Proteolytic activity of non-albicans Candida and Candida albicans in oral cancer patients

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SUMMARY

Oral Candida infections can be life-threatening in medically compromised patients. In particular non-albicans Candida strains are virulent. However, our knowledge is sparse on how proteolytic these strains are in patients with oral cancer. Our study aimed to investigate differences in proteolytic activity of non-albicans Candida and Candida albicans isolated from oral cancer patients. The hypothesis was based on anticipated different invasive capacity of the strains. Clinical and reference yeast samples from our laboratory were used for analyses. Candida strains were grown in yeast peptone glucose and the activity of Candida proteinases of broken cell fractions were analysed by MDFP-gelatin zymography. Fluorometric assay was used to compare activities of proteolytic enzymes and degradation assays were performed using CLDN 4 and plasma fibronectin. Clear differences were seen in the proteolytic activity between the studied non-albicans Candida and C. albicans strains. C. tropicalis had the highest proteolytic activity followed by strains of C. krusei and C. glabrata. The results confirmed our study hypothesis by showing differences between the non-albicans Candida and Candida albicans strains studied. Higher proteolytic activity may thus have an effect on the virulence of non-albicans Candida strains in oral cancer patients.

INTRODUCTION

The mouth is an important source of infections that may even be associated with mortality (Hämäläinen et al., 2005). Invasive Candida infections are particularly associated with high mortality in the aging population and non-albicans Candida are responsible for most of such cases (Guo et al., 2013; Tortorano et al., 2012). Candida may also play a role in the development of cancer (Meurman et al., 2011). In a follow-up study from Denmark, subjects with Candida infections were found to have a twofold increased risk of cancers found in mouth, tongue, oropharynx and esophagus (Nørgaard et al., 2013). Candida infections have been associated with oral epithelial dysplasia and neoplasia (Cawson, 1969, McCullough et al., 2002). C. albicans is the most frequently isolated strain from cancerous lesions followed by C. glabrata and C. tropicalis (Galle et al., 2013). A higher number of C. albicans genotype A strains were found in oral cancer patients, showing this organism as a possible significant risk marker in oral cancer patients (Alnuaimi et al., 2015). Non-albicans Candida strains, however, pose a further risk to cancer patients due their increasing antifungal agent resistance (Bugg et al., 2003; Kalantar et al., 2015). Furthermore, the non-albicans strains have been shown to be an important cause of systemic infections in cancer patients in general (Nucci et al., 1998; Schelenz et al., 2011). Oral mucosal fungal infections increase with the aging population. Yeasts are opportunistic pathogens and systemic yeast infections can be fatal in frail patients (Richardson et al., 2003). Oral candidosis is a common infection particularly in medically compromised patients, such as patients with HIV infection, endocrine disorders and nutritional deficiencies (Scully et al., 1994). Oral cancer patients’ immune defenses are often impaired emphasizing the risk for systemic infections. Furthermore, Candida strains like some other oral microorganisms metabolize ethanol to carcinogenic acetaldehyde (Tillonen et al., 1999; Meurman et al., 2008). Of the non-albicans strains C. glabrata in particular seems to produce acetaldehyde from ethanol which could be one pathogenic mechanism in the development of oral cancer (Nieminen et al., 2009). Previous studies have shown that non-albicans Candida degrade junctional and basement membrane proteins (Pärnänen et al., 2008). Fibronectin is a glycoprotein that attaches to other extracellular and cell surface proteins, such as collagen and integrins. Human plasma fibronectin has a molecular weight of 440 kDa and is produced by hepatocytes. Plasma fibronectin circulates in the blood and plays a role in blood clotting, wound healing and phagocytosis. Its structure is similar to locally produced cellular fibronectin in oral epithelial tissue (Tamkun et al., 1983). Tight junctional proteins form a seal between adjacent polarized epithelial or endothelial cells, which ensures proper tissue barrier function (Krause et al., 2008). CLDNs are integral membrane proteins found in tight junctions of all...
epithelia and endothelia. CLDNs are 20-27 kDa transmembrane proteins which span the bilayer four times, where the N- and C- termini are oriented towards the cytoplasm and there are two extracellular loop domains (Furuse et al., 1998; Morita et al., 1999). A basic property of CLDNs in the tight junctions is the paracellular sealing function, which is tissue, size and charge selective. CLDN4 is known to reduce paracellular cation permeability (Gerd et al., 2008). The present study examined the proteolytic activity of non-albicans Candida and Candida albicans isolated from oral cancer patients. We hypothesized that non-albicans yeasts present higher proteolytic activity compared to C. albicans. For comparison, laboratory Candida strains were investigated.

