Yeast Microflora and Halitosis in Oral Squamous Cell Carcinoma- Two Microbiological Aspects of a Disease

Summary of the PhD Thesis

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Szeged, 2017

Publications providing the basis of the thesis

I. **Berkovits C**, Toth A, Szenzenstein J, et al. Analysis of oral yeast microflora in patients with oral squamous cell carcinoma. *Springerplus.* 2016;5(1):1257.

Q1 IF: 0.982 (2015)

II. Szabo A, Tarnai Z, **Berkovits C**, et al. Volatile sulphur compound measurement with OralChroma(TM): a methodological improvement. *J Breath Res.* Jan 05 2015;9(1):016001.

Q1 IF: 4.177 (2015)

Cumulative impact factor: 5.159

Abbreviations

ASR-W – age-weighted standardized incidence rate
IACR – International Association of Cancer Registries
ICD – International Classification of Diseases
IL-8 – Interleukin 8
MALDI-TOF MS- matrix assisted laser desorption ionization-time of flight mass spectrometry
OSCC – Oral Squamous Cell Carcinoma
TNF-α – Tumornecrosis factor alpha
VOC- volatile organic compound
VSC- volatile sulfur compond

I. Introduction

Cancers of the oral cavity (ICD C00-C14) belong to the tumors of the highest morbidity and mortality. Their epidemiological characteristics are determined by geographical factors: they are more frequent in the developed countries than in the developing ones.

Unfortunately, Hungary appears to be in a leading position regarding both the morbidity and mortality of oral cancer. This is well reflected in the age-standardized incidence rates of GLOBOCAN 2012: the ASR-W[1] of Hungary in 2012 was 9.7 , while of the neighboring countries, Slovakia scored 6.5, Romania 5.4, and Austria only 4.2. In Europe, Greece scored the lowest (1.6). With a score of 9.7, Hungary is the first not only in Central Europe, but in the entire European region. Data of the International Association of Cancer Registries (IACR) and data from the national cancer registry of Hungary (Nemzeti Rákregiszter) show the same.

The causes of this unfortunate situation have not been clarified, and it probably cannot be explained by a few simple and well-known factors, such as smoking or excessive alcohol consumption (or the combination of these). These factors play a significant role in the pathogenesis of the oral cancers of the Hungarian population beyond doubt. However, the increasing representation of non-smoking and non-drinking elderly

[1] This value expresses the incidence of a given disease as compared to a standard world population, for a population of 100,000, considering that the disease does not occur at the same frequency in all cohorts.

women and young adults among the Hungarian oral cancer patients is definitely against such a simplifying explanation.

Certainly, this sadly notable position of Hungary on the international map of oral cancer is an incentive to the Hungarian researcher of oral health to focus on the causes, consequences and potential cures for oral cancer, especially squamous cell carcinoma (OSCC). This, however, is a vast field of study. In the present thesis I narrow my focus down to two oral cancer-related questions, both rooted in the microbiology of the oral cavity. One is considered by many as a cause or promoter of OSCC, the other is obviously a consequence.

Yeasts (especially Candida spp.) have long been suspected as causative agents of oral malignant and premalignant states. The thesis discusses the yeast microflora of the oral cavity in OSCC based on our own research, considering also the specific question of the role of lipase/protease activity in epithelial colonization. It is also known that the microbial composition of the oral cavity in OSCC differs from that of the healthy oral cavity both in qualitative and quantitative respects.

This also means that the composition of the exhaled air of OSCC patients is different from that of healthy subjects, which can lead to oral malodor (halitosis). This, of course, can lead to serious psychosocial consequences, which makes halitosis in oral cancer (and otherwise) an important problem to be addressed. At the same time, the altered composition of the exhaled air could offer a new diagnostic possibility, even if this topic is scarcely researched in oral cancer. One of the reasons for the relative lack of research in this area

is that the available methodologies are not precise and reliable enough. In the present thesis I describe a methodological improvement developed by our team and tested with OSCC patients.

II. Aims and hypotheses

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A. Regarding our study on candidiasis and OSCC, our aims were the following:

1. To describe the differences in oral yeast carriage between the OSCC-affected and healthy epithelium, with special attention to Candida strains.

2. To determine if the lipase/protease producing activity of the Candida strains is associated with the colonization rate.

We hypothesized that a bigger variety of yeast genera would be found on the neoplastic surface, and in higher numbers, as compared to the healthy surface, and we also hypothesized that the lipase/protease producing activity of the Candida strains would be associated with their ability to colonize the epithelium.

B. As for the halitosis study, our aims were:

1. To test an improved software version for OralChroma in a population of OSCC patients as compared to healthy controls, and to check the reliability

of the new softweae version by comparing the measurements to Halimeter measurements.

2. To enable OralChroma to identify and measure isoprene and acetaldehyde too, so as to enhance diagnostics based on this device. We set this goal considering that these compounds are present in the breath of healthy controls too.

3. To determine the exhaled air composition of OSCC patients and to compare it against that of healthy controls of excellent oral hygiene.

