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Characterisation of the Clinically Normal Tissue and Plasma in Patients with Squamous Cell Carcinoma of the Oral Cavity

Nima Attaran

Department of Medical Biosciences, Pathology
Department of Clinical Sciences, Otorhinolaryngology
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In Loving Memory of Mohsen Kolahdooz

"Even if the skies were shorter than my knees, I would not kneel."
— Cyrus the Great

Table of Contents

Abstract

Background

Oral cancer is often surrounded by epithelium that clinically appears normal but harbours genetic aberrations, including pre-cancerous changes, a phenomenon known as field cancerisation. In patients with squamous cell carcinoma of the oral cavity (SCCOC), it is crucial to study not only the tumour itself but also the clinically normal tissue that remains post-therapeutically. Additionally, liquid biopsy approaches, such as the analysis of plasma samples, have emerged as promising minimally invasive methods for detecting cancer-related alterations. The aim of this doctoral study is to characterize the clinically normal tissue and plasma in patients with SCCOC and to establish a panel of changes that may contribute to early detection, diagnosis or prognosis.

Materials & Methods

Microarray gene expression data of healthy tongue tissue, tumour and clinically normal tongue contralateral to tumour (NTCT) from patients with SCC of the oral tongue (SCCOT) were analysed. Reverse transcription quantitative PCR and immunohistochemistry were performed to validate microarray data and investigate protein expression, respectively. Data from whole exome sequencing and RNA sequencing were investigated to identify correlations between copy number variation and differential gene expression in paired tumour and NTCT samples. Finally, proteomics data based on the Olink explore 3072 platform were examined to compare plasma protein levels between healthy controls and patients with SCCOC. The prognostic impact of cancer-related alterations was assessed using Kaplan-Meier and Cox regression survival analysis.

Results

Focusing on transporter associated with antigen processing 1 (TAP1) and TAP2, two key factors in antigen presentation and immune evasion, our microarray data showed that TAP1 mRNA levels increased progressively from healthy controls to NTCT to tumour, whereas TAP2 mRNA levels were upregulated only in tumours. Notably, higher TAP1 mRNA levels in NTCT were associated with worse survival outcomes, while TAP1 levels in tumours provided no prognostic information. Immunohistochemistry confirmed elevated TAP protein expression in tumours. Similarly, TAP protein levels in tumours had no overall impact on survival but exhibited sex-specific associations. Further comprehensive analysis of microarray data revealed upregulation of apoptosis-related genes in NTCT. A positive correlation between copy number and mRNA levels was identified for the pro-apoptotic tumour suppressor Zinc Finger Protein 395 (*ZNF395*). Finally, plasma proteomics analysis revealed decreased levels of Secreted Frizzled Related Protein 4 (SFRP4) in SCCOC patients, with lower SFRP4 levels associated with worse survival outcomes.

Conclusions

We provide further evidence that NTCT harbours genetic aberrations, is susceptible to malignant transformations and contains biomarkers that may aid in early detection and prognosis. Plasma protein analysis, meanwhile, revealed systemic alterations with prognostic significance. Taken together, our findings demonstrate that molecular profiling of NTCT and plasma could improve our understanding of tumourigenesis and enhance early detection, risk stratification and personalized surveillance strategies for SCCOC patients.

Keywords: oral cancer, field cancerisation; plasma, biomarker

List of Publications

Paper I

Attaran N, Gu X, Coates PJ, Fåhraeus R, Boldrup L, Wilms T, Wang L, Sgaramella N, Zborayova K, Nylander K. Downregulation of TAP1 in Tumor-Free Tongue Contralateral to Squamous Cell Carcinoma of the Oral Tongue, an Indicator of Better Survival. Int J Mol Sci. 2020 Aug 27;21(17):6220.

Paper II

Attaran N, Coates PJ, Zborayova K, Erdogan B, Magan M, Sgaramella N, Nylander K, Gu X. Antigen peptide transporters are upregulated in squamous cell carcinoma of the oral tongue and show sex-specific associations with survival. Oncol Lett. 2022 Sep 21;24(5):390.

Paper III

Attaran N, Coates PJ, Zborayova K, Sgaramella N, Nylander K, Gu X. Upregulation of Apoptosis Related Genes in Clinically Normal Tongue Contralateral to Squamous Cell Carcinoma of the Oral Tongue, an Effort to Maintain Tissue Homeostasis. Head Neck Pathol. 2024; 18(1):89.

Paper IV

Attaran N, Magan M, Coates PJ, Zborayova K, Holmlund T, Nylander K, Gu X. Unveiling the prognostic potential of Wnt pathway antagonist SFRP4 in oral squamous cell carcinoma. *Manuscript*.

Abbreviations

cDNA	Complementary Deoxyribonucleic Acid
CNV	Copy Number Variation
CPP	Standardized Cancer Patient Pathways
DFS	Disease-Free Survival
DOD	Dead of Disease
DWD	Dead With Disease
FFPE	Formalin fixed paraffin embedded
HNC	Head and Neck Cancer
HPV	Human Papilloma Virus
ICD	The International Classification of Diseases
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
MDSCs	Myeloid-Derived Suppressor Cells
MHC	Major Histocompatibility Complex
NGS	Next-Generation Sequencing
NK cell	Natural Killer cell
NTCT	Normal Tongue Contralateral to the Tumour
OPMDs	Oral Potentially Malignant Disorders
PCR	Polymerase Chain Reaction
PEA	Proximity Extension Assay
RNA	Ribonucleic Acid
SCCHN	Squamous Cell Carcinoma of the Head and Neck
SCCOC	Squamous Cell Carcinoma of the Oral Cavity
SCCOT	Squamous Cell Carcinoma of the Oral Tongue
SFRP4	Secreted Frizzled Related Protein 4
SPT	Second Primary Tumour
TAP	Transporter associated with Antigen Processing
TCGA	The Cancer Genome Atlas
TNM	Tumour - Node - Metastasis
ZNF395	Zink Finger Protein 395

Svensk sammanfattning

Elakartade tumörer i munhålan omges av till synes frisk vävnad, vilken i själva verket innehåller genetiska avvikelser, inklusive precancerösa förändringar. Hos patienter med skivepitelcancer i munhålan (SCCOC) är det därför viktigt att inte begränsa studierna till själva tumören utan även studera den kliniskt normala vävnaden. Som ett komplement till de traditionella vävnadsbiopsierna kan även blod-, urin- och salivprov, så kallade "vätskebiopsier" användas för att upptäcka cancerrelaterade förändringar.

Det övergripande syftet med denna doktorsavhandling är att karakterisera den kliniskt normala vävnaden och blodet hos patienter med SCCOC och att fastställa en panel av förändringar som kan bidra till tidig upptäckt, diagnos och/eller prognos.

Vi analyserade genaktiviteten i vävnadsprover från helt frisk tungvävnad, tumör och vävnad från den motsatta till synes friska sidan av tungan från patienter med skivepitelcancer i den mobila delen av tungan. I dessa prover använde vi olika tekniker som RT-qPCR, immunfärgning och DNA- och RNA-sekvensering för att kartlägga specifika genförändringar, genuttryck och proteinnivåer.

Ett protein kallat *Transporter associated with Antigen Processing* (TAP1) vilket utgör en nyckelkomponent i antigenpresentation och undvikandet av immunsystemet, visade på stegvis ökande nivåer från friska kontroller till den normala vävnaden omgivande tumören och högst nivåer i själva tumören. Intressant nog visade det sig att höga TAP1-nivåer i den normala vävnaden omgivande tumören var relaterade till sämre överlevnad, medan nivåerna i själva tumören inte påverkade överlevnaden och därmed inte heller hade något prognostiskt värde.

Vidare konstaterade vi lägre nivåer i blod av det tumörhämmande proteinet *Secreted Frizzled Related Protein 4* (SFRP4) hos patienter med SCCOC, vilket i sin tur var kopplat till sämre överlevnad.

Detta avhandlingsarbete ger nya bevis för att den till tumören angränsande kliniskt normala vävnaden uppvisar genetiska förändringar och innehåller markörer som kan bidra till en tidig upptäckt och prognos. En molekylär karaktärisering av den till tumören angränsande och till synes normala vävnaden samt analys av plasmaproteiner förbättrar vår förståelse avseende tumörutveckling, ökar chansen till tidig upptäckt, förbättrar riskbedömning och möjliggör monitorering av patientspecifika behandlingsmetoder.

Introduction

Head and neck cancer

Head and neck cancer (HNC) refers to a heterogenous group of malignant tumours that according to International Classification of Diseases, 10th Revision (ICD-10), are anatomically classified into nine diagnostic categories, including tumours of the lips, oral cavity, oropharynx, nasopharynx, hypopharynx, larynx, nose and sinuses, salivary glands and malignant cervical lymph nodes of unknown primary tumour (Figure 1) [1].