**MATERIAL AND METHODS**

**Patient and laboratory samples**

The patients were in-patients undergoing routine treatment for oral cancer in our hospital. They were examined in the hospital ward and paraffin stimulated whole saliva samples were collected from the subjects according to Meurman and Rantone (1994). The study exclusion criteria were the following: patients with a previous one week hospitalization, HIV infection, malignancy other than oral cancer, and other immunosuppression than that of the oral cancer treatment protocol. The ethical committee of the Hospital District of Helsinki and Uusimaa had approved the study (Ethical permit #525/E6/2003). In addition to clinical samples, Candida isolates from our laboratory were used for comparison. These included the America Type Culture Collection (www.ATCC.org) species listed in Table 1.

**Candida isolates**

The identification of Candida was based on colony morphology on CHROMagar® Candida medium (CHROMagar, Paris, France), latex agglutination test (Bichro-Dubli Fu-mouze®, Fumouze Diagnostics, Levallois-Perret, France) and API ID 32C (Bio-Merieux, Lyon, France) assimilation tests (Scully et al., 1994; Chryssanthou et al., 2007).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
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<tbody>
<tr>
<td>C. albicans 16.1</td>
<td>Helsinki University Hospital, Helsinki, Finland</td>
</tr>
<tr>
<td>C. albicans ATCC 32723</td>
<td>Department of Oral and Maxillofacial Diseases Research Laboratory, Helsinki, Finland</td>
</tr>
<tr>
<td>C. glabrata 40</td>
<td>Helsinki University Hospital, Helsinki, Finland</td>
</tr>
<tr>
<td>C. glabrata ATCC 32725</td>
<td>Department of Oral and Maxillofacial Diseases Research Laboratory, Helsinki, Finland</td>
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<tr>
<td>C. krusei 96</td>
<td>Helsinki University Hospital, Helsinki, Finland</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>Department of Oral and Maxillofacial Diseases Research Laboratory, Helsinki, Finland</td>
</tr>
<tr>
<td>C. tropicalis 32.2</td>
<td>Helsinki University Hospital, Helsinki, Finland</td>
</tr>
<tr>
<td>C. tropicalis ATCC 750</td>
<td>Department of Oral and Maxillofacial Diseases Research Laboratory, Helsinki, Finland</td>
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The isolates were stored in 20% skim milk at -80°C. Four Candida species were included in the present study, both reference and clinical strains: C. albicans, C. glabrata, C. krusei and C. tropicalis (Table 1). We chose the Candida species most commonly found and those previously known as most proteolytic of the oral species based on our earlier investigations (Pärnänen et al., 2008; Pärnänen et al., 2009; Pärnänen et al., 2010; Nawaz et al., 2015). Other yeast species were excluded from the present study. Yeasts were grown in YPG medium containing 1% yeast extract, 1% peptone, and 1% glucose, at 37°C for 24 hours in a water bath with shaking. One millilitre of yeast (10⁷ cfu) suspension was washed twice (14000 rpm, 10 min, and 4°C) with TNC buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃). The cells were broken with 0.5 mm glass beads and Retsch Mixer Mill (Retsch GmbH, Haan, Germany) according to Nawaz et al. (2015).

To investigate the enzymatic activity of Candida proteinases, the cell-bound fractions were analysed by 2-methoxy-2, 4-dephenyl-3-(2H)-furane (MDPF, Fluka, Buchs SG, Switzerland) gelatin zymography (Nawaz et al., 2015). Briefly, cell-bound fractions (15µl) were incubated with sample buffer and run on 11% SDS-PAGE with MDPF labelled-gelatin (1 mg/ml). Staining was performed with 0.2% Coomassie Brilliant Blue and the gels were scanned with Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA).

**Fluorimetry**

The activities of proteolytic enzymes were analysed using fluorometric assay according to the manufacturer’s instructions in a black 96-well microtiter plate (Costar; Corning, NY, USA) using Perkin Elmer 2030 Multi label reader (Victor x-4). L-Arg 7-amido-4methylcoumarin hydrochloro-ride (AMC) (Sigma) was used as substrate. Ten microlitres of cell bound fractions were incubated with L-Arg- AMC in 182 µl of TNC buffer 150 µl of ethanol was used to stop the reaction after 15 min. Excitation wavelength 355 nm and emission wavelength 535 nm were used in order to measure the fluorescence using Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the Quantity one program (Nawaz et al., 2015).

