The role of *Candida albicans* candidalysin ECE1 gene in oral carcinogenesis

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Abstract
Oral squamous cell carcinoma is associated with many known risk factors including tobacco smoking, chronic alcoholism, poor oral hygiene, unhealthy dietary habits and microbial infection. Previous studies have highlighted *Candida albicans* host tissue infection as a risk factor in the initiation and progression of oral cancer. *C albicans* invasion induces several cancerous hallmarks, such as activation of proto-oncogenes, induction of DNA damage and overexpression of inflammatory signalling pathways. However, the molecular mechanisms behind these responses remain unclear. A recently discovered fungal toxin peptide, candidalysin, has been reported as an essential molecule in epithelial damage and host recognition of *C albicans* infection. Candidalysin has a clear role in inflammasome activation and induction of cell damage. Several inflammatory molecules such as IL-6, IL-17, NLRP3 and GM-CSF have been linked to carcinogenesis. Candidalysin is encoded by the ECE1 gene, which has been linked to virulence factors of *C albicans* such as adhesion, biofilm formation and filamentation properties. This review discusses the recent epidemiological burden of oral cancer and highlights the significance of the ECE1 gene and the ECE1 protein breakdown product, candidalysin in oral malignancy. The immunological and molecular mechanisms behind oral malignancy induced by inflammation and the role of the toxic fungal peptide candidalysin in oral carcinogenesis are explored. With increasing evidence associating *C albicans* with oral carcinoma, identifying the possible fungal pathogenicity factors including the role of candidalysin can assist in efforts to understand the link between *C albicans* infection and carcinogenesis, and pave the way for research into therapeutic potentials.

Keywords
*Candida albicans*, candidalysin, ECE1 protein, immunomodulation
1 | INTRODUCTION

Oral cancer is classified as the sixth most common cancer in the world and globally ranks as the eleventh most common cancer in males and nineteenth in females. Oral cancer incidence has been consistently higher in males compared to females over the last few decades. However, the gender ratio gap is slowly narrowing, owing to an increasing trend of tobacco smoking and alcohol consumption in females. According to The Global Cancer Observatory (GLOBOCAN), it is reported that the new cases of the lip, oral cavity and pharyngeal cancers in 2018 are 354,864 worldwide with 177,384 were reported deaths. Mortality rates for both lip and oral cancers are higher in developing countries, especially in females. The highest incidences of oral cancer have been reported in the Pacific (Melanesia), South-East Asia, and Central and Eastern Europe, while lowest incidences were reported in Eastern Asia and Western Africa. Although surgical advances and chemoradiotherapy have improved the quality of life of patients, the overall mortalities remain unchanged.

Oral squamous cell carcinoma (OSCC) has been classified as the most common type of oral cancer, accounting for greater than 90% of malignancies originating from the oral cavity. It has been reported that the overall 5-year survival rate is only 50% with possibilities of recurrence. The global prevalence of OSCC is highlighted in Malaysia, with a 1.5% increase in oral cancer incidence and mortality rates of 1.2% as reported by GLOBOCAN in 2018. The risk factors that are associated with OSCC include tobacco smoking, heavy alcohol consumption, poor oral hygiene, unhealthy diet and microbial infection. Due to the increased global prevalence of oral cancer, an in-depth multi-factorial analysis is required to understand the underlying mechanisms behind oral cancer occurrence and probable association with other risk factors, particularly biological causative agents.

1.1 | Role of C albicans in promoting oral carcinogenesis

Candida species, especially C albicans, are normal components of the human oral cavity. C albicans is an opportunistic fungus that is pathogenic in immunocompromised individuals (HIV, cancer or transplant patients). It possesses the distinct ability to grow polymorphically, that is as yeast and hyphal form (Figure 1). The pathogenic state of C albicans is commonly associated with biofilm and hypha formation. Colonization of C albicans along the gastrointestinal tract has been significantly linked to candidemia and is a risk factor for invasive candidiasis.