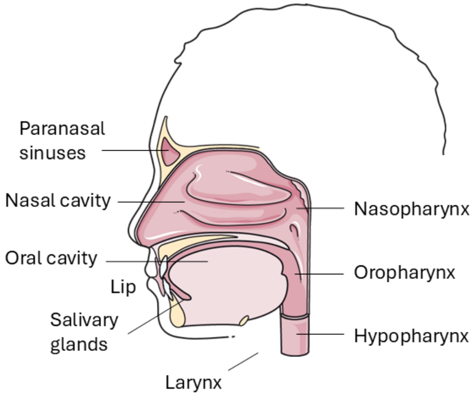

Fig. 1 Head and neck cancer regions. Modified from Servier Medical Art, licensed under a Creative Commons Attribution 4.0 International License. Source: https://smart.servier.com

Within each anatomical subgroup, there are further subcategorized tumours with very different growth patterns, risks of spreading, prognosis and treatments. This is the case with i.e. oral cavity cancer which is divided into six different tumour locations: tongue, gingiva, floor of the mouth, hard palate, buccal mucosa and the rest of the oral cavity [1]. According to the latest data from the Global Cancer Observatory (GLOBOCAN) 2022 (https://gco.iarc.fr/en) [2], HNC is the sixth most common cancer globally, accounting for 947 211 new cases, which represent 5% of all cancer diagnoses worldwide. There are geographic differences in the prevalence of HNC (Figure 2). Regions such as South Asia, Eastern Europe and South America have higher incidence rates, while Australia also experiences a notable burden of HNC.

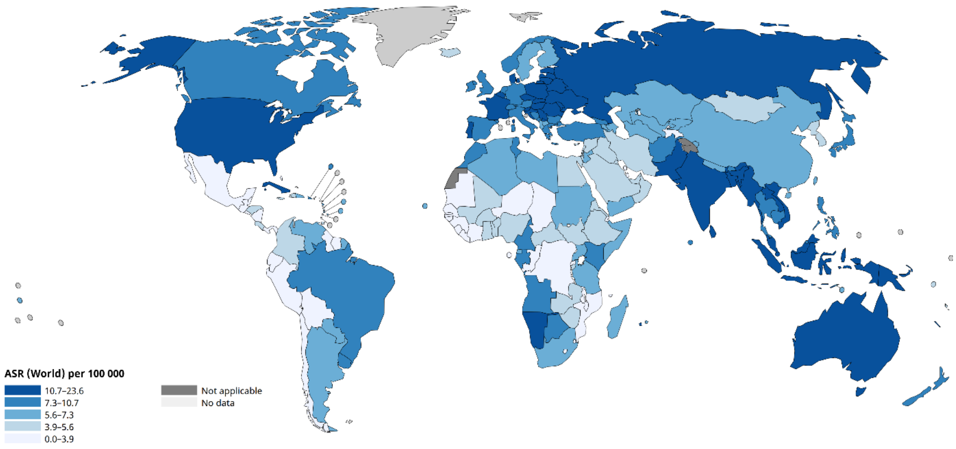

Fig. 2 Global prevalence of head and neck cancer [2].
Age-Standardized Rate (World) per 100.000, Incidence, Both sexes, in 2022.

In Sweden, the total cancer incidence is increasing, reaching above 60.000 cases annually, similar to the trend in HNC, which increased with 300 cases (25 %) during the time period of 2008–2016 [3]. In 2016 some 1500 new cases of HNC were reported to the Swedish Head and Neck Cancer Register (SweHNCR), corresponding to 2.6% of the total domestic cancer incidence [3].

Similar to most countries, the oral cavity is the most common subsite of HNC in Sweden, followed by oropharynx (tonsills, tongue base and remaining parts of the oropharynx) and larynx (Figure 3) [3].

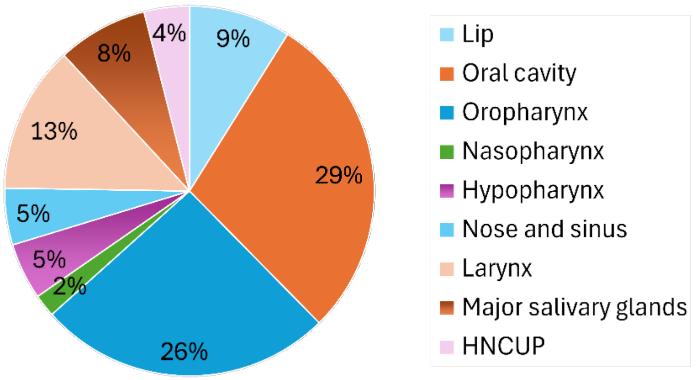

Fig. 3 Distribution of head and neck cancer cases in Sweden. 2008-2017 [4] HNCUP, Malignant cervical lymph nodes of unknown primary tumour.

The mean age of patients with HNC is approximately 65 years, with a male dominance in which approximately two-thirds are males [3].

The 5-year survival rate for all HNC incidences in Sweden during the period of 2008-2016 was 58 % [3] and the survival outcomes in general for HNC patients has improved over last decades, while the death rate within six months after diagnosis for curative patients remains at approximately 5% [5]. Most recently however it was reported that 6-month mortality rate after HNC diagnosis among Swedish patients treated with curative intent decreased from 4.7% (2008–2012) to 2.5% (2017–2020) which was explained by a significant reduction in the mean time of therapy initiation both for surgical and oncological treatment [5, 6]. Furthermore the increased risk of early death in this patient category was associated with male sex, older age, advanced disorder, increased WHO score as well as a hypopharyngeal tumour location [5].

In a Finnish retrospective, population-based analysis of all patients with squamous cell carcinoma of the head and neck (SCCHN) (n = 762) treated with curative intent at the Helsinki University Hospital (2012–2015) the rate of early death was found to be circa 10% with a median age of 70 years upon diagnosis and with advanced stage, smoking > 40 pack-years, heavy alcohol abuse as well as elevated thrombocyte levels were associated with increased odds of early death [6]. In addition only age, T4 class, N2 class and N3 class were observed being independent risk factors for early death [6]. Thrombocytes may facilitate cancer progression by immune cell binding, modulation of the immune response, inhibition of tumourlytic activity through aggregation around tumour cells and promotion of tumour growth and metastasis [6].

Oral cavity cancer

The Swedish incidence of Oral Cavity Cancer increased with some 30% during the time period 2008-2016 with an average of circa 3% annual increase and with 442 cases registered by the end of that period [3]. In the same period the male-to-female ratio was 1:1 with a few percentage less for females [3]. The median age upon diagnosis was 68 years for males, while 71 years for females [3, 4] .

The oral cavity is surrounded by the lips and is composed of two separate regions, the vestibule which is the area between the cheeks, teeth and lips, versus the oral cavity proper which is mostly filled with the tongue and bounded anteriorly and on the sides by the alveolar processes containing the teeth and posteriorly by the isthmus of the fauces [7].

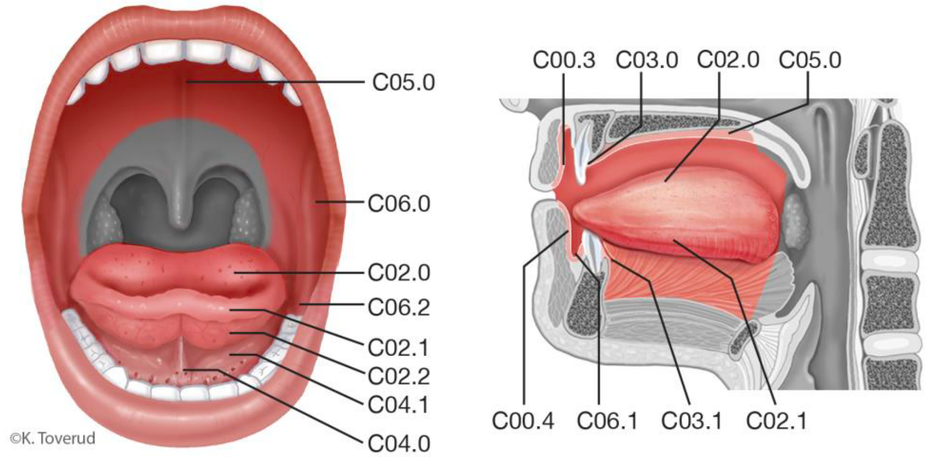

Fig. 4 Oral cavity and its subsites with ICD-10 codes. © K.C.Toverud CMI (certified medical illustrator).

Anteriorly, the roof is formed by the hard palate and posteriorly by the soft palate ending with the uvula [7]. The mylohyoid muscles constitute the floor of the oral cavity proper [7].