**CLDN degradation assay**

CLDN4 was used for the degradation studies. Fifteen microlitres of unbroken yeast cells (10⁷ cfu/ml) were incubated with 1 µl (0.55 µg/µl) CLDN4 at 37°C for 24 h. Un-incubated and 24 h incubated (37°C) CLDN4 (both without yeast) were used as controls. After incubation, the samples were centrifuged (14000 rpm, 10 min, and 4°C) and 15 µl of top supernatants were run on sodium dodecyl-sulphate polyacrylamide gel electrophoresis (4-20% Mini-PROTEAN® TGX™ Precast Gels; Bio-Rad Laboratories Inc., Carlsbad, CA, USA). After electrophoresis, the gels were stained and scanned with Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the Quantity one program. The degradation assays were performed three times, and the results were repeatedly identical.

**Fibronectin degradation assay**

Fifteen microlitres of unbroken yeast cells (10⁷ cfu/ml) were incubated with 3.3 µl (0.12 µg/µl) plasma fibronectin (pFn) at 37°C for 24 h. Un-incubated and 24 h incubated (37°C) fibronectin (both without yeast) were used as controls. Centrifugation, SDS-PAGE, silver staining and scan-
ning were performed as above with Pierce Silver Stain Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). SDS-PAGE analysis was performed three times and the results were repeatedly identical.

RESULTS

Patient data
Of the 106 patients enrolled in the study, 46% were women. The patients’ average age was 67.5 ±10.5 years (range from 31 to 91 years). Ninety-four percent of the patients had a diagnosis of oral squamous cell carcinoma, whereas 3% had adenocarcinoma and 3% had other malignancies of the mouth. Most of the patients harbored C. albicans (56.6%), while 28% showed no yeast growth. NAC was isolated in 17% of the samples. The most common NAC yeast found was C. dubliniensis (8% of samples) followed by C. tropicalis (3%), C. krusei (1%) and C. glabrata (1%).

Zymography
The cell bound fractions of all strains except C. albicans showed 20-100 kDa gelatinolytic activity at pH 7.6 (Figure 1). C. tropicalis was the most gelatinolytic when compared with the rest of the strains (Figure 2). The only clinical strain that showed slightly higher gelatinolysis than the respective ATCC strain was C. glabrata Cg40. C. albicans showed no gelatinolytic activity.

Fluorimetry
C. glabrata Cg40 showed the highest fluorescence followed by the rest of the Candida strains. Furthermore, C. glabrata Cg40 and C. krusei Ck96 appeared more L- arg-AMC degradative than the ATCC strains (Figure 3). Overall C. albicans as well as the non-albicans strains degraded this substrate.

Degradation assays
The degradation with unbroken yeast cells is seen as a reduced or disappeared intensity of the substrate bands. The

![Figure 1 - Gelatinolytic activities assessed by MDPF-gelatin zymography for cell fractions at pH 7.6. The activities of cell fractions in MDPF-gelatin zymography showed that C. tropicalis was the most gelatinolytic among all species. Order: lane 1: molecular weight standard (kDa), lane 2: C. albicans ATCC 32723; lane 3: C. albicans 16.1; lane 4: C. glabrata ATCC 32725; lane 5: C. glabrata 40; lane 6: C. tropicalis ATCC750; lane 7: C. tropicalis 32.2; lane 8: C. krusei ATCC 6258; lane 9: C. krusei 96.](image1)

![Figure 2 - Gelatinolytic activities of Candida strains studied. The higher optical density (Odu/mm²) indicates higher gelatinolytic activity.](image2)
Proteolytic activity of Candida in oral cancer patients

**DISCUSSION**

We investigated the proteolytic activities of non-albicans Candida compared with those of C. albicans from oral cancer patients with the hypothesis that the non-albicans strains show higher activity. As expected, C. tropicalis showed higher proteolytic activity than the other strains investigated. Our results are in line with our previous studies showing that non-albicans strains indeed have higher protease activity (Nawaz et al., 2015). C. glabrata and C. krusei appeared more L-arg-AMC degradative than ATCC strains. Overall C. albicans and the non-albicans strains degraded this substrate. The clinical strain of C. glabrata showed slightly higher gelatinolytic activity than the corresponding ATCC strain while C. albicans showed none. All the species were capable of degrading CLDN4 and fibronectin in various degrees.

