cancer patients: T1: carcinoma fundi oris l.d., T2: carcinoma linguae l.d., T3: carcinoma hypopharyngis l.s., T4: carcinoma radicis linguae l.d., T5: carcinoma gingivae l.s., T6: carcinoma hypopharyngis l.s., T7: carcinoma linguae l.d, T8: carcinoma fundi oris l.s., T9: carcinoma fundi oris l.d., T10: carcinoma linguae l.s., T11: carcinoma radicis linguae l.s., T12: carcinoma linguae l.s., T13: carcinoma labii superioris vestibularis l.d., T14: carcinoma labii inferioris vestibularis l.s., T15: carcinoma mandibulae ging. l.d., T16: carcinoma labii inferioris vestibularis l.d., T17: carcinoma fundi oris et gingivae l.s., T18: carcinoma linguae l.d., T19: carcinoma fundi oris l.d., T20: carcinoma hypopharyngis l.d.

**Oral samples**

Oral swabs were taken from a 1 cm² area from two different locations in the oral cavity (in the case of OSCC patients, both from the surface of neoplastic and healthy epithelium). Samples were inoculated on Sabouraud dextrose agar plates and Sabouraud broth, both incubated for 7 days at room atmosphere, in 32 °C. Direct samples that tested negative for yeast growth on agar plates after the first 3 days were subcultured from the liquid medium and incubated again for 7 days (referred to as "subculture"). Basic identification of the isolated yeasts was carried out based on macro- and micro-scopic morphology, catalase test and CHROMagar Candida plates (Becton–Dickinson, UK), next MALDI-TOF (Bruker Daltonics, Bremen, Gr) analysis was carried out from all of the isolates.

**MALDI-TOF-MS**

Sample preparation was carried out according to the Bruker protocols using three methods: (1) direct transfer (DT), (2) extended direct transfer (eDT), and (3) ethanol (EtOH)–formic acid (FA) extraction. In the DT method, a thin smear of biological material was placed onto a target plate, which was immediately overlaid with 1 μl of alpha-cyano-4-hydroxycinnamic acid matrix solution (HCCA; Bruker Daltonics, Gr.), prepared according to the protocol of the manufacturer. In the eDT method, the biomass was treated with 1 μl of 70 % FA on the target plate prior to the HCCA matrix overlay. In the EtOH–FA method, one or two loops of yeast biomass (1 μl volume, sterile inoculation loop) was used for the crude protein extraction, as described previously (Lacroix et al. 2014). One microliter of the crude protein extract was spotted onto the target plate, and after air-drying, it was overlaid with 1 μl of HCCA matrix solution. For all methods, each tested strain was spotted in duplicate. Standard commercially available Bruker Daltonics database (BDAL) was used in this study: individual spectra using the MALDI Biotyper automated FlexControl software version 3.0 (Bruker Daltonics). The MALDI-TOF MS identification results were automatically classified using the log-score values generated by the MALDI Biotyper software (Bruker Daltonics, Germany), performed according to the manufacturer's instructions. Scores higher than 1.7 indicated genus-level identification.

**Analysis of lipase and protease activity**

Extracellular lipase activity of Candida strains was examined on YNB-rhodamine B plates as previously described (Nemeth et al. 2013). Briefly, strains were inoculated onto YNB-rhodamine B (yeast nitrogen base) plates (Sigma-Aldrich) and incubated at 30 °C for 7 days. Lipase positivity was determined by the presence of pink halo around the colonies. For the detection of proteolytic activity, Candida strains were cultured on YCB-BSA (yeast carbon base—bovine serum albumin, Sigma-Aldrich) agar plates at 30 °C for 7 days, and proteolysis around the colonies was visualised by amidoblack (Sigma-Aldrich) staining (Nemeth et al. 2013).

**Statistical analysis**

All statistical analyses have been performed by GraphPad Prism 5.0 software. Fisher’s exact test, Mann–Whitney test or Wilcoxon signed-rank test were used wherever
results

20 patients with confirmed OSCC as well as 40 healthy controls were enrolled in the study. 18 (90 %) of the 20 OSCC patients and 12 (30 %) of the healthy controls had yeast isolated from their oral cavity (Table 1), indicating that the frequency of oral yeast colonization was significantly higher in OSCC patients compared to the control group (Fisher’s exact test, \( p < 0.0001 \)). Furthermore, OSCC patients had a significantly higher average fungal burden (mean ± SEM, 73.08 ± 33.39 CFU/cm²) in their oral cavity compared to healthy individuals (1.10 ± 0.78 CFU/cm², Fig. 1a), and samples taken from the neoplastic surface contained more yeast cells (77.38 ± 38.53 CFU/cm²) compared to the swabs taken from the healthy epithelium of the same individual (28.58 ± 19.18 CFU/cm², Fig. 1b). To assess the spectrum of yeast genera present in the oral cavity of healthy or OSCC patients, samples were subjected to peptide mass fingerprinting by matrix-associated laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). While the predominant fungal genus present in the oral cavity of both healthy and OSCC patients was Candida, we found that there was a higher diversity of yeasts in the oral swabs of cancer patients compared to healthy controls (Table 2). As the secretion of hydrolytic enzymes is an important virulence factor of Candida spp. (Nemeth et al. 2013; Stehr et al. 2004; Trofa et al. 2009; Monod and Borg-von 2002), we also examined whether the higher colonisation rate in the oral cavities of OSCC patients is associated with an increased prevalence of Candida strains with hydrolytic enzyme activity. To analyse the enzyme profiles of Candida strains, we have randomly chosen 40 isolates from healthy controls and 140 isolates from OSCC patients and tested their extracellular lipase and protease activity. However, we found that the enzyme production of isolates derived from the two different patient group did not differ significantly (Table 3).