The retromolar trigone constitutes the posterior part of the gingival mucosa of the mandible and due to nonexistent distinct barriers separating the buccal membrane and retromolar trigone, SCCs arising from these areas tend to invade one another [3, 8].

Histologically, the oral mucosa is composed of stratified squamous epithelium and forms the inner lining of the mouth [7].

Cancers in the different subtypes of the oral cavity are defined by ICD-10 as oral tongue cancer (C02.0, C02.1, C02.2, C02.3, C02.8 C02.9), gingival cancer (C03.0, C03.1, C03.9), cancer of the floor of the mouth (C04.0, C04.1, C04.8, C04.9), buccal cancer (C06.0, C00.3, C00.4), cancer of the hard palate (C05.0) and cancer of the remaining parts of the oral cavity (C06.1, C06.2, C06.8, C06.9) (Figure 4) [3].

Squamous cell carcinoma of the oral cavity

Approximately 90% of oral cavity cancers originate from the squamous cells lining the oral mucosa, which gives them the name of squamous cell carcinomas of the oral cavity (SCCOC) [9, 10].

Risk factors

SCCOC is a multifactorial disease with a wide range of behavioral, environmental, and biological risk factors contributing to its development.

Tobacco and alcohol

The most well-established risk factors for SCCOC are tobacco use and excessive alcohol consumption. These factors contribute to cancer development through multiple mechanisms, including direct DNA damage, genetic instability, impaired DNA repair and immune suppression [11, 12].

The primary carcinogenic substances in tobacco that demonstrate a dose-response relationship are polyaromatic hydrocarbons such as nitrosamines and benzopyrenes, found in both tobacco smoke and smokeless tobacco products [3].

Alcohol's contribution is primarily through the production of acetaldehyde and its effects on folate metabolism [13].

Longlasting alcohol consumption on a daily basis increases the risk for oral cancer significantly especially in females due to the lower stomach alcohol metabolism, resulting in higher blood alcohol concentrations and thereby increased acetal-dehyde in oral tissues, leading to epithelial atrophy and consequently its carcinogenic impacts [14].

The combination of chronic alcohol and tobacco consumption results in a synergistic effect, with a relative risk increase of 15-24 times depending on tumour sublocation, increasing mucosal carcinogenic permeability in the oral cavity leading to further mutagenic alterations and increased potential of invasiveness [12, 14].

Increased proliferation rate of oral epithelium due to smoking occurs even after cessation of smoking suggesting involvement of field cancerisation [15, 16], however cessation results in a substantial risk reduction, with the largest reductions among those who stop smoking prior to the age of 40 [17, 18].

Areca nut

Habitual chewing of *betel* which is a mix of leaves from climbing plant named *betel* and *areca nuts* and sometimes mixed with tobacco, is the main cause of oral cavity cancer in Southeast Asia. Its carcinogenic impact is due to tissue hypoxia, inflammatory processes, formation of reactive oxygen species (ROS), induction of autophaghia, promotion of epithelial-mesenchymal transition (EMT), DNA-injury which has been demonstrated to cause precancerous oral submucosal fibrosis as well as squamous hyperplasia in animal studies [19, 20].

Diet

Numerous studies have linked dietary habits to the development of various cancers, including oral malignancies. A pro-inflammatory diet, rich in fried food, processed foods and red meat, can create a chronic inflammatory environment in the body, leading to increased oxidative stress and impaired DNA repair [13, 21]. Inflammatory cytokines such as *Tumour Necrosis Factor-alpha* (TNF-α) and interleukin-6 (IL-6) can suppress immune surveillance and promote the growth and survival of cancer cells [13].

As a preventive measurement of risk reduction of oral cancers optimization of the nutritional intake needs to be highlighted, implying increased consumption of various fruits, vegetables and cereals to address the recommended allowance of micronutrients like vitamin A, C, E, calcitriol which is the active metabolite of vitamin D, antioxidants, zinc, β-carotene as well as folate are effective in prevention of oral cancer [21, 22].

Obesity

Adipose tissue is an active endocrine and immunological organ that secretes adipokines such as leptin, adiponectin, resistin, and other cytokines regulating inflammation and the local immune microenvironment [23].

Obesity, defined as adipose tissue dysfunction due to excessive fat accumulation and characterized by a subclinical, chronic low-grade inflammatory state, is linked to carcinogenesis through several mechanisms. These include alterations in adipokine pathophysiology, impaired insulin-like growth factor I (IGF-I) axis, insulin resistance, lipid uptake causing metabolic reprogramming, oxidative stress and the regulation of number and functions of the myeloid-derived suppressor cells (MDSCs) [23, 24].

MDSCs are immunosuppressive cells that can prevent T cell activation, inhibit natural killer (NK) cell's toxic effect and induce regulatory T cells. Although their numbers are minute under normal conditions, they are amplified during disease conditions such as in chronic low-inflammatory processes causing changes in the local immune microenvironment [23].

Poor oral hygiene

Several studies have provided strong evidences that microbiomes related to a poor oral health contributes to local chronic inflammatory disorders such as periodontitis with increased risk for developing SCCOC as well as cancer in the pharynx [25-31]. The microbial triggered carcinogenesis in periodontal disease is linked to expression of pro-inflammatory cytokines such as IL-1 and TNF- α [32].

Oral Potentially Malignant Disorders (OPMDs)

OPMDs constitute a group of pre-cancerous oral mucosal conditions with the capability of successive progression to SCCOC and includes leukoplakia, erythroplakia, erythroleukoplakia, proliferative verrucous leukoplakia (PVL), oral verrucous hyperplasia (OVH), oral submucous fibrosis, oral lichen planus, lichenoid dysplasia and oral dysplasia [12, 33, 34].

High-risk HPV

Human papillomavirus (HPV), particularly the high-risk strains such as HPV-16, has been classified as a high-risk carcinogen for oropharyngeal malignancies (palatine tonsils, soft palate and base of the tongue) by the International Agency for Research on Cancer (IARC) since 2007 [3, 20, 35]. HPV is the main cause of HNC in the United States as well as in Northern Europe, however it is responsible for less than 4% of SCCOCs [20].

The carcinogenic effects of HPV are attributed to impaired DNA-damage repair and anti-apoptotic activity. A key event in this process is the integration of HPV DNA into the host genome, which enables the expression of the viral oncogenes E6 and E7. The E6 protein binds to and degrades p53, a crucial protein that regulates the cell cycle and induces apoptosis in response to DNA damage. The E7 protein binds to and inactivates the retinoblastoma protein (pRb), which normally suppresses cell cycle progression [20, 35].

Autoimmunity, genetic predispositions and hereditary syndromes

A number of other conditions and disorders are associated with higher susceptibility of SCCOC, among which some autoimmune chronic diseases such as *scleroderma (systemic sclerosis)* can be mentioned, where the chronic inflammatory state and immunosuppression are believed to be the pathomechanism behind the higher risk [12, 36].

A number of genetic predispositions and hereditary syndromes are associated with increased susceptibility for HNC due to polymorphisms in DNA repair enzymes, apoptotic pathway components and cell-cycle controlling proteins as well as mutations in the *FANCA* genes normally coding proteins involved in DNA damage repair and contribute to genomic stability, resulting in *Fanconi anemia* with bone marrow failure and a 500-700 times higher risk for this patient category than the general population to develop HNC with the oral cavity being the commonest sublocation [20, 37, 38].

Among other genetical syndromes involved in DNA metabolism dysregulation of the oral epithelium and associated with an increased risk of OPMDs and SCCOC *Xeroderma pigmentosum*, *Bloom syndrome (BS; congenital telangiectatic erythema)* as well as *Dyskeratosis Congenita (Zinsser-Engman-Cole syndrome)* related to dysfunctional telomere maintenance should be mentioned [12, 20].

Epidemiology and clinical features

Age and sex

Globally SCCOC affects primarily patients over the age of 50, with a male-to-female ratio of circa 2:1 and with mortality rates varying significantly worldwide, influenced by geographic areas and demographic characteristics [39].

Moreover, an increase in the incidence of SCCOC is observed on a global scale among young adults (< 44 years old), with Asia having twice that in the US and believed to be strongly related to risk consumption of tobacco, betel quid chewing and alcohol [12]. In addition, a notable incidence of especially tongue cancer is seen in younger adults around the world [12, 40].

Prognosis

Interestingly the prognosis for the younger patients manifests similar or better overall survival compared to that in the elderly, even in cases of relapses, but worse 5-year disease-free survival (DFS) despite receiving similar therapy [12]. The relative 5-year survival of SCCOC in Sweden is 62%, but the difference is noticeable depending on tumour sublocation and tumour stage upon diagnosis, with malignancy of the hard palate having the best survival while patients with floor of the mouth cancer having the worst prognosis [3].

