New Microbiologica, 41, 4, 296-301, 2018, ISSN 1121-7138

FULL PAPER

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 MDPF-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.

Received April 25, 2018 *Accepted* September 16, 2018

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 (Bagg *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

Key words:
Non-*albicans Candida*, Proteolysis, Oral cancer, *Candida albicans*.

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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 Rantonen (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 Fumouze®, Fumouze Diagnostics, Levallois-Perret, France) and API ID 32C (Bio-Merieux, Lyon, France) assimilation tests (Scully *et al.,* 1994; Chryssanthou et al., 2007).

Table 1 - *Candida strains included in the study.*

Strain	*Source*
C. albicans 16.1	Helsinki University Hospital, Helsinki, Finland
C. albicans ATCC 32723	Department of Oral and Maxillofacial Diseases Research Laboratory, Helsinki, Finland
C. glabrata 40	Helsinki University Hospital, Helsinki, Finland
C. glabrata ATCC 32725	Department of Oral and Maxillofacial Diseases Research Laboratory, Helsinki, Finland
C. krusei 96	Helsinki University Hospital, Helsinki, Finland
C. krusei ATCC 6258	Department of Oral and Maxillofacial Diseases Research Laboratory, Helsinki, Finland
C. tropicalis 32.2	Helsinki University Hospital, Helsinki, Finland
C. tropicalis ATCC 750	Department of Oral and Maxillofacial Diseases Research Laboratory, Helsinki, Finland

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 milliliter of yeast (10^7cfu) 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 $CaCl_2$, 0.02% NaN_3). The cells were broken with 0.5mm 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)-furanone (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 (1mg/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 hydrochloride (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 (10^7 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 (10^7cfu/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. glabrata* (3%), *C. tropicalis* (3%), *C. parapsillosis* (2%), *C. sake* (2%), *C. krusei* (1%) and *C. quilliermondii* (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. *albican*s 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

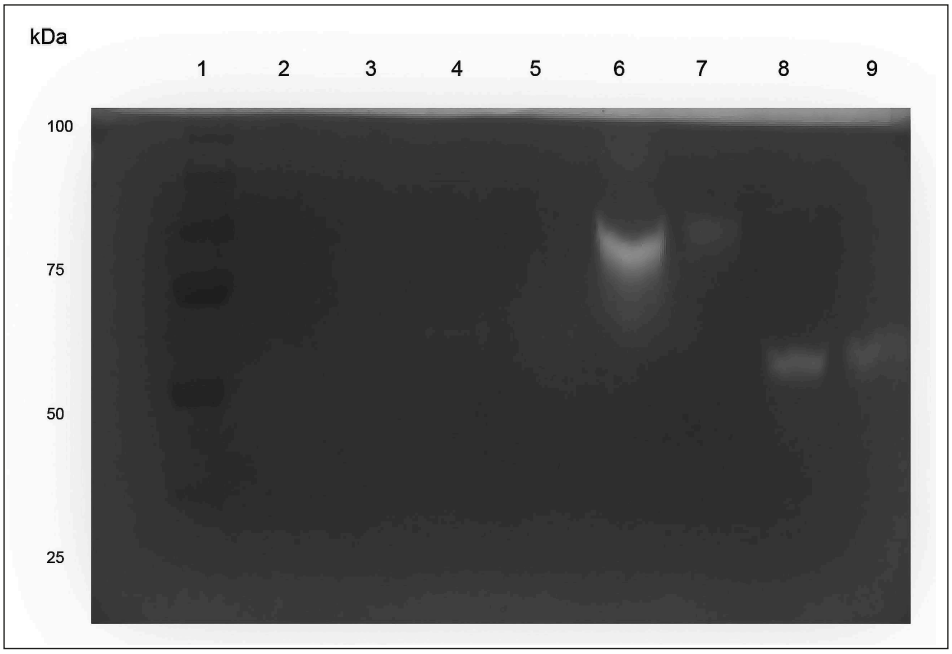

Figure 1 - *Gelatinolytic activities assessed by MDPF-gelatin zymography for cell fractions at pH 7.6. The activities of cell fractions in MDPF-gelatin zymography showed that* C. tropicalis *was the most gelatinolytic among all species. Order: lane 1: molecular weight standard (kDa), lane 2:* C. albicans *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.*

