Ph.D. THESIS

INVESTIGATION OF THE EFFECTS OF *CANDIDA* ON THE PROGRESSION OF ORAL SQUAMOUS CELL CARCINOMA

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Introduction

The head and neck tumor account for 2 to 4% of all cancer cases, which includes neoplasms that affect several regions of the oral cavity, pharyngeal sites, and salivary glands. Approximately 90% of oral neoplasms are squamous cell carcinomas (OSCC). OSCC is the 16th most common cancer worldwide and 6th in the United States. In Europe, the incidence of oral cancer is especially high in Central and Eastern Europe, and both morbidity and mortality rates are highest in Hungary. OSCC is treated by surgery, radiation, and chemotherapy. Chemotherapy and radiotherapy, when used simultaneously, provide a synergistic benefit against OSCC. Currently, the primary treatment mode for OSCC is surgery followed by radiotherapy or chemoradiotherapy depending on risk factors. Adverse effects include mucositis and myelosuppression, which also affect the composition, quantity, and complexity of the oral microbiota.

Candida albicans is a highly prevalent yeast in the oral cavity which proliferates and invades host mucosal tissues upon epithelial barrier dysfunction or disruption. *C. albicans* invades tissues via hypha formation and the production of associated hydrolytic enzymes and virulence factors. While these characteristics may endow *Candida* with a competitive advantage, it is the host's immune competence that ultimately determines whether clearance, colonization, or disease occurs.

There has long been a positive association between oral yeast carriage/dysbiosis and epithelial carcinoma. Notably, higher yeast carriage and diversity are observed in oral cancer patients than in healthy individuals, and oral fungal colonization in OSCC patients is higher on the neoplastic epithelial surface than on adjacent healthy surfaces. Furthermore, persistent oral candidiasis has been observed to lead to OSCC development in an elderly patient. Several other studies have indicated that *Candida* invasion promotes a hyperplastic epithelial response and that untreated *Candida* epithelial lesions may become dysplastic and transform into carcinoma. Thus, there is strong evidence supporting the idea that *Candida* promotes carcinogenic events in the oral cavity. However, *Candida* infection in cancer patients may also be considered the consequence of an altered immune status, because both myelosuppression and mucositis enable the development of oral candidiasis.

Aims

The results available in the literature clearly demonstrate the association between OSCC and oral candidiasis. In case of OSCCs, oral candidiasis often resulted by the tumor therapy. It is hypothesized that *Candida* may affect the progression of OSCC, however, this phenomenon has not been studied yet.

Therefore, we have set the following goals in our work:

1. Investigation of the effect of heat-inactivated *Candida* and zymosan (fungal cell wall component, general fungal stimulus) on the invasive and proliferative activity of OSCCs *in vitro*.

2. Investigation of the effect of live *Candida* on the invasive and proliferative activity of OSCC cells *in vitro*.

3. Investigation of the effect of *Candida* at the molecular level using transcriptomic analysis.

4. Developing an *in vivo* xenograft model to investigate the effect of oral candidiasis on OSCC cell progression

5. Investigation of the effect of oral candidiasis on OSCC cell progression using the newly established xenograft model.

Methods

***In vitro* methods**: HSC-2 and HO-1-N-1 cell lines; Wound healing assay; BrdU ELISA (Enzyme Linked Immunosorbent Assay); treatment of OSCCs with *Candida* cells and zymosan and lysis of OSCC cells for metabolomic analysis; analysis of metabolites with High-Performance Liquid Chromatography Coupled With High-Resolution Mass Spectrometry (HPLC-HRMS); Real-time cell analysis (time-lapse video); Matrix metalloproteinase (MMP) activity assay; total RNA isolation from OSCC cells *in vitro*; RNA sequencing; transcriptomic analysis; Causal analyses with Ingenuity Pathway Analysis (IPA); cDNS synthesis and qPCR for the validation of sequencing results; Western blot analysis of MMP10 and MMP1 proteins.

Development of a mouse xenograft model to investigate the effect of oral candidiasis on OSCC progression: immunosuppression of wild-type mice; anesthesia of mice; injection of HSC-2 cell to the tongue of the mice; inducing oral candidiasis in mice; determination of fungal burdens by colony counting; RNA isolation from xenograft tumor samples; histopathological staining of tumor samples (H&E, PAS, vimentin, E-cadherin, p63); RNA sequencing; transcriptome analysis.

Results

1. Investigation of the effect of heat-inactivated and live *Candida* fungi on OSCCs *in vitro*

In our work, we investigated whether *Candida* overgrowth in the oral cavity is able to affect the progression of oral squamous cell carcinoma. First we investigated the effects of heat-inactivated, live *Candida* and zymosan on OSCC progression *in vitro*. We could conclude that the proliferative activity of OSCC cells did not change either in the presence of HI-*Candida* cells and zymosan or in the presence of live *Candida* cells. However, the migration activity of tumor cells is significantly increased in the presence of HI-*Candida* cells and zymosan. Based on real-time cell analysis technique (time-lapse video), live *C. albicans* increased the numbers of detached, single HSC-2 cells compared to untreated controls.