Node involvement

Depending on the sublocation within the oral cavity, SCCOC tumours are detected at different stages at diagnosis, with regional spread to the lymph nodes (N1-N3) observed in 24% of cases [3, 41].

In Sweden, the most frequently affected sublocation is the oral tongue, accounting for circa 41% of all SCCOC cases (Figure 5). Within the oral tongue, the lateral borders are the most frequently involved sites. Due to the tongue's rich vascular and lymphatic supply, squamous cell carcinoma of the oral tongue (SCCOT) is often more aggressive and prone to early metastasis. More than one-third of these tumours have regional lymph node metastasis (N1-N3) already upon diagnosis, despite easy accessibility of the oral cavity for medical examination, significantly reducing the survival [3].

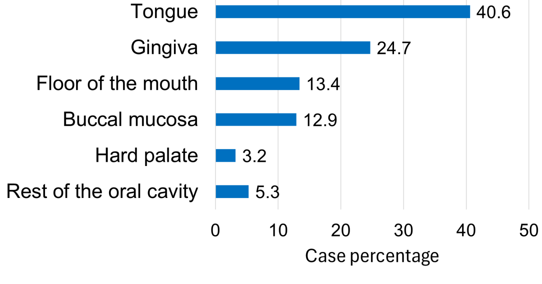

Fig. 5 Distribution of SCCOC cases in Sweden. 2008-2016, n=3540 [3].

Traditionally, the N classification has been considered to be the most important prognostic factor, where nodal positivity reduces the probability of survival radically [42]. Regarding the prognostic value of the N classification, it has been shown that in patients with HPV-positive tumours, the variables related to regional lymph node metastasis had a far less important role considering prognosis than in patients with HPV-negative tumours, implying that the nodal status is not a prognostic factor in patients with HPV-positive oral/oropharyngeal tumours [42].

Local recurrence and secondary primary tumour

Between 26% and 45% of SCCOC patients develop locoregional recurrence (LRR), a second primary tumour (SPT) or distant metastasis (DM) (also called second events) after the primary curative-intent therapy [43, 44].

The vast majority of all LRRs occurres within the first 2 years after treatment [43, 44]. Histological differentiation is related to tumour recurrence intervals implying that patients with a higher histological grading require more precise follow-up observation during the initial 2 postoperative years [44].

SPT, defined as a metachronous solid cancer that is neither a recurrence of the primary cancer nor a metastasis, developing at least six months after the initial cancer, is a leading long-term cause of death among survivors of primary SCCOC [45]. The median time to SPT diagnosis in HNC patients is approximately three years post-treatment, which further emphasizes the paramount importance of long-term surveillance in SCCOC management, as the risk of developing SPTs persists several years after initial treatment [46].

Clinical investigations

Standardized Cancer Patient Pathways

The Swedish concept of *Standardized Cancer Patient Pathways* (CPPs) (Figure 6) were introduced nationwide in Swedish healthcare back in 2015 with the purpose of reducing the time to cancer treatment and to improve diagnostics, satisfaction and equity of care between healthcare providers on a national level regarding cancer patients [47]. The CPP concept aim to continuously investigate the patient-flow in order to optimize it; to visualize the patient population undergoing CPPs, in terms of investigation time and indications of different outcomes including cancer diagnoses [47].

Each CPP basically defines the signs and symptoms required for *well-founded suspicion* (WFS) of cancer, principles for referral to a specialist in the field and requested pre-diagnostic investigations [47]. Several CPPs have a filter function, implying well-founded suspicion requires confirmation by a specialist [47]. The majority of CPPs have a lead time from well-founded suspicion to treatment initiation. All relevant data in this regard are recorded and monitored on a national basis [47].

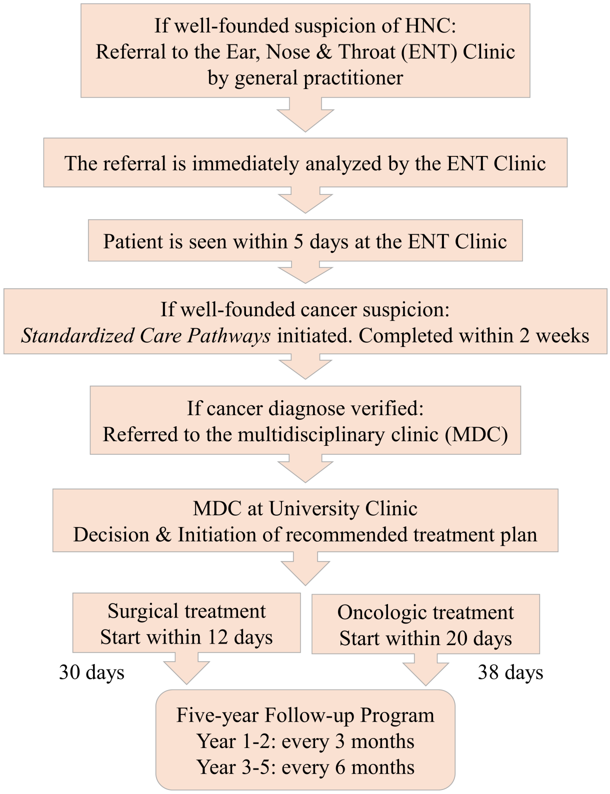

Fig. 6 Flow chart representing the Swedish concept of *"Standardized Cancer Care Pathways",* a fast track accelerated clinical pathway shortening the waiting duration and the care process between reasonable suspicion and the start of the treatment for all new HNC patients in accordance with *The Swedish National Program on Standardized H&N Cancer Care.* Source: [3]. Flowchart modified and translated from Swedish by the Thesis Author.

Radiological investigations [3, 48]

- Computer Tomography (CT) of the head & neck and thorax.
- Magnetic Resonance Imaging (MRI) of the tumour area and skullbase when T stage is difficult to assess with CT.
- Ultrasound with Fine Needle Aspiration Cytology (US-FNAC) of the neck lymph nodes.
- Cone Beam Computer Tomography (CBCT) if necessary to map bone involvement.
- For T3–T4 tumours, fludeoxyglucose-18 (FDG) Positron Emission Tomography (PET) scan, (FDG-PET-CT) is recommended.

Treatment strategies

The anatomy of the oral cavity plays a crucial role in the surgical management of oral malignancies, where a transoral approach is applied for excision of premalignant lesions and smaller sized superficial cancers in the oral tongue, anterior portions of the floor of the mouth and alveolus [49].

A more invasive approach is, however, required regarding the more posteriorly located tumours in order to obtain adequate perioperative field exposure upon surgery, where lip splitting paramedian mandibulotomy approach may be performed implying a longer incision through the lips and mandible, thereby enabling the exposure of the posterior lateral aspect of the oral cavity [49].

According to SweHNCR, during the time period of 2008-2016, 45% of the Swedish patients with oral cavity cancer (including all tumour locations and stages) underwent surgery exclusively, while 35% was treated with the combination-therapy of surgery and preoperative or postoperative radiotherapy, while chemotherapy in combination with other treatments were given 4% of the cases [3].

Biomarkers

Biomarkers are crucial components within the field of precision medicine and have sparked considerable interest in identifying specific biomarkers with the aim of enabling the enhancement of prevention, early diagnostics as well as optimizing personalized treatment strategies for cancer patients [50-52].

Being obtained frequently from body fluids or solid tissues, the biomarker concentrations are indicative of the quantified risk of disorders and/or the treatment outcome [51]. The field of biomarkers could be divided into diagnostic and prognostic biomarkers, however there is often an overlap between these groups [52].

Biomarkers in the peripheral blood & plasma proteins

The peripheral blood is a biomarker source of outmost importance including host and viral DNA, RNA, proteins, cells as well as numerous metabolites [51].

Furthermore in the search for cancer biomarkers, plasma proteins have long been considered a resource of paramount importance due to their easy accessibility, non- or minimally-invasiveness as option to tissue biopsies, as well as its long-term cost-effectiveness. Cancer-specific plasma proteins detectable at an early stage of malignancies may be used as a tool for real-time monitoring of the health status and therapy response, thereby improving the prognosis of cancer patients, especially as there are currently no clinically approved biomarkers for oral cavity malignancies [53].

Cytokines being soluble proteins and their receptors are functioning as biomarkers for immunological and inflammatory status, where i.e. higher concentration of soluble interleukin-2 receptor (IL-2R) has been shown in oral cancer patients compared to healthy controls and higher in stage III-IV than in stage I-II in this patient category [51].

The viral genome of HPV, a DNA virus belonging to the Papillomaviridae family, can be analyzed from peripheral blood applying PCR and is involved in the pathology of the squamous cell carcinoma of the oropharynx (SCCOP) [51, 52].

HPV status is considered to be the most reliable prognostic factor in SCCHN, although several other factors such as molecular and pathological characteristics as well as tumour location and clinical stage also have a great impact on patient outcomes [54].

