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New Insights into RAS in Head and Neck Cancer

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Abstract

RAS genes are known to be dysregulated in cancer for several decades, and substantial effort has been dedicated to develop agents that reduce RAS expression or block RAS activation. The recent introduction of RAS inhibitors for cancer patients highlights the importance of comprehending RAS alterations in head and neck cancer (HNC). In this regard, we examine the published findings on RAS alterations and pathway activations in HNC, and summarize their role in HNC initiation, progression, and metastasis. Specifically, we focus on the intrinsic role of mutated-RAS on tumor cell signaling and its extrinsic role in determining tumor-microenvironment (TME) heterogeneity, including promoting angiogenesis and enhancing immune escape. Lastly, we summarize the intrinsic and extrinsic role of RAS alterations on therapy resistance to outline the potential of

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Author Contributions

S.J, O.Z.N and M.E were involved in writing the article including designing the figures. S.J, O.C, I.K, J.H, A.J.R, J.R.G and M.E were involved in writing, and editing the article and figures.

Declaration of Competing Interests

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targeting RAS using a single agent or in combination with other therapeutic agents for HNC patients with RAS-activated tumors.

Keywords

Head and neck cancer; RAS activation; therapy resistance; progression; metastasis; pre-clinical and clinical targeting

1. Introduction

Kirsten rat sarcoma viral oncogene homolog (*KRAS*), neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*), and Harvey rat sarcoma viral oncogene homolog *(HRAS*) are the three widely expressed genes of the RAS family with high sequence similarities and overlapping functions [1]. RAS proteins, located on the inner side of the cell membrane, play a crucial role in transmitting signals to different effector molecules within the cell. This leads to the initiation of a series of interconnected pathways involving phosphorylation reactions, ultimately resulting in the activation of nuclear transcription factors [2] (Fig. 1). Mutations in *RAS* genes that lead to constitutive protein activation are commonly associated with carcinogenesis, with each cancer type displaying unique mutation profiles of the individual *RAS* genes [3].

Head and neck cancer (HNC) is a diverse disease that impacts different areas of the upper aerodigestive tract, including various histological types and anatomical locations [4]. According to National Cancer Institute, HNC is a collective name given to both squamous and non- squamous cell carcinoma of the head and neck region. Squamous cell carcinoma accounts for 90% of HNC collectively known as head and neck squamous cell carcinoma (HNSCC) that arises in the squamous cells that line the mucosal surface of the oral cavity, sinonasal cavity, pharynx, and larynx [5]. The non-squamous cell carcinomas of HNCs are rare and they arise in the salivary gland, sinus, muscles, bones, and nerves. The burden of HNC varies across countries and regions[6,7]. This variation has been correlated with exposure to tobacco-derived carcinogens, excessive alcohol consumption, or both, betel quid chewing, and infection with oncogenic viral strains, such as human papillomavirus (HPV), and Epstein Barr Virus (EBV) [6,8,9].

For many years, mutations in *RAS* (excluding *HRAS*) have been considered a relatively rare genomic alteration in HNC, so most studies have focused on more frequently occurring alterations such as *TP53* and *PIK3CA*. Therefore, *RAS* mutations were not typically considered in the diagnostic process for HNC. Accumulating genome wide studies revealed that RAS activation occurs in large cohort of HNC patients, despite these patients lack any oncogenic somatic RAS mutation[10,11]. However, in recent years, RAS-targeted therapies have gained momentum in cancer treatment, and the success of tipifarnib in *HRAS*-mutated HNSCC[12–14], and AMG510 in HNC patients[15] has prompted clinicians to reconsider RAS alterations as a potential target for therapy in a small fraction of patients with RAS hyperactivation (referred to as RAS-driven HNC). This review summarizes the four decades of accumulated evidence and provides a comprehensive understanding of RAS biology in HNC.

2. RAS alterations in head and neck cancers

Genetic alterations in *RAS* include a variety of mutational types including single nucleotide changes, duplication, and deletion. Within the single nucleotide changes in *RAS* genes, there exist point mutations at the DNA level, and single nucleotide polymorphism at the population level. Alterations in *RAS* genes, such as mutations, amplifications, and overexpression, have been observed in HNC, and a recent meta-analysis on the global prevalence of RAS in HNC published shown that the mutation spectra of *RAS* isoforms within the HNC sub-anatomical sites exhibit surprisingly distinctive codon mutation and amino acid substitution biases [16]. While the relationship between *RAS* mutations and anatomical sites in HNC requires further investigation, these data have important implications for the development of personalized treatment strategies for this disease.

2.1. RAS gene mutations

The discovery of *RAS* gene mutations in the early 1990s laid down the groundwork for understanding the role played by *RAS* oncogenes in HNC patients [17]. In the past four decades, more than 200 independent studies have reported the genetic alterations of *RAS* genes in HNC (Supplementary Table 1). Accordingly, mutations in *HRAS* appear to be more prevalent in HNC *vs*. those in *KRAS* and *NRAS* with 80% of the mutations occurring at one of three mutational hotspots, G12, G13, or Q61 (Fig. 2). However, other mutant variants have been identified at non-canonical codons including 19, 59, 117, and 146 in HNC (Fig. 2). Some non-canonical mutations have been shown to confer increased RAS activity, leading to increased tumor growth and metastasis. For example, mutations in codon 146 of *NRAS* have been associated with increased activity of the RAS signaling pathway and provide acquired resistance to cetuximab therapy [18]. The biological role of these non-canonical mutations found in HNC is yet to be studied and further research is needed to fully elucidate the effects of these mutations on HNC initiation, progression, and response to therapy.

Comprehensive analysis of *RAS* mutations in HNC has connected the mutational prevalence of a particular *RAS* isoform and the anatomical subsite (Fig. 3). Salivary and oral cavity cancers exhibit a higher prevalence of *HRAS* mutations compared to other HNCs[16]. These mutations are believed to arise due to distinct factors, including the embryonic origin of the affected tissues and various risk factors. During embryonic development, salivary gland tissue originates mostly from the ectoderm [19], while other HNCs such as squamous cell carcinoma arise from the endoderm. This difference in embryonic origin may contribute to the divergent occurrence of *HRAS* mutations between salivary glands and other HNCs.

In addition to the specific site of the tumor, other influential factors in HNCs encompass patient demographics and risk factors. Notably, certain risk factors have been associated with an increased prevalence of *HRAS* mutations in salivary gland and oral cavity cancers. For instance, studies have linked the habit of betel quid chewing to a higher frequency of *HRAS* mutations[20,21].

KRAS mutations are more likely found in sinonasal cancer [16]. The causal link between the aforementioned association remains incompletely comprehended, albeit it is hypothesized

to be correlated with the sinonasal tissue's exposure to environmental carcinogens. The respiratory epithelium lining the sinonasal cavity is persistently and pervasively exposed to inhaled environmental carcinogens, encompassing wood dust and an array of chemicals, notably polycyclic aromatic hydrocarbons, glues, formaldehyde, chromium, nickel, among others [22,23]. This exposure can lead to DNA damage and mutations, including KRAS mutations, which may contribute to the development of sinonasal cancer. In addition to environmental exposure, other factors such as genetic predisposition and viral infections may also play a role in the development of *KRAS* mutations in sinonasal cancer. In HNC, while HPV infection is most commonly linked to oropharyngeal cancers, it can also contribute to the development of cancers in other locations within the head and neck region. For example, HPV infection has been associated with non-keratinizing squamous cell carcinoma of the sinonasal cavity [23,24], and some studies have suggested that HPV infection may lead to the activation of the KRAS signaling pathway [25]. It is also observed in mice models that activation of an oncogenic *KRAS G12D* allele in the oral cavity induces oral tumor formation[26], and also exposure to carcinogens like 4-nitoquinoline 1- oxide [27] or 7,12-dimethylbenz(a)anthracene along with 12-O-tetradecanoylphorbol-13- acetate [28] could cause mutation in *KRAS* gene. Recently meta-analysis in HPV-positive HNC patients also revealed a significant association between HPV-positive status and *KRAS* mutations, with an OR of 2.09 (95% CI, 1.01–4.31) [16].

NRAS mutations arise more frequently in nasopharyngeal cancer [16]. One of the viral factors implicated in the development of nasopharyngeal cancer is EBV infection, which is found in nearly all cases of nasopharyngeal cancer [29]. EBV infection has been shown to promote the activation of the RAS signaling pathway [30], which can lead to the development of *NRAS* mutations in nasopharyngeal cancer [28]

2.2. RAS gene amplification and overexpression

In addition to oncogenic *RAS* point mutations, RAS activation in tumors results from the amplification and/or overexpression of *RAS* genes. *RAS* gene amplification can result in increased expression of RAS proteins and activation of the RAS signaling pathway, leading to tumor growth and resistance to chemo- and radiotherapy. According to the analysis of the Catalogue of Somatic Mutation in Cancer (COSMIC) database v98 on HNC cohorts, the copy number variation is 0.38%, 2.69%, and 0.38%; whereas overexpression is 3.83%. 9.77% and 1.72% for *HRAS*, *KRAS*, and *NRAS*, respectively [31]. In addition, the overexpression of *HRAS* and *KRAS* has been reported in tumor tissues from HNC patients [32–34], and the TCGA dataset provides supporting data for the overexpression of *RAS* genes in tumors compared to normal tissue.

3. Other RAS pathway activation in head and neck cancers

Besides *RAS* gene mutation and amplification, diverse mechanisms can activate RAS, such as the activation of receptor tyrosine kinases (RTKs), modifications in upstream signaling molecules like growth factor receptors or guanine nucleotide exchange factors (GEFs), epigegenetic alterations, which trigger downstream signaling events that ultimately lead to RAS activation. Additionally, deviant initiation of the downstream signaling cascade of the

RAS protein can also play a role in the activation of the mitogen-activated protein kinase (MAPK) pathway or phosphatidylinositol 3-kinase (PI3K)/AKT pathways.

3.1. Upstream activators of RAS signaling in head and neck cancers

RAS oncogenes can also be activated by increased upstream signaling. Multiple signaling pathways upstream of RAS have been identified, and the most prominent RAS pathway activators in HNC are described below (Fig 1).

3.1.1. Receptor tyrosine kinases (RTKs)—RTKs are one of the best-represented RAS activators - mediating cell-to-cell communication and controlling a wide range of complex biological functions, including cell growth, motility, differentiation, and metabolism. Hyperactivation of RTKs driven by gene amplification or mutation of the receptors and their ligands is a very common feature observed in HNCs. Among the RTKs, EGFR is one of the best-characterized activators of RAS signaling. EGFR is overexpressed and/or active in 80–90% of HNCs, and it plays a vital role in carcinogenesis and tumor progression [35]. Activation of EGFR stimulates proliferative and pro-survival intracellular signaling through aberrant activation of the RAS- RAF-MEK-ERK cascade, the phosphatidyl inositol 3-kinase (PI3K)/AKT/mTOR pathway, or the JAK/STAT pathway [36–39]. Other EGFR family members, ErbB2/HER2, ErbB3, and ErbB4, may also be overexpressed in HNC and are associated with RAS activation [40,41]. Other RTKs that are mutated in HNC include *IGF1R, MET, ROS1, ALK, DDR2, RET, AXL*, and *MERTK* [42–53], and prominent RTKs that are overexpressed in HNC include fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR) [54–62]. These activated RTKs switch on RAS signaling through GRB2 recruitment of SOS1, which activates the RAS protein through a conformational change induced by exchanging GDP for GTP [63].

3.1.2. G-protein coupled receptors (GPCRs)—Additional, widely studied RAS activators are G-protein coupled receptors (GPCRs, Fig. 1). Most GPCRs transduce signals through the activation of heterotrimeric G-proteins that are comprised of Gα and Gβ/Gγ subunits. Following activation through GTP binding, the Gα subunit dissociates from the Gβγ dimers, thus enabling the Gβγ dimers to regulate distinct signaling axes, including the PI3K/AKT [64] and RAS/MAPK pathways [65]. Many GPCR ligands, such as the GPR87 [66], PGE2 [67], bradykinin 2 [68], gastrin-releasing peptide [69], lysophosphatidic acid [70], CXCR7 [71], smoothened (Smo) [72], and their receptors [73,74] are known to be overexpressed and activated in HNC. Activation of these GPCRs induces HNC[64,75,76] growth via EGFR-dependent [77] or independent [78] pathways. In HNC, GPCRs function in a RAS-dependent manner, with the Gβ/Gγ subunit activating RAS signaling [79], either by non-RTKs [80] (including Src, Lyn, and Syk) or by RTKs [78]. In addition, mutations in the Gα, Gβ, and Gγ subunits, such as mutations in the *G-protein subunit β2* (*GNB2)*, are associated with HNC progression [81].