Discussion

Candida infection has long been implicated in cancer development, particularly in malignancies affecting the oral epithelium (Mohd et al. 2010). In this study, we found that the level of oral yeast carriage is significantly higher in patients with OSCC. It has been previously shown that there is positive association between oral yeast carriage and epithelial carcinoma (Nagy et al. 1998; Barrett et al. 1998; McCullough et al. 2002; Jahanshahi and Shiri 2015; Alnuaimi et al. 2015). Although the underlying mechanisms of how Candida species might promote carcinogenesis are still obscure, multiple mechanisms have been suggested to contribute to cancer development. These include the secretion of potential carcinogens, conversion of procarcinogens, damage of epithelial barriers by secreted proteolytic enzymes as well as the ability to induce chronic inflammation (Mohd et al. 2010). In a mouse model of carcinogenesis, C. albicans infection has

Table 1 Prevalence of oral yeast carriage in OSCC patients and healthy controls

| Oral yeast carriage | Positive, N (%) | Negative, N (%) |
|---------------------|-----------------|-----------------|
| Control patients (n = 40) | | |
| Original sample | 3 (7.5) | 37 (92.5) |
| Subculture | 12 (30) | 28 (70) |
| OSCC patients (n = 20) | | |
| Original sample | 10 (50) | 10 (50) |
| Subculture | 18 (90) | 2 (10) |

Table 2 Yeast genera identified in OSCC and control samples based on MALDI-TOF–MS analysis

| Genus           | No. of isolates (%) |
|-----------------|---------------------|
| Control (N = 12) | OSCC (N = 22)       |
| Candida         | 10 (83.3)           | 15 (68.2)         |
| Rhodotorula     | –                   | 2 (9.1)           |
| Saccharomyces   | –                   | 2 (9.1)           |
| Kloeckera       | –                   | 1 (4.5)           |
| Other yeast (unidentified) | 2 (16.7) | 2 (9.1) |
been shown to promote the development of 4-nitroquinolone 1-oxide (4NQO)-induced oral epithelial dysplasia, although the infection alone led only to the formation of hyperplastic lesions (Dwivedi et al. 2009). Here we tested a large number of yeast isolates from OSCC and control patients for extracellular enzyme production and found that there is no association between the lipase/protease producing capacity of strains and the higher colonization rate of neoplastic epithelium. This finding questions the role of fungal hydrolytic enzymes in the development of epithelial dysplasia, although further experiments are needed to thoroughly examine their effect on carcinogenesis. It has also been suggested that Candida infection is the consequence rather than the cause of malignant oral disorders, as the neoplastic epithelium represents an immunosuppressive microenvironment that may promote the survival of Candida spp. It has been recognized during the recent years that there is a delicate interplay between the innate immune system and commensal fungi that is responsible for maintaining the integrity of the mucosa. On the one hand, components of the innate immune system recognize the invading pathogens and limit the overgrowth of commensal fungi by intracellular killing (Romani 2011). On the other hand, contact with commensal microbes induces tolerance, that limits host damage caused by excessive inflammation (Romani 2011). Interleukin-10 (IL-10) is one of the most effective mediators of immune tolerance and it has been shown that patients with chronic candidal diseases often present high levels of this cytokine (Romani 2011). It has been shown that patients with OSCC have increased levels of salivary IL-10 and high expression of this cytokine in tumor cells has been associated with poor prognosis (Chen et al. 2013; Aziz et al. 2015). Therefore, the anti-inflammatory environment of neoplastic epithelium might support the proliferation of commensal yeasts by suppressing the activity of innate immune cells that are responsible for the limitation of microbial overgrowth. Using MALDI-TOF-MS analysis that represents a new and rapid method for the identification of yeasts in clinical samples (Pinto et al. 2011; Bader et al. 2011), we found that in addition to higher fungal burdens, the spectrum of isolated yeast genera was wider in samples derived from OSCC patients compared to healthy controls. This finding further strengthens the notion that the altered microenvironment associated with tumorigenesis leads to the development of a more diverse oral microflora. In conclusion, our results reveal important differences in the oral fungal flora of OSCC patients compared to healthy individuals, although further research is needed to elucidate the etiologic relationship between Candida infection and carcinogenesis.

