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Review

Molecular Pathways and Druggable Targets in Head and Neck Squamous Cell Carcinoma

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Simple Summary: Head and neck cancer remains a significant burden on patients and global health systems. Traditional approaches to therapy have included surgery, and more recently radiation and chemotherapies. Targeted immunotherapies are making significant inroads into improved outcomes, but only for small subsets of patients. Our ability to develop a wider range of targeted therapies rests on our understanding of the molecular pathways involved in carcinogenesis. This review paper summaries our current knowledge of the molecular pathways and druggable targets in head and neck oncology.

Abstract: Head and neck cancers are a heterogeneous group of neoplasms, affecting an ever increasing global population. Despite advances in diagnostic technology and surgical approaches to manage these conditions, survival rates have only marginally improved and this has occurred mainly in developed countries. Some improvements in survival, however, have been a result of new management and treatment approaches made possible because of our ever-increasing understanding of the molecular pathways triggered in head and neck oncogenesis, and the growing understanding of the abundant heterogeneity of this group of cancers. Some important pathways are common to other solid tumours, but their impact on reducing the burden of head and neck disease has been less than impressive. Other less known and little-explored pathways may hold the key to the development of potential druggable targets. The extensive work carried out over the last decade, mostly utilising next generation sequencing has opened up the development of many novel approaches to head and neck cancer treatment. This paper explores our current understanding of the molecular pathways of this group of tumours and outlines associated druggable targets which are deployed as therapeutic approaches in head and neck oncology with the ultimate aim of improving patient outcomes and controlling the personal and economic burden of head and neck cancer.

Keywords: head and neck cancer; squamous cell carcinoma; molecular pathways; biomarkers; personalised medicine; druggable targets; molecular therapies

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1. Introduction

Head and neck cancers (HNC) and their treatment can result in significant morbidity for patients. Patients are often diagnosed at advanced stages with lymph node metastasis. Therefore, the diagnosis of HNC at early cancerous stages has become vitally important. A greater majority of HNC are squamous cell carcinomas (HNSCC) arising from the epithelial tissue of the oral cavity, pharynx and larynx [1]. Numerous genetic aberrations

are involved in head and neck tumours. Additionally, various environmental factors including HPV infection and tobacco and alcohol exposure are associated with HNSCCs. HNSCC is a highly heterogeneous tumour type, hence personalised patient management should be based on both patient and tumour features [1].

HNSCC has traditionally been considered an environmental tumour mostly caused by tobacco and alcohol consumption and human papillomavirus (HPV) infection in Western populations [2–4]. There is evidence to suggest a critical role for genetic alterations contributing to the carcinogenesis of HNSCC [5]. This is demonstrated by a growing proportion of younger low-risk patients with suggested poorer prognosis and a distinctive clinical and histopathological pattern [6,7], in addition to genetic and prognostic differences between HPV-positive and HPV-negative HNSCC [8].

Currently the optimal treatment for HNSCC patients centres around a multidisciplinary approach including medical and surgical subspecialties such as surgery, medical oncology, radiation oncology, in addition to allied health disciplines. Targeted treatments have benefitted only a small subgroup of patients due to intrinsic and extrinsic drug resistance with limited drug efficacy [9]. This effect decreases with long-term follow-up [10]. Therefore, identification of new molecular targets and novel pharmacotherapies, as well as better patient selection, calls for new methods of identification and clinical validation of biomarkers.

In the past decade, massively parallel sequencing, also known as next generation sequencing (NGS) has enabled unbiased cancer genome sequencing in order to screen and search for new cancer genes at an unprecedented scale. NGS has been widely implemented for de novo whole genome, exome, and transcriptome sequencing for assessment of DNA copy number, re-arrangements, loss of heterozygosity, allele-specific amplification, methylation, transcription, aberrant splicing and RNA editing faster and more cost effectively than Sanger-based sequencing [11–13]. In recent years, NGS has bridged the gap between molecular screening and clinical applications with 96.1% accuracy in comparison to Sanger sequencing. In addition, it can reveal gene alterations at very low allelic frequency [14]. Not only has NGS helped identify new altered genes for novel biomarker development [15,16]; but by revealing specific gene alterations it has helped identify patients who are sensitive or resistant to particular therapies [17]. Genomic alterations collected by the International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA) projects, in addition to the Catalog of Somatic Mutations in Cancer (COSMIC) database provide the most comprehensive source of somatic mutations in cancer to date.