3.1.3. Integrin family—Evidence suggests that integrin engagement may activate RAS to regulate cell proliferation, adhesion, and migration (Fig. 1). Members of the integrin family, such as IGTB2, IGTB4, and IGTB6, play multiple roles in HNC,

including promoting tumor progression, metastasis, and radioresistance [82–85]. It is known that receptor-induced integrin-mediated cell adhesion to fibronectin triggers intracellular signaling events through c-Src and focal adhesion kinase (FAK), thereby activating multiple pathways upstream of RAS [86]. The phosphorylation of FAK through integrin-mediated mechanisms results in the RAS activation of the mitogen-activated protein kinase (MAPK) pathway, a process that has been linked to the proliferation and tumorigenic behaviour of HNC [87,88].

3.1.4. RAS-GEFs and RAS-GAPs—RAS-GEFs and RAS-GAPs play a pivotal role in regulating receptor-ligand-induced RAS activation (Fig. 1). RAS-GEFs comprises three functionally important families: RAS- GRFs, RAS-GRPs, and SOS-family GEFs, whereas RAS-GAPs comprises of six functionally important families: RASA (RAS p21 protein activator) 1/p120GAP 1, NF1, GAP1 (includes RASA2/GAP1^{m}, RASA3/GAP^{IP4BP}, RASA4/CAPRI, and RASAL1), synaptic RAS GTPase-Activating Protein (SynGAP) (has SynGAP1, DAB2IP, RASAL2, and RASAL3), IQ motif containing GTPase-Activating Protein (IQGAP) and Plexins [89]. Mutations in *RAS-GEF* and *RAS-GAP* family members have been shown to activate the RAS-MAPK pathway in multiple disease conditions [90]. Particularly the rare amplifications discovered in HNC arising from copy number variations or increased expression of RAS-GEFs are capable of hyperactivating RAS-ERK signalling. For instance, somatic mutations of SOS1, particularly base substitutions, producing amino acid changes or premature terminations in specific functional domains of SOS1 hyperactivates RAS signaling [11]. In addition to loss-of-function mutations, epigenetic regulation of RAS-GAPs have been implicated in prolonged activation of the RAS/RAF/MAPK signaling pathway in HNC [91,92]. Comprehensive analysis of the TCGA database revealed that synaptic *RAS GTPase activating protein 1 (SynGAP1/RASA5*) inactivating mutations appear to activate RAS pathway in HNC [93,94]. Furthermore, hyperactivation of wild-type RAS, but lacking *RAS* mutations, is associated with downregulation of either RAS protein activator-like (RASAL) or the RASAL inducer paired like homeodomain 1 (PITX1) in HNC[95]. Strikingly, it has been found that HNC tissues exhibit promoter hypermethylation of RASAL[96,97], indicating that silencing of GAP mRNA expression can contribute to oncogenic events that lead to increased wild-type RAS activity in HNC. These observations highlight the idea that infrequent mutations in elements of RAS signaling pathways, which may have previously been overlooked statistically, can contribute to tumor development based on their capacity to hyperactivate RAS signaling in HNC. The biological significance of most of the rare *RAS-GEFs* and *RAS-GAPs* mutations identified during genomic analysis has been studied in other cancers, and additional characterization of these factors on RAS activation status in HNC will shed more light into the biology of RAS-mediated oncogenic transformation.

3.1.5. Src homology-2 domain-containing protein tyrosine phosphatase-2 (SHP2)—SHP2 serves as an essential hub connecting several intracellular oncogenic signaling pathways, such as JAK/STAT, PI3K/AKT, RAS/RAF/MAPK[98]. In oral cancer, it has been found that SHP2 overexpression is associated with advanced tumor stage and lymph node metastasis ex vivo [99]. SHP2 binds to RTKs and activates RAS by dephosphorylating it, thus enhancing its interaction with RAF [100,101]. Gu et al.

demonstrated that SHP2 expression was associated with poor survival and promoted the growth of laryngeal cancer cells through the activation of the RAS-RAF-MEK-ERK pathway [102].

3.2. RAS downstream effector signaling in HNC

RAS effector proteins are characterized by the presence of a putative RAS binding domain (RBD) or a RAS association domain. Effector proteins are concentrated into plasma membrane signaling nanoclusters by activated RAS, where they can interact with multiple proteins and lipids to influence downstream pathways [103]. The downstream effectors of RAS in HNC that have been most widely studied are described below (Fig 1).

3.2.1. RAF—One of the first well-studied and characterized effectors of RAS is the protein serine/threonine kinase, RAF. CRAF1, BRAF, and ARAF are three RAF proteins that are closely related and can be activated by RAS-bound GTP [104,105]. Wakasaki et al. demonstrated that a c-Cbl-interacting protein of 85 kDa (designated CIN85), which is an adaptor protein that facilitates EGFR internalization, promotes transforming growth factor-alpha (TGF-α)-induced activation of RAS and phosphorylation of downstream molecules, such as c-RAF, MEK, and ERK, leading to the sustained proliferation of HNC [106].

3.2.2. Phosphatidyl inositol 3-kinases (PI3K)—Extensive research has provided comprehensive insights into the interplay between PI3K and RAS, elucidating their significance in diverse cellular processes across numerous cancer types [107]. RAS activation of PI3K signaling is essential for the formation of cutaneous tumors induced by activating mutations of *HRAS* [108]. According to the COSMIC database, 27.2% and 10.25% of HNC patients exhibit overexpression or mutational activation of *PIK3CA*, respectively [31]. Analysis of the TCGA dataset reveals a small subset of HNC patients in whom *PIK3CA* and *HRAS*-activating mutations co-exist, implying that synergistic signaling activates a single downstream target critical for HNC carcinogenesis. *HRAS* mutations may be a predictive biomarker for resistance to PI3K inhibitors [109–111]. In HNC, *RAS* mutations and *PIK3CA* amplification, but not *PIK3CA* mutations, have been associated with poorer progression-free survival [112].

3.2.3. RAS association domain family (RASSF)—The RASSF proteins are a group of RAS effectors possessing a RAS-association domain that is without any catalytic function. It has been observed that DNA methylation frequently leads to the inactivation of RASSFs, while their overexpression might trigger anti- proliferative and pro-apoptotic responses. The loss of expression of various members belonging to the RASSF family has been demonstrated to function as tumor suppressors in a variety of human tumors[113]. The best-studied RASSF family members in HNC are RASSF1A and RASSF5 (NORE1A). Hypermethylation of CpG islands in the *RASSF1A* and *RASSF5* promoter regions leading to epigenetic inactivation has been reported in HNC[113– 116]. This epigenetic inactivation triggers RAS-mediated downstream pathways essential for the proliferation and survival of tumor cells [114,117].

3.2.4. T lymphoma invasion and metastasis protein 1 (TIAM1)—The G proteins RAC and RHO cycle between GDP- and GTP-bound forms and are regulated by factors analogous to guanine nucleotide exchange factors and GAPs. RAS- GTP has been demonstrated to activate RAC and RHO proteins. Mechanistically, this happens through the interaction of activated RAS with the RBD of the TIAM1 protein, a guanine nucleotide exchange factor that facilitates the activation of RAC proteins. RAC activation leads to their binding and phosphorylation of p21-activated serine-threonine kinases, thus modulating various cellular processes needed for oncogenic transformation [118]. Studies have shown that the G proteins RAC and RHO contribute to HNC tumorigenesis [119–121]. In human HNC cell lines, it was identified that *HRAS* mutation leads to the persistent activation of RAC1 [122], controls the organization of the actin cytoskeleton and thereby regulating cell adhesion, polarity, and motility [123]. Moreover, p21-activated serine-threonine kinases are associated with aggressive tumor behaviour and poor prognosis of HNC [124–126].

3.2.5. Ral guanine nucleotide dissociation stimulator (RALGDS)—RALGDS is another well-known effector of RAS. Through RALGDS, RAS can stimulate RAS-like (RAL), resulting in the activation of phospholipase D1 and the CDC42/RAC-GAP-RAL binding protein 1 (RALBP1). Recent findings with dominant-negative *RAL* mutants in fibroblasts have been shown to impede RAS-mediated transformation in culture [127]. It has been observed that in HNC there is significant upregulation of RALA expression with higher tumor grade[128], thus linking hyperactivity of the RAS-RALGDS- RAL pathway to tumor progression. Future studies are required to explore the oncogenic transformation induced by RAS alterations in HNC through RALGDS-RAL signaling.

3.2.6. Phospholipase Cε (PLCε)—Phospholipase Cε is another effector of RAS catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol-1,4,5-triphosphate, which subsequently incites protein kinase C (PKC) activation and calcium mobilization. The activation of PLCε by RhoA-GTP, as observed by Bourguignon et al., can be obstructed by the overexpression of the PZD domain of the leukemia Rho-GEF (LARG) protein. Additionally, the authors suggest that the PZD domain of LARG may serve as a promising inhibitor of RhoA/PLCε-mediated production of inositol-3-phosphates as well as the release of Ca^{2+} from internal storage, thereby initiating signaling events for the development of HNC [129]. It has also been noted that *PLCE1* variants may acerbate the risk of HNC associated with tobacco and alcohol exposure [130].

3.3. Epigenetic activation of RAS genes

Mechanisms of epigenetic regulation in gene expression are associated with histone modification, DNA methylation, and expression of non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) [131,132]. Here we describe the epigenetic regulation of RAS genes and its activation in HNC.

3.3.1. Epigenetic regulation of *RAS* genes—Epigenetic regulation of RAS expression was drawn from the in-silico study of Johnson et al. on the let-7 miRNA family using *C. elegans* [28]. The study found that the 3’- untranslated regions (3’-UTRs) of KRAS, NRAS, and HRAS mRNAs contained multiple binding sites for the let-7a

miRNA. Modulating the expression of let-7 had an impact on the levels of RAS proteins. Notably, a specific polymorphism called let-7 complementary site 6 (LCS6) in the KRAS 3'-UTR region, identified as rs61764370, has been associated with a higher risk of tumor development and a worse prognosis in HNC [29,30]. Similarly to let-7, members of the miR-181 family were found to target all RAS family members (HRAS, KRAS, and NRAS). The down-regulation of miR-181 was identified as one of the mechanisms leading to the activation of oncogenic RAS in oral cancers [31]. Few other reports have demonstrated the modulation of RAS by miRNAs, but in many cases, the interaction was only predicted by computer algorithms and lacks experimental validation.

3.3.2. Epigenetic regulation of RAS activators and effectors—In addition to direct epigenetic regulation of RAS, there are indirect mechanisms through which RAS activation can be regulated. In HNC, Wang et al. identified the oncogenic miR-182, that downregulates RASA1 and Sprouty-related EVH1 domain containing 1 (SPRED1), which in turn, hyperactivate the RAS-MEK-ERK signaling pathway in oral cancer [133]. Other miRNAs that are found to activate the RAS pathway in HNC include miR-21 (via downregulation of RASA1) [134], miR-193b (via downregulation of the NF1) [135], and miR-214 (via downregulation of RASSF5) [136]. Downregulation of certain miRNAs in HNC were shown to be associated with receptor activation upstream of RAS signaling; for example, miR-98 downregulation enhances IGF1R-mediated RAS activation [137]. In addition to miRNAs, long non-coding RNAs (lnc-RNAs) are also known to epigenetically regulate RAS activation in HNC; for instance, maternally expressed gene 3 (MEG3) (which inhibits the expression of RASA1 by mediating the histone methylation of the promoter of the RASA1 gene by EZH2) [138] and H19 [139] activate the RAS-MAPK pathway [140–143]. The therapeutic potential of these epigenetic regulatory mechanisms of RAS targeting is another possible field that needs more exploration.

4. RAS-driven modulation of the tumor microenvironment

Tumor progression involves involves the disruption of crucial intracellular molecular processes along with abnormal signaling events triggered by the surrounding microenvironment. Various elements within the tumor microenvironment (TME) actively contribute to tumor growth, metastatic spread, colonization of secondary organs, reactivation of dormant micrometastases, and play a pivotal role in inducing resistance mechanisms, thereby influencing the efficacy of therapies [144–146]. Recent studies on RAS-induced tumorigenesis have started analyzing tumor intrinsic RAS activation effects on the components of the TME[147–149]. The outcomes of these conducted studies demonstrate that the activation of RAS in malignant cells has a far-reaching impact on the adjacent microenvironment, thereby altering the properties and functions of its constituents. RAS-mediated malignancy is thus markedly influenced by the mutual cross-talk with the surrounding TME (Fig. 4).

4.1. Extracellular matrix

Accumulating evidence links RAS activation with the composition and structure of the extracellular matrix (ECM) via the secretion of matrix metalloproteinases (MMPs)[150–

153]. It has been shown in rat and human embryonic fibroblasts that HRAS-mediated transformation and invasiveness are associated with enhanced MMP-9 expression at transcript and protein levels [154]. It has also been shown that *HRAS* gene expression through the secretion of MMP-2 exemplifies the invasiveness of MCF10A breast epithelial cells [155]. Similar upregulation of MMPs has been identified in murine models of RAS-driven HNC with an aggressive phenotype and in the human HNC *RAS* mutant cell lines HN31 and SCC9 [156,157]. This upregulation of MMPs is mediated through the activation of RAS- RAF/MEK/ERK signaling.

