Microenvironment-driven intratumoral heterogeneity in head and neck cancers : clinical challenges and opportunities for precision medicine

Microenvironment-driven intratumoral heterogeneity in head and neck cancers: clinical challenges and opportunities for precision medicine

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Abstract

Squamous cell carcinoma of the head and neck (SCCHN) is among the most prevalent cancer types worldwide. Despite multimodal therapeutic approaches that include surgical resection, radiation therapy or concurrent chemoradiation, targeted therapy and immunotherapy, SCCHN is still associated with a poor prognosis for patients with locally advanced or recurrent/metastatic (R/M) diseases. Although next-generation sequencing data from thousands of SCCHN patients have provided a comprehensive landscape of the somatic genomic alterations in this disease, genomic-based precision medicine is not implemented yet in routine clinical use since no satisfactory genetic biomarker has been identified for diagnosis, patient outcome prediction and selection of tailored therapeutic options. The lack of significant improvement in SCCHN patient survival over the last decades stresses the need for reliable predictive biomarkers and new therapeutic strategies for personalized clinical management of SCCHN patients. Targeting the SCCHN-associated microenvironment or the interaction of the latter with cancer cells may represent such paradigm shift in the development of new strategies to treat SCCHN patients, as exemplified by the recent implementation of immune checkpoint inhibitors to improve clinical outcomes by increasing anti-tumor immune responses in SCCHN patients. Several clinical trials are in progress in SCCHN patients to evaluate the activity of monoclonal antibodies and small-molecule inhibitors targeting the tumor microenvironment (TME) at different treatment settings, including combinations with adjuvant surgery, radiation therapy and chemotherapy. This review describes the current knowledge about the influence of the TME on intratumoral heterogeneity and clinical relapse in human SCCHN patients. More precisely, the role of hypoxia as well as the presence of non-cancer cells (e.g. cancer-associated fibroblasts and immune cells) on therapy response of SCCHN cells is highlighted. We also discuss relevant (pre)clinical models that may help integrate the microenvironment-tumor cell interplay in translational research studies for SCCHN. Finally, this review explores potential

therapeutic strategies that may exploit the crosstalk between TME and SCCHN cells in order to implement fundamental changes in the tumor treatment paradigm of patients with locally advanced or R/M SCCHN.

Keywords: Head and neck cancer, tumor microenvironment, intratumoral heterogeneity, therapy resistance, cancer-associated fibroblasts, precision medicine, metabolism

Abbreviations

5-FU, 5-fluorouracil; αSMA, alpha smooth muscle actin; ARCON, accelerated radiotherapy with carbogen and nicotinamide; CA, carbonic anhydrase; CAF, cancer-associated fibroblast; cfDNA, circulating-free DNA; CPS, combined positive score; CT, computed tomography; CTC, circulating tumor cells; ctDNA, circulating tumor DNA; CTLA-4, cytotoxic T lymphocyte antigen 4; DAHANCA, Danish Head and Neck Cancer Group; DDR1, discoidin domain receptor 1; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; EMA, European Medicines Agency; EMT, epithelial-to-mesenchymal transition; EORTC, European Organization for Research and Treatment of Cancer; FA, fatty acid; FAO, fatty acid oxidation; FAP, fibroblast activation protein; FAS, fatty acid synthesis; FAZA, ^{18}F-fluoroazomycin arabinoside; FDA, Food and Drug Administration; FDG, ^{18}F-fluoro-2-deoxy-D-glucose; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FMISO, fluoromisonidazole; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; HPV, human papillomavirus; IFN-γ, interferon gamma; IL, interleukin; MATH, mutant allele tumor heterogeneity; MCAM, melanoma cell adhesion molecule; MCT, monocarboxylate transporter; miRNA, microRNA; MRI, magnetic resonance imaging; NF, normal fibroblast; NGS, next-generation sequencing; NK, natural killer; NSG, NOD scid gamma; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival; OSCC, oral squamous cell carcinoma; OXPHOS, oxidative phosphorylation; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand 1; PDTO, patient-derived tumor organoid; PDTX, patient-derived tumor xenograft; PET, positron emission tomography; PFS, progression-free survival; PUFA, polyunsaturated FA; R/M, recurrent/metastatic; SCCHN, squamous cell carcinoma of the head and neck; scRNA-seq, single-cell RNA sequencing; TAM, tumor-associated macrophage; TAZ, transcriptional coactivator with PDZ-binding motif; TCGA, The Cancer Genome Atlas; TGF-β, transforming growth factor beta; TIGIT, T cell immunoglobin and immunoreceptor tyrosine based inhibitory motif; TiME, tumor immune microenvironment; TMB, tumor mutational burden; TME, tumor microenvironment; TPS, tumor proportion score; Treg, regulatory T cells; YAP, Yes-associated protein 1.

1. Introduction

Head and neck cancer is the sixth most common form of cancer worldwide, with more than 870,000 new cases and 440,000 deaths annually, accounting for 4.5% of cancer deaths (1). The majority of these cancers are squamous cell carcinomas (SCCHN) that originate from epithelial cells lining the mucosal surfaces in the oral cavity, larynx, oropharynx and hypopharynx (2). Oral squamous cell carcinomas (OSCC) represent the major subtype of SCCHN, with about two-thirds of the cases in developing countries. Exposure to tobacco-derived carcinogens and excessive alcohol consumption are the main risk factors for SCCHN development (about 75% of the cases). Additionally, oropharyngeal squamous cell carcinomas (OPSCC) are linked to prior infection with oncogenic strains of human papillomavirus (HPV), primarily HPV-16, and, to a lesser extent, HPV-18 and other strains. The incidence of HPV-positive OPSCC has been exponentially rising in Western countries and these cancers are now even more frequent than HPV-driven cervical cancers (3). SCCHN are often diagnosed at late stages, with loco-regionally advanced diseases for which treatment remains a clinical challenge. Indeed, despite aggressive multimodal therapeutic interventions including surgery (often with neck dissection), radiation therapy with or without concomitant chemotherapy (exclusive or postoperative), more than half of SCCHN patients experience loco-regional or distant relapse (4). Before the recent implementation of immunotherapy with two anti-programmed cell death-1 (PD-1) inhibitors, i.e. pembrolizumab and nivolumab (5, 6), the standard first-line protocol for R/M SCCHN, consisted of systemic chemotherapy associated with cisplatin or carboplatin, 5-fluorouracil (5-FU) and cetuximab (anti-epidermal growth factor receptor (EGFR) targeted therapy). This therapeutic regimen, known as the EXTREME protocol, confers a median overall survival (OS) of about 10 months (7). Today, for patients with R/M SCCHN expressing programmed cell death ligand 1 (PD-L1) (about 85% of the population), the first-line standard of care is pembrolizumab with or without chemotherapy with a median OS between 13-15 months.

However, no validated treatment options exist for the patients who progress after these treatments (unmet medical need). The lack of significant improvement in SCCHN patient survival over the last years stresses the need for reliable predictive biomarkers and ideally new therapeutic strategies for personalized clinical management of SCCHN patients.

The tumor microenvironment (TME) has emerged as an important factor that can contribute to disease progression and clinical relapse in SCCHN patients (**Figure 1**). A better understanding of the SCCHN biology, in particular the interplay between cancer cells and their surrounding TME, may allow the identification of novel biomarkers enabling patient stratification for clinical decision-making. This may also contribute to the development of innovative therapeutic avenues in order to prevent and/or overcome the current treatment failures in R/M SCCHN patients. In this review, we describe the current understanding and complexity of TME in SCCHN, and the mechanisms by which TME peculiarities contribute to intratumoral heterogeneity, therapy resistance and disease progression. In addition, we also summarize relevant (pre)clinical models that may help integrate the microenvironment-tumor cell interplay in translational research studies for SCCHN. Finally, this review explores potential therapeutic strategies that may exploit the crosstalk between TME and SCCHN cells in order to implement fundamental changes in the personalized clinical management of patients.

2. Precision medicine in head and neck cancers: clinical illusion or reality?

Precision medicine is defined as "a form of medicine that uses information about a person's own genes or proteins and environment to prevent, diagnose and treat disease" by the US National Cancer Institute (8-11). In oncology, clinicians generally aim to exploit patient-specific molecular alterations (e.g. gene mutations or amplifications) to identify treatments (the so-called targeted therapies) with the greatest probability of clinical benefit. However, despite intensive efforts in basic and translational research, SCCHN patients still poorly benefit from

precision medicine in routine clinical use since no satisfactory (genetic) biomarker has been identified for diagnosis, patient outcome prediction and selection of tailored therapeutic options (12-14). As a result, clinical decision-making for SCCHN patients is mostly based on tumor location and disease staging rather than specific tumor biology. The impact of HPV status on prognosis has been demonstrated, with better clinical outcomes in HPV-positive SCCHN (15, 16), thereby leading to a new staging system adopted in 2019 and the recommended use of p16 immunohistochemistry as a surrogate marker for HPV-induced OPSCC (17). Nevertheless, adaptation of the clinical management of SCCHN patients on the basis of HPV status has not been implemented yet.

2.1. Intratumoral genetic heterogeneity and molecular classification in SCCHN

Since the seminal work of Slaughter et al., in 1953 and the concept of field cancerization, according to which carcinogen-induced early genetic changes in the oral stratified squamous epithelium lead to the development of multifocal tumors (18), there has been compelling evidence in literature documenting a high degree of intratumoral genetic complexity and heterogeneity in SCCHN (19, 20). Several studies have provided data from next-generation sequencing (NGS)–based genetic profiling of SCCHN specimens and reported frequent chromosomal instability and somatic genomic alterations (e.g. gene amplifications in 1q, 3q, 5p and 8q regions) that differ with the anatomic site and HPV status of the tumors (21-23). Indeed, the most frequent genomic alterations in HPV-negative SCCHN are loss-of-function *TP53* mutations (84%) and *CDKN2A* inactivation (58%), while *PIK3CA* amplifications or mutations are the most common gene changes (56%) in HPV-positive OPSCC. Earlier studies had also demonstrated intratumoral heterogeneity at the molecular level in primary SCCHN samples by microsatellite marker testing as well as fluorescence *in situ* hybridization (24, 25). Interestingly, such genetic heterogeneity has been correlated with clinical outcomes in SCCHN

patients by the use of a mutant allele tumor heterogeneity (MATH) score that reflects the spatial distribution of mutant-allele fractions at tumor-specific mutated loci (26). High tumor MATH scores have been associated with poor OS as well as adverse treatment outcomes in clinically high-risk SCCHN patients (27, 28).

Genome-wide expression analyses have allowed the identification of four molecular subtypes of SCCHN that exhibit distinct gene expression patterns, including deregulation of the KEAP1/Nrf2 oxidative stress pathway (atypical), focal amplification of both *EGFR* and *CCDN1* genes (basal), differential utilization of the lineage markers SOX2 and TP63 (classical), and upregulation of epithelial-to-mesenchymal transition (EMT)-related genes (mesenchymal) (22, 29, 30) (**Figure 2**). In another study, De Cecco et al., have reported the existence of six different subtypes based on molecular signatures (immunoreactive, inflammatory, HPV-like, classical, hypoxia-associated and mesenchymal) (31). Interestingly, these different tumor subtypes have been associated with different patterns of sensitivity to oncogene-driven targeted therapy and radiotherapy (31, 32). Single-cell transcriptomic analyses have recently been carried out to reveal intratumoral heterogeneity in SCCHN, with the identification of distinct cancer cell subpopulations within the tumor bulk that may support therapy resistance and metastatic dissemination (33, 34). For example, single-cell RNA sequencing (scRNA-seq) profiles of primary tumors from 18 treatment-naive SCCHN patients, including matching lymph node metastases from five of these patients, have allowed to cluster malignant cells according to the expression of gene signatures related to cell cycle, hypoxia, stress and epithelial differentiation (35). Interestingly, a subset of SCCHN cells has been found to harbor a partial EMT (p-EMT) phenotype with the expression of classical mesenchymal markers and EMT-associated genes (e.g. vimentin, integrin alpha 5, transforming growth factor-beta (TGFβ)-induced) while maintaining the expression of epithelial markers such as cytokeratin-encoding genes and lacking most of the classical EMT-inducing transcription factors (e.g. ZEB1/2,

TWIST1/2, and SNAIL1). Such p-EMT gene program has been observed in cancer cells spatially localized to the leading edge of primary SCCHN in close proximity to cancer-associated fibroblasts (CAFs), thereby supporting a regulatory cross-talk between these two cell populations (36) (**Figure 2**). Although re-analysis of transcriptomic and clinical data from SCCHN specimens in The Cancer Genome Atlas (TCGA) has enabled to define p-EMT as a marker of worse clinical prognosis (i.e. association with the presence of nodal metastases, lymphovascular invasion and higher tumor grade), the clinical implication of intratumoral genetic heterogeneity is still largely unknown and requires further investigation.