Most known cancer genes have been found through primary cytogenetic analyses, although sequencing of cancer genomes has revealed new cancer genes including *BRAF*, *EGFR*, *ERBB2*, *PIK3CA*, *PPP2R1A*, and *JAK2* [18–23]. Specifically in relation to HNSCC, Agrawal and Stransky independently and simultaneously confirmed previously known HNSCC genome alterations such as mutations in *TP53*, *CDKN2A*, *PIK3CA*, *PTEN*, and *HRAS* [24,25] but also identified a novel gene *NOTCH1* [15,16]. *NOTCH1*, an important tumour suppressor gene, is second most common gene involved in HNSCC [26], and had not been identified by Sanger sequencing due to its large size (34 coding exons), despite it being previously shown to be important in functional studies in cutaneous SCC [27].

More targeted approaches have also been undertaken to explore the cancer genome of HNSCC. Mahjabeen et al. sequenced 17 exons of *XRCC1* in HNC patients and matched controls, and found two silent mutations in 45% and two missense mutations in 55% of cases. This accounted for a total mutation frequency of 87%. These silent mutations were distributed equally among smokers and non-smokers and amongst males and females [28]. Scheckenbach and colleagues sequenced exons and adjacent introns of *RAD51C* and showed five distinct heterozygous sequence alterations in 5.8% of HNSCC cases [29]. Transcriptional profiling of oropharyngeal SCC and matching normal samples showed *TP53* mutation along with CHEK2 and ATR over-expression (both p53 DNA damage repair pathway gene targets) in HPV-negative current smokers compared to past or non-smokers [30].

Given the heterogeneous nature of HNC generally and HNSCC specifically, there is great expectation that targeted therapies underpinned by deeper understanding of the molecular oncological pathways in these tumours, will result in better outcomes for patients. The promise of more effective therapeutic options for patients suffering from these tumours cannot be overstated, given the poor outcomes associated with advanced HNSCC. The potential of molecular profiling is tremendous, with the underlying goal of achieving "genome-informed personalised medicine" where a family-based disease history, gene expression pathways, and drug resistance and toxicity is considered in formulating a treatment plan [31]. Understanding of the mutational and genomic landscape of HNSCC [32] has enabled better use of therapies such as EGFR inhibitors and, more recently, immune checkpoint inhibitors [33], and will continue to drive innovations in targeted druggable approaches. This paper summarises the molecular pathways and druggable targets involved in head and neck cancer as a precursor to understanding current and emerging therapies in head and neck oncology, as detailed by us elsewhere [34].

2. Molecular Pathways and Druggable Targets

2.1. EGFR Pathway

Epidermal growth factor receptor (EGFR/HER1/ErbB1) is a 170-kDa transmembrane glycoprotein and one of four members of ErbB receptor tyrosine kinase family. Other members include HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). EGFR has an important role in initiating the signalling that directs the behaviour of epithelial cells and tumours of epithelial origin. The EGFR signalling pathway is crucial in mammalian cells and regulates proliferation, migration, differentiation, and apoptosis as well as intercellular signalling during development. Binding of ligands and growth factors including EGF, transforming growth factor (TGF)-α and amphiregulin induces both hetero- (i.e., EGFR-HER2) and homo-dimerisation (EGFR-EGFR) of tyrosine kinases, which leads to their intracellular phosphorylation and activation of downstream signal transduction cascades [35,36]. Signalling proteins which have src homology-2 (SH2) or phosphotyrosine binding (PTB) domains can bind to the EGFR family members after tyrosine phosphorylation. The most important activated pathways are Ras/Raf/MEK/mitogen-activated protein kinase (MAPK)/ERK, phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), and Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathways [37,38]. These pathways are implicated in tumour cell growth/survival, proliferation, local invasion, angiogenesis, metastasis, protein translation and cell metabolism [38,39]. EGF-bound EGFR can also translocate to the nucleus to function as a transcription factor and one of its nuclear targets, *CCND1*, encodes cyclin D1 which is involved in cell cycle progression [40]. Aberration of EGFR signal activation, transduction, duration and intensity can result in disruption of cancer cell homeostasis.