4.2. Cancer-associated fibroblasts

Fibroblasts in the tumor stroma can stimulate tumor initiation and progression by acting as master promoters as well as regulators of ECM remodeling dynamics and angiogenesis [158]. Preclinical evaluation using HPV-positive *HRAS* mutant murine HNC tumors revealed that cancerous cells frequently exhibit a surrounding stroma that is focally desmoplastic, indicating their capacity to foster a microenvironment that is rich in fibroblasts, a phenomenon that is commonly acknowledged to promote tumorigenesis by enabling the generation of an immunosuppressive milieu, facilitating resistance to therapy, and promoting tumor invasion[159]. *HRAS* mutant HNC cell line SCC9 exhibits an activated Hedgehog pathway [160,161], and this activation could mediates the activation of fibroblast through MAPK pathway via upregulation of the transcriptional activity of GLI1, as observed in other RAS driven cancers such as pancreatic cancer [162]. Nuclear localization of GLI1 in cancer- associated fibroblasts and activation of the Hedgehog pathway was found to be linked to oral carcinogenesis [163]. This finding is supported by the known association of GLI1 activation with distant metastases and poor outcomes in patients with HNC [164]. GLI1 binds to the interleukin 6 (IL-6) promoter in fibroblasts of the TME, thereby increasing IL-6 expression [165] and modulating STAT3. Modulation of STAT3 contributes to the development of premalignant lesions and malignant transformation [166].

4.3. Endothelial cells and angiogenesis

RAS promotion of tumor-associated angiogenesis has been extensively studied [167– 171]. Studies using *RAS* mutant models suggest that RAS regulate inflammatory cytokines that promote neovascularization specifically vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8). HNC cell line analysis of angiogenic heterogeneity showed that *HRAS* mutated lines (SCC9) exhibited higher VEGF levels than other RAS wild-type lines [172]. Another study using a xenograft model of mutant *HRAS* (UM-SCC17B) displayed high microvessel density, indicating the prominent role of mutant *RAS* in promoting tumor-associated angiogenesis [173]. Recent studies in *HRAS*-mutated HNC that include patient/cell line-derived xenografts and syngeneic murine models have provided evidence that the farnesyl transferase inhibitor tipifarnib inhibits RAS-activation-mediated angiogenesis and vasculogenesis in vivo [174,175]. Gene enrichment analysis of *HRAS* wild type vs. mutant HNC showed that multiple pro-inflammatory genes are upregulated in *HRAS* mutants [176], which are known to stimulate angiogenesis. For example, RAS-induced IL-8 secretion promotes both tumor inflammation and the recruitment and growth of endothelial cells in RAS-driven cancers [177]. Furthermore, IL-8 is a transcriptional target of the HRAS,

KRAS, MAPK, and PI3K signaling pathways [178]. In addition to IL-8 and VEGF, oncogenic RAS is also known to promote the production of the CXC chemokines [179], CXCL1 [180] and CXCL5 [181,182], resulting in enhanced invasion and angiogenesis in HNC. Combination of antiangiogenic agents with immunotherapy have demonstrated promising results [183,184] and are currently under evaluation in large randomzied studies (NCT04428151-LEAP-009; NCT04199104-LEAP-010, NCT02501096).

4.4. Immune cells

The interaction between cancer and immune cells in stromal tissue plays a crucial role in tumorigenesis. Multiple immunomodulatory mechanisms have been identified in RAS-mutated cancers (as reviewed in [185]), but RAS-mediated immune modulation in HNC remains to be explored. Among the few studies that have been done, a transcriptional study, linking HRAS mutational status in HNC with immune signatures demonstrated that neutrophils, natural killer cells, plasmacytoid dendritic cells, regulatory T cells (Tregs), $CD8^+$ tumor-infiltrating lymphocytes (TILs), and B cell gene signatures were significantly higher in HRAS-mutated tumors [176]. Recently, our group found that in murine RAS mutant HNC models express colony-stimulating factor 1 (CSF-1) that promote myeloid-derived suppressor cells (MDSCs) accumulation and subsequently suppresses $CD8^+$ T cells activation [27]. In line with our findings, Saloura et al. showed that a high $CD8^+$ T cell inflamed phenotype is enriched in tumors with HRAS mutations [186]. In a distinct model of HNC with *KRAS* mutation, Judd et al. discovered that a significant proportion of $CD4^+$ T-cells that infiltrated tumors generated from *KRAS* mutant MOC-2 tumors were immunosuppressive $FOXP3^+$ T-regulatory cells (Tregs). Moreover, antibody-mediated inhibition of these Tregs resulted in attenuated tumor growth [187]. Studies using murine *HRAS* mutant HNC cell line - MOC1, demonstrated that the reduction of polymononuclear MDSC intensified the anti-tumor T cell response of anti-CTLA-4 therapy, ultimately leading to the generation of immune memory in MOC-1 [188]. A further investigation employing MOC-2 demonstrated that $CD11b^+/Gr1^-/F4/80^+$ tumor-associated macrophage (TAM)s were absent in metastatic tumor deposits originating from MOC-2 induced tumors. This observation indicates that TAMs have a biological impact on primary disease rather than metastatic disease in this particular model [187].

5. Clinical and therapeutic relevance of RAS alterations in HNC

Multiple studies on HNC have identified *HRAS* mutation as an adverse prognostic factor for disease-free survival [112], as well as progression-free survival, and overall survival rates [20,189]. Other studies have shown that mutations in *HRAS* are associated with distant metastasis and advanced tumor grade [190]. A meta-analysis compiling the findings of 149 studies with over 8,500 HNC patients showed that mutations in *HRAS* were associated with advanced disease [16]. In accordance, it is also noted that most of the currently available *HRAS* mutant HNC cell lines are isolated from metastatic sites [namely UM-SCC9, HN31 and HSC-6 (from the lymph node metastasis) [191,192] and UM-SCC17B (from the metastatic neck tumor)[193] or from advanced stages of the primary tumor [namely ORL214 [194] and VU147T [195]]. Most of these human *HRAS* mutant lines are proved to form regional lymph node and distant metastasis in immunodeficient mice[175,196–198].

Recently, we showed that HRAS mutation drives metastasis through negative regulation of the Hippo pathway, activation of YAP1 that regulates AXL expression to enhance cell invasiveness capacity [175]. In HNC, recent reports have revealed that both *HRAS* overexpression and mutation could impart resistance to chemotherapeutics, such as cisplatin [199]. There have been many mechanisms postulated for intrinsic resistance to cisplatin through the activation of MAPK pathway (Fig. 5). Through the RAS/PI3K/RAC1 pathway, HRAS has been shown to increase DNA repair [200] and promote the up- regulation of ERCC1, an important enzyme involved in nucleotide excision repair [201], thereby providing protection against platinum-based anticancer drugs such as cisplatin. Studies using HNC cell lines showed that mutations in HRAS promote resistance to the EGFR tyrosine kinase inhibitor (TKI), erlotinib [202], and Rampias et al., have shown that *HRAS* mutations are linked to a poor response to cetuximab therapy [37]. The suggested explanation for the development of resistance to EGFR TKIs in HNSCC involves the persistent activation of signaling pathways downstream of mutated *RAS* genes, facilitated by a specific set of genes including *CCND1, c-MYC, BCL-XL, and BCL-2*[37].

KRAS amplification has long been identified as a major determinant of the proliferation of HNSCC cells and keratinocytes [203], as amplification of *KRAS* in HNSCC contributes to tumor growth. KRAS overexpression is correlated with progressive dedifferentiation in laryngeal cancer [204] implicating an aggressive phenotype. Clinically, a SNP in the 3′-untranslated region of *KRAS* (rs61764370, *KRAS*-variant: TG/GG) is a potential predictive biomarker for poor response to cisplatin in recurrent and metastatic HNC [205]. Liquid biopsy analysis of cetuximab-treated HNC patients showed that the patients developed *KRAS*- activating mutations in the course of treatment with significant disease progression [18]. Moreover, data acquired with a cetuximab-resistant HNC cell line corroborates the finding that the overexpression of RAS family members and the loss of radiosensitization in resistant cells are associated with acquired resistance to cetuximab [206]. In addition to RAS mutations and overexpression, multiple RAS downstream proteins such as dual specificity phosphatase 1 (DUSP1), activator protein 1 (AP-1) were identified to confer resistance to cetuximab therapy in HNC [207,208]. Studies using *KRAS* mutant murine lines from our group [27,209] as well as others[187,210] revealed that these lines are highly aggressive and could metastasize to lymph nodes and lungs implicating the involvement of *KRAS* mutation in deciding the tumorigenicity and metastasis of HNC. Thus, RAS-mediated metastasis and drug resistance remain considerable problems in HNC treatment.

Due to the low frequency of *NRAS* mutation in HNC, only a few studies assessed its role in the context to prognosis[16]. However, evidence from nasopharyngeal cancer showed that NRAS mutation is associated with metastasis and relapse [211].

Cancers that are driven by RAS frequently exhibit the presence of immune checkpoint molecules on their cellular surfaces [212] The aforementioned molecules exhibit the capacity to engage in binding with their corresponding receptors located on immune cells that are present within the TME, including CD4 helper T-cells, CD8 cytotoxic T-cells, and natural killer cells. This interaction results in a consequential reduction in the functional capacity of the immune cells, leading to functional exhaustion and a decreased ability to eliminate cancer cells. Furthermore, oncogenic RAS also hinders interferon signaling

and antigen presentation by suppressing MHC expression [213–215]. These features were commonly predictive of the response to immunotherapeutics, such as anti-programmed cell death protein 1 (PD-1). Murine models of HNC harboring RAS-activating mutations are found to be resistant to anti-PD-1 therapy [27,216–218]. Possible mechanisms underlying anti-PD-1 resistance by RAS-driven HNC are through the RAS-mediated upregulation of oxidative metabolism and a consequent hypoxic environment [217] or by the upregulation of secretory factors, such as CSF-1[27], that modulate the immune microenvironment to favor immunosuppression. These studies support the notion that treatment decisions should not be based solely on *RAS* mutations but also need to consider RAS pathway activation status as well as the immunosuppressive TME.

6. RAS targeting studies in head and neck cancers

The study of RAS biology in HNC over four decades has given rise to the current assumption that targeting RAS would be a potentially effective therapeutic strategy for HNC. The multiple approaches to targeting RAS – both direct and indirect (RAS activity) inhibitors – are reviewed in brief below (Fig. 6).

6.1. Direct RAS inhibitors

In recent years, the major strategy for directly targeting RAS proteins is by inhibiting the SOS-RAS interaction, thereby trapping RAS in its inactive conformation, targeting the guanine nucleotide-binding site of RAS and hindering RAS effector interaction. This strategy is facilitated by using either antisense oligonucleotides or RAS-mutant-specific inhibitors. In this section, we describe the suitability and availability of these strategies for RAS-driven HNC.

6.1.1. Antisense oligonucleotides targeting RAS—Targeting mutant RAS in cancer through the use of small molecules or oligonucleotides represents a highly promising approach for directly inhibiting its function. Using short synthetic antisense oligonucleotides specific for sequences in the mRNAs for mutated RAS proteins bind to cRNA, and inhibit mutant protein production. Protein production is halted either by promoting degradation of the mRNA by directing RNaseH to the RNA–DNA duplex or by interfering with the translation process. Small interfering RNAs (siRNAs) have considerable therapeutic potential as inhibitors of mutant-specific RAS owing to their capacity to target a vast array of potential sites. Nonetheless, their efficacy may be constrained by their brief half-life in the circulatory system and within cells. To surmount this limitation, localized administration of siRNAs directly at the tumor site can be employed, enabling the concentration of siRNA in the tumor microenvironment. Among the RAS- directed antisense oligonucleotides that have exhibited substantial efficacy, ISIS 2503, an antisense oligonucleotide specific to HRAS, has displayed promise in phase I clinical trials for advanced solid tumors [219]. As for KRAS, the antisense oligonucleotide AZD4875 has been tested in a clinical trial (NCT03101839).