2.2. Genomic testing and the quest for biomarker-driven therapies in SCCHN

Several clinical trials have been launched with the aim to match patients with advanced cancers, including SCCHN, with targeted therapies according to data from genomic testing (37-40). Although oncogenic mutations have been identified in 30%-50% of cancer patients, only few of them (about 5-15%) have received treatment that was selected based on genomic analysis. Interestingly, a study reporting the implementation of NGS-based genetic tumor profiling in the routine clinical care of 213 SCCHN patients has correlated genomic alterations with clinical outcomes (41). The authors have identified that *PIK3CA* amplification and *RAS* mutations are associated with dismal prognosis and even more strikingly, they have reported that among eight patients with advanced, treatment-refractory SCCHN, enrolled on clinical trials with a compound that matched tumor profiling results, four of them achieved a partial response. Another group performed NGS on 53 patients with SCCHN and included 13 patients in molecular guided therapies. Four patients with alterations in *PIK3CA* or *PTEN* were treated with PI3K inhibitors, one patient with a *MAPK1* mutation with an ERK inhibitor, one patient with a *HRAS* mutation with a farnesyltransferase inhibitor, and one patient with a *SMARCB1* deletion with an EZH2 histone methyltransferase inhibitor (42). More recently, the European

Organization for Research and Treatment of Cancer (EORTC) has launched the first international umbrella clinical trial (EORTC 1559 HNCG: UPSTREAM study; NCT03088059) in which patients with R/M SCCHN were allocated to receive standard of care, targeted therapy, or immunotherapy according to a comprehensive sequencing-based molecular tumor characterization (43). Other recent studies have shown that mutations in the *HRAS* proto-oncogene, found in 4-5% of patients with R/M SCCHN, make tumors more vulnerable for treatment with tipifarnib, a potent and highly selective inhibitor of farnesyltransferase (44, 45). A recent preclinical study has also shown that *NOTCH1* loss-of-function mutations (observed in 10-19% of SCCHN) are correlated with sensitivity to PI3K/mTOR pathway inhibitors (46). Given the high frequency of *PIK3CA* genomic alterations in SCCHN, biomarker-driven assays have also been performed with PI3K inhibitors in pre-clinical models of *PIK3CA*-mutant head and neck tumors. Increased sensitivity towards dactolisib (BEZ-235; PI3K/mTOR dual inhibitor) and alpelisib (BYL719, specific inhibitor of PI3Kα) has been observed in *PIK3CA*-mutant patient-derived SCCHN xenografts and cell lines, respectively (47, 48). Nevertheless, results from a clinical trial have been somewhat disappointing, with a limited antitumor activity of alpelisib observed in 19 human patients with *PIK3CA*-altered SCCHN (49). Importantly, a recent preclinical trial using copanlisib, a pan-class I PI3K inhibitor, as monotherapy or in combination with cetuximab, has also shown increased activity in the combination group especially in cetuximab-resistant SCCHN. However, the response rate was not related to *PIK3CA* mutation status but instead to PI3K signaling activity as assessed through gene expression profiling (50).

Altogether, these observations illustrate the complexity (and limitations) associated with genomic-based tumor profiling for the identification of clinically actionable mutations or biomarkers for patient stratification and for the development of precision medicine protocols for SCCHN patients (51). Integrating and exploiting TME conditions and associated (cancer)

cell phenotypes may help to increase our understanding of non-genetic mechanisms of SCCHN progression and identify novel predictive biomarkers or therapeutic avenues (52-54).

3. Roles of stromal and immune compartments in SCCHN progression

There is now growing evidence that cancers, including SCCHN, are dynamic ecosystems in which subclonal cancer cell populations undergo phenotypic changes in order to adapt to harsh microenvironmental conditions and therapy-induced stress (55). SCCHN cells can behave cooperatively or competitively with surrounding cells such as CAFs, endothelial cells and infiltrating immune cells to support disease progression.

3.1. Cancer-associated fibroblasts as key components of the TME in SCCHN

Cancer-associated fibroblasts (CAFs) are important players of the TME in SCCHN with diverse functions, including matrix deposition and remodeling, extensive reciprocal signaling interactions with cancer cells and functional interplay with infiltrating immune cells (56, 57). Although resident and bone marrow-derived mesenchymal stem cells have been identified as precursors of the SCCHN stroma (58), the origin of CAFs is still poorly understood, mostly due to the lack of fibroblast-specific markers (59). SCCHN are frequently associated with large, spindle-shaped stress fiber activated fibroblasts and a desmoplastic stroma (60), so that CAFs generally form the predominant non-malignant cell type in the SCCHN microenvironment. The ratio of CAFs to SCCHN cells actually increases with the disease stage (61), sometimes even reducing treatment response by increasing associated fibrosis, as observed in a clinical window-of-opportunity study with cetuximab (62, 63). High CAF density is indeed correlated with histopathological criteria of poor prognosis in SCCHN (64). CAFs have also been shown to act as the leading cells opening the path for SCCHN cells in the extracellular matrix (ECM) (65), thereby actively participating in the spread and resistance mechanisms of tumor cells.

Communication between CAFs and tumor cells is supported by signaling molecules, secretion of growth factors, interleukins and metabolite exchanges (**Figure 3**). Several proteins secreted by CAFs (but not or barely by normal fibroblasts (NFs)), including MFAP5 (66), periostin (67, 68), hepatocyte growth factor (HGF) (60, 69), carboxypeptidase E (70) and interleukin-6 (IL-6) (71), have been reported to promote growth, migration/invasion and stemness capacities in SCCHN cells *in vitro* as well as metastasis in *in vivo* preclinical models. Various microRNAs (miRNAs), including miR-7, miR-145, miR-196, miR-335 and miR 382-5p are also overexpressed in CAFs and contribute to SCCHN progression by either regulating the functional phenotype of CAFs (72, 73) or by promoting an aggressive behavior in SCCHN cells via exosome-mediated paracrine effects (74, 75). On the contrary, expression of miR-3188 and miR-34a-5p in CAF-derived exosomes is reduced, thereby leading to increased expression levels of the anti-apoptotic protein B-cell lymphoma 2 and AXL tyrosine kinase receptor, respectively and promotion of proliferation and migration capacities in SCCHN cells (76, 77). Importantly, SCCHN cells can promote CAF proliferation and migration by secreting basic fibroblast growth factor (bFGF) (78). Preclinical investigations have also shown an implication of CAFs in therapy resistance (57). As such, decreased sensitivity of SCCHN cell lines to cisplatin and anti-EGFR therapy (cetuximab) has been described in co-culture models with CAFs (79-82), thereby highlighting the role of a signaling crosstalk between CAFs and SCCHN for disease progression.

3.2. Intratumoral heterogeneity of immune microenvironment in SCCHN

SCCHN is among the most inflamed, immune-infiltrated cancers, especially with CD8+ tumor-infiltrating lymphocytes and natural killer (NK) cells (83). Even though anti-PD-1 antibodies (pembrolizumab and nivolumab) have recently been approved in the clinical management of R/M SCCHN, only a small fraction of patients (18%) benefits from immunotherapy (84). Chen

et al., have recently proposed a novel stratification of SCCHN patients based on the immune cell composition and expression of inflammatory markers (85). More precisely, they have observed that 40% of SCCHN show enriched inflammatory response, enhanced cytolytic activity, and active interferon-γ signaling and they have identified two distinct subgroups, namely "Exhausted Immune Class" and "Active Immune Class", characterized by the expression of markers of exhausted or active immune response, respectively. By using scRNA-seq in mouse and human melanoma and head and neck cancers, Davidson et al., have identified three temporally distinct stromal populations, namely "immune", "desmoplastic" and "contractile" subsets, each displaying unique functional signatures and supporting disease progression (86) (**Figure 4**). While "immune" and "desmoplastic" cells are predominant in early tumors, "contractile" cells are mostly observed at later stages. Importantly, the complement component C3 is specifically expressed in "immune" cells and its cleavage product C3a has been found to support the recruitment of C3aR-positive macrophages, thereby highlighting important immune-stromal interactions that promote immune infiltration and tumor growth. Such heterogeneity for the immune cell type composition has also been revealed by transcriptomic profiling of about 6,000 single cells from 21 SCCHN and further application of two newly developed deconvolution algorithms (CIBERSORTx and MuSiC) to bulk RNA-seq data from more than 500 SCCHN samples available in TCGA (87). Different T cell subpopulations have been identified in SCCHN, with the presence of regulatory T cells (T_{reg}) being correlated with improved survival. Tumor necrosis factor receptor superfamily member 4 has been found to be differentially expressed in the core T_{reg} subpopulation and it is correlated with greater survival, thereby suggesting that this receptor may play a key functional role to support T_{reg}-associated outcomes in SCCHN patients. In a TCGA study, gene expression profiling of 1,368 patients with squamous cell carcinomas, including SCCHN, has identified six robust immune subtypes, with different patterns in tumor genetic alterations, tumor-

infiltrating immune cell composition and function (*i.e.* promoting or suppressing immune response) and clinical outcomes (88) (**Figure 4**). Cillo et al., have also determined the gene expression profiles of >130,000 single cells from peripheral and intratumoral immune populations from patients with HPV-negative, HPV-positive SCCHN and healthy donors (89). They report that the transcriptional signature of immune cells within SCCHN differs according to the HPV infection status, as reported in earlier studies (90, 91). Indeed, while $CD8^+$ T cells and $CD4^+$ T_{reg} cells are relatively similar, helper $CD4^+$ T cells and B cells are quite divergent in either carcinogen- or virus-mediated SCCHN. Interestingly, this study shows that a gene expression signature specific for $CD4^+$ T follicular helper cells correlates with longer progression-free survival (PFS) in SCCHN patients. These analyses highlight the differences in immune lineages present in the TME of HPV-negative and -positive SCCHN and the need to implement such transcriptomic profiling for the design of immunotherapy in these patients. Still, the association of different tumor immune landscapes with (non-)response to immunotherapy is unclear and needs further investigation to be translated towards clinical use.

Recently, spatial transcriptomics/proteomics approaches have been used to enable simultaneous capture of the distribution and localization of the different components of the TME and thus to better understand its interaction in response to treatment. Indeed, by using quantitative single-cell spatial proteomics analyses in the tumor immune microenvironment (TiME) of nine matched primary and recurrent HPV-negative SCCHN, Blise et al., have demonstrated that a low mixing score in tumor regions (i.e. high spatial compartmentalization of malignant and immune cell populations) is correlated with longer PFS (92). This study also revealed distinct spatial immune landscapes, associated with clinical outcomes, at the neighborhood of mesenchymal cells expressing alpha smooth muscle actin (αSMA). Finally, they also reported that samples from the same patient are more similar to each other than samples from different patients (with same anatomic site), thereby highlighting inter-patient heterogeneity for SCCHN

and the potential prognostic value of TiME cellular heterogeneity and spatial organization in those cancers (93).

3.3. Roles of CAFs on immune cell function in SCCHN microenvironment

Compelling evidence in literature describe functional interactions between stromal and immune cells within the SCCHN microenvironment and supports the existence of immunosuppressive roles for CAFs, thereby hampering the clinical response to immunotherapy. A recent study by Kieffer et al., has aimed to address the heterogeneity and specific roles of the immunosuppressive subpopulation of CAF-S1 (i.e. fibroblast activation protein $(FAP)^{high}$, $CD29^{med\text{-}high}$, αSMA^{high}) in primary resistance to immunotherapy in cancer patients (94). By using scRNA-seq on more than 19,000 single CAF-S1 from breast cancer, the authors have identified eight different CAF-S1 clusters among which three subsets display an inflammatory ("iCAF") phenotype and five clusters correspond to the myofibroblastic ("myCAF") subpopulation previously reported in pancreatic cancers (**Figure 4**). Importantly, existence of the five most abundant CAF-S1 clusters has been validated by flow cytometry in other cancer types, including SCCHN, thereby highlighting their biological relevance. Myofibroblasts, characterized by high expression of genes encoding either ECM proteins or proteins involved in the TGF-β signaling pathway, showed to be indicative for primary resistance to immunotherapies. Abundance of these two specific CAF-S1 clusters correlated with an immunosuppressive environment. Mechanistically, CAF-S1 with high expression of genes coding ECM proteins increase the fraction of $FOXP3^{high}$ T cells and stimulate both PD-1 and cytotoxic T lymphocyte antigen 4 (CTLA-4) protein levels at the surface of $CD4^+$ $CD25^+$ T lymphocytes, which in turn promote the TGF-β signaling pathway in CAF-S1. Takahashi et al., have also investigated the impact of CAFs on tumor-associated macrophages (TAMs) in OSCC models. They have shown that $CD14^+$ monocytes overexpress TAM markers when co-cultured

with the supernatant of OSCC-derived CAFs. Furthermore, such CAF-educated monocytes exhibit increased expression levels for *ARG1*, *IL10*, and *TGFB1* genes and hamper T cell proliferation. In clinical samples of patients with OSCC, infiltration of CAFs has been associated with the presence of $CD68^+$ and $CD163^+$ macrophages and correlates with lymphovascular invasion, lymph node involvement, and tumor stage (95). CAF-induced immunosuppressive properties are also favored by dysfunction of cytotoxic T cells (96, 97) and recruitment of T_{reg} (94, 96). In SCCHN, it has been shown that αSMA^+ FAP^+ CAFs inhibit the proliferation of $CD8^+$ T cells and promote the recruitment of $CD4^+$ $CD25^+$ T cells by secreting TGF-β and IL-6 (97). Furthermore, when comparing the effects of SCCHN-derived CAFs and NFs on the functions of T cells, proliferation of the latter is more suppressed by CAFs or their supernatants than by NFs and expression levels of cytokine genes, including those for IL-6, CXCL8, TNF, TGF-β1, and VEGFA, are higher in CAFs. Altogether, these studies highlight the intricate interplay between stromal and immune cells within SCCHN TME and how it evolves in space and time along tumor progression.