The tissue expression of the tumour suppressor protein p16, encoded by the gene cyclin-dependent kinase inhibitor 2A (*CDKN2A*), is considered to be a surrogate biomarker for HPV with up to 90% concordance between the HPV and p16 detections. p16 levels are associated with better therapy outcomes, whereas p16-negativity is related to poor prognosis [51].

Another important virus is the Epstein–Barr virus (EBV), recognized as a key pathogen in different HNC, specially associated with nasopharyngeal carcinoma, where diagnostic biomarkers may confirm the presence of a disorder [52].

Human genomic DNA, extracted from peripheral blood mononuclear cells (PBMC) provide information regarding status of health and disorders, including occurence of oral malignancies due to its involvement in DNA repair pathways, involving base excision repairs, nucleotide excision repairs and double-strand break repairs [51].

Biomarkers in cancer tissues

Tissues that have undergone malignant transformation harbours an important source of molecular alterations with role in all hallmarks of carcinogenesis including uncontrolled cell cycle, invasiveness, metastasis and anti-apoptosis [51].

Somatic mutations takes place in the DNA of cancerous cells; which is illustrated by studies such as one where a next-generation sequencing (NGS) of hundred cancer-related genes revealed that a higher tumour mutational burden was observed in HPV-negative SCCHN patients [51, 55].

IL-24, an immunological protein mainly expressed in the cytosol and measurable by immunohistochemistry (IHC) method has been shown to correlate with a better outcome and lower incidence of second primary oral cancer [51, 56].

High levels of immunological checkpoint proteins, such as Programmed Death-Ligand 1 (PD-L1) and PD-L2 levels in SCCOC tissue samples are correlated with poor OS [51, 57, 58], while PD-L1 in metastatic sites are higher than in the primary tumour site [57].

Biomarkers in tumour environment

The tumour microenvironment (TME) is composed mostly of immune and stromal cells, involved in a dynamic and intricate interaction with the tumour cells impacting the cancer progression, thereby functioning as predictive indicator of immunotherapeutical response [50, 51].

There is a load of accumulating evidence suggesting that tumour-infiltrating cells, which can be evaluated in ordinary routine Hematoxylin-Eosin (HE)-stained histological slides from various SCCHN subsites, contribute additionally to evaluate tumour immune response [50].

A higher concentration of tumour-infiltrating lymphocytes (TILs) such as CD3 or CD8 T cell and CD57 NK cell infiltrates has been observed being linked to an improved OS and progression-free survival (PFS) of oral cancer patients, while preponderance of tumour infiltrates rich in Treg or CD4+ Th2 lymphocytes inversely correlates with patient outcome [51, 59].

Biomarkers of other origin

Saliva constitutes part of the oral microenvironment, is in close contact with oral malignancies and is easily accessible for cost-effective screening, it harbours components that are applicable as biomarkers [51]. Valine and lactic acid are metabolites that may indicate occurrence of oral cancers [60]. Aminoacids such as lycine, proline, ornithine and citrulline have been revealed to be related to early stage SCCOC [61].

Field cancerisation

In 1953 Albeit Slaughter and colleagues analyzed a population cohort of 783 patients with SCCOC, publishing the paper "Field cancerisation in oral stratified squamous epithelium", introducing the field cancerisation model, suggesting that tumours arise from an expanded pool of genetically altered preneoplastic cells [62, 63]. SCCOC is preceded by premalignant alterations in the mucosal epithelium characterized by tumour-associated genetic changes, also named precancerous fields [64], thereby partly explaining the high incidence of local recurrence and multiple second primary tumours seen in this patient category (Figure 7). This paradigm has further been modified to include exposure of the tissue microenvironmental milieu to damaging and mutagenic agents that can influence all stages of tumour evolution and is termed "*etiologic field effect*" [65].

Multiple clonal populations with distinct genetic and/or epigenetic alterations can coexist within the cancer field [66, 67]. Clonal competition, the evolutionary dynamics between different cell clones, occurs as these clones compete for space, nutrients and survival signals [66, 67]. Over time, some clones outgrow others due to fitness advantages such as faster growth, immune evasion or drug resistance [66, 67].

Transcriptome profiling of different cancer types, including those of prostate and colon has shown aberrant gene expression in non-tumour cells in tumour-bearing patient cohort compared to corresponding controls from healthy individuals [68-70].

By examining gene expression in non-tumour samples (NAT) across eight tissues (bladder, breast, colon, liver, lung, prostate, thyroid and uterus) and corresponding tumour types, Aran et al. reveal altered pathways shared among NATs across these tissue types, characterized a set of 18 genes that were specifically activated in NATs and based on the findings suggested a pan-cancer mechanism where pro-inflammatory signals from the tumour stimulate an inflammatory response in the adjacent endothelium [69].

In the case of SCCOT, it can therefore be of great value to study not only the tumour itself but also the NTCT to clarify field cancerisation and to map alterations indicative of a tumour, prior the presence of clinical manifestations. This would enrich our lacking knowledge at status quo regarding the pathomechanism behind the primary, secondary and recurrence development of SCCOT and other oral malignancies which often result in rather invasive and mutilating surgical interventions.

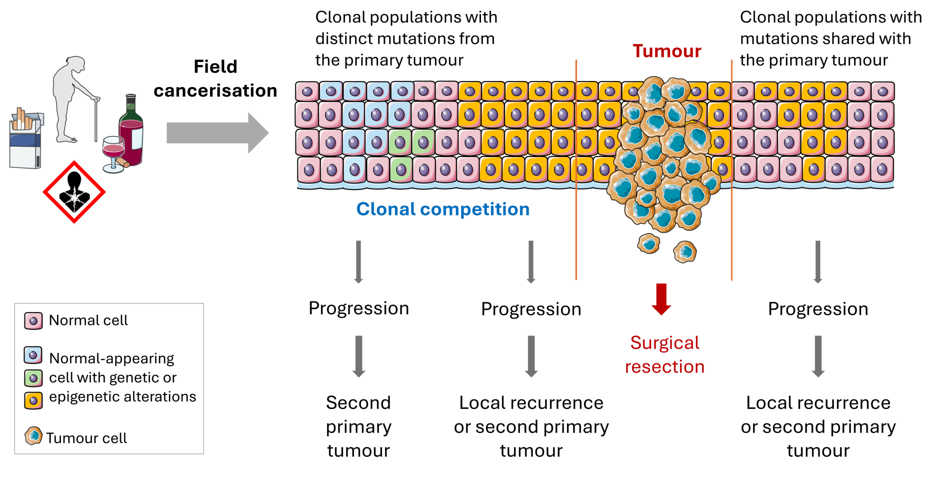

Fig.7 Field cancerisation. Figure produced by Dr. Xiaolian Gu using images adapted from Servier Medical Art (https://smart.servier.com), licensed under a Creative Commons Attribution 4.0 International License.

Antigen presentation and anti-tumour immunity

Major histocompatibility complex

The human immune defense machinery is comprised of several defense barriers. The very first line of defense is comprised by various physical and chemical barriers that prevent pathogen entry. If a pathogen overcomes these barriers, it will face the innate immune system constituting the second defense barrier with its immediate pathogen-oriented response through immune cells i.e. granulocytes, macrophages, NK cells as well as via the plasma protein cascade of the complement system [71].

The adaptive immune system specialized on endogenous and exogenous pathogens is comprised of the humoral and cellular paths respectively. The humoral antibody-mediated response based on B-lymphocyte´s antigen/pathogen recognition in the blood-lymphatic environment where their activation results in the release of i.e. B-cell receptor antibodies causes the antigen degradation mediated by cells such as macrophages [71, 72].

The cellular path, regulatory or cytotoxic, is orchestrated by T-lymphocytes recognizing antigenic peptides on the host cell surface by major histocompatibility complex (MHC), where the antigen presentation follows either the MHC class I or MHC class II dependent pathway [71, 72].

MHC class II molecules on the surface of specialized antigen-presenting cells present exogenous peptide fragments to CD4+ T helper cells, resulting in B cell antibody production, whereas MHC class I molecules on nucleated cells present endogenous or mutated peptides to CD8+ cytotoxic T lymphocytes (CTL), leading to the elimination of cells with non-self antigens [71, 72].

The level of expressed MHC I on the cell surface under physiological condition varies depending on the cell type; i.e. lymphocytes and dendritic cells which are of hematopoietic origin typically exhibit high baseline MHC I antigen presentation components and consequently without any stimulation have high levels of MHC I molecules on their cell surface [73].

MHC I expression tends to increase in all cells upon stimulation by interferon-gamma (IFN-γ), a key immune-regulatory cytokine, during a T cell response or infection. This mechanism is of paramount importance and is well-documented in studies of mice with defects in the IFN pathway, resulting in an increased cancer incidence [73].