In the present study, 17% of the oral cancer patients harbored non-albicans Candida strains which is in agreement with earlier studies (Davies et al., 2002; de Freitas et al., 2013). Previously, non-albicans strains were isolated in particular from patients who had received radiotherapy to the head and neck due to cancer (Redding et al., 1999, 2004; Karbach et al., 2012).

In general, Candida species are prevalent in oral cancer patients, and their increase may be due to the compromised immune system of the host due to the underlying disease process and its treatment (De Sousa et al., 2016). Indeed, the clinical species investigated here showed higher proteolytic activity compared to laboratory strains which may indicate higher virulence of the clinical isolates. Basement membrane integrity is essential for the normal function of the epithelium. Fibronectin is an adhesion protein and its degradation might cause changes in the adhesive functions and altered mobility of epithelial cells. Studies performed with various Candida strains have suggested that Candida degrades fibronectin (Morschhäuser...
CLDN4 is a potential marker for predicting the outcome of patients with oral squamous cell carcinoma (De Vicente et al., 2015). Previous researchers have used extracellular hydrolyses such as proteinases and phospholipases as major facilitators to explain the complex host tissue invasion and the disease process (Mane et al., 2012). Based on previous research and our present findings, tools might be developed in the future for diagnosing Candida infections accurately and in explaining the underlying processes of Candida invasion in detail. At the molecular level, it is important to understand and verify the interaction mechanisms between basic tissue structural proteins and Candida yeasts. The main strength of the present study is the homogenous patient material from a Caucasian population derived from the 1.5 million inhabitants of the Helsinki University Hospital area. However, further studies are needed to compare differences in the prevalence of Candida species and their proteolytic activities in patients with and without cancer. Moreover, more complex settings are needed to explain in detail the interaction of non-albicans Candida and also other yeast strains with oral mucosal cells. Molecular level understanding may offer new means for assessing the risk that yeasts pose on oral cancer development and progression during cancer treatment.

Conflict of Interest
The authors declare that they have no conflict of interest.

Acknowledgements
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References


et al., 1997; dos Santos et al., 2005; Pärnänen et al., 2009). Our results support these findings particularly regarding the Candida strains isolated from the oral cancer patients. CLDNs are important transmembrane proteins within tight junctions that promote cell-to-cell adhesion by forming intercellular strands comprised of various claudin combinations (Furuse et al., 1999). Loss of the cell-to-cell adhesion is considered a primary step in the process of microbial invasion and cancer metastasis, making the adhesion proteins important predictive biomarkers for both metastasis and recurrence. CLDN4 is a component of the tight junctions important for sealing cellular sheets and controlling paracellular ion flux. Hence CLDN4 degradation may cause changes which may promote the spread of infection and indicate invasiveness of the Candida strains here observed. CLDN can have either cancer-promoting or tumor-suppressing functions, leading to complex relationships between CLDN expression and patient prognosis (Tabaries et al., 2017). Previous studies have shown that...
Furuse M., Sasaki H., Tsukita S. (1999). Manner of interaction of hetero-
genous claudin species within and between tight junction strands. J Cell Biol. 147, 891-903.
sp. in oral cancer and oral precancerous lesions. New Microbiol. 36, 283-288.
Structure and function of claudins. Biochim Biophys Acta - Biomem-
branes. 1778, 631-45.
in intensive care units in china: A multicentre prospective observa-
antibiotic resistance pattern caused oropharyngeal candidiasis among
Candida colonization and susceptibility of Candida strains after head
Mane A., Gaikwad S., Bemblark S., Risbud A. (2012). Increased expres-
sion of virulence attributes in oral Candida albicans isolates from hu-
Oral yeast carriage correlates with presence of oral epithelial dyspla-
sia. Oral Oncol. 38, 391.
linked to cancer? Oral Dis. 17, 779-84.
Meurman J.H., Kantonen P. (1994). Salivary flow rate, buffering capacity,
and yeast counts in 187 consecutive adult patients from Kuopio, Fin-
lan. Scand J Dent Res. 102, 229-34.
Meurman J.H., Uttamaj J. (2008). Oral micro-organisms in the etiology of
family encoding four-transmembrane domain protein components of
tight junction strands. Proc Natl Acad Sci. 96, 511-6.
of human subendothelial extracellular matrix by proteinase-secret-
ty and cytokine up-regulation by non-albicans Candida albicans. Arch
Microbiol. 197, 533-7.