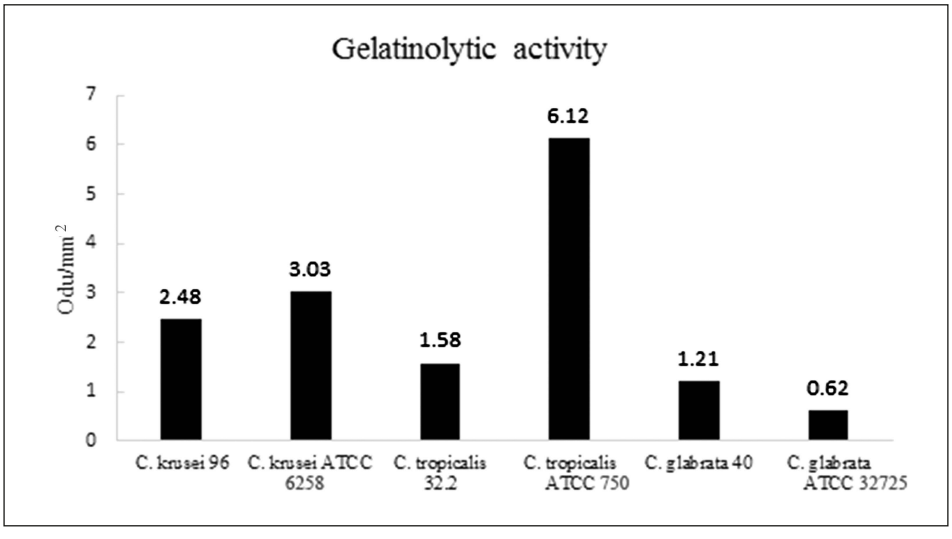

Figure 2 - *Gelatinolytic activities of* Candida *strains studied. The higher optical density (Odu/mm²) indicates higher gelatinolytic activity.*