As the MHC I molecule and most components of the MHC I antigen presentation machinery are non-essential for cell survival, tumour cells can downregulate them by epigenetic silencing or lose this immunological presentation (as cancer cells are genetically unstable). This does not affect their growth or metastasizing potential but enables them to evade immune surveillance by CD8+ CTLs [73].

Transporter associated with antigen processing

A key factor in the immune defence against malignant cells and the process of cytotoxic T cell activation is the ATP-dependent heterodimeric transporter associated with antigen processing, TAP1/TAP2 complex with its nucleotide-binding domain (NBD) and a multiple transmembrane spanning domain which brings antigen peptide substrates into the endoplasmic reticulum, where they are processed and loaded onto MHC-I for export to the cell surface [74, 75].

Given its central role in the conventional antigen processing pathway, TAP is a major target for immune evasion strategies. Viruses such as herpes simplex virus, cytomegalovirus, Epstein-Barr virus suppress TAP1 expression to evade immune surveillance; moreover loss of function mutations in TAP are observed in tumours [76, 77].

By downregulating the expression of TAP, causing loss of MHC I complexes on the surface, tumour cells are enabled to escape the recognition by the MHC class I restricted CD8+ CTL [74], which otherwise upon recognition of cancerous cells, eradicate these by perforin or FAS-dependent pathways as well as via incitement of inflammatory response [73].

In most studies performed previously, a downregulated TAP1 protein level has been shown in various tumour tissues such as in early stage breast cancer [78], SCCHN [79-81] and to an higher extent in metastatic compared to primary SCCHN [75]. However in a few other publications regarding other malignancies than HNC, contradictory observations has been made; thus i.e. in esofageal malignancy, upregulation of TAP1 protein in malignant tissues was observed to be an unfavorable prognostic factor [82]. Similarly, based on The Cancer Genome Atlas (TCGA), an extensively curated, publicly available dataset comprising RNA sequencing data and matched clinical information from a high number of patients, a higher TAP1 and TAP2 expression level in more advanced stage breast cancer suggests an indicator of aggressiveness [83].

Aims

Overall aim

To characterize the clinically normal tissue and plasma in patients with SCCOC and to establish a panel of changes which may contribute to early detection, diagnosis and prognosis.

Specific aims

Paper I

To investigate the RNA expression patterns of TAP1 and TAP2 in SCCOT and clinically normal tongue contralateral to tumour (NTCT), in order to assess their involvement in field cancerisation and evaluate their prognostic value.

Paper II

To investigate the protein expression levels of TAP1 and TAP2 in SCCOT and benign hyperplastic tongue lesions, in order to confirm their upregulation in SCCOT and explore their potential as prognostic markers.

Paper III

To identify the transcriptional profiles in NTCT, in order to improve our understanding of field cancerisation and its role in cancer development.

Paper IV

To identify SCCOC-specific alterations in plasma proteins and evaluate the prognostic significance of these systemic markers.

Material and Methods

Study participants and samples

All patients included in this study were diagnosed at the Department of Otorhinolaryngology and Head & Neck Surgery, Norrland's University Hospital, Umeå, Sweden. Following written informed consent, tissue biopsies and/or blood samples were collected prior to treatment. Archived formalin-fixed, paraffin-embedded (FFPE) tissues were obtained from the Department of Clinical Pathology, Umeå University Hospital. No written consent was required for the use of archived tissues. All procedures were conducted in accordance with the principles of the Declaration of Helsinki and were approved by the Regional Ethics Review Board.

Project I & III comprised 14 healthy individuals and 31 SCCOT patients (15 men and 16 women) with a median age of 64 years (range 19–87 years). Among patients with a status of "alive disease-free", the minimum follow-up time was 5 years.

In healthy controls, biopsies were taken from the lateral border of the tongue. In patients, biopsies were collected from the tumour and from NTCT. Tumour and NTCT samples were obtained simultaneously during the surgical procedure. In total, 14 healthy control samples, 23 NTCT samples and 29 tumour samples were successfully collected for downstream experiments. Paired NTCT and tumour samples were available from 21 patients. In the remaining cases, paired sampling was not possible due to practical considerations, such as prioritization of tumour tissue for diagnostic purposes or patient unwillingness to provide clinically normal tissue.

Project II included tongue tissue samples from a total of 95 patients; 17 of these had benign hyperplastic tongue lesions, while 78 had SCCOT of whom 37 were men and 41 women with a median age of 64 years at diagnosis (range 19-89 years). For SCCOT patients with a status "alive disease-free", the minimum follow-up time was 5 years. FFPE tissue samples from these patients were used in this project.

Project IV included 74 individuals; 41 of these were patients with SCC from various sublocations of the oral cavity, including the oral tongue, floor of the mouth, gingiva and buccal mucosa (age range 28-87 years), while 33 were healthy individuals (age range 20-83 years). Blood samples were collected at diagnostic time, prior treatment measurements.

Methodology applied

Microarray (project I & III)

Microarray technology is a useful and cost-effective method that has been widely applied for high-throughput gene expression analysis, enabling the simultaneous measurement of thousands of genes in a single experiment.

To measure RNA expression levels, RNA is first converted into complementary DNA (cDNA), which is then labeled with a fluorescent dye and hybridized to a pre-designed set of probes on a chip. Each probe corresponds to a specific gene or genome region. RNA expression levels are quantified based on the intensity of hybridization [84-86].

In our study, RNA expression profiling data, previously generated using the Illumina HumanHT-12 v4 Expression BeadChip (Illumina Inc., San Diego, CA, USA), were analyzed.

In *project I* we analysed the previously published microarray results [83, 87] in order to see the up- or downregulation of TAP1mRNA-level in NTCT, healthy controls and in tumour samples in relation to each other.

In *project III* we investigated the microarray gene expression data of healthy controls, NTCT and SCCOT samples with the aim of characterizing transcriptional profiles in NTCT.

Quantitative RT-PCR (project I)

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) is a powerful high sensitivity and high specificity method of quantitating alterations in gene expression and amplifying and quantifying targeted DNA molecules [88].

RT-qPCR is applied in numerous contexts including in connection with microarray validation, pathogen detection, gene expression analysis and genetic testing [89].

Reverse Transcription step of RT-qPCR transcribes RNA into cDNA by reverse transcriptase whereafter cDNA is used as the template for the qPCR reaction [89].

PCR amplification is combined with fluorescent detection so that the product accumulation is monitorable and measurable in real-time as the reaction progresses, with product quantification after each PCR cycle [88, 89].

In our *project I* we applied RT-qPCR to measure gene expression levels in healthy controls and pairs of Tumour / NTCT samples (QuantStudio 6 Flex real-time PCR system).

Custom primers used were TAP1 (forward: CTGGACTCCCTCAGGGCTAT, reverse: GGTTTCCGGATCAATGCTCG).

Reference genes used were RPL13A (forward: CACGAGGTTGGCTGGAAGTA, reverse: ACGTTCTTCTCGGCCTGTTT) and GAPDH (forward: GCCCTCAACGACCACTTTGT, reverse: TTACTCCTTGGAGGCCATGTG). All primers were obtained from Thermo Fisher Scientific.

Immunohistochemistry (project II)

IHC is one of the most widely applied techniques in clinical diagnostics of abnormal cells in tumourous tissues and in basic research to describe the presence, localization and abundance of target antigens/proteins and biomarker and differentially expressed proteins in intact cells and tissues quantitatively by utilizing antigen-antibody interaction, in which antibodies are used to specifically bind to target antigens [90, 91].

In order to visualize the antigen-antibody complexes by microscopy, antibodies are conjugated with a detectable probe; in chromogenic IHC antibody is conjugated to an enzyme, i.e. a peroxidase (the combination being termed immunoperoxidase) that can catalyse a colour-producing reaction or as in immunofluorescence where fluorescent conjugated antibodies are used [91, 92].

Formaldehyde fixation of the specimen may “mask” target antigens in the tissue sample whereby the binding between the primary antibody and its epitope might be prevented due to methylene bridge formations upon fixation cross-linking proteins and thereby masking antigenic sites [93].

Via the process of antigen retrieval, an “unmasking” of the antigens within a sample takes place, by either a *heat-induced epitope retrieval* (HIER) or *protease-induced epitope retrieval* (PIER) to reverse antigen masking, breaking the methylene bridges, exposing the antigenic sites and thereby allowing the antibodies to bind [93]. *Epitope*, also named antigenic determinant, constitutes the antigen part recognized.