There has been a growing body of evidence supporting the link between microorganisms and various types of cancer, including the oral cavity. In normal host physiology, the microbiome coexists homeostatically; however, this balance can be altered by extrinsic factors such as a weakened immune system due to disease or prolonged cancer treatment. Upset of the balanced interactions could lead to outgrowth of opportunistic pathogens over beneficial microbes, resulting in host immunological reactions that may lead to oncogenesis.

Oral carcinoma begins as epithelial dysplasia (cell damage), a state in which the normal structure of epithelial cells is altered to an abnormal proliferative state. Dysplasia results from cell injury followed by chronic inflammation. Epithelial dysplasia is characterized by altered proliferation of the damaged squamous cells on the epithelial surface, which leads to degradation of the basal membrane. The damaged cell undergoes apoptosis, or transforms into a malignant state, resulting in local destruction and distant invasion. The ability of cancerous cells to invade underlying connective tissues followed by migration to distant sites to form metastases is characteristic of carcinoma.

Infection-induced inflammation has been reported as a risk factor in oral carcinogenesis. A common factor that leads to inflammation in the oral cavity is the increase in pro-inflammatory cytokines due to microbial infection of the oral mucosa. Cytokines are signalling molecules that regulate differentiation, proliferation and other crucial functions in human inflammatory cells. Cytokine signals are received at the cell surface not only as a single message, but also as complex, subtle, synergistic and antagonistic combinations that coordinate processes, including stimulation of haematopoiesis, orchestration of directed leucocyte migration (chemokines), activation of various inflammatory cells, stimulation of lymphocyte development and maturation, as well as processes associated with the immune response. Nonetheless, under certain circumstances such as failure to resolve an injury, an excessive immune cell infiltration is provoked that may persistently generate cytokines. As a result, the host may respond to the high cytokine expression by enhancing cancer formation and progression. Cytokines that are involved in inflammation include interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-18, tumour necrosis factor (TNF)-α, IFN-γ and GM-CSF.
C. albicans has been recognized as an independent risk factor in the development of oral carcinoma. C. albicans has also been suggested to be associated with oral leukoplakic lesions, a pre-cancerous state of oral cancer. Candida-linked leukoplakia presents as a chronic oral Candida infection (candidiasis) with increased malignancy potential compared to non-Candida leukoplakia. The majority of non-homogenous leukoplakias that are most often invaded by C. albicans have higher malignant transformation potential than the homogenous leukoplakias. C. albicans invades leukoplakias when there is an imbalance between C. albicans virulence factors and host defences, which commonly occurs in immunocompromised individuals. Thus, a defect in the immune system and overexpression of the C. albicans virulence traits represent a risk factor for C. albicans infections that have malignant potential.

Colonization by C. albicans is initiated with the adhesion of the fungus to the host cell surface with a subsequent morphological switch from yeast to hyphae. Alnuaimi et al. established that there is a high frequency of oral yeast presence with high levels of oral colonization in oral cancer patients compared to non-oral cancer individuals. The study further showed that C. albicans genotype A was implicated explicitly in oral cancer patients. This finding is supported by a previous study by McCullough et al. reporting that oral yeast density is strongly related to epithelial dysplasia and squamous cell carcinoma, which suggested that progression from leukoplakia into dysplasia is induced by C. albicans. C. albicans associated with dysplasia may represent a secondary infection of a pre-existing altered epithelium.

C. albicans is more commonly isolated from oral biofilms on OSCC sites when compared to the control sites.

2 | ROLE OF CANDIDALYSISN IN C. ALBICANS PATHOGENICITY AND AS A CANCER RISK FACTOR

Due to the increasing evidence associating C. albicans with oral carcinoma, recent efforts have focused on identifying fungal pathogenicity factors at the molecular level. A novel C. albicans toxin, namely candidalysin, was recently elucidated. This toxin is encoded by the ECE1 gene initially associated with fungal filamentation ability and host cell adhesion.