Moderate secreted MMP activity was observed after treatment with heat-inactivated fungal cells and zymosan. Treatment with live *C. albicans* cells significantly increased MMP activity in both cell lines. However, no change was observed in the case of live *C. parapsilosis* treatment. The results of the metabolomic analysis showed a moderate change in the amount of some metabolites in the presence of HI-*Candida* cells and zymosan, but no correlation was observed between the changes. In the presence of live *C. albicans*, the amount of succinate and aspartate in tumor cells was significantly increased.

2. *In vitro* transcriptomic analysis

In vitro transcriptomic analysis confirms that the presence of live *C. albicans* cells increases the progression-related processes of OSCCs on the molecular level as well, primarily in case of HSC-2 cell line. The results are consistent with migration, MMP activity, and metabolomic analysis showing that HI-*Candida*, zymosan, and live *C. parapsilosis* cause no or mild change in OSCC cell progression. The phenotypic changes of OSCC cells observed in the presence of live *C. albicans* cells are supported by transcriptomic studies due to elevated expression levels of genes involved in the metabolism of succinate and aspartate oncometabolites.

Furthermore, a significant increase in the expression levels of four MMP genes (MMP1, MMP10, MMP9, MMP3) was observed, which is also consistent with the results of secreted MMP activity assays. Analyzing the literature data, we identified OSCC progression marker genes which expression is significantly altered in the presence of *C. albicans*. Fourteen OSCC progression marker genes (ATF3, F3, FOS, FOXC2, HBEGF, IL6, INHBA, JUN, LIF, PHLDA1, PLAUR, PTHLH, SEMA7A, VEGFA) were found, which upregulated by live *C. albicans* cells in both HSC-2 and HO-1-N-1 cells.

In case of HSC-2 cell line, a considerable number of gene expression changes were identified that allowed for further analysis. Examination of the differentially expressed genes, we also identified activation of signaling pathways involved in OSCC proliferation,

invasion, tumor growth, angiogenesis, and metastasis. Ingenuity Pathway Analysis (IPA) with built-in causal analyses was also used to investigate activation patterns of several intracellular signaling pathways based on the coherent regulation of their molecular elements. IPAs predicted the activation of tumor-related pathways, including the tumor microenvironment pathway, as well as the significant activation of several prognostic features, such as metastasis, invasion, angiogenesis, and proliferation of tumors based on the *C. albicans* stimulus-derived differentially expressed genes (DEGs) in HSC-2 cell line.

3. Investigation of the effect of oral candidiasis on the progress of OSCC cells *in vivo*

In order to investigate the effect of oral candidiasis on the progression of OSCC, we successfully established a new *in vivo* xenograft mouse model in which OSCC and oral candidiasis were induced in the tongue of immunosuppressed mice. Next, we compared the groups of animals with and without oral candidiasis. Based on histopathological examinations, we found that *C. albicans* caused severe inflammation. Inflammation can promote tumor progression and metastasis by producing cytokines that promote tumor progression. Epithelial-mesenchymal transition (EMT) and thrombosis due to oral candidiasis were also observed in histopathological examinations. EMT plays a key role in the processes of tumor invasion and metastasis. Thrombosis has also

been shown to promote tumor progression, but the exact mechanisms are not known yet. To confirm the role of oral candidiasis in tumor progression, immunohistochemical staining of p63, E-cadherin, and vimentin was performed. The p63 protein is encoded by the TP63 gene, which is a homologue of the TP53 gene (p53 protein). Overexpression of p63 is a prognostic marker in squamous cell carcinomas. Results show that oral candidiasis resulted in higher level of expression and localization of p63 protein in the nucleus in the *in vivo* samples. In squamous cell carcinoma, vimentin expression is associated with metastasis and poor prognosis. More vimentin-positive cells were detected in histopathological samples in case of oral candidiasis than in the control samples. A previous study showed that the amount of reduced E-cadherin is a marker to detect increased tumor invasion capacity. E-cadherin expression decrease was observed in the tumor samples as a result of oral candidiasis in the *in vivo* tumor samples. Elevated p63, vimentin, and decreased E-cadherin expression of the tumor cells in the presence of *C. albicans* suggest that *C. albicans* overgrowth increase the EMT process, suggesting a poorer outcome in terms of patient survival.

Histopathological results were confirmed by *in vivo* transcriptomic results. Five genes have been identified that play a role in the progression of OSCC and have increased gene expression levels in oral candidiasis (MMP1, MMP10, COL5A2, SERPINB4, CRABP2). It is important to highlight MMP1 and MMP10, as *in vitro* results have also shown that *C. albicans* increases all secreted MMP activity.

Major findings:

1. Heat-inactivated *Candida* as well as zymosan affect the metastatic properties of OSCC cells.

2. Live *Candida* affects the metastatic properties of OSCC cells.

3. Live *C. albicans* stimulus activates genes and signaling pathways involved in the invasive processes of OSCC *in vitro*.

4. We successfully established a new xenograft mouse model to investigate the effect of oral candidiasis on OSCC progression.

5. Oral candidiasis promotes the progression of OSCC *in vivo*.

6. Oral candidiasis increases the progression of OSCC by affecting the expression of various genes *in vivo*.

List of publications:

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