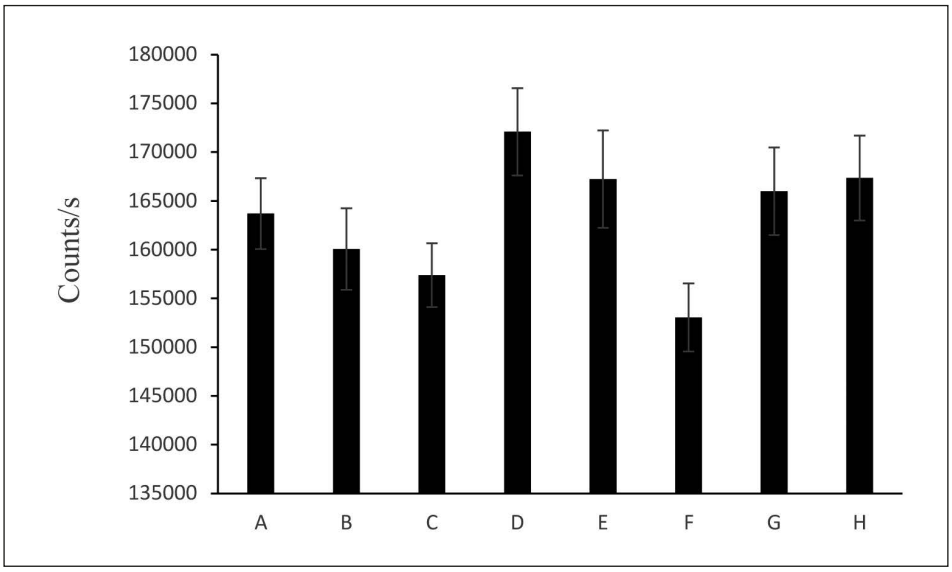

Figure 3 - *Fluorimetric analysis of the* Candida *strains studied. The average counts obtained are on y-axis while the yeast samples are on x-axis. (A)* C. albicans *ATCC 32723; (B)* C. albicans *16.1; (C)* C. glabrata *ATCC 32725; (D)* C. glabrata *40; (E)* C. tropicalis *ATCC 750; (F)* C. tropicalis *32.2; (G)* C. krusei *ATCC 6258; (H)* C. krusei *96.*

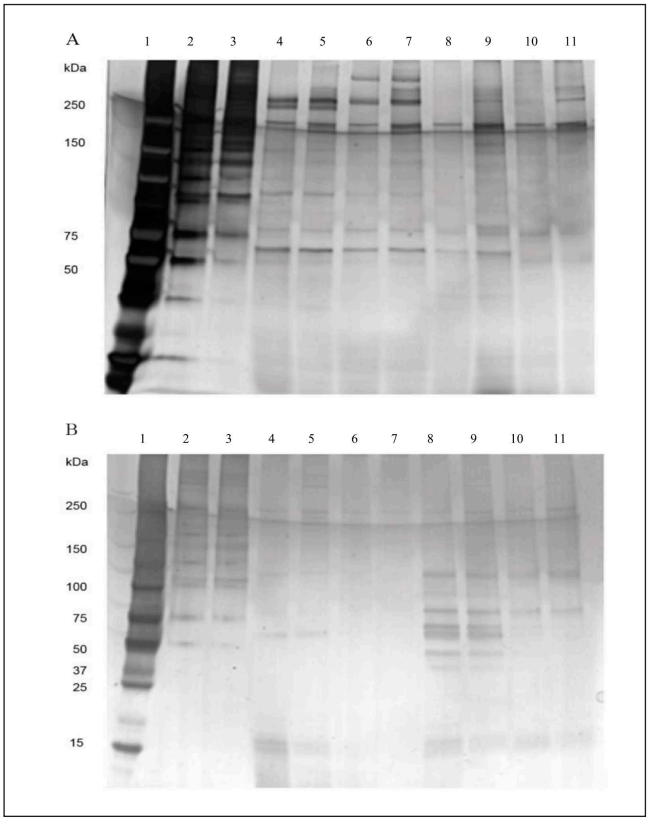

Figure 4 - *SDS-PAGE. Plasma fibronectin (pFn) incubated with* Candida *fractions. (A) Samples in wells 2-11. Lane 2: intact unincubated pFn. Lane 3: incubated pFn. Lane 4:* C. krusei *ATCC 6258. Lane 5: pFn* + C. krusei *ATCC 6258. Lane 6:* C. krusei *96. Lane 7: pFn* + C. krusei *96. Lane 8:* C. tropicalis *ATCC 750. Lane 9: pFn* + C. tropicalis *ATCC 750. Lane 10:* C. tropicalis *32.2. Lane 11: pFn* + C. tropicalis *32.2. (B) Lane 2: intact unincubated pFn. Lane 3: incubated pFn. Lane 4:* C. albicans *ATCC 32723. Lane 5: pFn* + C. albicans *ATCC 32723. Lane 6:* C. albicans *16.1. Lane 7: pFn* + C. albicans *16.1. Lane 8:* C. glabrata *ATCC 32725. Lane 9: pFn* + C. glabrata *ATCC 32725. Lane 10:* C. glabrata *40. Lane 11: pFn* + C. glabrata *40. The molecular weight standard on the left in kilodaltons (kDa).*

degradation of fibronectin and CLDN4 is seen in *Figure 4A* and *Figure 5A* by strains of *C. krusei* and *C. tropicalis,* respectively, while the degradation of fibronectin and CLDN4 is visible in *Figure 4B* and *Figure 5B* by strains of *C. glabrata* and *C. albicans*.