3.4. Roles of tumor-associated macrophages on therapy response in SCCHN

As main components of the innate immunity, TAMs reside in the TME and have major roles in tumor growth and response to therapy, by orchestrating the adaptive immune response. They can switch between two very distinct phenotypes, classically designated as M1 and M2. The first one is regarded as pro-inflammatory and exerts anti-tumoral effects, while the latter confers anti-inflammatory tumor-promoting properties. Several studies have investigated the prognostic value of TAM markers, namely CD68 and CD163, in SCCHN patients. While infiltration of $CD68^+$ TAMs has been associated with poor prognosis and radioresistance in HPV-negative SCCHN (98, 99), other studies have shown that the M2-like marker CD163 could better predict poor prognosis in SCCHN patients (100, 101). Fu et al., have analyzed the

specific content of TAMs in 52 SCCHN clinical samples and have found a significantly higher proportion of $CD163^+$ (M2) TAMs compared to $iNOS^+$ (M1) TAMs in HPV-negative tumors, unlike HPV-positive tumors which had less $CD163^+$ cells (102). $CD163^+$ macrophages secreted more heparin binding-EGF that conferred resistance to radiation therapy by activating DNA damage response in the HPV-negative SCCHN cell line Cal27. Tomita et al., have accumulated *in vitro* evidence for an exosome-based communication between TAMs and OSCC cells responsible for decreased sensitivity to 5-FU and cisplatin (103). Another study has identified an increased proportion of the $CD163^+$ $CD206^+$ (M2) TAMs, as well as an enhanced gene expression of M2-related cytokines IL-4, IL-5 and TGF-β1, together with a decrease in $CD8^+$ T cells in lymph node biopsies of 15 patients with SCCHN after 2 weeks of radiation therapy (104). The authors have reported a rescue of immune response and radiotherapy efficacy by targeting STAT3, thereby preventing M2 macrophage activation. Furthermore, recent studies in SCCHN have shown a positive correlation between PD-1/PD-L1 expression and $CD163^+$ TAMs (97, 101), which is predictive of poor prognosis. Several groups have reported a role of TAMs in the modulation of immune response in SCCHN via the production of immune suppressive molecules, such as IL-10 and PD-L1 (105-107). Altogether, these observations identify TAMs as potential therapeutic targets to enhance treatment efficacy in SCCHN patients (99, 108).

4. TME-mediated hypoxic and metabolic pressures in SCCHN microenvironment

Along tumor progression, SCCHN cells constantly face microenvironment-driven phenotypic pressures (e.g. limited access to nutrients and/or oxygen, local accumulation of protons and the presence of tumor-associated stroma cells) forcing them to adapt and optimize their fitness to maintain their survival and growth capacities. It is tempting to speculate that local niches within the TME may influence the phenotype of clonal populations and confer survival benefit to (pre-

existing) residual cells when therapy is applied, in particular *via* the supply of SCCHN cells with specific growth factors (e.g. HGF, EGF, TGF-β) and the acquisition of stemness-related traits (e.g. slow-proliferating state, p-EMT) that will collectively allow the selection of the fittest clone(s) with tumor-initiating potential.

4.1. Hypoxia as TME selection barrier during SCCHN progression

Together with cervical and lung cancer, SCCHN is among the most hypoxic tumor types (109), with median tumor oxygen tension (pO_2) levels below 14 mmHg, as detected by Eppendorf histography (110) or polarography (111) in SCCHN patients. Of note, pO_2 levels have been detected at approximately similar levels in HPV-positive and -negative tumors of the same stage (109, 112). High levels of hypoxia in SCCHN correlate with poor prognosis and resistance to treatments, including radiation therapy (113-116) and chemotherapy (117). In the context of anti-EGFR targeted therapy, high levels of hypoxia-associated factors have been correlated with higher risk of relapse, and co-localization of EGFR and hypoxia markers has been associated with poor outcome (118, 119). However, most studies on EGFR-targeting agents support a markedly increased antitumor potency of both anti-EGFR monoclonal antibodies and tyrosine kinase inhibitors under hypoxic conditions (120-123). Together with the demonstrated antiproliferative and proapoptotic effects, the antiangiogenic activity of cetuximab and its antibody-dependent cellular cytotoxicity are now believed to contribute to its overall antitumor activity (124, 125).

Hypoxia induces the expression of hypoxia-inducible factor 1α (HIF-1α), a subunit of the transcription factor HIF, which drives expression of a range of genes encoding proteins that play pivotal roles in cell proliferation, energy metabolism, angiogenesis, survival, cell cycle arrest, pH regulation, cell migration as well as chemoresistance (126-131). There is a growing body of evidence that HIF-1α is overexpressed in the vast majority of SCCHN patients, with

reported rates varying from 30% to 75% (132-134). Several research groups have examined the correlation between HIF-1α expression and outcome of SCCHN patients and have concluded that in SCCHN, HIF-1α overexpression is directly correlated with worse tumor-node-metastasis classification, poor patient prognosis, and resistance to radiation therapy (135, 136). Moreover, it has been demonstrated that dysregulated expression of HIF-1α contributes to locally aggressive behavior, enhanced invasive potential, intensification of angiogenesis and lymph node metastasis (133, 137, 138). Overexpression of HIF-1α in SCCHN cells is related to resistance to carboplatin treatment (133). This was confirmed in a clinical study, showing that high HIF-1α expression was a predictor of poor clinical response for locally advanced SCCHN patients treated with cisplatin plus radiotherapy (139). Likewise, it has been shown that increased levels and nuclear translocation of the cellular oxygen sensor prolyl hydroxylase PHD2 (which under normoxia marks HIF-1α for degradation) are associated with less differentiated and highly proliferative SCCHN (140).

4.2. TME-mediated intratumoral metabolic heterogeneity during SCCHN progression

Tumor metabolism perfectly illustrates the influence of TME peculiarities on cancer cell phenotypes. Metabolic needs and preferences evolve along tumor progression and cancer cells exhibit high capacities of metabolic reprogramming to maintain production of biosynthetic precursors, ATP and reduced cofactors (NADH, NADPH) (141-143). Like most aggressive tumors, SCCHN exhibits a high rate of glycolysis to meet their metabolic demands (144, 145). The outcome of increased glycolysis in tumors can be visualized by ^{18}F-fluoro-2-deoxy-D-glucose (FDG) positron emission tomography (PET)/computed tomography (CT) (FDG-PET/CT) (146-148). Molecular imaging studies using FDG-PET/CT have demonstrated a high glucose uptake and an increased glycolysis in SCCHN (149, 150). Importantly, Walenta et al.,

have also shown that elevated levels of tumor-derived lactate in primary SCCHN are associated with a high risk of metastatic spread (151). As a consequence of the exacerbated glycolysis in tumor cells and the disorganized vasculature within the TME, acidosis is now considered a hallmark of the microenvironment in solid tumors, including SCCHN, with mean values of extracellular pH ranging from 6.2 to 6.8 (152). Acidosis is not only a metabolic collateral effect of uncontrolled cell proliferation but indeed, when established in the TME, acidosis can act as a driving force to shape tumor cell (metabolic) phenotypes (153). Under chronic acidic conditions, a dramatic shift from the preferential use of glucose towards glutamine and fatty acid (FA) metabolism is observed in tumor cells, including SCCHN cells (154). In particular, the unexpected concomitance of glutamine-derived FA synthesis (FAS) and FA oxidation (FAO) has been reported in acidosis-exposed cancer cells. The simultaneous activation of FAS and FAO is rendered possible by a sirtuin-mediated histone deacetylation and consecutive downregulation of acetyl-CoA carboxylase 2, making mitochondrial fatty acyl-CoA degradation compatible with cytosolic lipogenesis (155). Acidosis-driven FA metabolic dysregulation in cancer cells is associated with disease progression (156). Indeed, acidic pH promotes autocrine TGF-β2 signaling, which in turn favors the formation of lipid droplets that represent energy stores readily available to support anoikis resistance and cancer cell invasiveness *in vitro* as well as metastasis *in vivo*. We have found that n-3, but also remarkably n-6 polyunsaturated FA (PUFA), selectively induced ferroptosis in cancer cells, including SCCHN cells, under ambient acidosis (157). We have also reported that a n-3 long-chain PUFA-rich diet significantly delayed mouse tumor growth when compared with a monounsaturated FA-rich diet, an effect further accentuated by administration of diacylglycerol acyltransferase inhibitors or ferroptosis inducers (157). These data suggest dietary PUFA as a selective adjuvant antitumor modality that may efficiently complement pharmacological approaches to target acidosis-driven tumor cell phenotypes. Imaging of tumor acidosis is a

promising strategy to improve SCCHN detection, surveillance and staging. ONM-100, a nanoparticle-based fluorescent reporter of acidosis, has recently been described as a useful clinical tool for fluorescence-guided surgery to assess tumor margins in human patients with head and neck, colorectal and breast cancers (158). Importantly, ONM-100 allowed the detection of five occult lesions, including one SCCHN, otherwise missed by the standard-of-care surgery or pathological analysis (158). Similarly, two other studies have revealed the great potential of acidosis-based imaging strategies to improve cancer detection. Huang et al., have shown that occult nodules (10-20 mm^3) in orthotopic HN5 and FaDu SCCHN xenografts could be detected by using PET imaging with pH-sensitive ^{64}Cu-labelled polymers (159), while Zhao et al., have reported real-time tumor-acidosis guided detection and surgery of occult nodules (<1 mm^3) in SCCHN-bearing mice, via the use of a transistor-like pH-activatable fluorescent nanoprobe (160).

Intratumoral metabolic heterogeneity in SCCHN also involves a functional crosstalk between CAFs and tumor cells. CAF-secreted HGF has been documented to promote lactate production by SCCHN cells via upregulation of glycolysis, while SCCHN-secreted bFGF increased mitochondrial oxidative phosphorylation (OXPHOS) in CAFs (161). When considering stroma, the model describes how, within a tumor, lactate released by glycolytic cancer cells can be captured by more oxidative CAFs to be used as a fuel (161, 162). A metabolic reprogramming of NFs through caveolin-1 downregulation, elevation of monocarboxylate transporter (MCT)-4, enhanced lactate production and oxidative stress has been described in a co-culture model with OSCC cells (163). From scRNA-seq studies, OXPHOS heterogeneity in head and neck cancers has been validated. Like in other cancer types, the authors have been able to distinguish two CAF subpopulations, FAP^{high} and melanoma cell adhesion molecule $(MCAM)^{high}$, similar to CAF-S1 and CAF-S4, respectively. They reported that FAP^{high} CAFs exhibit enhanced glycolysis, as well as enriched arachidonic and linoleic acid metabolism, while $MCAM^{high}$

CAFs rely on OXPHOS and tricarboxylic acid cycle (164). As mentioned earlier, CAFs also have an impact on cancer progression by inducing fibrosis and ECM stiffness. Bertero et al., found that CAFs induce activation of mechanotransduction, relocalize Yes-associated protein 1 (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) to the nucleus, and activate the transcription of glutaminase 1, lactate dehydrogenase A, aspartate/glutamate transporter SLC1A3, along with known YAP-dependent genes such as connective tissue growth factor and cysteine-rich angiogenic protein 61, both implicated in the composition of the ECM (165). In this way, they have shown that ECM stiffening mechanoactivates glycolysis and glutamine metabolism and thus coordinates non-essential amino acid flux within the tumor niche. Specifically, CAF-derived aspartate sustains cancer cell proliferation, while cancer cell-derived glutamate balances the redox state of CAFs to promote ECM remodeling. Park et al., have also demonstrated that glycolysis responds to architectural features of the actomyosin cytoskeleton through the TRIM21-modulated degradation of phosphofructokinase, thus coupling cell metabolism to the mechanical properties of the surrounding tissue (166).