We hypothesized that our enhanced software would allow a more precise determination of exhaled air components than the default software and that the measurement of isoprene and acetaldehyde would also become possible. Regarding the exhaled air of OSCC patients, we hypothesized that our results would corroborate the results of previous studies, especially concerning elevated VOC concentrations. As for acetaldehyde and isoprene, we expected different levels between controls and OSCC patients.

III. The Oral Yeast Microflora of Patients with Oral Sqamous Cell Carcinoma

III.1. Background

The role of *Candida* spp. in various types of cancer is widely recognized today, but it has not always been so. Even though the link was suggested already from the 1960s, research in this direction gained a new momentum only in the last few decades. *Candida* spp. appear to be carcinogenic primarily through promoting the malignant transformation of premalignant lesions.Candida albicans appears to have a distinguished role in human pathogenesis. As for the carcinogenic effect itself, the same mechanisms are assumed as with other microbial factors, that is: the direct production of carcinogenic compounds, turning procarcinogens into carcinogens, or the induction of chronic inflammation. Naturally, these do not exclude one another.

A major question according to *Candidae* and OSCC is how the fungus colonizes the epithelium. The literature offers two main hypotheses in this respect. The first one of these is that *C. albicans* produces enzymes (especially aspartate proteases), by which it degrades the surface of the epithelial cells and opens up the way for the hyphae towards the inside of the cells and between them. This hypothesis we also tested in the first study for this thesis (below). It was also observed that *C. albicans* can stimulate keratinocytes by Als3 invasin in a way that they emit pseudopodium-like structures, which, in turn, pull the fungus inside the cell. Colonization, however, does not depend solely on *C. albicans*. It was shown that the type

and level of differentiation of the keratocyte are also influencing factors.

III.2. Methods

Sixty 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. Patient eligibility criteria were a histologically confirmed diagnosis and no prior treatment for OSCC. Controls were recruited from outpatients free of oral mucosal pathology who arrived for routine procedures (e.g. tooth extraction).

Oral swabs were taken from a 1 cm^2 area from two different locations in the oral cavity (in the case of OSCC patients, both from the surface of neoplastic and healthy epithelium).

Beyond the counting of colony forming units (CFUs), the samples were analyzed with MALDI-TOF and the extracellullar enzyme production of the isolated yeasts was also assessed.

III.3. Results

Eighteen (90 %) of the 20 OSCC patients and 12 (30 %) of the healthy controls had yeast isolated from their

oral cavity, indicating significantly higher colonization in OSCC patients compared to the controls (Fisher's exact, $p < 0.0001$). OSCC patients also had a significantly higher average fungal burden (73.08 ± 33.39 CFU/cm^2) in their oral cavity compared to healthy individuals (1.10 ± 0.78 CFU/cm^2), and samples taken from the neoplastic surface contained more yeast cells (77.38 ± 38.53 CFU/cm^2) compared to the swabs taken from the healthy epithelium of the same individual (28.58 ± 19.18 CFU/cm^2).

According to the MALDI-TOF analysis, *Candida* was the dominant genus in both groups, but the yeast microflora of the OSCC patients was more diverse (i.e. it contained genera not found on the healthy epithelium).

The analysis of enzymatic activity indicated no significant difference between the protease/lipase producing activity of *Candidae* isolated from the healthy and OSCC samples.

III.4. Summary and interpretation

In this study we sought to characterize the oral yeast microflora of OSCC patients, with special attention to protease/lipase production as a proposed differentia specifica of carcinogenic yeasts.

First of all, we found that the level of oral yeast carriage was significantly higher in OSCC patients. This came as no real surprise, as the association between oral yeast carriage and epithelial carcinoma had previously been pointed out by several authors. In this sense, our results corroborate those of earlier studies.

Second, we tested the extracellular protease/lipase producing capacity of Candida strains to see if correlation can be found between the enzyme production and the

colonization rates. We found no such correlation, which is against the role of fungal hydrolytic enzymes in the development of epithelial dysplasia.

Third, using MALDI-TOF-MS that represents a new and rapid method for the identification of yeasts in clinical samples, 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 provides further support to the assumption that the altered microenvironment associated with tumorigenesis leads to the development of a more diverse oral microflora.

Considering the literature and our results we propose that the altered immunological environment in OSCC opens up the way toward colonization by yeasts, even in the case of genera which would normally be suppressed. This also explains why extracellular hydrolytic enzyme production shows no correlation with colonization rates: while enzyme production is indeed an important virulence factor, higher colonization rates in OSCC also require an altered immunological millieu.

IV. A methodological improvement for halitometry tested in OSCC patients

IV.1. Background

Halitosis is defined as an unpleasant odor that emanates from the oral cavity with intraoral and/or extraoral origin. Halitosis can be physiologic (putrefaction on the dorsoposterior region of the tongue without disease) or pathologic (oral or extraoral).Intraoral contributing factors are responsible for halitosis in 90% of the cases.

From a chemical point of view, malodor comes about as a result of the microbial degradation of organic substrates present in the saliva, in the crevicular fluid exudate, oral tissues and retained debris. During this process, volatile sulphur compounds (VSCs; H_2S, CH_3SH, $(CH_3)_2S$), diamines (e.g. cadaverine, putrescine) and phenyl compounds (indole, skatole) are formed. It is these substances, and especially VSCs that are responsible for the malodor.