6.1.2. RAS mutant-specific inhibitors—By selectively targeting specific mutations in RAS, it becomes possible to adopt a more precise approach in inhibiting the oncogenic

function of RAS while preserving the normal function of the wild-type protein. Currently, there is a dearth of FDA-approved medications that effectively inhibit RAS. Nevertheless, remarkable strides have been made in the advancement of direct KRAS drugs that primarily concentrate on the glycine-to- cysteine mutation at residue 12 (G12C) in KRAS. Divergent from KRAS G12D and KRAS G12V, the KRAS G12C mutant protein harbors the capacity to fluctuate between GDP- bound and GTP-bound states while concomitantly preserving its interaction with downstream effectors [220]. This distinction makes it possible to target the reactive cysteine residue and specifically block the protein, trapping it in the inactive state. As G12 mutations in KRAS are prevalent, RAS-G12C specific inhibitors may be active in a subset of KRAS-mutant HNC (Fig. 1). The following first-in-class RAS G12C-specific inhibitors are in phase 1/2 clinical trials for solid tumors harboring the KRAS G12C mutation: AMG 510 (NCT03600883 and NCT04185883), MRTX-849 (NCT03785249), JNJ-74699157 (NCT03114319), and LY3499446 (NCT04165031), with more in the pipeline. Only AMG 510 (NCT04185883) is currently in a clinical trial for HNC. Other KRAS G12 mutant specific inhibitors are in the pipeline and the most promising among them is the KRAS G12D specific inhibitor KS-58 [221] and MRTX1133 [222], as G12D mutation is the most prominent amino acid substitution observed in HNC (Fig. 2). Wide-ranging therapeutic implications and the need for further clinical research in patients with KRAS-activated HNCs are provided by the recent discovery of pan-KRAS inhibitors that blocked nucleotide exchange to prevent the activation of wild- type KRAS and a variety of KRAS mutants, including G12A/C/D/F/V/S, G13C/D, V14I, L19F, Q22K, D33E, Q61H, and A146A/T [223].

6.2. Indirect RAS targeting

Indirect targeting of RAS is achieved mainly through inhibition of upstream activators, inhibition of RAS association with the plasma membrane, and inhibition of effector signaling pathways. Here, we elaborate the various indirect RAS targeting strategies that has been employed both preclinically and clinically in HNC.

6.2.1. Inhibitors of RAS upstream activators—RAS signaling is activated by multiple mechanisms in HNC. Despite the infrequent occurrence of RAS mutations, it remains a prevalent characteristic in HNC, thus prompting the utilization of upstream activator blockade as a therapeutic strategy to impede downstream RAS signaling cascades. Numerous inhibitors have been formulated, targeting proteins preceding RAS, and are presently undergoing multiple stages of clinical trials (Fig. 6). The upstream RAS activator inhibitors include inhibitors of RTK, GPCR, SOS, SHP, and integrins, which are known to activate RAS pathway in HNC.

RTK inhibitors are the most extensively studied inhibitor upstream of RAS. The only Food and Drug Admistration (FDA) approved RTK targeted therapy for HNC is the EGFR inhibitor, cetuximab. Cetuximab has only modest single agent activity, but does have defined role in recurrent/metastatic setting with chemotherapy[224], and in combination with radiation for cisplatin-ineligible locoregionally advanced disease [225], however remains inferior to platinum-based chemoradiotherapy [226–228]. As cetuximab is inefficient for RAS-driven HNC, studies were subsequently directed to target other members of the EGFR

family. Other EGFR inhibitors that have undergone clinical trials in HNC are shown in Table 1. RAS-mutated HNC are largely insensitive to most of these EGFR tyrosine kinase inhibitors (EGFR TKIs). Multiple resistance mechanisms both intrinsic and extrinsic mechanisms activated by mutant *RAS* contribute to cetuximab/EGFR TKI resistance. In addition to EGFR inhibitors, there are other RTK inhibitors for MET, RET, VEGFR, FGFR, etc., that have undergone clinical trials in HNC (Table 1). Unfortunately, most of these RTK inhibitors showed limited efficacy and resistance to therapy developed due to adaptive feedback mechanisms (Fig. 5). As a result, combinatorial therapy with multiple approaches, including chemoradiation or immunotherapy (Table 1), may be required to address drug resistance and improve treatment efficacy.

Other RAS upstream inhibitors target GPCR signaling. As an increasing body of data links GPCRs to RAS activation, the pharmacological manipulation of these receptors has become increasingly attractive as a means to target RAS indirectly. Chemokine receptors (CXCR), in particular, CXCR4, which is the receptor for CXCL12 (SDF-1), are rapidly emerging therapeutic targets [229]. Ligands inhibiting CXCR4 such as AMD3100 (NCT04058145), are being evaluated for their efficacy [230]. Maussang et al. showed that nanobodies directed against the chemokine receptor CXCR7 reduce HNC growth in vivo [71]. Other GPCR inhibitors in clinical trials for HNC are GDC-0449 (NCT01835626, NCT02465060), CXCL8/IL-8 (NCT04848116), and sonidegib (NCT04007744).

As evidence suggests that both RTKs and GPCRs are overexpressed in HNC and are capable to activate RAS through the recruitment of GRB2 to SOS, SOS appears to be a particularly suitable target for RAS-driven malignancies. Many potential small molecules have been identified that can interrupt the RAS-SOS interaction and hence disrupt RAS activation and downstream signaling. Currently, the SOS1 inhibitor BI-1701963 is in a clinical trial for HNC (NCT04111458).

The SHP2 protein-tyrosine phosphatase plays a crucial role in cellular signaling by mediating the RAS pathway. It is believed to function by activating SOS1, which in turn regulates the loading of RAS-GTP. SHP2 inhibitors are being explored and are at different stages of investigation, ranging from preclinical studies to clinical trials. JAB-3068 (NCT03518554, NCT03565003, NCT04721223), TNO155 (NCT04000529), JAB-3312 (NCT04121286, NCT04045496) are in ongoing early phase of clinical trials for HNC.

Recent studies have shown that the activation of integrin signaling pathways plays crucial roles in cancer progression and resistance through the activation of multiple downstream molecules, including RAS in HNC. Current treatment strategies targeting integrin are designed to interfere with integrin-ligand interactions to block downstream RAS activation. Pre-clinical evaluation using primary cells and established HNC cell lines with the integrin inhibitor, cilengitide showed promising results [231]. The integrin inhibitors that have completed clinical trials in HNC include cilengitide (NCT00705016) and PF-06940434 (NCT04152018). In the context of a phase I/II clinical trial, denoted as ADVANTAGE (NCT00705016), that aimed to appraise the efficacy of cilengitide combined with cetuximab, cisplatin, and 5-fluorouracil (5-FU) in patients afflicted with recurrent and/or metastatic HNC, it was ascertained that the incorporation of cilengitide alongside

cetuximab and platinum- based chemotherapy was deemed well-tolerated. Further, the administration of this regimen did not elicit any dose-limiting toxicity or unexpected adverse events. Based on the phase I findings, a safe dose of cilengitide (2000 mg) was selected for the subsequent randomized phase II trial, which aimed to assess progression-free survival [232]. Despite these efforts, the phase II trial did not demonstrate improvements in median overall survival or objective response rates with the addition of cilengitide [233].

As most of these new class of upstream activator inhibitors of RAS are undergoing phase I clinical trials, and the impact of each approach remain unknown.

6.2.2 Inhibitors of RAS association with membrane—RAS proteins are functional and capable of interacting with downstream effectors exclusively when they are linked to the plasma membrane. Consequently, in the event that the post-translational modifications that are accountable for guiding RAS proteins to the membrane are obstructed, they would indeed become inactive. The pivotal function played by farnesyltransferases in facilitating the localization of RAS to the plasma membrane has resulted in their identification as potential targets during the preliminary stages of the search for compounds that have a direct effect on RAS activity. Numerous highly effective inhibitors of farnesyltransferase were discovered during screening endeavors, and two of them, namely tipifarnib (NCT03719690, NCT02383927, NCT04997902) and lonafarnib, underwent evaluation in clinical trials. Tipifarnib, in particular, showed promising therapeutic potential in the treatment of HRAS-driven HNC [13,174,234]. In a clinical trial of phase II, which encompassed individuals diagnosed with recurrent or metastatic HNSCC, and with a mutant *HRAS* variant allele frequency of 20% or higher, administering tipifarnib led to an objective response rate of 55%, a median progression-free survival of 5.6 months, and a median overall survival of 15.4 months. The safety profile of tipifarnib was found to be tolerable and manageable[13]. Mechanistically, tipifarnib treatment affected both tumour cells and endothelial cells, which led to a reduction in MAPK pathway signalling, an inhibition of proliferation, the induction of apoptosis, and a significant abrogation of neovascularization [174]. Whereas clinical trial using lonafarnib (NCT00073450, NCT00038584) was less effective in treating HNC. Despite efficacy in inhibiting tumor growth in preclinical models of HRAS-driven cancers, farnesyl transferase inhibitors showed no clinical efficacy in KRAS- driven cancer. This clinical finding may be attributed to the prenylation of NRAS and KRAS by geranylgeranyltransferase type-1 in the absence of farnesyltransferase, a modification that does not occur in HRAS [235]. It is also evident from clinical trials that response to tipifarnib depend on tissue type and could varies among various subtypes of HNC, for instance between recurrent, metastatic HNSCC vs salivary gland cancers. Resistance to tipifarnib can occur through reactivation of RAS signaling by RAS geranylgeranylation following the inhibition of farnesyltransferases or due to adaptive mechanisms such as mutations in NF1 and GNAS genes [236]. The resistance that is encountered while targeting farnesyl and geranylgeranyltransferases may be overcome through the inhibition of other enzymes that contribute to the post-translational modification of RAS proteins. Specifically, phosphodiesterase-δ (PDEδ), which is a prenyl-binding protein, plays a significant role in facilitating the translocation of RAS from the golgi apparatus and endomembranes to the plasma membrane. Such strategies have not been studied to date in HNC.

6.2.3. Inhibitors for RAS downstream effectors—The identification of RAS effector proteins and recurrent oncogenic mutations in downstream RAS pathway constituents (BRAF, MEK, ERK, and PI3K pathway members) has engendered the generation of several inhibitors, which are presently in varying phases of clinical evolution (Table 1). Inhibitors of RAF, MEK, ERK, PI3K, AKT, and mTOR as well as dual-target inhibitors have been tested or are undergoing clinical evaluation in HNC patients. Despite concerted efforts, targeted therapies that show promise are often beset by the swift onset of resistance. For instance, targeting mTOR by everolimus to induction chemotherapy was not beneficial in a phase I/II clinical trial. Resistance mechanisms to these therapies can arise from stress-adaptive pathways, and this may be more pronounced in cancers driven by oncogenic RAS, as their survival is dependent on cellular stress responses. Therefore, when treating a RAS-driven cancer cell, it may be necessary to inhibit these stress-adaptive pathways during initial therapy to reduce tolerance and prevent the development of stress-adaptive mechanisms. Thus, targeting RAS effector signaling alone might not be sufficient for clinical efficacy. An alternative strategy would be combination treatments targeting both the RAS and RAS effector signaling pathways and such combinations are now under clinical evaluation in HNC (Table 1). Two such ongoing preclinical study that shown promising output includes the combination of inhibitors that targets ERK[237] and PI3K[238–240] along with tipifarnib.

6.2.4. Inhibition of RAS-effector interaction—Disruption of RAS-effector interaction is another alternative targeting strategy. It has been reported, for example, Rigosertib, a non-ATP-competitive multi-kinase inhibitor, has been shown to act as a RAS mimetic compound. It binds to the RBD of several RAS effectors, including RAF, RALGDS, and PI3K. This binding inhibits RAS-dependent cell transformation both in vitro and in vivo [241]. Clinical trials of rigosertib (NCT01807546, NCT02107235) as an HNC therapeutic are under way. In a phase I trial, two patients with recurrent/metastatic HNC had objective responses, currently being evaluated in combination with cisplatin/radiotherapy[242].

7. Future strategies to target RAS in HNC

RAS activation modulates the TME during tumorigenesis and in response to therapy. The potential approaches targeting TME along with oncogenic RAS could be particularly effective. For instance, the inhibition of upstream activators or downstream effectors of RAS signaling induces immunologic changes in tumor cells (e.g. increase in the expression of MHCs and tumor-associated antigens, and decrease in the production of immunosuppressive cytokines), that impairs antitumor immunity thus delimiting treatment efficacy. Studies using MEK inhibitors in HNC models have shown that these inhibitors prevent the proliferation and activation of T cells causing a relapse of disease [243]. Strategies to target RAS in HNC focusing on inhibition of RAS vulnerabilities (synthetic lethal interaction strategies targeting tumor metabolism), and harnessing of the immune system has shown promising.

7.1. Targeting of RAS vulnerabilities

Synthetic lethal methodologies have been employed in order to discern genes that are crucial for the operation of mutated RAS within cancer cells, while concurrently remaining non-essential in cells that harbor the wild-type RAS. RAS signaling establishes an oncogenic cell state that gives rise to adaptive changes to oncogenic stress including metabolic stress, DNA damage and replicative stress, oxidative stress, and proteotoxic stress that are necessary for cancer cell proliferation and survival.