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 Candida* indeed have higher protease activity (Nawaz *et al.,* 2015). *C. glabrata* 40 and *C. krusei* 96 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* 40 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

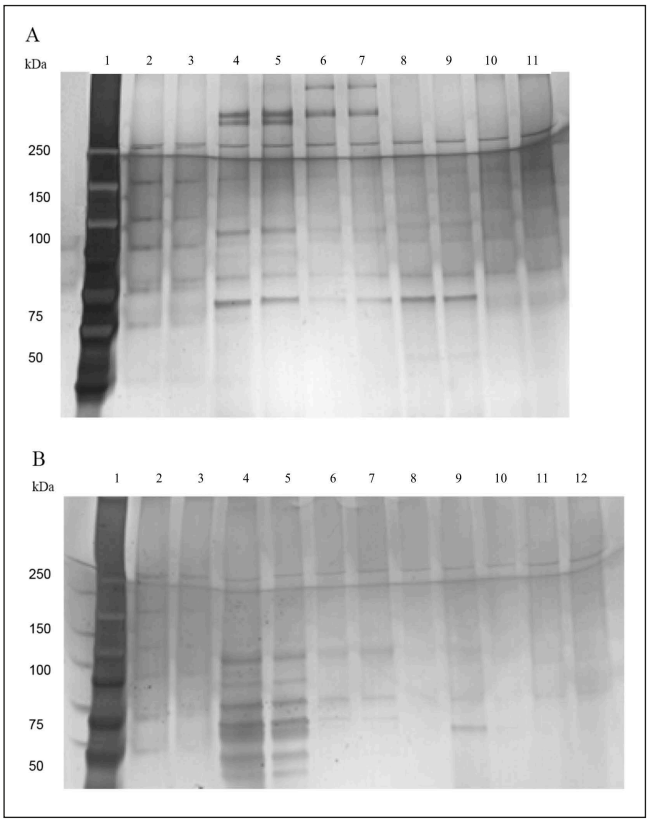

Figure 5 - SDS-PAGE. CLDN4 incubated with *Candida* fractions. (A) Samples in wells 2-11. Lane 2: intact unincubated CLDN4. Lane 3: incubated CLDN4. Lane 4: *C. krusei* ATCC 6258. Lane 5: CLDN4 + *C. krusei* ATCC 6258. Lane 6: *C. krusei* 96. Lane 7: CLDN4 + *C. krusei* 96. Lane 8: *C. tropicalis* ATCC 750. Lane 9: CLDN4 + *C. tropicalis* ATCC 750. Lane 10: *C. tropicalis* 32.2. Lane 11: CLDN4 + *C. tropicalis* 32.2. (B) Lane 2: intact unincubated CLDN4. Lane 3: incubated CLDN4. Lane 4: CLDN4 + *C. glabrata* ATCC 32725. Lane 5: *C. glabrata* ATCC 32725. Lane 6: *C. glabrata* 40. Lane 7: CLDN4 + *C. glabrata* 40. Lane 8: *C. albicans* ATCC 32723. Lane 9: CLDN4 + *C. albicans* ATCC 32723. Lane 10: CLDN4 + *C. albicans* ATCC 32723. Lane 11: *C. albicans* 16.1. Lane 12: CLDN4 + C. *albicans* 16.1. The molecular weight standard on the left in kilodaltons (kDa).

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 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 hydrolases 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

The study was carried out during 2015-2017 at the Department of Oral and Maxillofacial diseases, Helsinki University Hospital in Finland. The study was supported by a grant from the Helsinki University Hospital (Y1149SUL26) and by the Finnish Medical Society to J.H.M. Chancellors. A travel grant from the University of Helsinki was given to A.N.

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