ECE1 encodes a large 271 amino acid pre-protein that is cleaved by Kex8p enzyme into eight smaller peptides (Ece1-I to Ece1-VIII). Ece1-II is considered to be the active peptide, acting as an epithelial immune activator, and contributes to the cytolytic activity of C. albicans. This region of Ece1p was considered the first fungal peptide toxin discovered in C. albicans specifically and human fungal pathogens in general and was named “candidalysin.” Candidalysin is an amphipathic, α-helical peptide that inserts itself into the host cell lipid bilayer membrane readily thus exerting a cytolytic ability.

During infection, C. albicans switches morphology from budding yeast to hyphal form. At the initial stages of the immune response, macrophages recognize the pathogen-associated molecular patterns (PAMPs) on the C. albicans cell surface. The recognition phase allows macrophages to identify the invading C. albicans before engulfment of the cells into macrophage bodies for phagocytosis. Although macrophages readily engulf C. albicans in its yeast and short hyphal forms, most C. albicans still retains its ability to form hyphae. Elongation of the hyphae as C. albicans infection progresses exerts tension onto the macrophage membrane. While some macrophages can withstand the pressure and successfully engulf C. albicans, some are killed through the direct piercing action exerted across the macrophage membrane. Macrophage death allows C. albicans to escape, survive and outgrow other macrophages. Studies have shown that ECE1 is expressed during macrophage phagocytosis. Nonetheless, expression of ECE1 was demonstrated to be insignificantly correlated to the rate of macrophage phagocytosis, elongation of candidal filaments, rates of fungal survival and outgrowth after engulfment.

Apart from the aforementioned release of candidalysin in the previous subsection, alternative route of immunogenesis was proposed towards C. albicans through formation and activity of inflammasomes. Earlier studies have reported that C. albicans triggers inflammasome-mediated cell death. Some mechanisms proposed for inflammasome activation include intracellular penetration and lysosomal disruption and hyphal formation. With the discovery of candidalysin, a potential new mechanism for inflammasome activation was added. The inflammasome is a multiprotein complex system that comprises oligomers of NLR, LRR domains, ASC adaptor protein and self-cleaved caspase-1.

Mature caspase-1 enzymes induce the production of active IL-1β and IL-18, essential promoters for inflammatory signalling pathways of NF-kB and MAPK. Similarly, candidalysin is an inducer for NF-kB and MAPK pathways. However, induction of these pathways by candidalysin does not require the presence of caspase-1 or release of IL-1β from primary macrophages. Hence, candidalysin action is independent of formation and maturation of caspase-1 and IL-1β; however, it is associated with the activation of non-canonical inflammasomes, caspase-8 enzymes.

3 | CANDIDALYSISN ASSISTS TISSUE INVASION AND CAUSES EPITHELIAL DAMAGE

The ability to grow hyphae is an essential virulence attribute of C. albicans; the morphogenic switch from yeast form to hyphal form is the most important characteristic of C. albicans pathogenesis. Hyphae are associated with the majority of C. albicans virulent activities, such as adherence, cellular invasion, acquisition of nutrients and inducing expression of other virulence traits. Hyphal invasion is intuitive and is achieved through two mechanisms: endocytosis and active penetration across the epithelial lining. However, the exact link between hyphal formation and induction of the virulent properties of C. albicans has been unclear, as an invasion of the hyphae does not necessarily inflict damage or harm on the host cells. It only serves to disrupt the original alignment of
the epithelial structure. Moyes et al\textsuperscript{25} reported that hyphal formation alone was insufficient to elicit cell damage and activate the host immune response. Instead, the ECE1 gene responsible for the release of the toxic protein candidalysin from the hypha was required to induce epithelial damage and elicit host inflammatory response due to \textit{C albicans} infection.\textsuperscript{25}

Hence, the secretion of candidalysin from invasive hyphae is hypothesized to be the missing link between invasion of \textit{C albicans} and activation of the host's innate immune response.\textsuperscript{25,30} Candidalysin has been implicated in initiating epithelial damage and the host response hence activating trigger signals including expression of cytokines that can contribute to carcinogenesis. In the absence of candidalysin, the initiation of epithelial damage and innate host response would not be possible and triggering signals that support the progression, that is expression of cytokines that contribute to carcinogenesis, would not be expressed.