In our *study II* we stained the tissue sections with a TAP1 polyclonal antibody (1114-1-AP, Proteintech, Chicago, IL, USA) or a TAP2 polyclonal antibody (ASJ-BB69A9; Nordic BioSite, Sweden) at a dilution of 1:100 and 1:50 respectively by an automated Ventana Benchmark Ultra staining machine with the ultra-VIEW

DAB Detection Kit for visualization (Ventana Medical Systems, Inc., Tuscon, AZ, USA). Lymphocytes were used as internal positive controls and the stained samples were photodocumented by a Panoramic 250 Flash III scanner (3DHISTECK Ltd., Hungary).

Whole Exome Sequencing (project III)

Whole exome sequencing (WES) is a genomic technique for sequencing all protein-coding gene regions [94]. WES identifies genetical variants altering protein sequences, thus enables the detection of copy number variations (CNVs) as well as single nucleotide variants (SNVs) whereafter the combined data can further be analyzed and is a method applied in academics as well as in the clinical diagnostics [94]. WES is performed in two consecutive steps; where initially acquisition of high-quality genomic DNA (gDNA) takes place from biological samples [94, 95]. In the second step the exonic DNA is sequenced applying any high-throughput DNA sequencing technique [95].

Currently two types of NGS technologies are available; namely DNA amplification-based sequencing (Illumina, Ion Torrent) versus single molecule real-time sequencing (Pacific Biosciences, Oxford Nanopore) [94]. The WES data used in our study were produced by the Illumina HiSeq platforms (Illumina Inc., San Diego, CA, USA). CNVs were identified using GATK copy number variant discovery tools [96, 97].

RNA-sequencing (project III)

RNA-sequencing, which has replaced microarray technology, applies NGS to detect and quantify various RNA populations in a biological sample, thereby providing a picture of gene expression in the sample, so called transcriptome [98, 99].

The RNA-sequencing data used in our study were produced by the Illumina HiSeq platforms. Alignment, assembly and quantification of RNA-sequencing data were performed using HISAT2 [100], StringTie [101] and RSEM (RNA-Seq by Expectation–Maximization) [102], respectively.

Proximity Extension Assay (project IV)

The Proximity Extension Assay (PEA) is a robust platform developed for the analysis of secreted proteins and biomarker discovery and validations in serum and blood plasma combining antibody specificity with the sensitivity and amplification capabilities of Polymerase Chain Reaction (PCR) [103]. PEA enables simultaneous analysis of several thousands target proteins, applying a minute volume (microliters) of blood with high sensitivity protein detection and quantification, far beyond detection capability of mass spectrometry [104].

This technology is based on 2 proximity probes, each consisting of an antibody linked to a unique DNA oligonucleotide and once these probes bind to their target protein, they are brought into close vicinity of each other, thereby allowing DNA oligonucleotides to hybridize and form a double-stranded DNA molecule which in turn can be extended by a DNA polymerase [103].

Detection of numerous proteins simultaneously, so called sample multiplexing, is enabled by adding unique sample indices during a subsequent PCR step resulting in generation of a unique DNA sequence directly quantifiable by real-time PCR or NGS, translating the protein presence into a measurable DNA signal [103]. NGS counts is proportional to the protein concentration in the sample, enabling its relative quantification [103].

In *study IV* proteomics analysis of plasma samples were performed at Olink Proteomics AB (Uppsala, Sweden) where we measured the proteins included in the Olink Explore 3072 platform via PEA.

Statistical Analysis

For statistical analysis all tests were conducted in IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA), where a two-sided p-value < 0.05 was considered significant.

Survival analysis (project I, II, IV)

- *Cox Regression model* was applied for survival analysis.
- *Kaplan–Meier* with log-rank tests were performed to compare survival curves.
- *Cancer-Specific Survival (CSS)* was defined as the time from diagnosis to death due to the specific cancer type.
- *Disease-Free Survival (DFS) or Disease-Free Intervall (DFI)* was defined as the time from completion of treatment to recurrence or death.
- *Overall Survival (OS)* was considered the time from histopathological diagnosis to death from any cause or last follow-up.

Fisher's exact test (project I, II)

Fisher's exact test is used to find out whether there is a significant association between two categorical variables in a 2×2 table.

Differential RNA expression analysis (III)

To determine the degree of differential expression between groups, fold change in mRNA levels and false discovery rate were calculated using the ComparativeMarkerSelection tool in GenePattern [35].

PCA and PLS–DA (project III)

Unsupervised principal component analysis (PCA) (SIMCA 16, MKS Data Analytics Solutions, Umeå, Sweden) provided an overview of transcriptional profiles of our study cohort in project III, while supervised partial least-square-discriminant analysis (PLS–DA) identified discriminant factors between the cohort groups examined. Genes with "variable influence of projection" (VIP) ≥ 1 and "correlation coefficient between model and original data" p(corr) > 0.9 were selected as the topmost discriminant factors.

Spearman Correlation analysis (project I, II, III)

Nonparametric Spearman correlation analysis was used to measure the strength and direction of association between two variables based on the ranked values of the data.

Differential protein abundance analysis (project IV)

This analysis was used between patients and controls applying Stat Analyzer, were proteins with a normalized protein expression (NPX) difference > 0.5 or < -0.5 and an adjusted p-value < 0.05 were considered as differentially expressed and their impact on patient survival was assessed by Kaplan-Meier and Cox regression survival analysis.

Results

Paper I

Previously published microarray results were investigated, revealing a significant upregulation of TAP1 mRNA in NTCT compared to healthy controls, which was even further upregulated in the tumour samples. For TAP2 no corresponding findings were seen, while a significant correlation was observed between TAP1 and TAP2 mRNA levels. RT-qPCR analysis of TAP1 was performed to validate the microarray findings, confirming a significant correlation between microarray and RT-qPCR.

Univariate Cox regression showed that low expression of TAP1 in NTCT was significantly related to better overall and disease-free survival. No correlation with survival was found regarding TAP1 levels in tumour samples.

Controlling for the effects of cytolytic activity (a previously identified NTCT-based prognostic marker), age at diagnosis, sex, clinical stage, tumor size and nodal status, multivariate Cox regression showed that TAP1 levels in NTCT remained an independent prognostic factor for both overall and disease-free survival.

In Kaplan-Meier analysis, dividing patients into TAP1-high and TAP1-low groups based on the median TAP1 level in NTCT revealed that patients with lower TAP1 mRNA levels had better overall and disease-free survival. No significant correlations were observed between TAP1 levels and the aforementioned clinical covariates. Dividing patients into TAP1-high and TAP1-low groups based on the median TAP1 level in tumour, no significant difference was found in overall and disease-free survival.

Using data from the TCGA head and neck squamous cell carcinoma cohort, we confirmed the clinical significance of TAP1 in non-tumour samples.

To summarize, the main results from this project shows that TAP RNAs are upregulated in NTCT, indicating field cancerisation and such field changes confer prognostic information.

Paper II

TAP1 and TAP2 protein levels were found to be highly correlated and significantly upregulated in SCCOT compared to benign lesions. Both proteins exhibited cytoplasmic localization and were detected in all SCCOT specimens.

The proportion of TAP1- or TAP2-positive tumour cells was > 80% in almost all tumours, while 25.6% and 23% of tumours exhibited weak staining intensity for TAP1 and TAP2, repectively.

Neither TAP1 nor TAP2 staining intensity was related to any clinicopathological factors such as age, sex, tumour stage, nodal status, TNM stage (T category describing the primary tumour site and size, N category the regional lymph node involvement, while M category refers to the presence or otherwise of distant metastatic spread [105]), Worst Pattern of Invasion (describing the tissue infiltration by cancer cells at the tumor/host interface [106]), Lymphocytic Response (quantified as the density of lymphocytes at the host/tumor interface *according to Brandwein-Gensler et al [107]*) or degree of differentiation (refering to the extent to which a tumour resembles its tissue of origin, with well-differentiated tumours being less aggressive than poorly-differentiated ones [108]). No significant differences in survival outcomes were observed between patients with intermediate /strong TAP expression and those with weak TAP expression.

In patients < 70 years old and with early stage SCCOT, male patients were shown to have a better outcome than female patients and the best outcome was observed in male patients with intermediate/strong TAP expression.

In summary the most important findings of *project II* was the fact that TAP proteins really are upregulated in tumours and are not undergoing downregulation, which thereby confirmed the results from *project I*.

Paper III

Applying PCA, NTCT transcriptional profiles were shown to differ from tumour samples, while similar to the controls. Furthermore, using PLS-DA, 61 mRNAs distinguishing NTCT from healthy tongue tissue were selected as topmost discriminatory factors; 22 mRNAs upregulated versus 39 mRNAs downregulated in NTCT compared to healthy controls.