Cancers driven by RAS mutations heavily rely on the upregulation of metabolic processes to sustain their aberrant cell growth, thereby highlighting the potential for targeting the metabolism of RAS-mutated malignancies. The metabolic reprogramming induced by RAS mutations leads to increased glutaminolysis, glycolysis, autophagy, and macropinocytosis, which collectively support the energetic and biomass requirements for uncontrolled proliferation [244]. The activation of oncogenic RAS in cancer cells induces metabolic reprogramming, leading to an increased reliance on glutamine as a crucial nutrient for both catabolic and anabolic pathways [245]. Glutamate metabolism is being investigated as a therapeutic target [245]. It has been shown, for example, glutaminase inhibitor- CB-839, is potent in curtailing the growth of RAS-driven HNC [246] and in combination treatment with ionizing radiation leads to an enhanced anti-tumor response in preclinical HNC models[247].

Oncogenic RAS redirects glucose metabolism towards the hexosamine biosynthetic pathways by upregulating various crucial glycolytic enzymes. It also stimulates the nonoxidative pentose phosphate pathway to facilitate augmented nucleic acid biosynthesis [244]. Targeting glycolysis in RAS-driven malignancies makes sense given the interaction between oncogenic RAS and glycolysis. Recently, it was proven preclinically that treatment with glucose transporter-1 inhibitor - BAY-876 sensitize cisplatin resistant oral squamous cell carcinoma cells[248]

Several investigations have indicated that autophagy is escalated in the scenario of RAS transformation, thus proposing another possible target for *RAS* mutant tumors[249]. Cancer stem cell phenotype of *NRAS* mutant Cal27 cells is found to be regulated by autophagy through the noncanonical FOXO3/SOX2 axis [250]. It has been demonstrated by Cheong et al. that RAS-driven PI3K signaling amplifies the levels of casein kinase 1α, which subsequently phosphorylates and obstructs the nuclear localization of FOXO3A, a transcription factor that positively regulates the expression of crucial autophagy genes [251] Preclinical evaluation using chloroquine – an autophagy inhibitor indicate that chloroquine may be considered a potent therapeutic agent against HNC.

RAS-driven tumors consume extracellular protein through the process of macropinocytosis [252]. The protein that has been internalized is broken down through proteolytic degradation, resulting in the production of amino acids, including glutamine, which can be utilized in central carbon metabolism. Researchers are now combining substances that can trigger macropinocytosis cell surface receptors with anticancer drugs that do not rely on macropinocytosis. By forming drug conjugates of such nature, the macropinocytosis pathway is employed as a means of delivering anticancer drugs to enhance cancer

treatment. For instance in HNC, silmitasertib-induced macropinocytosis is used for cisplatin intracellular uptake to enhance cell apoptosis in oral squamous cell carcinoma [253].

Oncogenic RAS induce DNA damage and replication stress. The presence of RAS- induced replicative stress leads to the activation of the DNA damage response (DDR) pathway. This activation involves the involvement of ataxia telangiectasia and Rad3-related protein (ATR) and checkpoint kinase 1 (CHK1), which in turn initiates cell-cycle arrest and DNA repair processes. Additionally, it promotes the activation, reactivation, or stabilization of replication origins or forks to mitigate the level of genomic instability[254,255]. In preclinical studies, the potent and selective CHK1 inhibitor, CCT244747, increased the sensitivity of head and neck cancer (HNC) cell lines to radiation in laboratory settings, leading to a delay in tumor growth in cancer xenograft models and improved overall survival [256,257]. The in vivo assessment of prexasertib, a CHK1 inhibitor, was conducted in a syngeneic mouse model of HNSCC (MTE-Ras). This mouse model was created by transforming mouse tonsil epithelial cells with HRAS expression and inducing PTPN13 knockdown to mimic tobacco- induced HNSCC. This evaluation demonstrated that prexasertib not only increased the infiltration of innate and adaptive immune markers within the tumor immune microenvironment but also enhanced tumor sensitivity[258]. Evidences have also shown that ATR inhibition using AZD6738 on HNC cells including *RAS* mutant lines sensitizes cells to cisplatin[259].

RAS driven cancers rely on reactive oxygen species (ROS) for their proliferation [260]. The results of the in vitro and in vivo evaluation on the anti-tumorigenic effect of natural anti- oxidant vitamin C using *RAS* mutant oral cancer cell line (Cal27 and SCC9) supported the above statement [261]. Stockwell and colleagues conducted a synthetic lethal small molecule screening using isogenic fibroblasts with either $HRAS^{G12V}$ mutant or wild-type (WT) genes. Through this screening, they discovered a set of small molecules that induce ferroptosis, a form of non-apoptotic cell death characterized by the iron-dependent accumulation of harmful lipid ROS in cells harboring oncogenic *RAS* mutations[262]. Recent studies also demonstrates that *HRAS* mutant HNC cells are sensitive to ferroptosis inducers like erastin, ML-162[263] .

Proteotoxic stress refers to a condition where cells are unable to maintain proper protein homeostasis, leading to the accumulation of misfolded or damaged proteins. Many cancer cells live with high levels of proteotoxic stress, and this condition could be exploited from a therapeutic perspective. For instance, in a recent study focused on screening drugs, inhibitors targeting heat shock protein 90 (HSP90) proteins and AXL were found to exert negative impact on the growth of chemoresistant cell lines, in comparison to therapy-naïve parental control cell lines [264]. Consistent to this findings, it was identified in HNC, that HSP90 inhibitor, AUY922, sensitize Cal27 cells to concurrent cisplatin radiotherapy[265] and AXL inhibitor R428, sensitize VU-147T cells to radiotherapy[266].

Despite targeting RAS vulnerabilities showed good anti-tumorigenic activity and synergism with conventional therapies preclinically, clinical translation of these targeted therapies are yet to be performed in HNC.

7.2. Levaraging the immune microenvironment

Apart from altering cellular metabolism, RAS mutations can also impact immune cells within the TME and influence the immune response. Utilizing the immune system to target *RAS*-mutant cancers has shown promising outcomes in both preclinical models and patients. Combining anti-PD1 with RAS inhibitors, particularly inhibitors of the RAF-MEK-ERK pathway, may be useful in treating RAS-activated malignancies because of the potential interaction between RAS signaling and the immunological milieu[267,268]. Evidence from our lab showed that MEK1/2 inhibition in concert with anti-PD-1 therapy in *KRAS* mutant murine HNC caused tumor regression and hence enhanced the survival of tumor-bearing mice [27].

7.3. Other Immunotherapeutic approaches

Recent progress in understanding how tumors are recognized by the immune system and the development of viral vectors has allowed for the insertion of synthetic genes into T cells. This, along with the consistent mutation profile of RAS, enables the mass production of T cells that target tumor-specific antigens. These T cells can be engineered with T-cell receptors (TCRs) or chimeric antigen receptor (CAR) T cell therapies [269]. A significant development is the discovery of CD8+ T cells in a patient with metastatic colorectal cancer that can recognize mutant KRAS [270]. After these cells were expanded outside the body and reinfused into the patient, a decrease in metastatic burden was observed, indicating the potential success of immunotherapies targeting RAS. Another approach is to use adoptive cellular therapy to target specific mutations, which involves taking tumor-infiltrating lymphocytes (TILs) from the patient, selecting and expanding them, and then reinfusing them back into the patient [271]. This strategy is being tested for treating RAS-driven rectal and pancreatic cancer (NCT03745326 and NCT03190941). Additionally, a mRNA-based cancer vaccine (V941) that targets common KRAS mutations (G12D, G12V, and G12C) is being studied for colorectal and pancreatic cancer (NCT03948763). However, these approaches have not yet been approved or specifically studied for RAS-driven HNC.

7.4. Proteolysis targeting chimeras (PROTACs) against mutant RAS

PROteolysis TArgeting Chimeras (PROTACs) has emerged as potential approach to target and degrade mutant RAS. PROTACs are hetero-bifunctional molecules composed of a warhead that engages a protein of interest and an E3 ligase ligand connected via a suitably designed linker. Using this approach, bifunctional molecules combine mutant RAS with an E3 ligase, forming a ternary complex, enabling E3 ligase to ubiquitinate the mutant RAS and subsequently allowing the proteosomal degradation machinery to recognize and degrade mutant RAS. In a study by Bond et al., a novel endogenous $KRAS^{G12C}$ degrader called LC-2 was developed. LC-2 combines MRTX849, which acts as a warhead, with a VHL E3 ligase ligand. They demonstrated that LC-2 forms a covalent bond with KRASG12C using the MRTX849 warhead and recruits the E3 ligase VHL. This mechanism induces rapid and sustained degradation of $KRAS^{G12C}$, resulting in the suppression of MAPK signaling in cell lines carrying both homozygous and heterozygous $KRAS^{G12C}$ mutations. [272]. This technology has been applied to a variety of therapeutically relevant RAS upstream and

downstream targets, and a wide range of novel PROTAC designs for targeted delivery or activation have been developed but, yet to be studied in RAS-driven HNC.

7.5. Combinatorial therapies

Accumulating evidence suggests that targeting RAS using a single agent is not an effective strategy for the treatment of most of the *RAS*-mutant cancers. The resistance observed in RAS-driven cancers is primarily attributed to negative feedback mechanisms, the overexpression of alternative key regulator proteins, and the reflexive activation of downstream partners of RAS. These factors collectively contribute to the development of resistance against targeted therapies (Fig. 5). For instance in case of resistance to KRAS G12C inhibitors, one study suggested that signals from either EGFR or AURKA can keep the KRAS G12C protein in its active form, allowing it to evade treatment with KRAS G12C inhibitors [273]. However, another study found that activation of wild-type RAS by multiple RTKs, rather than just one, is responsible for resistance to KRAS G12C inhibitors (ARS-1620 and sotorasib) in various cancer cell lines [274]. Similar mechanisms of resistance could exist in HNC, hence blocking such escape mechanism along with EGFR or other RTK inhibitors could be beneficial to overcome such resistance. Consequently, combinatorial approaches that target mechanisms of resistance and RAS pathway regulators and effectors will likely be required for long-term, efficacious therapeutic response. Ongoing preclinical and clinical research has started using a combination of inhibitors that targets kinases such as MEK, ERK, PI3K, AKT, mTOR, EGFR and/or immune checkpoints along with RAS direct inhibitors (Table 1). Results from these studies will provide more safer and less toxic combinations that could achieve both targeting RAS activation and overcoming RAS-mediated resistance mechanisms.

8. Clinical prospects for RAS targeting as a precision therapy for HNC

The RAS network in HNC is extensive and complex, with multiple interconnected pathways involved in cellular development, evasion of cell death, and metastasis. HNCs that rely on RAS signaling for survival are frequently aggressive, leaving the clinician with few therapeutic choices. Due to the difficulty of quantifying RAS-related signaling events in tumors and the inevitable development of drug resistance to targeted therapies, the treatment of RAS-driven HNC is challenging. Here, we have examined studies in HNC that suggest that dysregulation of numerous network nodes can activate the RAS pathway and that gene expression and mutation signatures can be utilized to quantify RAS network activation. Detection and exploitation of RAS mutations/activation for molecular targeted therapy and as a diagnostic and prognostic marker could account for a new therapeutic approach in HNC. Scientific evidence suggest the importance of targeting RAS as a precision medicine for a relatively larger subset of HNC population. This review has also opened new avenues for designing treatment modalities by considering the understanding of the RAS-driven TME modulation during the oncogenic process. Taking the vast knowledge on the RAS oncobiology in HNC, future RAS targeting should concentrate on designing potent inhibitors or strategies that significantly hamper the multifacet oncogenic activity of RAS and its cooperating partners with the least side effects.