EGFR family members are involved in a wide range of human diseases from psoriasis, cancer, inflammation, heart diseases to Alzheimer's disease. Mutations in EGFR have been associated with adenocarcinoma of the lung, glioblastoma, and head and neck epithelial tumours. Mutations leading to overexpression lead to constant activation and consequently uncontrolled cell division, and its family members are implicated in about 30% of all epithelial cancers. Over expression of EGFR and its hetero-dimerisation with HER2 have been shown to be of prognostic value in HNSCC [41–44]. EGFR overexpression is mainly at the transcriptional level, as there is only up to 7% and 30% *EGFR* gene point mutation and amplification reported in HNSCC cases, respectively [44–47]. A mutant variant of EGFR (EGFRvIII) with 2–7 exons missing in the extracellular region has been reported in 42% of HNSCC patients [48]. Interestingly, *EGFR* gene copy number has not been found to be an efficient biomarker for EGFR-directed therapy [49] and it has been associated with poorer prognosis and patient outcome [49,50]. Hence EGFR and HER2 are feasible targets for cancer therapy, and agents targeting these molecules have been FDA-approved for HNSCC treatment, demonstrating increased response rates and increased overall survival when combined with standard therapy [51].

2.2. PI3K/AKT/mTOR Pathway

The PI3K/AKT/mTOR signalling pathway is one of EGFR downstream signalling networks and plays an essential role in cellular regulatory mechanisms including cell growth, differentiation, survival, proliferation, migration and glucose metabolism [52–54]. The PI3K family of lipid and protein kinases is divided into three classes, Class I, II and III, based on their primary structure, regulation, and lipid substrate specificity; but only Class I kinases function as second messengers in intracellular signalling pathways and are most frequently associated with oncogenesis. Class I PI3K is a heterodimer protein comprised of two main subunits (with several isoforms) of 110-kDa (p110) and 85-kDa (p85) which mediate enzyme catalytic and regulatory activity, respectively [35,55]. The p110 subunit exists in three isoforms, p110 α (encoded by *PIK3CA*), β (encoded by *PIK3CB*) and δ (encoded by *PIK3CD*), while the p85 subunit is encoded by three genes, *PIK3R1* (p85α), *PIK3R2* (p85β) and *PIK3R3* (p85γ) [55].

Following activation by receptor-associated tyrosine kinases (RTKs) such as EGFR, the catalytic subunit phosphorylates 3′-hydroxyl group of phosphatydilinositol 1,4-bisphosphate (PIP_2) to form phosphatydilinositol 1,4,5-triphosphate (PIP_3). Then, PIP3 calls pleckstrin-homology domain-containing proteins including phosphoinositide-dependent protein kinase 1 (PDK1) and AKT to the plasma membrane which results in phosphorylation of AKT by PDK1 and mammalian target of rapamycin complex 2 (mTORC2). Activation of AKT and, consequently, mTORC1 activates ribosomal protein S6 kinase 1 (S6) and inactivates eukaryotic translation inhibition factor 4E-binding protein 1 (4E-BP1) resulting in protein synthesis, cell growth and proliferation [35,56]. However, the tumour suppressor phosphatase and tensin homology (PTEN) tightly regulates the cellular level of PIP_3 by converting it to PIP_2 through its lipid phosphatase activity, and therefore counteracting the activation of AKT and its downstream pathways [57].

Genetic aberrations of the PI3K/AKT/mTOR pathway are very common in head and neck cancers [58]. In particular, as genome sequencing findings in 2011 revealed, pathway activation is frequently (6% to 20% of HNSCCs) mediated by mutations in *PI3KCA* which codes for p110α with more than 80% of mutations occurring in exon 9 (helical domain) as well as mutations in exon 20 (kinase domain) and exon 4, especially through the mechanisms of gene amplification and low-level copy number increase [15,16]. In addition, copy number increase in 3q26 has been reported as a common and early oncogenic event in almost half of HNSCC cases [59,60] which has been associated with a more invasive phenotype [61], vascular invasion [62] and higher chance of lymph node metastasis [60]. Additionally, *PTEN* mutations have been reported in 10% of HNSCC cases [63]; however, mutations may not be the primary mechanism for *PTEN* loss in HNSCC [64]. Moreover, the mTOR pathway can be independently activated from EGFR activation or mutant p53 presence, especially in patients with HPV-positive tumours [58].