Performing functional enrichment analysis of the downregulated genes using g: Profiler, Cellular Component (CC) ontology of “cytoplasm” was the only enriched term identified, while regarding the upregulated genes, several enriched terms in Biological Process (BP) and CC ontology were revealed with increased manifestation of “positive regulation of nitrogen compound metabolic process” in NTCT. The 12 genes contributing to this process were apoptosis-related. Compared to healthy controls, Zinc Finger Protein 395 (ZNF395), a pro-apoptotic tumour suppressor, was the only gene manifesting increased mRNA level in NTCT whereas decreased in SCCOT.

Considering the common loss of chromosome 8p (where *ZNF395* gene is located) in SCCOT, the impact of *ZNF395* CNV on gene expression was also examined by using the dataset of our previous genomic study [109] comprising both RNA sequencing data and CNV data of NTCT and tumour samples from 15 patients with SCCOT, showing a positive correlation between copy number and mRNA level.

Paper IV

A total of 2923 plasma proteins were successfully quantified. PCA showed a high degree of overlap between patients and controls, implying similar protein expression profiles.

Applying differential abundance analysis and a predefined cutoff value, resulted in the identification of 20 proteins with the most significant differential abundance between SCCOC and controls. Among these SFRP4 (Secreted Frizzled Related Protein 4) showed significant downregulation in patients and was the only protein significantly associated with survival outcome. After categorization of patients into high- and low-expression groups based on the median protein level, SFRP4-high patients had better overall and disease-free survival.

Adjusted for tumour size and the nodal status, SFRP4 was demonstrated to be an independent prognostic factor both with respect to OS and DFS.

Discussion and Future Perspectives

The research compiled in this thesis presents a multidimensional characterization of clinically normal tissue and plasma in patients with squamous cell carcinoma of the oral cavity (SCCOC) and with the primary focus on establishing a panel of changes which may contribute to early detection, diagnosis and prognosis.

By combining a vast spectra of state-of-the-art laboratory techniques, genetical databases and various analyzing methods including transcriptional profiling, immunohistochemistry, proteomics and survival analyses, this work provides a more nuanced understanding of the pathological processes occurring not only in the tumour itself but also in the surrounding clinically normal tissue.

The findings highlight the key role of the anti-tumour immune response, apoptosis regulation and microenvironmental components in shaping cancer initiation, progression and patient outcomes and further underscore the need to redefine our perception of tumour biology and prognostic biomarker landscapes in oral oncology.

A common and central theme throughout this thesis is the molecular divergence between histologically normal tissue distant from the malignancy and the truly healthy oral epithelium. In our projects, tumour-free tongue tissue contralateral to the primary SCCOT, also referred to as NTCT, was found harboring transcriptional alterations of paramount significance, particularly involving immune activation and apoptosis regulation. These molecular aberrations, despite the absence of visible or microscopic pathology, affirm the concept of field cancerisation as well as the etiological field effect extending beyond the surgical margins and having direct implications regarding both prognosis and surgical/radiotherapeutical planning in SCCOC patients.

Molecular changes in NTCT represent a promising target for early detection strategies, prognostic risk assessment and perhaps even field-targeted interventions aimed at reducing recurrence or secondary tumour formation.

The findings from our first two studies highlight the significance of TAP expression in cancer, particularly in NTCT. Increased immune cell infiltration in the tumour microenvironment can produce IFN-γ and lead to upregulation of TAP in NTCT. Elevated TAP levels in NTCT may also be linked to increased mutation burden and genomic instability, which are characteristic of field cancerisation. Field cancerisation refers to the process by which a tissue surrounding a primary tumor acquires genetic changes, making it more susceptible to malignant transformation. Upregulation of TAP mRNA in NTCT suggests the utility of TAP as an early warning signal, enabling timely intervention before overt tumor development occurs. However, the challenges in translating these findings into clinical practice underscore the need for further research into the molecular mechanisms governing TAP expression and its role in cancer progression.

To our best knowledge, we have performed the very first study to investigate TAP proteins in a cohort of SCCOT patients, revealing increased TAP protein expression in tumour cells. In most previous studies, downregulation of TAP1 protein levels has been observed across various tumour tissues. However, in our study, we found an elevation of TAP mRNA and protein in tumour samples. These findings suggest that loss of TAP expression is not a common mechanism of tumour immune evasion in SCCOT. In addition, sex-specific immune modulation of TAP1 in SCCOT was suggested. Given the growing use of immunotherapy in cancer treatment, it is worth investigating whether increasing TAP expression could serve as an immune-restoring approach, particularly in male patients.

Comprehensive transcriptional profiling in the third study revealed that the top discriminant genes distinguishing NTCT from healthy controls were involved in the regulation of cell death. The simultaneous upregulation of both pro- and anti-apoptotic genes may indicate cellular competition, which could influence the trajectory of potential tumour development.

In our last study, we aimed to identify plasma protein alterations specific to SCCOC and assess their prognostic significance. Among the differentially expressed plasma proteins, SFRP4 emerged as a particularly promising biomarker. SFRP4 belongs to the secreted frizzled-related protein family, which modulates Wnt signaling pathways. Wnt signaling plays a crucial role in regulating cell proliferation, migration, differentiation and survival. Dysregulation of this pathway contributes to various diseases, including cardiovascular conditions, neurodegenerative disorders, metabolic syndromes, autoimmune diseases and cancer [110]. The altered plasma levels of SFRP4 observed in SCCOC patients may reflect disruptions in Wnt signaling in cancer cells or in other components of the tumour microenvironment.

Importantly, altered circulating SFRP4 levels may indicate biological processes occurring beyond the tumour itself or the tumour microenvironment. Tumours interact with the immune system, inflammatory networks and systemic metabolism in complex ways. Altered circulating SFRP4 levels may reflect these broader changes in which Wnt signaling is actively invloved, offering prognostic information that complements tumour-localized assessments such as histopathology and staging. Further studies are needed to elucidate whether SFRP4 plays an active role in SCCOC development or primarily serves as a marker of systemic tumour-related processes.

Considering some general limitations and challenges of the various studies comprising this thesis, lack of detailed information with regard to some variables and clinicopathological factors and patient habits could be mentioned, as well as insufficiency of medical records on more specific smoking/tobacco habits, level of alcohol consumption, absence of follow-up data as well as limited study cohorts. In addition, the profile mapping of the molecular landscapes of solid tumours have traditionally been based on tissue samples obtained through biopsies, which constitutes sampling bias as it only provides a snapshot of tumour heterogenicity and often can not be reobtained [111].

Future research should focus on analyzing larger sample sizes, including multiple biopsies from the tumour vicinity in order to gain deeper insight into the etiologic field effect. Comparative studies involving patients with other oral malignancies and OPMDs are also recommended. In addition genomic profiles of circulating cell-free tumour DNA (ctDNA) have been revealed to closely match their primary corresponding tumours [111] and has gained increasing interest in precision oncology [112]. Liquid biopsies in form of circulating nucleic acids may be applied as an efficient and cost-effective tool for monitoring therapy responses, assess emergence of various drug resistance as well as quantifying minimal residual disease [111]. The molecular profiles collected from ctDNA can be further complemented with those gained via analysis of circulating tumour cells (CTCs), RNA, proteins and lipids contained within vesicles such as exosomes [111]. Moreover several biofluids such as sweat, urine, saliva, pleural effusions and cerebrospinal fluid, which all can be collected non-invasively may contain tumour-derived genetic information [111, 113, 114].

General remarks and endnote

Through this thesis we have provided evidence that clinically normal epithelium can harbor early, prognostically relevant molecular changes and that peripheral blood and plasma indeed may serve as an outstanding informative matrix regarding the health status of the individuals and having a unique personalized surveillance and real-time monitoring capability.

By extending the investigative lens beyond tumours and into their surrounding fields and microenvironment, we have established a panel of changes that may have unlocked some novel possibilities for early detection, diagnosis and prognosis in the fight against head and neck cancer.

Conclusions

Paper I

TAP mRNA upregulation in NTCT may be driven by increased mutation burden and genomic instability associated with field cancerisation. Assessment of TAP expression levels have potential utility in prognosis, early detection and monitoring of disease progression.

Paper II

We confirmed the upregulation of TAP proteins in tumours. In addition, we found that TAP levels in the tumour have low prognostic value in general, highlighting the greater utility of NTCT-based biomarkers.

Paper III

Due to field cancerisation, NTCT is susceptible to malignant transformation, where tissue homeostasis is maintained at least partly through regulation of apoptosis. Loss of the pro-apoptotic *ZNF395* gene may initiate cancer development.

Paper IV

SFRP4 protein is a potential circulating prognostic marker for SCCOC patients. Its altered levels may reflect systemic biological changes that are either driven by the tumour or contributes to tumour development, suggesting prognostic value beyond the tumour microenvironment.

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It is my sincere hope that the novel discoveries presented in my PhD thesis will contribute to better outcomes for future head and neck cancer patients and help pave the way for improved strategies in early diagnosis, treatment and prognosis in the field of head and neck oncology.

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