Supplementary Material

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Abbreviations

AP1	activator protein 1
AURKA	aurora kinase A
CAR-T	chimeric antigen receptor T cell
CHK1	checkpoint kinase 1
COSMIC	catalogue of somatic mutation in cancer
cRNA	complementary ribonucleic acid
CSF1	colony stimulating factor 1
CTLA-4	cytotoxic T lymphocyte antigen 4
CXCR	C-X-C motif chemokine receptor
CXCL	chemokine (C-X-C motif) ligand
DNA	deoxy ribonucleic acid
DUSP	dual specificity phosphatase 1
EBV	epstein barr virus
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
FAK	focal adhesion kinase
FGFR	fibroblast growth factor receptor
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
G-CSF	granulocyte colony stimulating factor

GM-CSF	granulocyte macrophage colony stimulating factor
GPCR	G protein coupled receptor
GRB2	growth factor receptor bound protein 2
GTP	guanosine triphosphate
HPV	human papillomavirus
HNC	head and neck cancer
HNSCC	head and neck squamous cell carcinoma
HRAS	harvey rat sarcoma viral oncogene homolog
HSP 90	heat shock protein 90
IGF1R	insulin like growth factor receptor 1
IL	interleukin
KRAS	kirsten rat sarcoma viral oncogene homolog
lncRNA	long non-coding ribonucleic acid
MAPK	mitogen activated protein kinase
MIP-1α	macrophage inflammatory protein 1 alpha
MCL-1	myeloid cell leukemia 1
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MEG-3	maternally expressed gene 3
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
miRNA	micro ribonucleic acid
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
PD-1	programmed cell death protein 1
PD-L1	programmed cell death ligand 1
PDGFR	platelet derived growth factor receptor
PI3K	phosphatidyl inositol 3-kinase
PITX1	paired like homeodomain 1
PKC	protein kinase C

PLCε	phospholipase Cε
PROTAC	proteolysis targeting chimeras
RAS	rat sarcoma viral oncogene
RALGDS	ral guanine nucleotide dissociation stimulator
RBD	RAS binding domain
RASSF	RAS association domain family
RASAL	RAS protein activator like
RTK	receptor tyrosine kinase
SHh	sonic hedgehog
SHP2	src homology-2 domain-containing protein tyrosine phosphatase-2
siRNA	small interfering ribonucleic acid
SOS1	son of sevenless homolog 1
TAMs	tumor associated macrophages
TCGA	the cancer genome atlas
TCR	T cell receptor
TGFβ	transforming growth factor beta
TKI	Tyrosine kinase inhibitor
TNFα	tumor necrosis factor alpha
TIAM1	T-lymphoma invasion and metastasis-inducing protein 1
TME	tumor microenvironment
Tregs	T regulatory cells
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

Data Availability

No data was used for the research described in the article.

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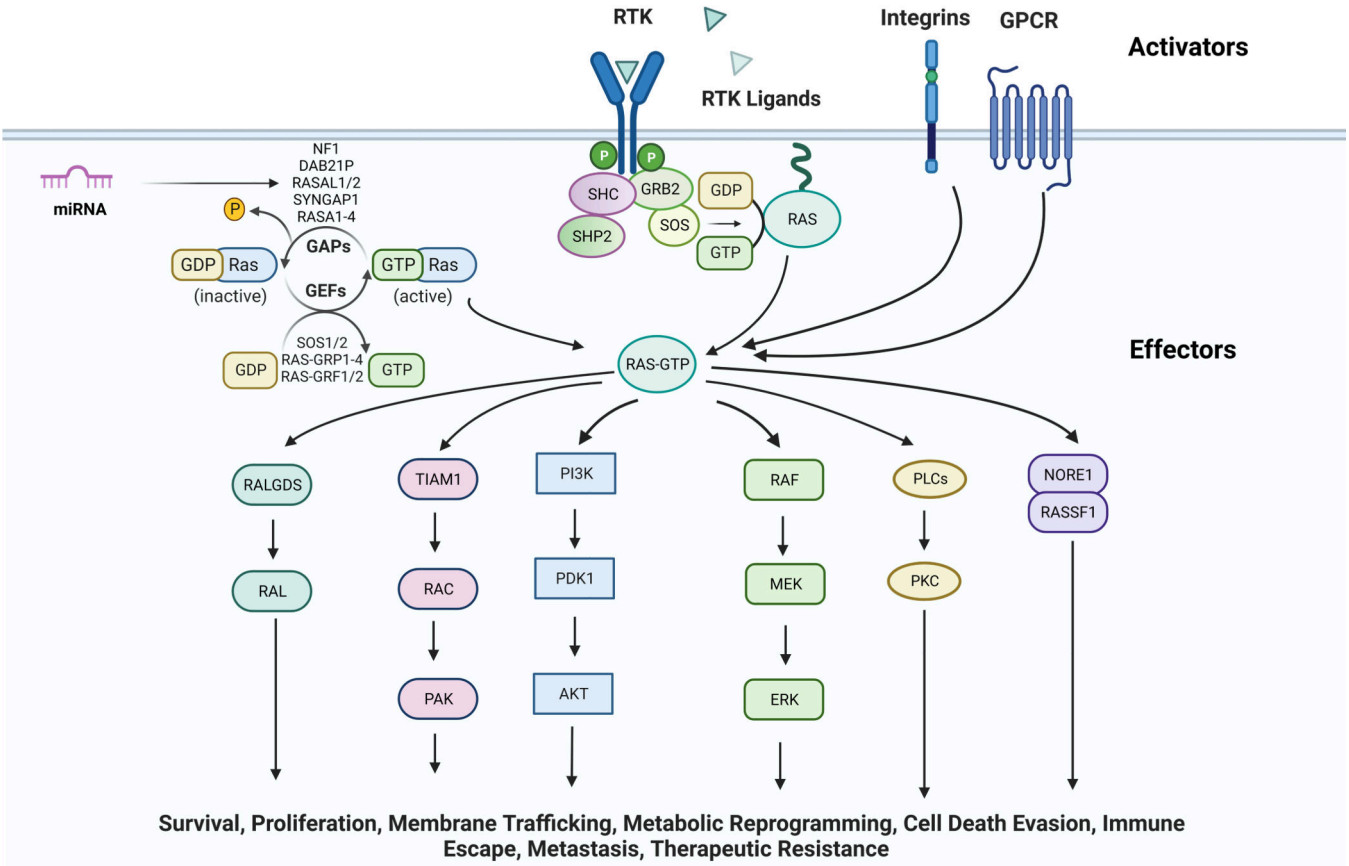

Figure 1: Overview of RAS signaling with upstream activators and downstream effector pathways in HNC.

The activation state of RAS is tightly regulated by the hydrolysis of bound GTP, facilitated by GTPase activating proteins (GAPs), and the exchange of bound GDP with fresh GTP, catalyzed by guanine nucleotide exchange factors (GEFs). RAS mutations or ligand binding to receptor tyrosine kinases (RTK) triggers downstream effector signaling pathways, which control the transcription of genes promoting cell cycle progression and survival in cancer cells. This involves the formation of complexes between activated, autophosphorylated RTKs and the GEF SOS, facilitated by the adaptor proteins GRB2 and possibly SHC. The recruited SOS is then directed to the plasma membrane, where RAS resides. The SHC-GRB2-SOS complex facilitates the exchange of GDP to GTP, converting RAS into its active conformation, thus propagating signaling through various RAS pathways, including the prominent RAF/MEK/ERK cascade. SHP2 also plays a crucial role in RAS activation, acting upstream of SOS. Additionally, RAS activates the phosphatidylinositol 3-kinase (PI3K)–3-phosphoinositide-dependent protein kinase 1 (PDK1)–AKT pathway, which frequently regulates cellular survival. Furthermore, activated RAS activates phospholipase C (PLC), and its hydrolytic products modulate calcium signaling and protein kinase C (PKC). RALGDS and TIAM1 serve as exchange factors for RAL and RAC, respectively. Among RAL's effectors is phospholipase D (PLD), an enzyme that regulates vesicle trafficking. Moreover, the RAS effectors NORE1 and RASSF1 are involved in apoptosis through the MST/Hippo tumor suppressor pathway, leading to programmed cell death. miRNA also

regulate RAS activity through the modulation of RAS regulators such as RAS-GAPs. (This scheme is created using BioRender.com).

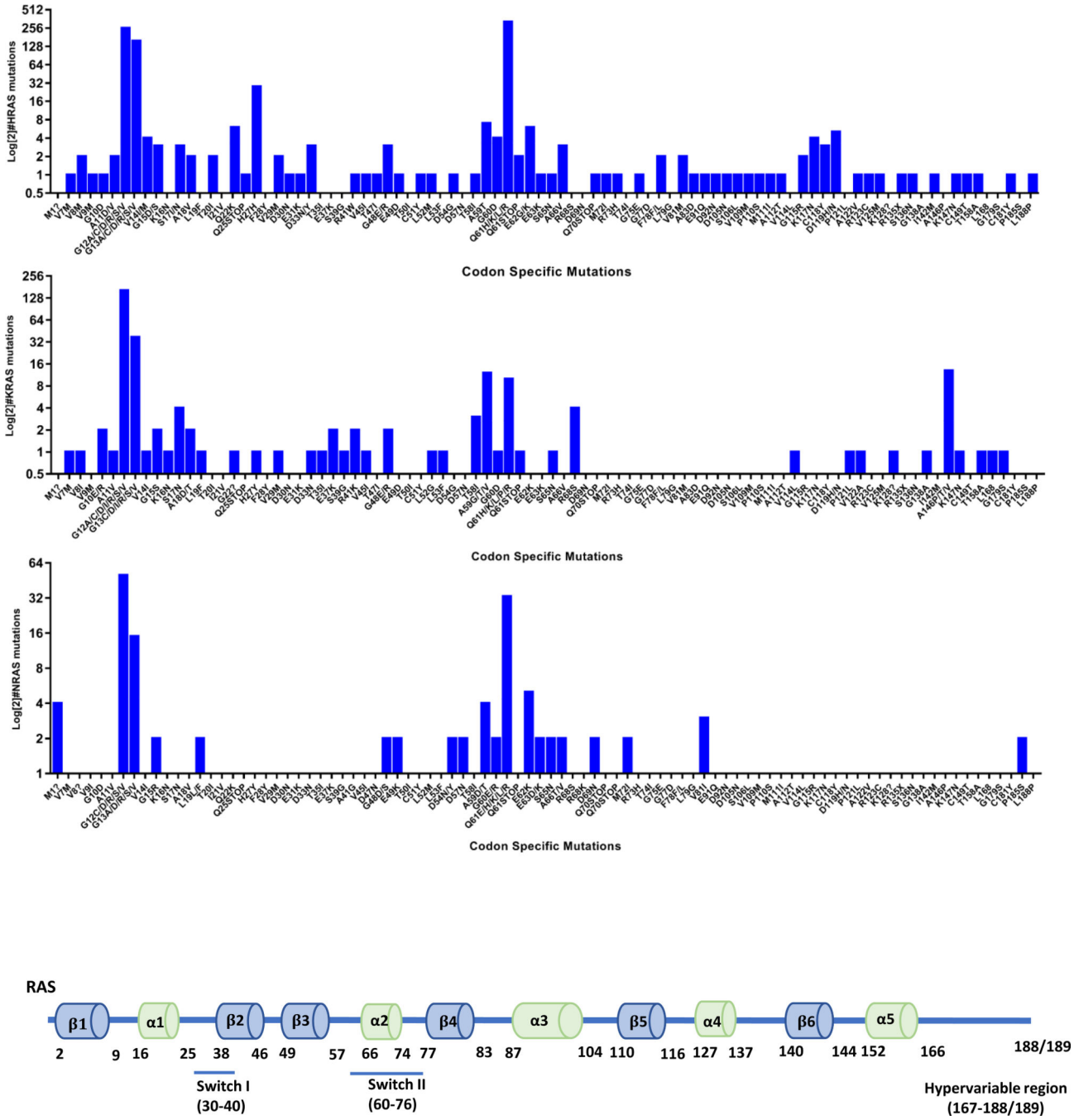

Figure 2: *RAS* mutation burden, hotspots, and functional domains in head and neck cancer.
Top graphs indicate mutation burden per codon of the RAS proteins HRAS, KRAS, and NRAS, respectively; the data are plotted on a logarithmic scale of 2. Structure and switch of RAS. Structure of RAS proteins, including the effector lobe (aa 1–86), allosteric lobe (aa 87–165), and hypervariable region (HVR) (aa 167–188/189). Switch I (aa 30–40) and switch II (aa 60–76) are located in the effector lobe and function in effector binding and GEF or GAP binding. The HVR domain contributes to RAS binding to cell membranes.