2.3. RAS/RAF/MEK/MAPK Pathway

The RAS/MEK/ERK (MAPK) signal transduction pathway activates many important cellular mediators leading to cell growth, proliferation, differentiation, migration, invasion and survival [65–67]. Ras is a guanosine nucleotide binding protein (GTPase) localised at the intracellular side of the plasma membrane and is activated by TRKs including EGFR. The *Ras* gene family consists of three members, *HRAS* (Harvey), *KRAS* (Kristen) and *NRAS* (neuroblastoma) [26,35,68]. Once Ras becomes activated, it converts from guanosine diphosphate-bound (GDP-bound) inactive state to guanosine triphosphate-bound (GTP-bound) active state which activates RAF (RAF1) pathway as well as PI3K pathway [69]. RAF exists in three isoforms including ARAF, BRAF and CRAF (RAF1) [70]. Although they all activate MEK, it has been shown that they might be differentially activated by oncogenic RAS [71]. Activated RAF phosphorylates MEK1 and MEK2 kinases that, in turn, activate MAP kinases ERK1 and ERK2, which may either phosphorylate cytoplasmic targets or translocate into the nucleus to target genes affecting mechanisms such as cell growth, proliferation and survival [26,35,72].

Oncogenic RAS mutations are implicated in almost 30% of all cancer types (http://cancer.sanger.ac.uk, accessed on 7 February 2021) [69] which prevents switching of GTP and GDP, keeping RAS in its constitutive GTP-bound active state and consequently active downstream signalling results in proliferation and survival [73]. *KRAS* mutations have been reported for almost 90% of pancreatic adenocarcinomas, occurring mainly in exon 12 as well as in colorectal and lung cancers while *HRAS* and *NRAS* mutations are rarely seen in those conditions. However, *NRAS* and *HRAS* mutations are frequently seen in melanoma and salivary gland tumours, respectively [74]. The work of Stransky and Agrawal independently revealed that *HRAS* mutations are one of the most common mutations (6th and 8th) and were found in 4–6% of HNSCC cases [15,16].

Among all RAF mutations, *BRAF* mutations have been associated with many cancers including nervous system, melanoma and thyroid (http://cancer.sanger.ac.uk, accessed on 7 February 2021). One of the most common *BRAF* mutations is a single-base missense substitution of T to A at nucleotide 1799 in exon 15 (known as hot spot), that substitutes valine for glutamic acid at amino acid 600 (V600E) in the kinase domain of protein which results [75,76] in impaired kinase activity in a RAF1-dependant manner and generating kinase resistance to feedback inhibition and activating the MAPK pathway [77]. In 2003, Weber et al. performed direct DNA sequencing on 89 HNSCCs and showed that somatic *BRAF* mutations were relatively rare (approximately 3%; 3/89 cases), and all mutations were in exons 11 and 15. In addition to BRAF V600E mutations, *BRAF* mutations in 1403 nucleotide, resulting in substitution of G to T and therefore replacing alanine with glycine, were detected. They also found that heterozygous mutations of *KRAS2* occur in 6% of HNSCC within exons 12 and 13. Their results confirmed that *KRAS2* and *BRAF* mutations do not co-exist in HNSCC; hence, oncogenic *KRAS2* activates wild-type *BRAF*, but mutated *BRAF* does not require *KRAS2* for activation, suggesting simultaneous mutations may be redundant [65].

2.4. NOTCH Pathway

As with PI3K/AKT and MAPK, NOTCH pathway is a conserved signal transduction cascade which affects cell function such as self-renewal capacity, cell differentiation and survival in a cell- and context-specific manner [35,78]. The NOTCH family consists of four receptors which are bound to the cell membrane (NOTCH1-4) and interact with two families of ligands including Delta-like (Dll1, DllL3, Dll4) and Jagged (Jag1 and Jag2) ligands [79]. Ligand binding to NOTCH receptors leads to NOTCH cleavage by TNFα-converting enzyme (TACE) and γ-secretase which releases the NOTCH intracellular domain (NICD). The NICD consists of several domains including JM, RAM, anykrin repeats (ANK) and transcriptional activation domain (TAD); however, TAD is lacking for Notch 3 and Notch 4. Adjacent to the carboxyl-terminus of the NICD lies the PEST domain, which can be ubiquitinated by the FBXW7-containing E3 ubiquitin ligase complex resulting in NICD destruction [78]. The NICD regulates transcription of target genes such as *HRT* and *HES* families through the NOTCH ANK and NOTCH RAM motif interactions with transcriptional machinery partners, including the CBF1, Su(H), Lag-1 (CSL) DNA-binding transcription factor and Mastermind transcriptional coactivators (MAML1–3), respectively [80–83].