Table 3  Extracellular lipase/protease activity of Candida strains isolated from OSCC or control patients

| No. of isolates (%) | lip+ | lip− | prote+ | prote− | lip+/prote+ | lip−/prote− | lip+/prote− | lip−/prote− |
|---------------------|------|------|--------|--------|-------------|-------------|-------------|-------------|
| Control (N = 40)    |      |      |        |        |             |             |             |             |
| N                   | 13   | 27   | 15     | 25     | 6           | 7           | 9           | 18          |
| %                   | 32.5 | 67.5 | 37.5   | 62.5   | 15          | 17.5        | 22.5        | 45          |
| OSCC (N = 140)      |      |      |        |        |             |             |             |             |
| N                   | 53   | 87   | 66     | 74     | 37          | 16          | 29          | 58          |
| %                   | 37.86| 62.14| 47.14  | 52.86  | 26.43       | 11.43       | 20.71       | 41.43       |

References

Alnaimi AD et al (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(2):139–145
Aziz S et al (2015) Salivary immunosuppressive cytokines IL-10 and IL-13 are significantly elevated in oral squamous cell carcinoma patients. Cancer Invest 33(7):318–328
Bader O et al (2011) Improved clinical laboratory identification of human pathogenic yeasts by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect 17(9):1359–1365
Barrett AW, Kingsmill VJ, Speight PM (1998) The frequency of fungal infection in biopsies of oral mucosal lesions. Oral Dis 4(1):26–31
Cawson RA (1966) Chronic oral candidiasis and leukoplakia. Oral Surg Oral Med Oral Pathol 22(5):582–591

Acknowledgements

AG was supported by Janos Bolyai Fellowship of the Hungarian Academy of Sciences. AG, AT, JSZ, AG analysed data, AT, AG prepared the manuscript. All authors read and approved the final manuscript.

Author details

1 Department of Oral Surgery, University of Szeged, Tisza Lajos krt. 64, Szeged 6720, Hungary. 2 Department of Microbiology, University of Szeged, Közép fasor 52, Szeged 6726, Hungary. 3 Institute of Clinical Microbiology, University of Szeged, Semmelweis u. 6, Szeged 6725, Hungary.

Competing interests

The authors declare that they have no competing interests.

Received: 20 December 2015   Accepted: 27 July 2016
Published online: 04 August 2016
Chen CJ et al (2013) High expression of interleukin 10 might predict poor prognosis in early stage oral squamous cell carcinoma patients. Clin Chim Acta 415:25–30
Dwivedi PP, Malviya S, Dongar-Bartzoglou A (2009) A novel immunocompetent murine model for Candida albicans-promoted oral epithelial dysplasia. Med Mycol 47(2):157–167
Hebbar PB, Pai A (2013) Mycological and histological associations of Candida in oral mucosal lesions. J Oral Sci 55(2):157–160
Jahanshahi G, Shirani S (2015) Detection of Candida albicans in oral squamous cell carcinoma by fluorescence staining technique. Dent Res J (Isfahan) 12(2):115–120
Lacroix C et al (2014) Evaluation of two matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) systems for the identification of Candida species. Clin Microbiol Infect 20(2):153–158
McCullough M et al (2002) Oral yeast carriage correlates with presence of oral epithelial dysplasia. Oral Oncol 38(4):391–393
Mohd Bakri M et al (2010) Revisiting the association between candidal infection and carcinoma, particularly oral squamous cell carcinoma. J Oral Microbiol. doi:10.3402/jom.v2i0.5780
Monod M, Borg-von ZM (2002) Secreted aspartic proteases as virulence factors of Candida species. Biol Chem 383(7–8):1087–1093
Nagy KN et al (1998) The microflora associated with human oral carcinomas. Oral Oncol 34(4):304–308
Nemeth T et al (2013) Characterization of virulence properties in the C. parapsilosis sensu lato species. PLoS ONE 8(7):e66704
Pinto A et al (2011) Matrix-assisted laser desorption ionization-time of flight mass spectrometry identification of yeasts is contingent on robust reference spectra. PLoS ONE 6(10):e25712
Pires FR et al (2013) Oral squamous cell carcinoma: clinicopathological features from 346 cases from a single oral pathology service during an 8-year period. J Appl Oral Sci 21(5):460–467
Romani L (2011) Immunity to fungal infections. Nat Rev Immunol 11(4):275–288
Sardi JC et al (2013) Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol 62(Pt 1):10–24
Stehr F et al (2004) Expression analysis of the Candida albicans lipase gene family during experimental infections and in patient samples. FEMS Yeast Res 4(4–5):401–408
Trofa D et al (2009) Acetylsalicylic acid (aspirin) reduces damage to reconstituted human tissues infected with Candida species by inhibiting extracellular fungal lipases. Microbes Infect 11(14–15):1131–1139