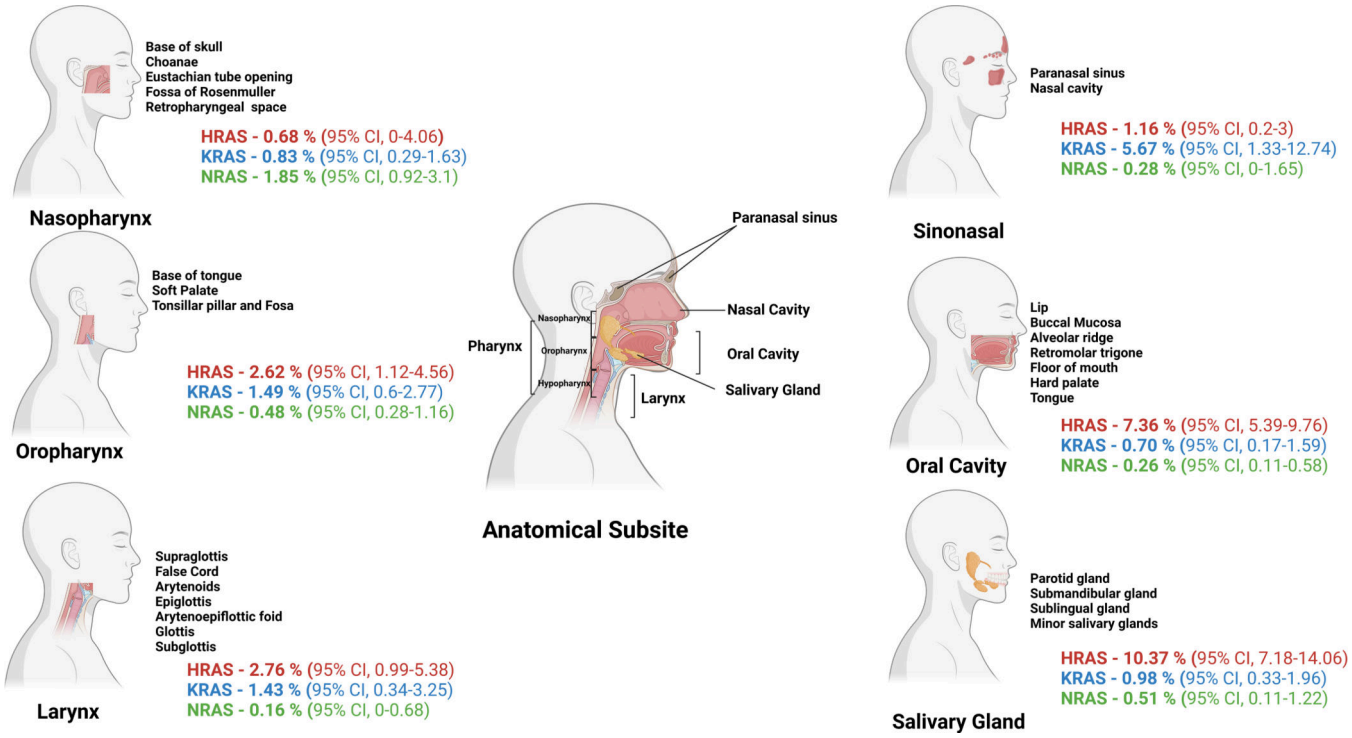

Figure 3: Distribution of *RAS* isoform mutations in various anatomical subsites of HNC.
(This illustration is created using BioRender.com).

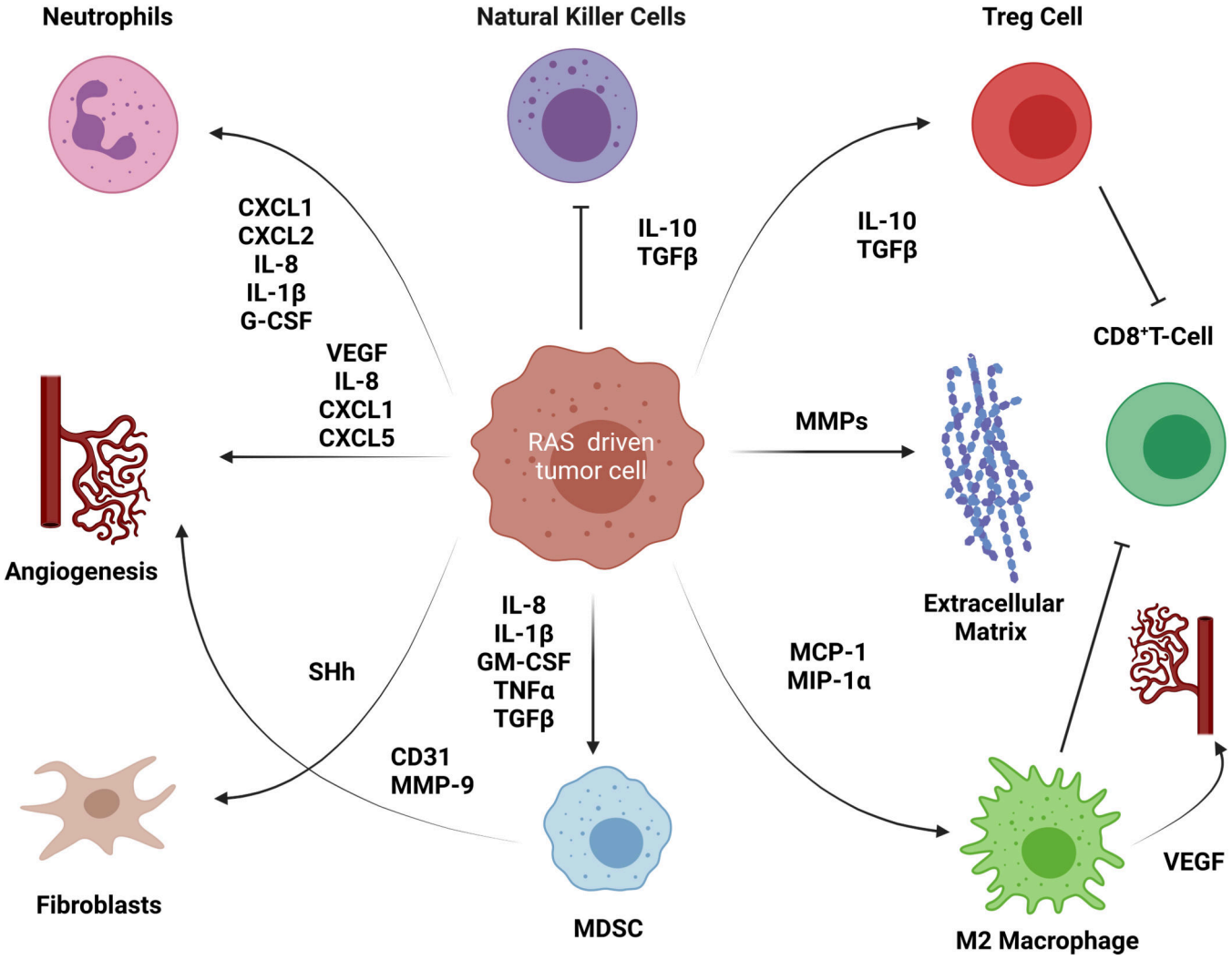

Figure 4: Overview of tumor microenvironment modulation by RAS activation in HNC.

The intricate paracrine effect of RAS-driven HNC has been shown to promote tumor progression by influencing the properties of tumor microenvironment components. Tumors are indeed infiltrated with immune cells, and the interaction between the tumor and the immune system is a crucial aspect of the tumor microenvironment. RAS-driven HNC cells secrete molecules that will promote the recruitment of M1 macrophages and neutrophils. The induction of a less reactive and more tolerogenic environment is achieved through recruitment of MDSCs, inhibition of $CD8^+$ cytotoxic lymphocyte activation, inhibition of natural killer (NK) cells, and induction of Treg differentiation. Fibroblast activation, endothelial cell recruitment, and blood vessel formation as well as ECM remodeling represent other microenvironment alterations led by mutant *RAS* cells. (This illustration is created using BioRender.com).

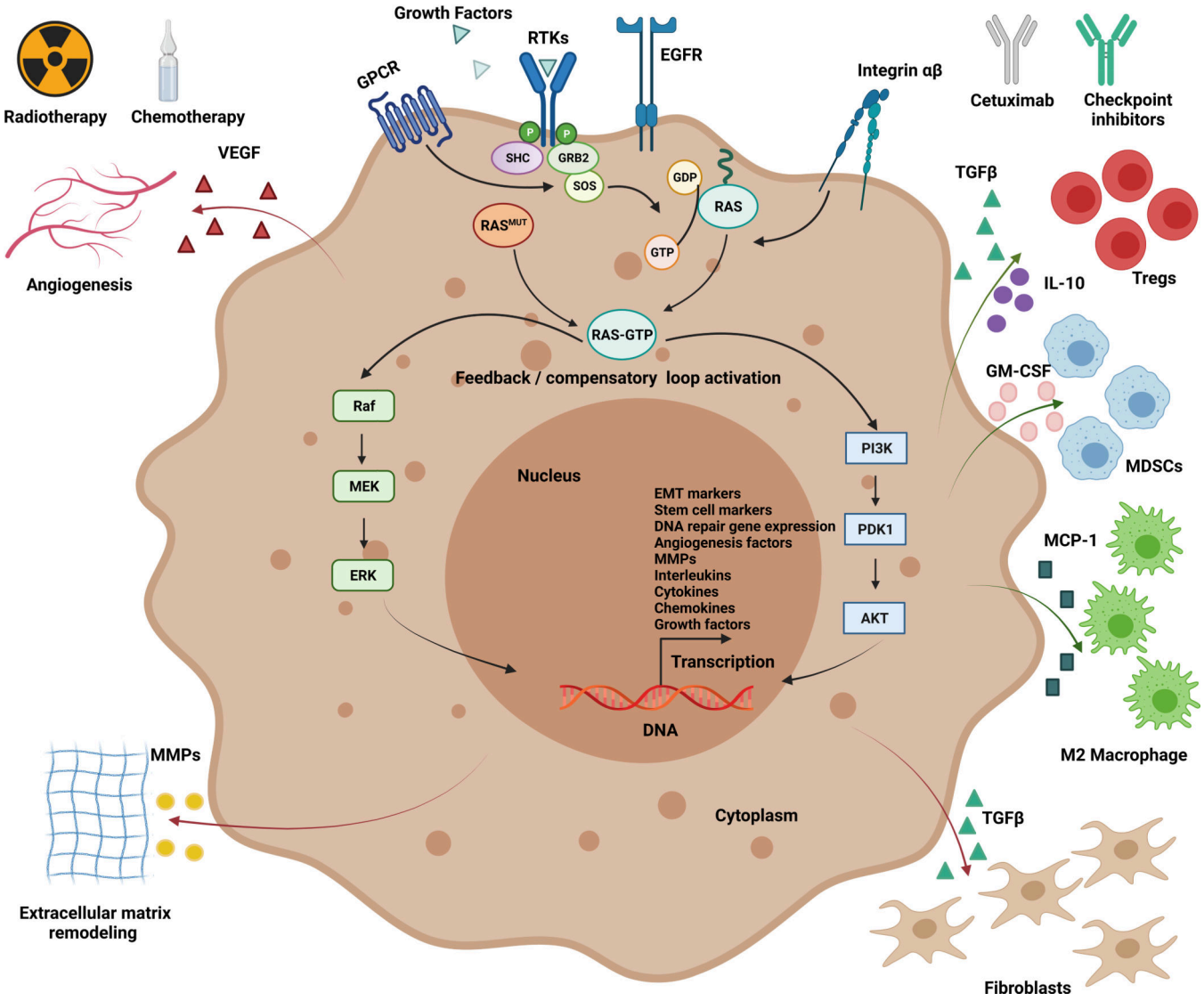

Figure 5: Schematic representation of potential resistance mechanisms to therapies activated by RAS alteration/activation in HNC.

The mechanism involves the reactivation of multiple receptor tyrosine kinases located upstream of RAS. When growth factors bind to RTKs like ERBB3, EGFR, and ERBB2, they induce phosphorylation and recruitment of the GRB2-SOS complex. This complex facilitates the removal of GDP from RAS, enabling RAS to bind GTP and become active. Additionally, parallel pathways, such as the PI3K-AKT-mTOR signaling pathways, are activated. Furthermore, transcription factors responsible for controlling epithelial and mesenchymal transition, as well as stemness, are activated. Moreover, there is an upregulation of angiogenesis factors, extracellular matrix components, cytokines, growth factors, and chemokines, which modulate the behavior of fibroblast and immune cells within the tumor microenvironment. (This scheme is created using BioRender.com).