Candidalysin damages the epithelial cell, which causes the pro-interleukins IL-1\(\alpha\), IL-1\(\beta\), IL-6, IL-23, granulocyte-macrophage colony-stimulating factor (GM-CSF), CCL20 and \(\beta\)-defensins to be released. The pro-interleukins trigger differentiation of antigen-presenting cells (APCs) including squamous cell carcinoma antigen (SCCA), which induces the interleukin IL-17 in \(\gamma\delta\)T, CD3\(+\), CD4\(+\), ILC3 and TCR\(\alpha\beta\)\(+\) cells (Figure 2).\textsuperscript{42,43} Furthermore, candidalysin triggers the release of epithelial danger responses such as phosphorylation (\(\alpha\)-MKP1) and c-Fos.\textsuperscript{44}

Chronic inflammatory infiltration also arises from severe damage of the epithelial cell layer (dysplasia).\textsuperscript{15} The initial cell injury leads to a pre-cancerous lesion, to the formation of the malignant neoplasm, which may develop into cancer.\textsuperscript{15} It has been reported before that epithelial dysplasia characterized by abnormal proliferation of the oral squamous cells is a critical factor in oral cancer. This correlates with the reports of McCullough et al\textsuperscript{26} linking oral yeast density to epithelial dysplasia and squamous cell carcinoma.

### 4 CANDIDALYSIN ACTIVATES IMMUNOMODULATORY MOLECULES

IL-17 is an essential cytokine in innate and adaptive host cell responses to \textit{C albicans} infection. The release of candidalysin is profoundly correlated to an upregulation of IL-17 secretion, expression of antifungal-related genes and the proliferation of TCR\(\alpha\beta\)+ cells. Acute \textit{C albicans} infection triggers the secretion of IL-17 from \(\gamma\delta\)T and TCR\(\alpha\beta\)+ cells.\textsuperscript{42} Null mutation of the ECE1 gene markedly reduced IL-17 secretion in TCR\(\alpha\beta\)+ cells in comparison with wild type strains and ECE1 recombinants as observed in mouse models.\textsuperscript{31} Upon secondary infection of \textit{C albicans}, the expansion of TCR\(\alpha\beta\)+ cells is independent of TCR signalling via recognition of \(\beta\)-glucan expressed on hyphal cell wall by dectin-1 or Clec7\(\alpha\), CARD9 and TLR2 pathways.\textsuperscript{42} Mutation of these receptors was insignificant in the proliferation of TCR\(\alpha\beta\)+ cells and clearance of fungal load. Previous studies report that candidiasis significantly subsided upon inoculation of homozygous ECE1 mutants of strains into mice and zebrafish models.\textsuperscript{25,30} Deleted ECE1 \textit{C albicans} strains were also unable to thrive in immunocompetent hosts and were less virulent in immunocompromised hosts.\textsuperscript{25,31}

Candidalysin has been reported to excite granulocyte-macrophage colony-stimulating factor GM-CSF, an essential molecule in carcinogenesis.\textsuperscript{45-47} GM-CSF is a 127 amino acid monomer with mass ranging from 14 to 35 kDa, depending on the amount of glycosylation in vivo. In mature haematopoietic cells, GM-CSF activates the effector functions of granulocytes, monocytes/macrophages and eosinophils. It is produced and released by various cell lines in response to immune and/or inflammatory stimuli, including activated T cells, B cells, mast cells, endothelial cells and fibroblasts. In addition, GM-CSF has also been shown to possess a functional role in non-haematopoietic cells by inducing human endothelial cells to migrate and proliferate.