Today, there are three primary methods to assess halitosis: the organoleptic method, gas chromatography and sulfide monitoring. The organoleptic method depends entirely on the subjective olfactory sensation of the clinician or a trained judge. Sulfide monitoring (Halimeter) is affordable and the device is portable, but it is weak at distinguishing between individual VSCs, instead, it allows the measurement of the total level of VSCs. In fact, Halimeter is almost insensitive to dimethyl sulfide. Gas chromatography has the ability to distinguish between VSCs, and this method is objective enough to be fit for scientific purposes; however, it used to be way too

expensive for scientific purposes. A change was brought about by Hanada and co-workers, who combined a semiconductor gas sensor and a compact gas cromatograph system, which became known as OralChroma, a portable, commercially available gas cromatograph. While this device is quite reliable, it still has its weaknesses: while the hardware meets the requirements of an accurate gas cromatograph, the software often assigns VSC peaks erroneously, and therefore yields false results.

IV.2. Methods

Thirty-five volunteers participated in the study. The volunteers were either healthy controls with excellent oral hygiene (11 females, 10 males, average age: 35.6 years), or OSCC patients (2 females, 12 males, average age: 59.8 years). Exclusion criteria included antibiotic treatment within four weeks prior to the measurements, and the consumption of onions, garlic or alcohol in two days prior to the measurements. All measurements were performed at least three hours after the last meal, drink or oral hygienic measure (e.g. toothbrushing, flossing, etc.). All measurements were carried out in triplicate, in each case between 8:30 AM and 12:30 PM.

The two most common devices used in clinical breathalysis - OralChroma (Abimedical Corporation, Japan) and Halimeter (Interscan Corporation,CA, USA) - were used. For the preparation and calibration of the devices, see the text of the thesis.

A new software (written in LabVIEW, National Instruments, TX, USA) was developed to simplify and

accelerate the re-evaluation of the OralChroma chromatograms. The code reads the files that are automatically generated by OralChroma when a measurement is saved, and detects local maxima in ±10 s intervals of the expected peak locations (i.e. at 30, 60, 100, 150, 250, 350 s). Then it fits the sum of six Gaussian (18-parameter) functions using the Levenberg–Marquardt method.

IV.3. Results and summary

As for the methological goals of the study, we have succeeded in enabling OralChroma to assign peaks more correctly and precisely, and as a result of the improvement, the concentrations of both CH_3SH and $(CH_3)_2S$ could be determined. It is notable that $(CH_3)_2S$ was detected at a very small concentration, showing both the sensitivity of the hardware and the ability of the software to exploit the possibilities of the hardware. The new software is also capable of assessing the isoprene and acetaldehyde content of exhaled air.

As for the composition of exhaled air in controls and OSCC patients, the results were partially expectable: as demonstrated by several studies before, the main VSCs were found to be elevated in OSCC. In this sense, our measurements corroborated the results of previous studies. The results regarding acetaldehyde, though, were somewhat surprising. In an OSCC sample, acetaldehyde is expected to be elevated, smoking and alcoholism being the most important risk factors of the disease, and also considering the practical observation that the patients

often fail to quit either habit when they get to know about their condition. Unfortunately, we did not assess the smoking and alcohol consumption of our patients, but given that they were chosen in a way as to be representative of the Hungarian OSCC population, it is less than likely that they quit cigarettes and drinking all of a sudden when they learned about the diagnosis. This finding necessitates further examination. The concentration of isoprene in the control group was half of that of the study group, while the difference in acetaldehyde concentration was negligible as compared to that. This raises the possibility that a decrement in isoprene concentration (for reasons unknown at this point) could be a marker, but measurements with a larger study group are definitely necessary. The ratio of the two components (i.e. isoprene and acetaldehyde), though, was rather similar – and significantly different – in the two groups, which supports the similarity of the two groups. As can be seen, therefore, our results regarding isoprene concentrations in the exhaled air of OSCC patients and controls can be regarded as preliminary results, but they show a direction for further research.

V. Acknowledgements

First of all, I would like to express my deepest gratitude to my supervisors. Professor Katalin Nagy helped me with her ongoing support and professional help throughout the years I spent at the University of Szeged. Dean Kinga Turzó made it possible for me to conduct research at the Faculty while working as a clinician.

I am also grateful to Dr. Attila Gácser, who allowed me to access advanced technologies, so that I could study fungi in the oral cavity in an up-to-date fashion. In this respect I am also indebted to Professor István Sonkodi who helped me with patient selection and kindly allowed me to work with some of his patients.

A distinguished gratitude goes out to my co-authors at the Faculty of Science, University of Szeged for having introduced me to the latest methods and problems in breath research. Of them, I am especially grateful to Dr. Anna Szabó for her help with the present thesis.

The faculty and staff at the Department of Oral Surgery, Faculty of Dentistry, University of Szeged were the people whom I worked with every day. They were always a great help and it was a pleasure to work with them. Thanks to all of you.