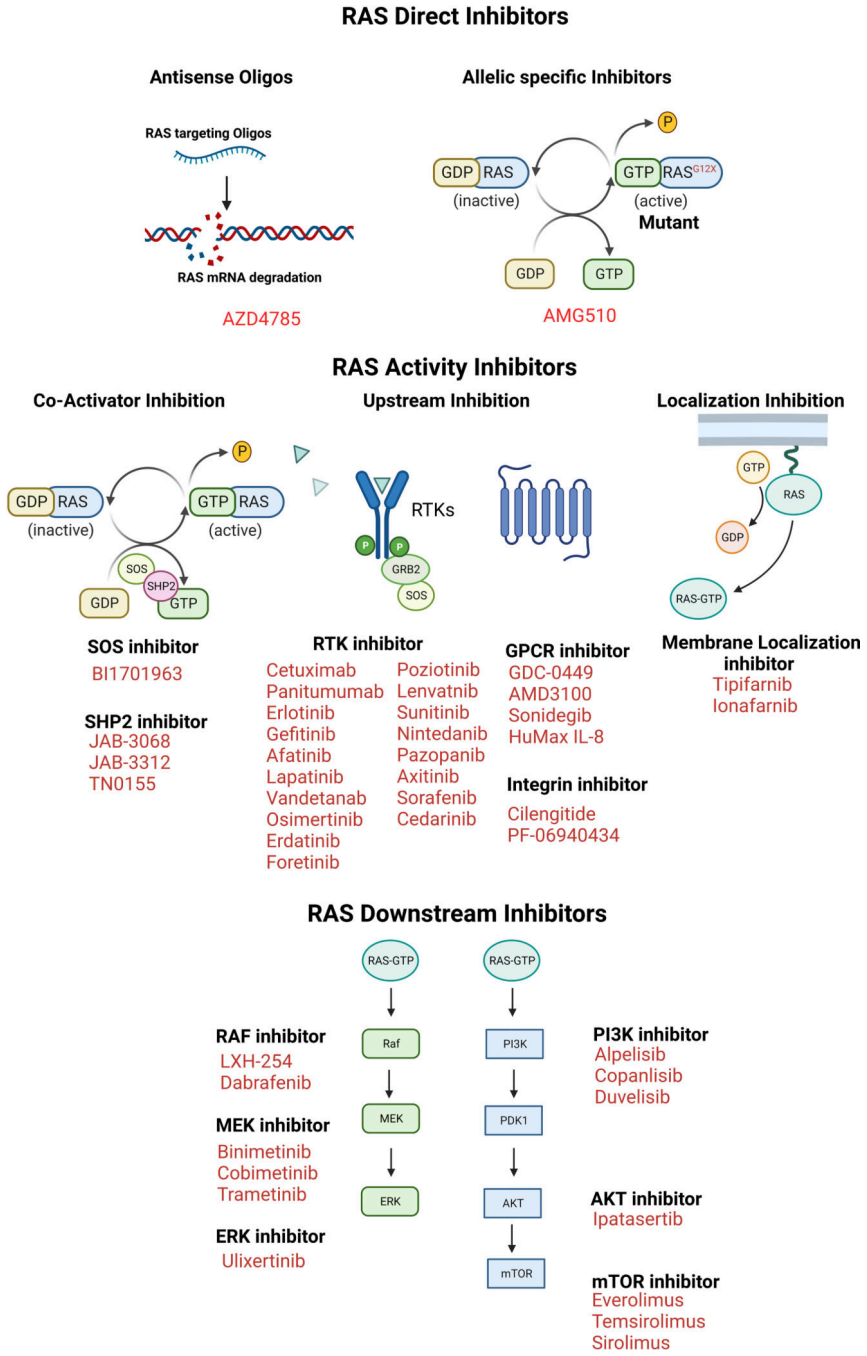

Figure 6: Clinical drugs targeting RAS or its activation and/or its downstream pathways in HNC.

Different strategies employed to target RAS includes (a) Targeting RAS directly, especially G12C covalent binders and RNA interference of mutant RAS mRNA; (b) Targeting upstream molecules of RAS –RTK, GPCR, Integrin, SOS and SHP2; (c) Targeting membrane localization of RAS; (d) Targeting downstream effectors- RAF, MEK, ERK, PI3K, AKT, and mTOR. (This illustration is created using BioRender.com).

Table 1:

RAS targeting strategies in clinical trials for HNC

RAS targeting strategy	Target	Single Agent/Combination	Clinical Trial no	Status
Direct RAS Targeting				
Antisense Oligos				
AZD4785	KRAS	Single	NCT03101839	Completed
RAS mutant specific inhibitor				
AMG 510	KRAS G12C	Single	NCT04380753	Active NR
		Single	NCT04185883	Recruiting
		Combination with anti-PD1/ Midazolam	NCT03600883	Recruiting
Indirect RAS targeting				
RAS Upstream inhibition				
RTK inhibitor				
Cetuximab	EGFR	Single	NCT03769311	Recruiting
		Combination with hedgehog inhibitor	NCT01255800	Completed
		Combination with Cisplatin or radiotherapy	NCT00089297	Completed
		Combination with Cisplatin or radiotherapy	NCT00713219	Completed
Panitumumab	EGFR	Single	NCT04511078	Recruiting
		Single	NCT00446446 NCT02643056	Completed
		Combination with Chemotherapy	NCT01264328 NCT00756444	Completed
		Combination with Chemoradiotherapy	NCT00500760 NCT00547157 NCT00513383	Completed
Erlotinib	EGFR	Single	NCT00570232 NCT00281866	Completed
		Combination with Chemotherapy	NCT00030576 NCT01927744 NCT00055770 NCT01316757 NCT01064479	Completed Active NR Completed Completed Completed
		Combination with chemoradiotherapy	NCT00304278 NCT00113347	Completed
		Combination with celecoxib	NCT00970502 NCT00400374	Completed
		Combination with other RTK inhibitors	NCT00140556 NCT00055913 NCT00101348	Completed
		Combination with mTOR inhibitor	NCT00942734	Completed
		Combination with Src inhibitor	NCT00779389	Completed
		Combination with HDAC inhibitor	NCT00738751	Completed

RAS targeting strategy	Target	Single Agent/Combination	Clinical Trial no	Status
Gefitinib	EGFR	Single	NCT00024089 NCT01185158 NCT00519077 NCT00015964	Completed
		Combination with Chemotherapy	NCT00352105 NCT00255476 NCT00206219	Completed
		Combination with chemoradiotherapy	NCT00228488 NCT00239304 NCT00242749 NCT00229723	Completed
Dacomitinib	EGFR	Single	NCT01449201	Completed
Poziotinib	EGFR	Single	NCT02216916	Completed
			NCT03292250 NCT02216916	Unknown
Afatinib	EGFR		NCT01427478 NCT01415674 NCT03695510 NCT01538381	Completed Active NR Completed
		Combination with other EGFR inhibitor	NCT02979977 NCT00514943 NCT03088059	Recruiting Completed Recruiting
		Combination with chemoradiotherapy	NCT01783587	Completed
		Combination with chemotherapy	NCT01721525 NCT01856478	Completed Active NR
		Combination with immunotherapy	NCT03695510	Active NR
Lapatinib	EGFR	Single	NCT00098631 NCT00114283 NCT00371566	Completed
		Combination with other EGFR inhibitor	NCT01184482	Completed
		Combination with chemotherapy	NCT00498953 NCT01044433	Completed
		Combination with chemoradiotherapy	NCT01711658 NCT01612351 NCT00387127 NCT00424255	Active NR Active NR Completed Completed
Osimertinib	EGFR	Single	NCT02465060	Recruiting
		Combination with immunotherapy	NCT04140526	Recruiting
Apatinib	VEGFR	Single	NCT02989259	Recruiting
		Combination with chemotherapy	NCT03654612 NCT02943252 NCT03096184	Unknown
		Combination with radiotherapy	NCT03539172	Unknown
		Combination with chemoradiotherapy and immunotherapy	NCT04393506	Active NR
		Combination with immunotherapy	NCT04393506 NCT04440917 NCT05069857	Active NR Recruiting Recruiting
Axitinib	VEGFR	Single	NCT02762513 NCT01469546	Completed
Cediranib	VEGFR	Single	NCT00458978 NCT00243347	Completed

RAS targeting strategy	Target	Single Agent/Combination	Clinical Trial no	Status
Foretinib	VEGFR	Single	NCT00725764	Completed
Lenvatinib	VEGFR			
		Combination with EGFR inhibitor	NCT03524326	Active NR
		Combination with chemoradiotherapy and immunotherapy	NCT05007106 NCT04977453 NCT04199104 NCT04428151	Recruiting
Nintedanib	PDGFR, FGFR, VEGFR	Single	NCT02558387 NCT03292250 NCT02558387	Unknown
Vandetanib	VEGFR, EGFR	Single	NCT01414426	Completed
		Combination with chemoradiotherapy	NCT00450138	Completed
Erdafitinib	FGFR	Single	NCT02465060	Recruiting
Rogaratinib	FGFR	Single	NCT01976741	Completed
AZD4547	FGFR	Single	NCT02465060	Recruiting
Pralsetinib	RET	Single	NCT03037385	Recruiting
Imatinib	PDGFR, KIT	Single	NCT00180921	Unknown
VMD-928	NTRK	Single	NCT03556228	Recruiting
Crizotinib	ALK, ROS1	Single	NCT02465060	Recruiting
Sunitinib	Multiple RTKs	Single	NCT00387335 NCT02465060	Completed Recruiting
		Combination with radiotherapy	NCT00437372	Completed
Pazopanib	Multiple RTKs	Single	NCT01377298	Unknown
		Combination with EGFR inhibitor	NCT01716416	Completed
Sitravatinib	Multiple RTKs	Single	NCT02219711	Active, NR
Sorafenib	VEGFR, PDGFR, RAF kinase	Single	NCT00096512 NCT00199160	Completed
		Combination with EGFR inhibitor	NCT00939627 NCT00815295	Completed
		Combination with chemotherapy	NCT02035527 NCT00703638 NCT00494182	Completed Completed Active NR
GPCR inhibitor				
Vismodegib	PTCH, SMO	Single	NCT02465060	Recruiting
		Combination with Radiotherapy	NCT01835626	Completed
AMD3100	CXCR4	Combination with immunotherapy	NCT04058145	Withdrawn
Sonidegib	SMO	Combination with immunotherapy	NCT04007744	Recruiting
HuMax-IL8	IL8	Combination with immunotherapy	NCT04848116	Recruiting
Integrin Inhibitor				
Cilengitide	Integrin alpha v	Combination with chemotherapy	NCT00705016	Completed
PF-06940434	Integrin alpha v beta 8	Single / combination with immunotherapy	NCT04152018	Recruiting
SOS1 Inhibitor				
BI 1701963	SOS1	Single/Combination with Trametinib	NCT04111458	Recruiting

RAS targeting strategy	Target	Single Agent/Combination	Clinical Trial no	Status
SHP Inhibitor				
JAB-3068	SHP2	Single	NCT03518554 NCT03565003	Recruiting
		Combination with immunotherapy	NCT04721223	Recruiting
TNO155	SHP2	Combination with immunotherapy	NCT04000529	Recruiting
JAB-3312	SHP2	Single	NCT04121286 NCT04045496	Recruiting
Membrane Localization Inhibitor				
lonafarnib	Farensyl transferase	Single	NCT00038584	Completed
Tipifarnib	Farensyl transferase	Single	NCT03719690 NCT02383927	Recruiting Completed
		Combination with PI3K inhibitor	NCT04997902	Not yet recruiting
RAS downstream Inhibition				
RAF inhibitor				
LXH-254	RAF	Single/ Combination with immunotherapy	NCT02607813	Active NR
Dabrafenib	RAF	Single	NCT02465060	Recruiting
MEK Inhibitor				
Binimetinib	MEK	Single	NCT02465060	Recruiting
Cobimetinib	MEK	Single/ Combination with immunotherapy	NCT03264066	Completed
Trametinib	MEK	Single	NCT02465060	Recruiting
ERK Inhibitor				
Ulixertinib	ERK	Single	NCT04566393	Available
			NCT02465060	Recruiting
PI3K inhibitor				
Alpelisib	PI3K alpha	Single	NCT03138070 NCT02145312 NCT03601507	Unknown Unknown Recruiting
		Combination with EGFR inhibitors	NCT02282371	Completed
		Combination with chemotherapy	NCT02051751	Completed
		Combination with Chemoradiotherapy	NCT02282371 NCT02537223	Completed
		Combination with Farensyl Transferase inhibitor	NCT04997902	Recruiting
Copanlisib	PI3K alpha and delta	Single	NCT02465060	Recruiting
		Combination with immunotherapy	NCT03735628	Active NR
Duvelisib	PI3K delta and gamma	Combination with chemotherapy	NCT05057247	Not yet Recruiting
GDC-0032	PI3K alpha, delta and gamma	Single	NCT02465060	Recruiting
AKT inhibitor				

RAS targeting strategy	Target	Single Agent/Combination	Clinical Trial no	Status
Ipatasertib	AKT	Single	NCT02465060	Recruiting
mTOR inhibitor				
Everolimus	mTOR	Combination with EGFR inhibitor	NCT00942734 NCT01637194 NCT01283334	Completed
		Combination with chemotherapy	NCT01333085 NCT00935961 NCT01283334	Completed
		Combination with chemoradiotherapy	NCT00858663	Completed
Sirolimus	mTOR	Single	NCT01195922 NCT02646319 NCT00375245	Completed
Temosirolimus	mTOR	Single	NCT01172769	Completed
		Combination with EGFR inhibitor	NCT01256385	Completed
		Combination with chemotherapy	NCT01016769	Completed
		Combination with chemoradiotherapy	NCT01326468	Completed
Dual target Inhibitor				
CC-115	DNA PK/mTOR	Single	NCT01353625	Completed
Gedatolisib	P13K/mTOR	Combination with CDK inhibitor	NCT03065062	Recruiting
Bimiralisib	P13K/mTOR	Single	NCT03740100	Completed
RAS mimentic				
Rigosertib	RBD	Single	NCT01807546	Completed
		Combination with Cisplatin or radiotherapy	NCT02107235	Completed

NR- Not Recruiting