5. Preclinical SCCHN models to integrate TME in precision medicine approaches

Relevant preclinical models, which reflect the disease at the genetic, histological, and functional levels, are needed to recapitulate the intratumoral heterogeneity and explore the intimate relationship between TME, cancer cell phenotypes and response to therapy in SCCHN. About 300 SCCHN cell lines have been described in the literature, with the majority being HPV-negative (167). Many of these cell lines have been established at early passage and therefore adequately represent common gene mutations observed in clinical SCCHN specimens (168-170). Large-scale drug screens and pharmacogenomics profiling of cell line panels, such as the Cancer Cell Line Encyclopedia, have addressed compound sensitivity in SCCHN cells to identify mechanisms of growth inhibition and to guide the selection of preclinical models for

translational research (171, 172). However, for cell lines, culture conditions are usually far from those existing *in vivo* and when TME is part of the equation, all cancer cells usually undergo exposure to the same microenvironmental tumor peculiarities in 2D culture conditions (i.e. cancer cell monolayers), still making these models poorly representative of tumor heterogeneity and clinical response. For instance, SCCHN cell lines cultured in 2D or 3D conditions, with or without CAFs, have been shown to display distinct responses to anti-EGFR therapy (173), thereby highlighting the need for accurate drug screening models to better reflect the physiology of the tissue of origin and improve drug efficacy prediction for SCCHN patients. In this setting, patient-derived tumor xenograft (PDTX) and organoid (PDTO) models of SCCHN have been recently developed and used as surrogate models in preclinical studies to predict clinical response to therapy (**Figure 5**) (174).

5.1. Patient-derived tumor xenograft models of SCCHN

PDTX models of SCCHN are generated through the direct implantation of a small, minimally processed patient-derived tumor fragment into immunodeficient mice either subcutaneously or into an orthotopic site such as the tongue or the floor of the mouth. Advantages of orthoxenografts include the possibility to assess the local invasion of primary tumors and the development of patient-like distant metastases, and to study tumor-host interactions in their anatomical context, as well as the site-specific effect on therapy response (175). Several studies have reported the establishment of SCCHN PDTX models, with an engraftment success rate around 60% in most cases (**Table 1**). This rate is frequently higher for models derived from patients exhibiting perineural invasion (176) and lymph node metastases (177-179) and it correlates with worse patient outcomes. Of note, while athymic nude mice have been typically used as initial hosts for SCCHN PDTX models, more highly immunogenic mouse strains, such as NOD scid gamma (NSG) have shown higher engraftment efficiencies (180). PDTX models

retain important molecular and histological characteristics of SCCHN, as extensively reviewed elsewhere (181, 182). Besides a high concordance in gene expression levels and genetic alterations with the matched primary tumors (183), PDTX models also recapitulate the heterogeneous and complex TME including stromal and vascular architecture (177-179).

Several studies have shown that the response rates in PDTX models correlate with those observed in clinics, both for targeted agents and chemotherapeutic drugs. For instance, Kang et al., have reported that the response to the pan-HER2 inhibitor afatinib in two PDTX models mirrored the one in the donor patients (177). An additional model exhibited resistance to the pan-PI3K inhibitor buparlisib, as observed in the donor patient (177). Another study has shown that a woman with heavily pre-treated SCCHN was unresponsive to afatinib and metformin and that the matching PDTX model was also insensitive to these treatments (184). Campbell et al., have also reported a PDTX model of SCCHN, with an *EGFR* gene amplification, that exhibits disease progression under treatment with the MEK inhibitor trametinib, after an initial response, as observed in the donor patient (185). Chia et al., have described the generation of a living biobank of patient-derived primary cultures for primary, metastatic or recurrent SCCHN obtained from human patients (186). A large-scale screening of anti-cancer therapeutics in these cell cultures and matched PDTX models identified unexpected treatment strategies that could be then translated to the clinics for two different patients, thereby supporting the idea that such patient-derived models may serve for clinical decision-making in SCCHN patients.

However, the use of PDTX models often lacks the desired effect on management as they may not be available in a clinically relevant time frame (187). Apart from being more expensive, pre-clinical models agree with those in 2D, the absence of many components of the immune system in these mice, and the loss of endogenous human immune cells upon propagation of the tumor over multiple passages, limit the utility of such models to explore the role of the immune system in tumor progression and to test novel immune-based therapies. Moreover, a progressive

loss of human stroma in PDTX models is observed over passages (179, 184). Another caveat for the use of PDTX models is that intratumoral evolution can occur during engraftment and passaging, thereby potentially altering the response to treatments. Finally, the utility of such PDTX models is also severely limited by the fact that they cannot be easily used to interrogate therapeutics or genetic vulnerabilities in high-throughput screen format, thereby highlighting the need for other preclinical models of SCCHN useful for precision medicine.

5.2. Patient-derived tumor organoid models of SCCHN

Patient-derived 3D culture models of SCCHN represent relevant approaches to recapitulate the complex cell-cell interactions under the influence of TME conditions, while minimizing the cost, time and ethical issues associated with PDTX animal models. Several studies have reported the development of tumor-derived spheroids upon *in vitro* expansion of cancer stem cells from dissociated primary SCCHN in serum-free culture conditions (188-190). In the last decade, major advances in 3D culture technology have led to the development of PDTO that can be described as miniaturized avatars of a patient's tumor in culture (**Figure 5**) (191). PDTO have been established for a variety of cancers, including SCCHN (192-198) (**Table 2**) and they have been shown to recapitulate histological and genetic features, as well as drug response, of the original tumor. They have been validated as relevant *ex vivo* models for HPV-driven mucosal pathology, either through culturing of an HPV-positive tumor specimen (193) or upon HPV infection of oral mucosa organoids (195). SCCHN organoid models reveal differential responses to standard of care therapies, including chemotherapeutic agents (e.g. cisplatin, carboplatin, 5-FU and docetaxel), anti-EGFR targeted therapy (cetuximab) and radiotherapy (193, 195, 197), thereby mirroring the inter-patient differences observed in clinics. Importantly, the clinical response of seven patients treated with radiotherapy correlated with the treatment outcome in the corresponding organoids (195). Additionally, drug screens on SCCHN

organoids revealed a sensitivity to experimental therapies, such as EGFR-targeted photodynamic therapy (194), but also to targeted agents (e.g. BRAF inhibitor vemurafenib or mTOR inhibitor everolimus) already clinically approved for other cancer types. These observations may inspire the use of PDTO models as a personalized approach for the clinical management of SCCHN patients. However, further validation is required on larger prospective cohorts of patients and matched organoid models to confirm the ability of the latter to predict clinical response. In this perspective, a validation observational study (ONCODE-P2018-0003) has been launched in the Netherlands to assess the response to standard first-line therapies in about 80 SCCHN organoid models and to compare it with the clinical outcome in the corresponding patients.

One of the current limitations for the widespread use of PDTO in precision medicine is the lack of stromal and immune cells. As emphasized above, CAFs and immune cell populations are major components of SCCHN microenvironment and have a strong impact on the response to therapies and the progression of the disease. As an alternative model, the air-liquid-interface method has been shown to preserve "*en bloc*" cancer cells with tumor stroma, including CAFs and functional native immune cells (T and B cells, myeloid cells, macrophages and NK cells) (199). Direct co-cultures of tumor organoids with autologous CAFs or peripheral blood lymphocytes have also been reported (200-204) and may represent amenable and useful tools to better predict clinical response and to recapitulate functional T cell activation and tumor-killing responses to immunotherapies (**Figure 5**).

6. Implementing TME profiling for clinical decision-making in SCCHN patients

6.1. TME-related biomarkers in SCCHN

Currently, TME-related biomarkers are not used in clinical routine except one which is used to select first-line therapy with anti-PD-1 targeted agents in R/M SCCHN patients. Nivolumab was the first anti-PD-1 agent to improve OS in R/M SCCHN after first-line platinum-based therapy in the CheckMate 141 trial (7.7 months *vs* 5.1 months with chemotherapy) (5, 205). Clinical benefit was even greater in patients with a PD-L1 tumor proportion score (TPS) ≥1%. Benefits have also been documented with pembrolizumab in R/M SCCHN, as a second-line treatment in the KEYNOTE-040 trial (206) and later as a first-line therapy in the KEYNOTE-048 trial (84). Both studies showed in their sub-analysis, an increased activity depending on PD-L1 status. The KEYNOTE-048 trial was able to demonstrate superiority of pembrolizumab monotherapy to the previously used EXTREME standard of care protocol for a PD-L1 combined positive score (CPS) (i.e. PD-L1 positivity on tumor and immune cells) ≥1 (12.3 *vs* 10.4 months) and ≥20 (14.7 *vs* 11 months). The medication was approved by the European Medicines Agency (EMA) alone or in combination with platinum and 5-FU in all patients with a CPS score ≥1% while the Food and Drug Administration (FDA) approved it regardless of the PD-L1 score. However, several studies have reported an intratumoral heterogeneity of PD-L1 expression, making the utility of such a biomarker rather challenging (207, 208). Regarding efficacy of immunotherapy in SCCHN, the total mutational load, using a cut-off of ≥102 mutations per exome, was evaluated in the KEYNOTE-012 trial and demonstrated a positive correlation with immunotherapy response (209). This has also been described in a clinical study including patients with SCCHN in which higher tumor mutational burden (TMB) and CD8+ T cell infiltration predicted anti-PD-1/L1 therapy benefit among HPV-negative SCCHN. Jiang et al., investigated the TMB and immune cell infiltration in 546 SCCHN patients obtained from TCGA independently of HPV status and observed worse survival outcomes in patients with high TMB (210). They identified that macrophages, CD8+ cytotoxic and CD4+ memory T cells were the most commonly infiltrated subtypes of immune cells regardless of TMB status.

Finally, in the KEYNOTE-012 trial, an interferon-γ (IFN-γ)-related six-gene signature (including *IDO1*, *CXCL10*, *CXCL9*, *HLA-DRA*, *STAT1* and *IFNG* genes) was also investigated as potential biomarker. The study has shown that this gene signature has a high negative predictive value and may facilitate patient selection (6). However, TMB and IFN-γ gene signature are not used as biomarkers of immunotherapy response.

Hypoxia-related markers including PET tracers, perfusion-based methods such as CT and dynamic contrast-enhanced magnetic resonance imaging (MRI) (211, 212) or specific gene signatures have been investigated in different trials. Two interesting PET tracers are ^{18}F-fluoromisonidazole (FMISO) and ^{18}F-fluoroazomycin arabinoside (FAZA). FMISO-PET has validated residual tumor hypoxia in SCCHN, upon radiochemotherapy, as a major driver of therapy resistance (213). Another study has shown that repeated FMISO-PET-CT allows longitudinal assessment of SCCHN tumor characteristics, and is a prognostic tool after radiochemotherapy, distinguishing tumors leading to either earlier or later disease progression (214). Moreover, long-term follow-up from 2009 to 2011 (Danish Head and Neck Cancer Group (DAHANCA) 24 trial, NCT01017224) recently confirmed that SCCHN tumors with a high uptake of the hypoxia-specific tracer FAZA had a significantly higher risk of locoregional failure (215). In addition to the studies focusing on hypoxia imaging, lower locoregional control was also shown to be correlated with the presence of a hypoxic gene expression profile in biopsies from SCCHN patients taken prior to cisplatin-based radiochemotherapy (114). Hypoxia-induced radioresistance can be reduced in SCCHN by the use of the hypoxic modifier nimorazole, as shown in the DAHANCA 5 trial. A 15-gene hypoxia classifier has been validated as a prognostic signature in retrospective series (216). The clinical relevance of this signature is highlighted by the decision to conduct a double-blind randomized multicenter phase III study (EORTC-1219-ROG-HNCG/DAHANCA-29 trial; NCT01880359). This study is designed to prospectively evaluate if nimorazole, a radiosensitizer, can improve the effect of

accelerated concomitant chemo-radiotherapy with cisplatin on the locoregional control rate in patients with newly diagnosed HPV-negative stage III-IV carcinomas of the larynx, oropharynx or hypopharynx. Patients are stratified according to the 15-gene signature in order to determine if the treatment benefit is larger in patients who exhibit a hypoxia-related gene profile.