Interestingly, GM-CSF can also stimulate the proliferation of a number of tumour cell lines, including breast cancer cell lines. Cancer cells have been shown to produce GM-CSF.\textsuperscript{48} Therefore, increased expression of this cytokine can be used as a biomarker in the detection of OSCC. Further investigations shall be conducted to determine the correlation between the activities of both candidalysin and GM-CSF, particularly in carcinogenesis. Successful elucidation of the role of candidalysin in upregulating the expression of GM-CSF will enhance researchers’ understanding of the underlying mechanism of cancer induced by \textit{C albicans}. From the establishment of a relationship between candidalysin and GM-CSF, the pharmacokinetics and pharmacodynamics properties of candidalysin may be

**Figure 2** Candidalysin induces pro-inflammatory cytokines that drive differentiation of T-helper 17 cell (TH17) cells such as \(\gamma\delta\)T, CD3\(+\), CD4\(+\), ILC3 and TCR\(\alpha\beta\)+ to produce IL-17 against invading \textit{C albicans}
exploited as a switch or regulator to inhibit further development of oral cancer in patients.

Candidalysin is a trigger for NLR family pyrin domain containing protein 3 (NLRP3) inflammasome activation and subsequently cytolsis. NLRP3 is the most well-characterized member of the NLR family of proteins. The NLRP3 inflammasome pathway is initiated through several mechanisms, including the viability of interacting cells, upregulation of K⁺ efflux pumps, generation of reactive oxygen species (ROS) and lysosomal destabilization. Candidalysin activates NLRP3 activation via upregulation of K⁺ efflux pumps. Blocking of the K⁺ efflux pumps with glibenclamide prevents candidalysin from inducing secretion of mature IL-1\(\beta\) that is essential for triggering the cascade of subsequent immune attacks to induce pyroptosis (Figure 3).

The action of the NLRP3 inflammasome has been widely reported as both pro-carcinogenic and anti-tumorigenic. Polymorphism of NLRP3 intensifies cancerous features such as hyperplasia, angiogenesis, metastasis and promotion of DNA damage in many cancer episodes (37; 50). Nevertheless, expression of NLRP3 is vital in the maturation of natural hepatic killer (NK) cells and acts as a negative regulator in chemical-induced colon and skin cancers. NLRP3 also helps maintain normal homeostasis of intestinal cells and promotes activation of CD8+ T cells against tumour. These antagonistic properties played by NLRP3 act in a coordinated, delicate manner to balance between promoting and fighting against cancer. NLRP3 was shown to correlate with the magnitude of OSCC progression positively. Significantly high expressions of NLRP3 and IL-1\(\beta\) have been observed in tissue isolated from patients with OSCC. In a recent study, NLRP3 displayed a novel role in attenuating the tumour-suppressive effect of 5-fluorouracil in OSCC. The expression of NLRP3 corresponds to the production of IL-1\(\beta\) in OSCC samples. Knocking out NLRP3 gene by introducing shNLRP3 restores the apoptotic ability of 5-fluorouracil on OSCC. It is also predicted that higher expression of NLRP3 in OSCC will display more aggressive cancer phenotypes among patients. Therefore, molecular and functional studies of NLRP3 can be further explored to determine its mechanisms in carcinogenesis and therapeutic potentials.

5 | CONCLUSION

Candidalysin promotes tissue invasion and epithelial damage, which activates epithelial signalling pathways MAPK and PI3K. The two pathways activate GM-CSF and NLRP3, which, alongside
hypha formation, are integral to epithelial damage. Severe dysplasia of the epithelium has been associated with chronic inflammatory infiltration, which later develops into cancer. Candidalysin activates several major immune response pathways associated with cell growth, proliferation, survival, angiogenesis, differentiation and motility, all of which are molecular biomarkers related to oral carcinoma and increased risk of OSCC. Epithelial membrane damage and activation of the immune response pathways are the two primary mechanisms by which candidalysin contributes to the induction and development of oral carcinogenesis. However, much remains to be understood of the role that candidalysin has on other pathogenicity factors in *C. albicans*, which requires further research.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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