TME-driven metabolic preferences are also exploited for SCCHN tumor imaging. A prospective study on SCCHN patients over 15 years has shown that high lactate levels in tumor tissues, assessed by metabolic bioluminescence imaging, might serve as a predictive marker for overall and recurrence-free survival (217). By using PDTX models of SCCHN, Mignion et al., have reported that changes in ^{18}FDG-PET and diffusion-weighted MRI could predict early response to cetuximab, whereas choline spectroscopy could not (218). In a follow-up study, the authors have also shown that metabolic imaging with hyperpolarized ^{13}C-pyruvate can serve as a predictive marker of response or resistance to anti-EGFR therapy in SCCHN (219). By using nuclear magnetic resonance spectroscopy to profile metabolic changes in SCCHN cells, sensitive or resistant to anti-EGFR tyrosine kinase inhibitor, another group has observed elevated levels of glycerophosphocholine in drug-resistant SCCHN cells cultured in 2D and 3D conditions as well as in mouse xenografts (220). Longitudinal studies assessing the metabolic characteristics of SCCHN and correlating them with patient outcome are now needed to evaluate the utility of such metabolic biomarkers for patient follow-up.

Liquid biopsies have been explored as potential biomarkers in SCCHN either by investigating circulating tumor cells (CTC), circulating tumor DNA (ctDNA) or exosomes. The impact of CTC as biomarkers remains debated and has to be further elucidated. Only a few studies on generally small cohorts and different methodologies have been published with variable results. Nevertheless, changes in CTC levels have been reported to be highly correlative to treatment response (221) and progression of disease (222) in SCCHN patients. Besides this, PD-L1, PD-L2 and CD47 expression levels have been assessed in CTC derived from patients with R/M

SCCHN. Even though PD-L1 expression in the tumor tissue did not correspond completely with that in the CTC, this approach looks promising and could be integrated in future clinical applications (223). The concept of ctDNA has been investigated in head and neck cancer studies as diagnostic and prognostic tool. High levels of circulating-free DNA (cfDNA) have been detected in 200 patients with SCCHN especially in patients with oropharynx carcinoma, stage IV disease and advanced nodal disease (224). Furthermore, HPV-related oropharyngeal cancers are characterized by cfDNA of HPV (225, 226) in plasma and saliva which is useful for screening of disease and early recurrence or response to therapy in combination with conventional imaging-based examinations (227). Finally, exosomes are extracellular vesicles, released by cells and implicated in intercellular communication through the transport of proteins, miRNA, mRNA, and DNA. In the context of immunotherapy, levels of PD-L1 carried by exosomes have been measured and have shown a correlation with tumor stage and lymph node status in a study including 40 patients with SCCHN (228). Exosomes have also been explored in 18 patients with SCCHN enrolled in a phase I trial with cetuximab, ipilumumab and radiation therapy to determine whether tumor-derived and/or T-cell derived exosomes can predict outcome. The authors showed that tumor-derived exosomes and T cell-derived circulating exosomes, instead of immune cells, were useful to monitor patient response to anticancer therapy. This opens the way for including exosomes as a non-invasive tumor and immune cell biomarker in SCCHN (229).

6.2. TME-targeting therapeutic strategies in SCCHN

As our understanding of the TME increased over the last decades, significant efforts have been made to identify, develop and test novel agents which interfere with the TME. In the next sections, we will discuss four different therapeutic strategies that primarily target the TME (**Figure 6**).

Targeting the hypoxic TME in SCCHN

Over the past decades, a number of hypoxia-modifying strategies have been examined, with little to moderate success, including the use of hyperbaric oxygen therapy or carbogen breathing (i.e. 95% oxygen plus 5% carbon dioxide), which increases blood oxygen levels. Janssens et al., assessed the effects of accelerated radiotherapy with carbogen and nicotinamide (ARCON), which increases the vascular perfusion in a large, randomized phase III trial of ARCON versus accelerated radiotherapy alone in 345 patients with locally advanced SCC of the larynx. However, the 5-year local control rates were identical in both treatment arms (79% vs. 78%), whereas locoregional control was in favor of ARCON (230). As the ARCON regimen is technically complicated to deliver and proper patient selection is difficult, based on the inconclusive results from the phase III trial, this strategy is not yet in routine clinical use.

In addition, two hypoxic cell radiosensitizers, misonidazole and nimorazole, have been reported. These compounds mimic the effects of oxygen due to their electron affinity, resulting in increased DNA damage and restoration of radiosensitivity. Whereas the clinical use of misonidazole was limited because it caused significant peripheral neuropathy in 26% of the SCCHN patients (231), nimorazole in combination with accelerated fractionation radiotherapy and concomitant chemoradiotherapy with weekly cisplatin has become standard for most SCCHN patients in Denmark (232). Currently, further studies of the DAHANCA assess the value of using hypoxia imaging for optimal patient selection (233). Moreover, two ongoing phase III trials (NCT01950689 and NCT01880359) are evaluating hypoxic gene expression scores as classifiers for nimorazole radiosensitization of SCCHN; the results of which might have an important impact on the future of the hypoxic cell radiosensitizers approach.

Another promising strategy to exploit tumor hypoxia is the use of hypoxia-activated cytotoxic prodrugs, such as tirapazamine. However, despite compelling results both *in vitro* and *in vivo* (234), a clinical study including patients with advanced SCCHN could not provide evidence that the addition of tirapazamine to chemoradiotherapy improves OS (235). Therefore, interest is growing in developing novel hypoxia-specific cytotoxins with more potent antitumor activity and one of the most recent and revolutionary strategies encompasses nanoparticles containing these hypoxia-activated prodrugs (236).

Finally, targeting HIF activity could prove useful for the disruption and reversal of adverse hypoxia-induced effects (237). For example, BAY 87-2243, a novel small molecule inhibitor of mitochondrial complex I and hypoxia-induced HIF-1 activity, has been shown to reduce tumor hypoxia in head and neck cancer xenografts (238). When administered prior to radiotherapy, this compound improves local tumor control due to radiosensitization, thus opening the door for new upcoming clinical trials in head and neck cancer patients. Importantly, the need for predictive biomarkers (especially those assessing pretreatment hypoxia) to guide clinical development of hypoxia-targeting agents is paramount in order to maximally explore elimination and/or exploitation of tumor hypoxia (239).

Targeting the tumor immune microenvironment in SCCHN

As mentioned above, immunotherapy shows selective effects on R/M SCCHN patients. Indeed, in the curative setting, the addition of immunotherapy to standard therapies has yet to improve outcomes for patients with locally advanced SCCHN (240). A phase Ib study evaluating the safety and efficacy of pembrolizumab addition to cisplatin-based chemoradiotherapy, showed encouraging OS and PFS outcomes (241). Nevertheless, these promising efficacy results have not been confirmed by the more recently reported phase III trial, JAVELIN Head and Neck

100, examining the PD-L1-blocking monoclonal antibody avelumab plus chemoradiation in patients with locally advanced SCCHN (242); this trial was closed early after it was found that PFS would not be improved in the experimental avelumab arm. In this light, optimal dose sequencing and timing of drug combinations is of utmost importance to affect the magnitude and duration of immune-mediated antitumor activity. Several studies aiming to test immunotherapy in combination with other treatment strategies for different stages of the disease are still under investigation (243). To gain insights in the biological activity of PD-1 inhibitors and to identify predictive biomarkers, several neoadjuvant studies administrating the drug before curative surgery have been performed and translational research is ongoing (244-247). Furthermore, other immune checkpoint molecules are being explored, including CTLA-4, T-cell immunoglobulin mucin protein 3 (TIM-3), lymphocyte activation gene 3 (LAG-3), T cell immunoglobin and immunoreceptor tyrosine based inhibitory motif (TIGIT), glucocorticoid-induced tumor necrosis factor receptor (GITR) and V-domain Ig suppressor of T cell activation (VISTA) (248).

Apart from the huge efforts to activate T cells, it is interesting to note that in comparison to other immune-infiltrated tumor types, both HPV-positive and HPV-negative SCCHN are marked by the highest levels of NK cell infiltration. Moreover, increased infiltration of NK cells, more specifically $CD56^{dim}$ NK cells, is associated with improved disease-free survival and OS, independently of HPV status (83, 249). We and others recently reviewed NK cell chartering immunotherapeutic strategies in SCCHN (250, 251), including: (i) targeting of negative immune checkpoint molecules (such as the PD-1/PD-L1 axis, the TIGIT pathway, the C-Type Lectin NK Cell Group 2 (NKG2) subfamily pathway and the killer-cell immunoglobulin-like receptor (KIR) pathway) in order to prevent immune escape; (ii) targeting of immune agonists (such as CD137 and toll-like receptors) in order to allow positive immune checkpoint therapy; (iii) cytokine-based immune potentiation using e.g. IL-2, IL-12, IL-15 or

IL-21; and (iv) combinations with immunomodulatory drugs such as PARP inhibitors and thalidomide derivatives. In addition, several clinical trials are underway investigating adoptive NK cell transfer for the treatment of solid tumors, including SCCHN (NCT03319459). A recent and promising approach for adoptive NK cell therapy is the genetic engineering of NK cells using transduction of chimeric antigen receptors (CAR-NK), which has shown promise in lymphoid tumors (252), and which is now being investigated in solid tumors, including SCCHN (NCT03415100).

Targeting CAFs in SCCHN

As previously discussed, CAFs are key components of the TME in SCCHN that secrete various growth factors and other proteins, which promote tumor cell proliferation and stemness as well as metastasis. Therefore, therapies targeting these CAFs are promising, although it remains challenging to identify CAFs, and CAF-directed therapies for that matter, due to the lack of CAF-specific markers (253). Different approaches to target CAFs directly or indirectly have been studied preclinically, including inhibition of CAF differentiation, activation and interruption of the signaling crosstalk between CAFs and tumor cells.

Regarding the inhibition of CAF differentiation and activation, Hanley et al., demonstrated that NOX4 regulates the differentiation of fibroblasts to myCAFs in SCCHN and multiple other tumor types. Remarkably, pharmacological inhibition of NOX4 in CAFs with GKT137831 suppressed the tumor-promoting functions of myCAFs and reversed the CAF phenotype into a more fibroblast-like phenotype (254). Co-cultures of fibroblasts and SCCHN cells, which were treated with the fibroblast growth factor receptor (FGFR) inhibitor D173074 demonstrated reduced cell proliferation compared to untreated co-cultures. In addition, FGFR inhibition in SCCHN xenografts resulted in significantly reduced tumor growth and stromal compartment

compared to untreated controls (255). Moreover, it has been shown that c-Met is activated by CAF-secreted HGF and the treatment of the c-Met inhibitor PF-02341066 combined with the FGFR inhibitor AZD-4547, reduced CAF-induced SCCHN tumor proliferation both *in vitro* and *in vivo* (256). Inhibition of CAF activation by targeting FAP with FAP5-DM1 also reduced tumor growth and induced complete regression in xenografts from different tumor types, including SCCHN (257). Interestingly, activated CAFs are also known to be involved in resistance to anti-EGFR therapies. For example, Yegodayev et al., showed that the TGF-β pathway was upregulated in CAFs originating from cetuximab-resistant PDTX of SCCHN, whereas the opposite applied for CAFs from cetuximab-sensitive xenografts. Inhibition of these TGF-β-activated CAFs by the SMAD3 inhibitor SIS3, sensitized resistant xenografts to treatment with cetuximab (258).

Another approach to target the TME is to interrupt the crosstalk between tumor cells and CAFs. In this regard, IL-6 secretion by CAFs was identified as an important mediator of the crosstalk, and blocking the IL-6 receptor with tocilizumab resulted in tumor growth inhibition and modified gene expression levels of STAT3 and ERK1/2 in a SCCHN patient-derived xenograft model (259). Similarly, tocilizumab in combination with cisplatin efficiently reduced tumor growth in mice injected with SCCHN cell lines that were co-implanted with human endothelial cells (260). As it has been shown in co-culture experiments that the expression of MCTs, which are responsible for the transport of lactate, is upregulated in both SCCHN cells and CAFs, and MCTs are important players in the signaling crosstalk between CAFs and tumor cells, targeting MCTs might also be a promising therapeutic strategy for SCCHN treatment (162). In this context, inhibition of MCT1 with AZD3965 led to a small reduction in tumor volume in SCCHN xenografts, but combined treatment with AZD3965 and simvastatin significantly reduced tumor growth compared to either treatment alone (261). Alternatively, combination treatment of matrix metalloproteinase activity with cetuximab has been reported to reduce CAF-

mediated protective effects in a preclinical model of SCCHN (81). Finally, Kumar et al., demonstrated that treatment with the HGF-neutralizing antibody ficlatuzumab significantly diminished SCCHN cell proliferation by neutralizing CAF-secreted HGF (262), hereby also interfering, at least in part, with the crosstalk between CAFs and SCCHN cells.

Although an increasing interest has grown towards the role of CAFs in SCCHN and multiple preclinical studies have focused on CAFs as a therapeutic target, multiple questions remain to be answered before these studies can be translated to the clinical setting (263). However, we believe that there is a future for CAF-targeted therapies, once our understanding of the different CAF phenotypes further develops.

Targeting the abnormal ECM network of the TME in SCCHN

The ECM consists of various macromolecules, such as collagens, fibronectins, glycoproteins, laminins and glycoproteins, and forms a highly structured 3D network that provides support to surrounding cells. The ECM is also involved in cell-to-cell communication. It can influence a wide range of cell signaling pathways via binding to receptors (e.g. integrins) or via growth factors that are immobilized in the ECM (264, 265). Although tumor cells also synthesize products for the ECM, CAFs are the main source of ECM components (266). Even small changes in the composition of the ECM can have a marked impact on cellular processes, potentially leading to sustained cell growth, therapy resistance, angiogenesis, and metastasis (264, 265). Therefore, targeting the ECM to improve the therapeutic outcome of patients might be interesting and has gained more attention. Unfortunately, like CAF targeting, it also remains challenging to target the ECM. For example, the clinical evaluation of an anti-microtubule agent coupled to an antibody against the cell surface adhesion receptor CD44, which is involved in cell-matrix interactions by hyaluronan, was terminated due to a lack of tumor selectivity, which

led to severe adverse events (267). Nevertheless, efforts are still being made to efficiently target an abnormal ECM. It has recently been shown that knockdown of lysyl hydroxylase 2, which is involved in the formation of stable cross-links between collagen in the ECM, resulted in a significantly decreased invasiveness in preclinical models of SCCHN (268).

However, the majority of studies in SCCHN that aimed to alter the tumor ECM have focused on disrupting the interactions between the ECM and tumor cells (e.g. using laminin and integrin inhibitors) and downstream signaling [e.g. focal adhesion kinase (FAK) inhibitors], rather than targeting the ECM components themselves. Preclinical results for integrin inhibitors are promising, as illustrated by the study of Eke et al., who showed that targeting β1 integrins with different antibodies increased the sensitivity to radiotherapy of 8 SCCHN cell lines grown in a 3D laminin-rich ECM–based cell culture model. In addition, β1 integrin targeting resulted in significant growth inhibition of SCCHN cells *in vivo* (269). As α integrin subunits interact with β1 integrin, targeting α integrins might give similar results. Indeed, genetic knockdown of α3 integrin radiosensitized SCCHN cells and reduced cell survival in the same 3D culture model as mentioned above. Interestingly, dual α3 and β1 integrin targeted therapy showed to be superior compared to α3 integrin inhibition alone (270). Other combination strategies with β1 integrin have also been studied, including the combination of a β1 integrin inhibitor, cetuximab and irradiation, which resulted in increased cytotoxicity and radiosensitivity compared to either treatment alone (271).

Regarding the downstream signaling of ECM-cell interactions, multiple studies demonstrated that inhibition of FAK, with or without co-targeting of EGFR, is also a promising therapeutic strategy in SCCHN (272-274). In addition, complete knockdown of discoidin domain receptor 1 (DDR1, a collagen-activated receptor tyrosine kinase) in SCCHN cell lines grown in the presence of collagen resulted in a significantly reduced migratory and invasive potential, suggesting a promising role of specific DDR1 inhibitors for the SCCHN treatment (275).

Lastly, microRNAs have also been studied for their usage as therapeutic agents, since various microRNAs are aberrantly expressed and known to be involved in the intercellular communication between tumor cells and the SCCHN TME (264). In this regard, Kinoshita et al., have demonstrated that the frequently downregulated tumor suppressor microRNA-29 is involved in the regulation of laminin-integrin signaling and silencing of its target genes laminin γ2 and α6 integrin results in a significant inhibition of SCCHN cell migration and invasiveness (276).

Concluding remarks and perspectives

Despite an increasing arsenal of therapies, the clinical outcome for SCCHN patients is still dismal, particularly in advanced stages and in the recurrent or metastatic setting. The lack of reliable genetic biomarkers in SCCHN has dampened the enthusiasm of the oncology community for the implementation of NGS-based precision medicine in the clinical management of R/M SCCHN patients. In line with the recent implementation of immunotherapy, integrating and exploiting TME peculiarities may help break with the current treatment paradigm in order to provide SCCHN patients with a new mode of personalized medicine. Main challenges in the identification of new reliable prognostic biomarkers and effective therapeutic agents for SCCHN reside in the high intratumoral heterogeneity and the capacity of malignant cells to develop mechanisms of resistance over time. As discussed in this review, several agents targeting hypoxia, ECM network, as well as immune and stromal cells, have been developed and are under current preclinical investigation in SCCHN. Further studies are now needed to identify combinatorial or sequential therapeutic strategies with the greatest potential to circumvent escape mechanisms or at least delay the development of resistance. Patient-derived models of SCCHN, such as xenografts and organoids, will help determine the therapeutic avenues likely to benefit the most to patients. The contribution of the oral

microbiota in SCCHN progression is also being increasingly recognized and will deserve more attention in future studies to fully recapitulate the bio-ecological niche along the upper aerodigestive tract and to address how microbiota-targeting strategies might be implemented for precision medicine in SCCHN patients. As the rate of HPV-related SCCHN is increasing and the most common oncogenic HPVs, HPV-16 and -18, are covered by FDA-approved HPV vaccines, clinicians are struggling to answer whether or not HPV-positive SCCHN could be prevented by vaccination campaigns worldwide. Finally, the identification of TME-related biomarkers for clinical decision-making in SCCHN patients will also undoubtedly benefit from the recent development of machine learning methods, including artificial intelligence-based predictive analysis, to analyze large-scale multi-omics datasets. We are thus convinced that all these new insights and novel technologies will help to better understand the SCCHN microenvironment biology and will pave the way for the implementation of new personalized modalities for disease detection, surveillance and treatment so that improving outcome and quality of life of SCCHN patients.

Declaration of Competing Interest

The authors have no conflict of interest to disclose.

Acknowledgments

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Figure legends

Figure 1: Tumor microenvironment heterogeneity during SCCHN progression. The microenvironment of SCCHN evolves during disease progression, with a higher complexity of stromal and immune cell components as well as ECM remodeling in late tumor stages. Apart from the influence of CAFs and immune cells, the development of gradients for oxygen, pH and nutrients creates metabolic pressure within the TME that contributes to the intratumoral phenotypic heterogeneity, thereby supporting invasion/metastasis, immune escape and therapy resistance. CAF, cancer-associated fibroblast; ECM, extracellular matrix; MMP, matrix metalloproteinase; PD-1, programmed cell death-1; TAM, tumor-associated macrophage.

Figure 2: TME-driven intratumoral heterogeneity of SCCHN cells. Single-cell RNA sequencing of SCCHN specimens has allowed the identification of distinct molecular subtypes of cancer cells exhibiting specific gene signatures. A partial EMT phenotype has been observed in a subset of SCCHN cells spatially localized to the leading edge of primary SCCHN in close proximity to CAFs, while an αSMA^{low}-expressing CAF subpopulation has been shown to promote cancer cell proliferation. CAF, cancer-associated fibroblast; EMT, epithelial-to-mesenchymal transition; HPV, human papillomavirus; SCCHN, squamous cell carcinoma of head and neck; scRNA-seq, single-cell RNA sequencing; SMA, smooth muscle actin.

Figure 3: Functional crosstalk between tumor cells and CAFs in the SCCHN microenvironment. The interplay between SCCHN cells and CAFs is supported by a bidirectional exchange of cytokines, signaling molecules and growth factors (**A**), by a metabolic symbiosis (**B**), and miRNA transfer (**C**) that modulate the phenotype of both cell populations and contribute to disease progression. CAF-derived ECM also induces phenotypic changes in SCCHN cells by mechanotransduction, and forms tracks that promote SCCHN cell migration

and invasion (**D**). αvβ3, alpha v beta 3 integrin; Asp, aspartate; bFGF, basic fibroblast growth factor; CAF, cancer-associated fibroblast; EGF, epidermal growth factor; EMT, epithelial-to-mesenchymal transition; ECM, extracellular matrix; FAK, focal adhesion kinase; GF, growth factor; GLS1, glutaminase 1; Glu, Glutamate; GLUT, glucose transporter; GSK3β, glycogen synthase 3 kinase beta; HGF, hepatocyte growth factor; HIF-1α, hypoxia-inducible factor 1 alpha; IL-6, interleukin 6; MCT, monocarboxylate transporter; MMP, matrix metalloproteinase; OPN, osteopontin; OXPHOS, oxidative phosphorylation; PAR-4, protease-activated receptor 4; Pyr, pyruvate; RASSF2, Ras association domain family member 2; TCA, tricarboxylic acid cycle; TGF-β, transforming growth factor beta;; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Figure 4: Intratumoral heterogeneity of stromal and immune cells in SCCHN. Single-cell RNA sequencing of SCCHN specimens has identified distinct subtypes of immune cells and CAFs based on the expression of specific markers, and exhibiting different functional features. Three temporally distinct stromal populations have been reported, with "immune" and "desmoplastic" cells being predominant in early-stage tumors while "contractile" cells are mostly observed at later stages, thereby inducing a shift from an inflammatory to immunosuppressive TME in SCCHN. αSMA, α smooth muscle actin; C3, complement 3; CAF, cancer-associated fibroblast; CSF1, colony stimulating factor 1; CXCL12, C-X-C motif chemokine ligand 12; FAP, fibroblast activation protein; iCAF, immune CAF; IFN-γ, interferon-γ; IS, immune subtype; MCAM, melanoma cell adhesion molecule; myCAF, myofibroblastic CAF; NK, natural killer; PDGFRA, platelet-derived growth factor receptor A; PDPN, podoplanin; TAM, tumor-associated macrophage; TGFβ, transforming growth factor β; TIL, tumor-infiltrating lymphocyte.

Figure 5: Patient-derived models of SCCHN for precision medicine. Xenograft and organoid models are derived from clinical specimens (surgical resection or biopsy) freshly obtained from SCCHN patients. Adequate protocols for tissue processing allow the initiation and expansion of organoid models, isolation of tumor-derived stromal and/or immune cells, and tumor engraftment in immunodeficient mice for xenograft models. If generated in a clinically relevant time frame, data from multi-omics analysis and drug screening may be correlated with clinical data in order to identify assay-guided treatments for personalized medicine of SCCHN patients. BMA, basement membrane extract; CAFs, cancer-associated fibroblasts; ctDNA, circulating tumor DNA; PBMC, peripheral blood mononuclear cells; PDTO, patient-derived tumor organoids; SCCHN, squamous cell carcinoma of head and neck.

Figure 6: Therapeutic strategies to target the TME in SCCHN. Several therapeutic agents, including monoclonal antibodies (mAb) and small-molecule inhibitors, have been tested in preclinical and clinical studies to target diverse components of the SCCHN TME. The molecules approved in clinics are indicated in red. Molecules indicated in green, blue and orange are currently being investigated in phase 1, phase 2 and phase 3 clinical trials, respectively. ATR kinase, ataxia-telangiectasia and Rad3-related protein kinase; CAF, cancer-associated fibroblast; CTLA4, cytotoxic T lymphocyte antigen 4; CXCL, C-X-C motif chemokine; CXCR, CXCL receptor; DDR2, discoidin domain-containing receptor 2; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; FAP, fibroblast activation protein; HGF, hepatocyte growth factor; Hsp90, heat shock protein 90; iCAF, immune CAF; IL-2R, interleukin 2 receptor; mTOR, mammalian target of rapamycin; myCAF, myofibroblastic CAF; NK, natural killer; PARP, poly-adenosine diphosphate-ribose polymerase; PD-1, programmed cell death 1; PD-L1, PD ligand 1; PI3K, phosphatidylinositol-3-kinases; SCCHN, squamous cell carcinoma of the head and neck; TAM, tumor-associated

macrophage TKI, tyrosine kinase inhibitor; TLR8, Toll-like receptor 8; TNF-R, tumor necrosis factor receptor; VEGFR, vascular endothelial growth factor receptor.

Figure 1

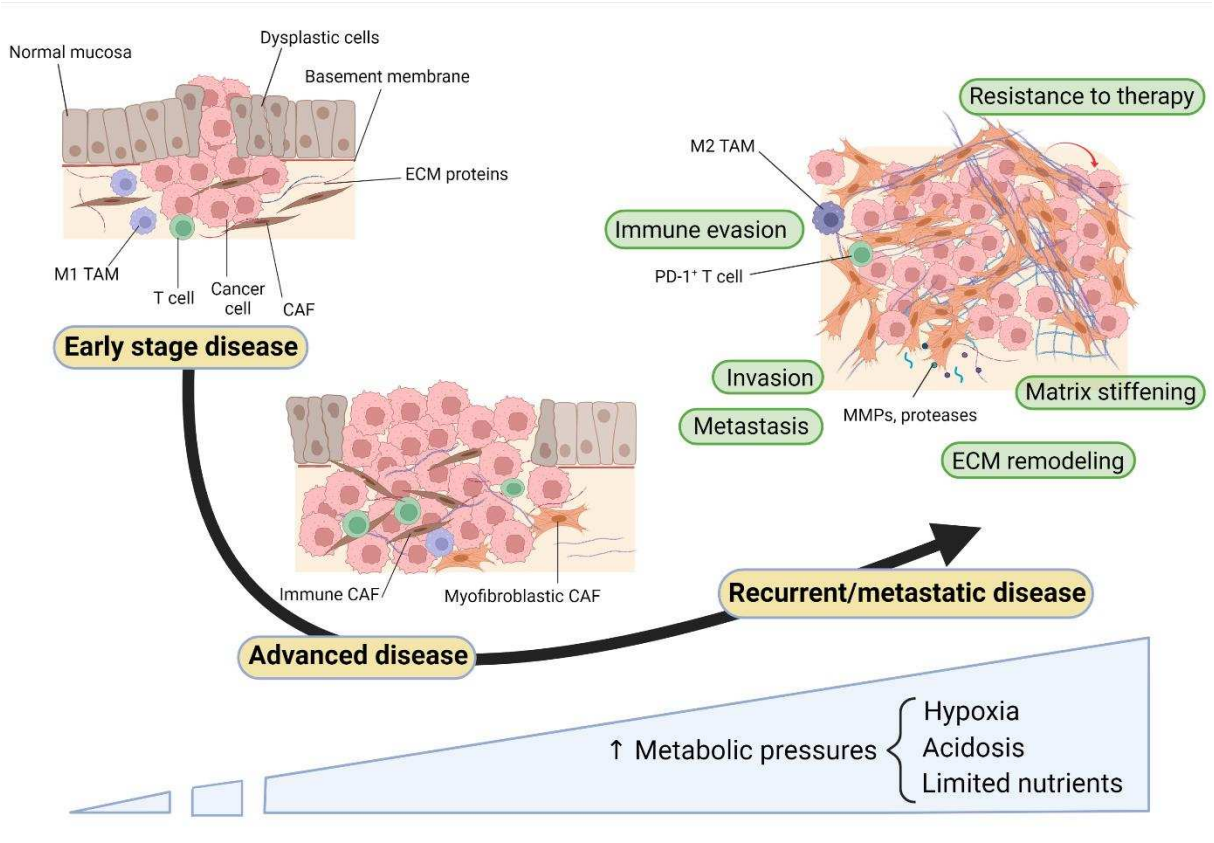

Normal mucosa
Dysplastic cells
Basement membrane
ECM proteins
M1 TAM
T cell
Cancer cell
CAF
Early stage disease
Immune CAF
Myofibroblastic CAF
Advanced disease
Recurrent/metastatic disease
Resistance to therapy
M2 TAM
Immune evasion
PD-1⁺ T cell
Invasion
Metastasis
MMPs, proteases
Matrix stiffening
ECM remodeling
↑ Metabolic pressures
Hypoxia
Acidosis
Limited nutrients

Figure 2

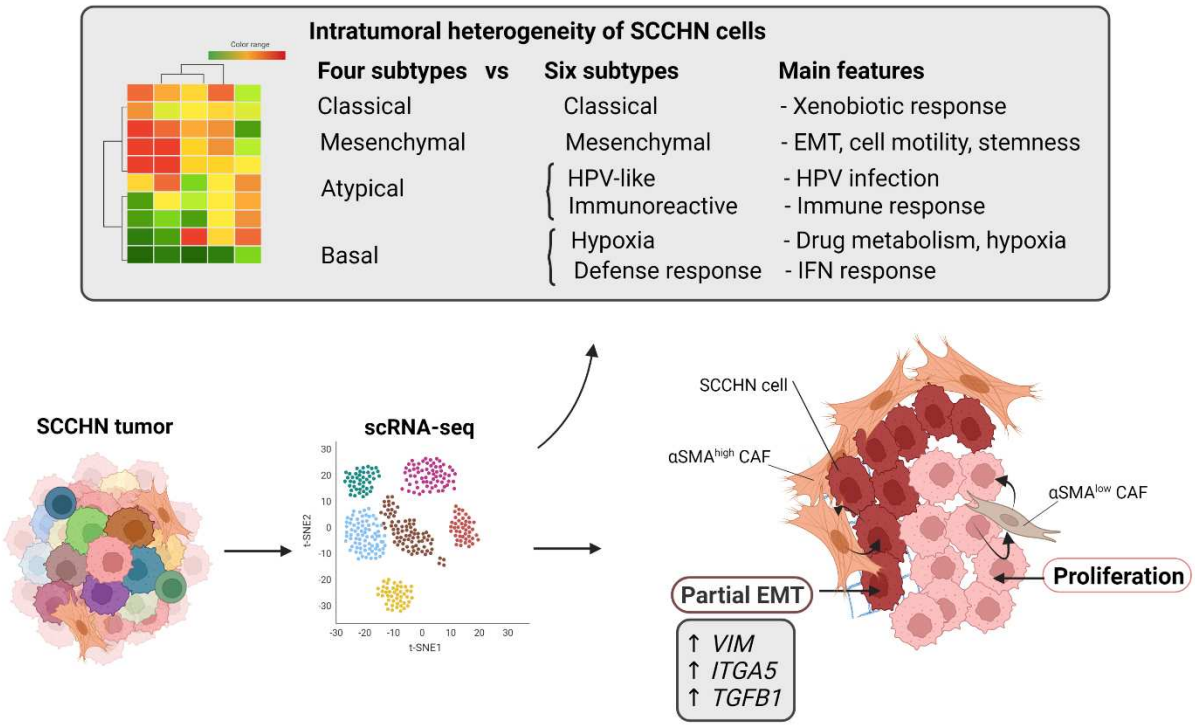

Intratumoral heterogeneity of SCCHN cells
Four subtypes vs Six subtypes
Main features
Classical
Classical
- Xenobiotic response
Mesenchymal
Mesenchymal
- EMT, cell motility, stemness
Atypical
HPV-like
- HPV infection
Immunoreactive
- Immune response
Basal
Hypoxia
- Drug metabolism, hypoxia
Defense response
- IFN response
SCCHN tumor
scRNA-seq
t-SNE1
t-SNE2
SCCHN cell
αSMA^high CAF
αSMA^low CAF
Partial EMT
Proliferation
↑ VIM
↑ ITGA5
↑ TGFB1

Figure 3

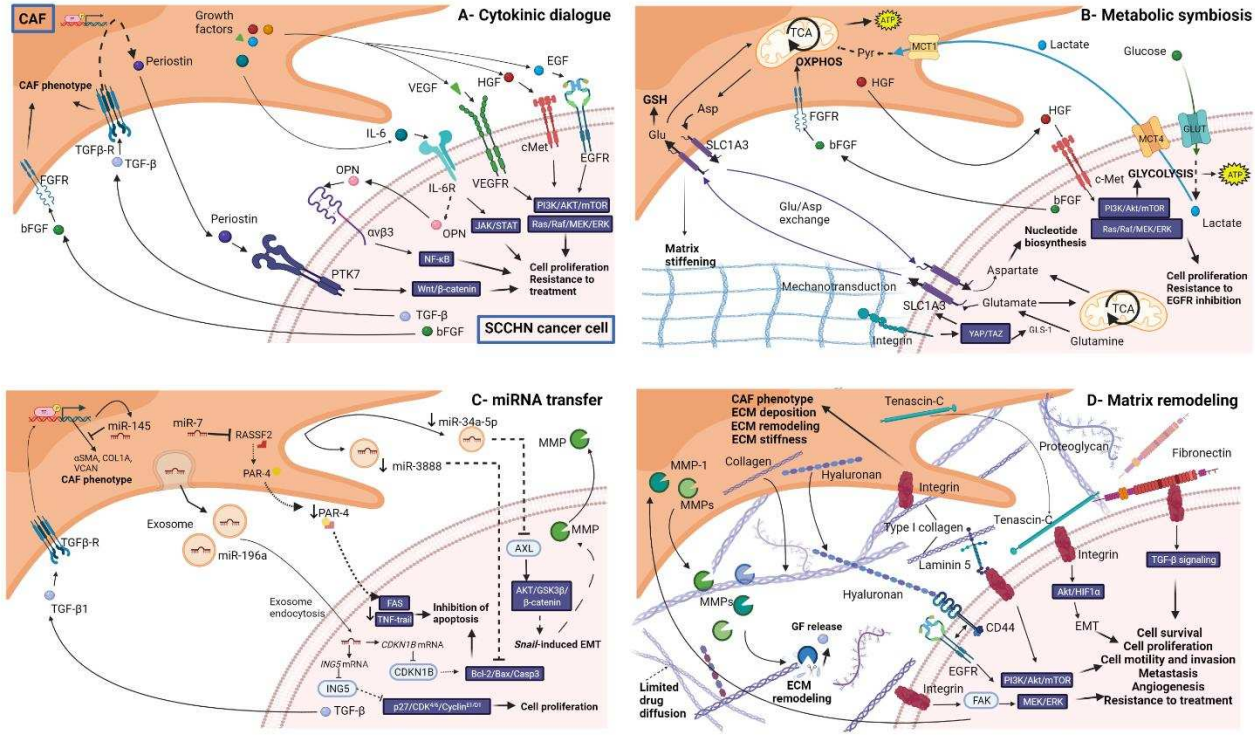

A- Cytokinic dialogue
CAF
Growth factors
Periostin
CAF phenotype
TGFβ-R
TGF-β
FGFR
bFGF
EGF
VEGF
HGF
IL-6
cMet
EGFR
OPN
IL-6R
VEGFR
αvβ3
PI3K/AKT/mTOR
JAK/STAT
Ras/Raf/MEK/ERK
NF-κB
PTK7
Wnt/β-catenin
Cell proliferation
Resistance to treatment
SCCHN cancer cell
B- Metabolic symbiosis
TCA
ATP
OXPHOS
Pyr
MCT1
Lactate
Glucose
GSH
Asp
Glu
SLC1A3
HGF
FGFR
bFGF
MCT4
GLUT
GLYCOLYSIS
c-Met
Glu/Asp exchange
Matrix stiffening
Nucleotide biosynthesis
Mechanotransduction
Aspartate
Glutamate
Glutamine
GLS-1
YAP/TAZ
Integrin
Cell proliferation
Resistance to EGFR inhibition
C- miRNA transfer
miR-145
miR-7
RASSF2
αSMA, COL1A, VCAN
CAF phenotype
PAR-4
Exosome
miR-196a
TGFβ-R
TGF-β1
miR-34a-5p
miR-3888
MMP
AXL
AKT/GSK3β/β-catenin
Snail-induced EMT
Exosome endocytosis
FAS
TNF-trail
Inhibition of apoptosis
CDKN1B mRNA
ING5 mRNA
CDKN1B
Bcl-2/Bax/Casp3
ING5
p27/CDK4/6/Cyclin D/D1
Cell proliferation
D- Matrix remodeling
CAF phenotype
ECM deposition
ECM remodeling
ECM stiffness
Tenascin-C
Proteoglycan
Fibronectin
MMP-1
Collagen
Hyaluronan
Integrin
MMPs
Type I collagen
Laminin 5
TGF-β signaling
Akt/HIF1α
EMT
CD44
GF release
ECM remodeling
Limited drug diffusion
EGFR
FAK
PI3K/Akt/mTOR
MEK/ERK
Cell survival
Cell proliferation
Cell motility and invasion
Metastasis
Angiogenesis
Resistance to treatment

Figure 4

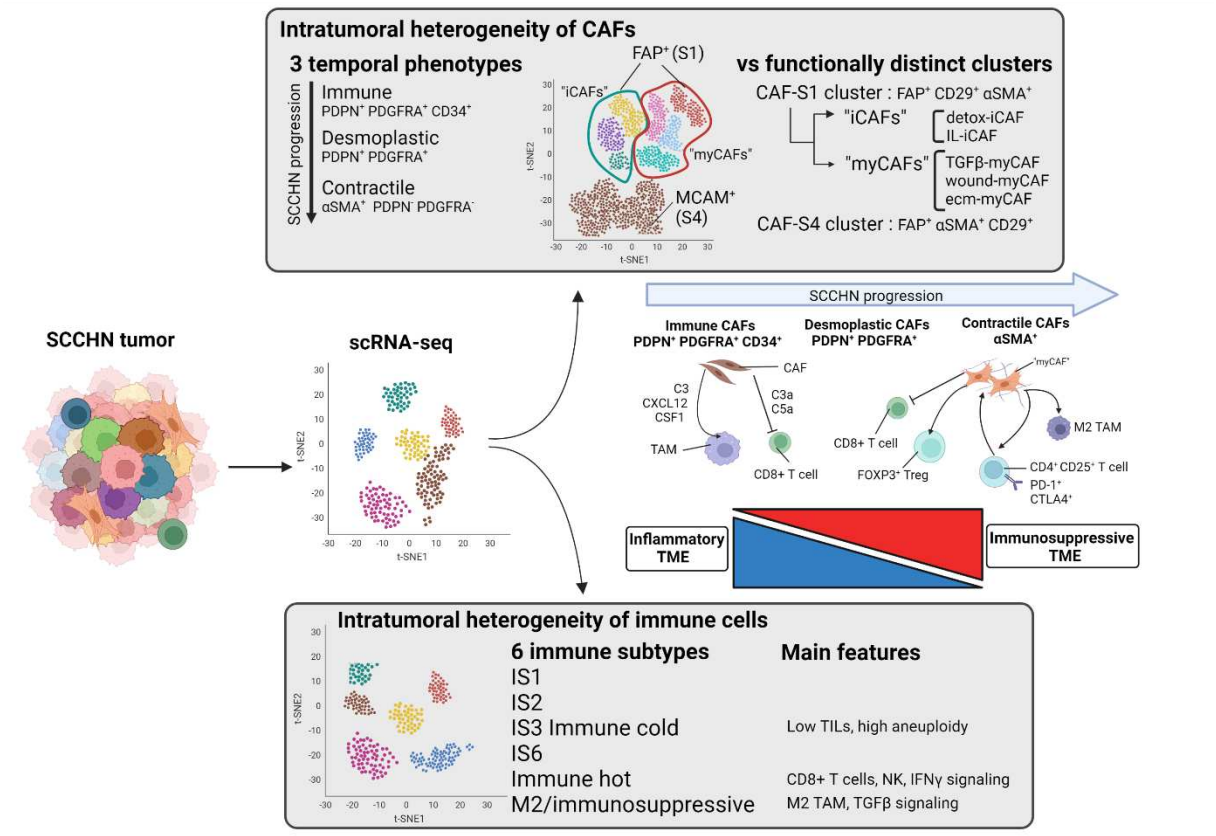

Intratumoral heterogeneity of CAFs
3 temporal phenotypes
SCCHN progression
Immune
PDPN+ PDGFRA+ CD34+
Desmoplastic
PDPN+ PDGFRA+
Contractile
αSMA+ PDPN- PDGFRA-
FAP+ (S1)
"iCAFs"
"myCAFs"
MCAM+ (S4)
t-SNE1
t-SNE2
vs functionally distinct clusters
CAF-S1 cluster : FAP+ CD29+ αSMA+
"iCAFs"
detox-iCAF
IL-iCAF
"myCAFs"
TGFβ-myCAF
wound-myCAF
ecm-myCAF
CAF-S4 cluster : FAP+ αSMA+ CD29+
SCCHN tumor
scRNA-seq
SCCHN progression
Immune CAFs
PDPN+ PDGFRA+ CD34+
Desmoplastic CAFs
PDPN+ PDGFRA+
Contractile CAFs
αSMA+
CAF
C3
CXCL12
CSF1
C3a
C5a
TAM
CD8+ T cell
"myCAF"
M2 TAM
CD8+ T cell
FOXP3+ Treg
CD4+ CD25+ T cell
PD-1+
CTLA4+
Inflammatory TME
Immunosuppressive TME
Intratumoral heterogeneity of immune cells
6 immune subtypes
Main features
IS1
IS2
IS3 Immune cold
Low TILs, high aneuploidy
IS6
Immune hot
CD8+ T cells, NK, IFNγ signaling
M2/immunosuppressive
M2 TAM, TGFβ signaling

Figure 5

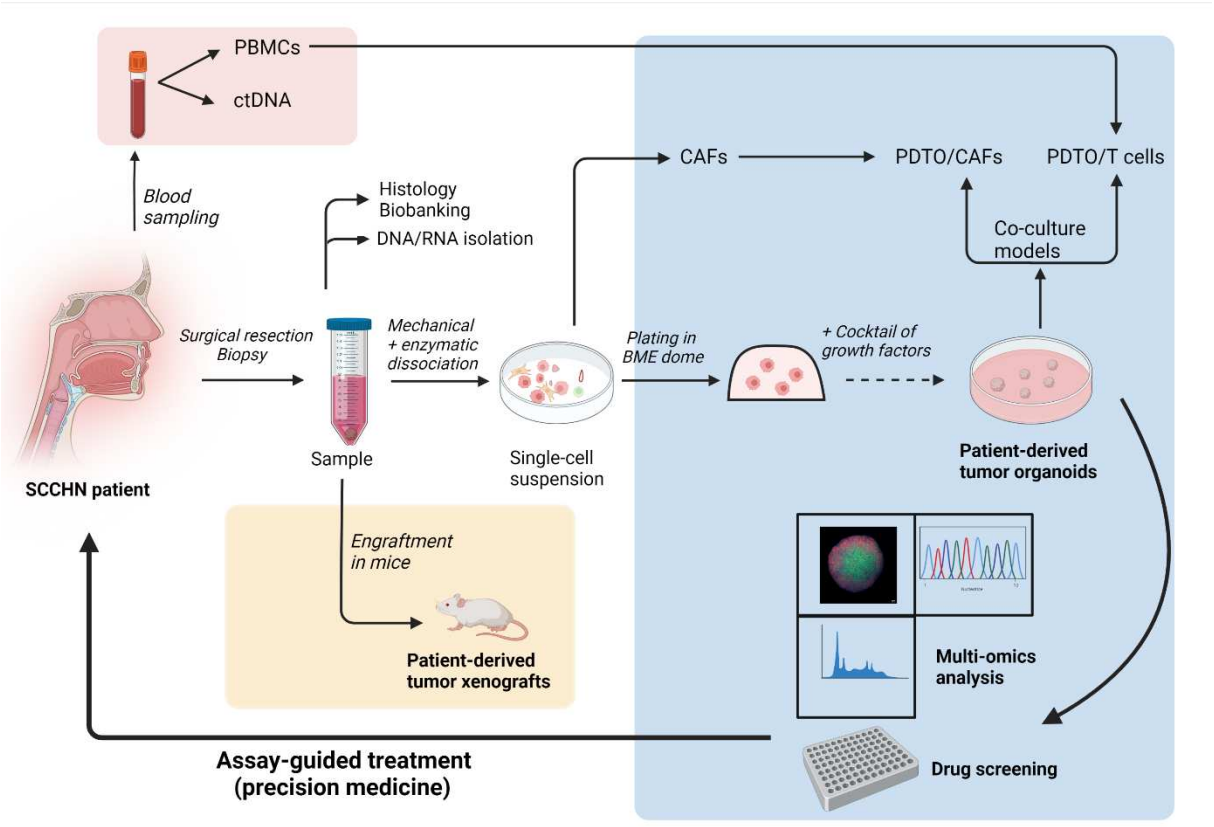
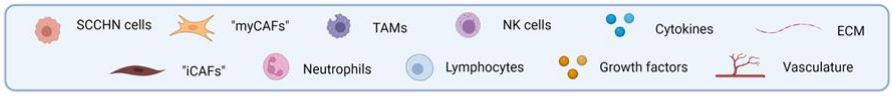

PBMCs
ctDNA
Blood sampling
Histology
Biobanking
DNA/RNA isolation
CAFs
PDTO/CAFs
PDTO/T cells
Co-culture models
Surgical resection
Biopsy
Mechanical
+ enzymatic
dissociation
Plating in
BME dome
+ Cocktail of
growth factors
SCCHN patient
Sample
Single-cell
suspension
Patient-derived
tumor organoids
Engraftment
in mice
Patient-derived
tumor xenografts
Multi-omics
analysis
Drug screening
Assay-guided treatment
(precision medicine)

Figure 6



Targeted Therapies:

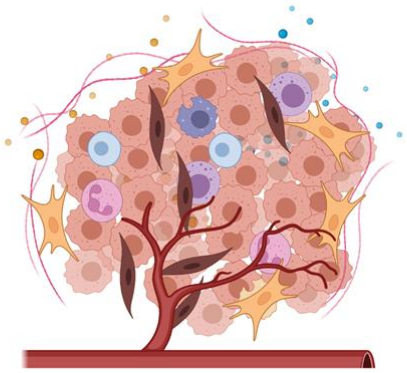
- anti-EGFR mAb: cetuximab, zalutumumab, panitumumab, nimotuzumab, dacomitinib
- anti-OX40 mAb: MEDI0562
- anti-TNF-R mAb: urelumab, utomilumab
- anti-B7-H3 mAb: enoblituzumab

- EGFR TKI: afatinib, gefitinib, lapatinib, erlotinib, poziotinib
- c-Met TKI: foretinib
- multi-target TKI: sunitinib, crizotinib, sorafenib, vandetanib

- anti-PI3K: buparlisib, sonolisib, PX-866, IRX2, alpelisib, IPI-549, copanlisib
- mTOR inhibitors: temsirolimus, everolimus, gedatolisib
- Ras inhibitor: tipifarnib
- PARP inhibitor: olaparib
- ATR kinase inhibitor: ceralasertib
- anti-IDO1: linrodostat, epacadostat
- IL-2R agonist: ALKS 4230

Immune checkpoints inhibitors:

- anti-PD1: pembrolizumab, nivolumab, MEDI4736, camrelizumab, cemiplimab
- anti-PDL1: avelumab, durvalumab, atezolizumab
- anti-CTLA4: ipilimumab, tremelimumab



: Phase 1 studies
: Phase 2 studies
: Phase 3 studies
: FDA/EMA-approved drugs

Tumor-associated macrophages:

- TLR8 agonist: motolimod
- CXCR2 inhibitor: AZD5069

Extracellular matrix:

- DDR2 inhibitor: dasatinib
- Hsp90 inhibitor: AT13387, danetespib

Cancer-associated fibroblasts:

- anti-HGF mAb: ficlatuzumab
- FAP inhibitor: RO6874281, AVA6000
- CXCR4/CXCL12 inhibitor: AMD3100, ulocuplumab

Vascularization:

- anti-VEGFR mAb: bevacizumab
- VEGFR TKI: sunitinib, nintedanib, lenvatinib

Hypoxia and metabolism:

- Mevalonate synthesis inhibitor: statins
- Hypoxia: radiosensitizer (e.g nimorazole)

Table 1: Characteristics of patient-derived tumor xenograft models of SCCHN. NI, not indicated.

HPV-negative		HPV-positive		Unknown HPV status		Anatomical site of SCCHN	Mouse strain	Reference
No of models	Success rate	No of models	Success rate	No of models	Success rate			
11	56%	9	24%			Oropharynx	NSG	(176)
50	45%	2	14%			Oral cavity	NSG	(185)
16	59%	5	45%			Larynx, pharynx, tonsil, floor of mouth, lip	Nu/Nu	(277)
10	59%	1	100%			Tongue, oropharynx, maxilla, buccal mucosa	NSG and NOD scid	(278, 279)
5	50%	7	29%			Tongue, tonsil, larynx	NSG	(280)
				5	17%	NI	Nu/Nu	(179)
19	79%	8	80%			Oral cavity hypopharynx, tongue, tonsil	NSG	(281, 282)
3		3		17	60%	Tongue, tonsil gingiva, floor of mouth, glottis	C.B-17 scid	(283, 284)
12	28%	3	16%			Tongue, oropharynx, hypopharynx, glottis and parotid gland	Nu/Nu, NOG, NSG	(177)
54	82%	4	80%	3	60%	Oropharynx, pharynx, oral cavity, larynx	NSG	(180)
161	66%					NI	NSG	(178)
				24	58%	NI	NSG	(285)
				63	43%	Oral cavity, tongue, floor of mouth	NSG	(186)
				36	68%	Oropharynx	NSG	(184)

Table 2: Characteristics of patient-derived tumor organoid models of SCCHN.

HPV-negative		HPV-positive		Unknown HPV status		Anatomical site of SCCHN	Treatments on PDTO	Reference
No of models	Success rate	No of models	Success rate	No of models	Success rate			
				4	100%	Tongue, cheek	MCT1 inhib. (CHC, siRNA)	(192)
10	29.4%	3	33.3%			Buccal mucosa, gingiva, tongue, tonsil, larynx	Cisplatin, docetaxel	(193)
				31	~60%	Floor of mouth, tongue, gingiva/alveolar process, pharynx, larynx, salivary gland, and neck	Nutlin-3, cisplatin, carboplatin, cetuximab, radiotherapy, alpelisib, vemurafenib, niraparib, everolimus, AZD4547	(195)
				3	75%	Tongue, mandible, oropharynx	Nivolumab	(199)

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