So far, a dual biological activity of Notch signalling has been reported in solid tumours. Preliminary reports of *NOTCH1* mutations in T cell acute lymphoblastic leukaemia (TALL) and chronic hematopoietic cancers were implicated as oncogenic [84–87]. Genome sequencing of HNSCC suggested *NOTCH1* acts as a tumour suppressor gene, and was the second most frequently mutated with an incidence of 15–19% [15,16]. In 2016, several *NOTCH1* somatic mutations were identified using whole-exome sequencing and were validated in a 13-year cohort of 128 HNSCC patients [88].

Recent integrated analysis has identified the deficiency of NOTCH pathway in 66% of HNSCC patients [89]. NOTCH1 signalling promotes terminal differentiation of keratinocytes and is negatively regulated by EGFR pathway. EGFR-activated c-jun suppresses

p53 and NOTCH1 whereas blockade of EGFR induces keratinocyte differentiation in cutaneous SCC [90]. Moreover, in basal epithelial cells, NOTCH1 has been inhibited by p53-related transcription factor p63 (TP63) where its downregulation during terminal differentiation results in NOTCH1 upregulation. Overexpression and amplification of TP63 has been reported in many HNSCC cases [91]. Additionally, it has been observed that *NOTCH1* is significantly reduced in HPV-positive cervical carcinoma cells as expression of activated NOTCH1 results in strong growth inhibition of these cells via down-modulation of transcription of the E6/E7 viral genes. Thus, NOTCH1 expression is likely to play a protective role in late stages of HPV-induced carcinogenesis [92]; however, its role in HPV-induced HNSCC should be investigated further.

Not only can *NOTCH1*, *NOTCH2* and *NOTCH3* (3–5% of cases) mutations contribute to modulate the NOTCH pathway [15,16], but JAG1, JAG2, MUMB and MAL1 chromosomal aberrations have been reported to be involved as well [89]. *FBXW7* mutations have been identified in 5% of HNSCC for the first time, mainly in a hotspot known to block the degradation of active *NOTCH1* [15,16]. It is noteworthy that mutations in *FBXW7* may also target other cancer-related proteins including cyclin E and c-myc [15].

2.5. MET Pathway

Mesenchymal-epithelial transition factor (MET) is a receptor tyrosine kinase (RTK) which is encoded by *c-MET* proto-oncogene located on chromosome 7q21-q31 [93,94]. MET contains several functional domains including juxtamembrane (JM) (regulatory domain), receptor tyrosine kinase domain (TK) and semaphorin (SEMA) which binds to its ligand, hepatocyte growth factor (HGF) [95,96]. MET-HGF binding induces MET dimerisation, autophosphorylation and activation of TK catalytic activity which activates other cellular signalling pathways including RAS/RAF/ERK, PI3K/AKT/mTOR, JAK/STAT and NOTCH which result in cell growth, motility and survival [94,96–98].

Overexpression of MET has been detected in solid tumours such as 57% of SCC [99,100] which has been associated with enhanced cell motility, angiogenesis and invasion/metastasis as well as aggressive phenotype and poor prognosis [95,96]. MET/HGF overexpression and increased MET copy number have been found in over 80% and 13% of HNSCC cases, respectively [101–103]. HPV-positivity of head and neck tumours is usually accompanied by p16 positivity and can have very different outcomes when compared to HPV-negative cancers [104]. Although in some studies Met expression has been reported to be a prognostic biomarker in HPV-negative HNSCC [105,106], others have found no statistically significant correlation between c-Met positivity and p16 positivity [103].

MET mutations for HNC have been reported mostly in the TK domain with up to 25% of lymph node metastasis association [96,107]. Although a large number of mutations in JK domain have been detected for cancers including small-cell lung cancer (SCLC) [99,108], not many reports are available for mutations in SEMA and JM domains in HNSCC [102].

2.6. JAK/STAT Pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway mediates mechanisms including cell growth, proliferation, differentiation, survival, angiogenesis and inflammatory/immune responses via transmitting signals from the plasma membrane to the nucleus [109]. The JAK family is a non-receptor tyrosine kinase and consists of four members including JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) which each has seven regions known as Janus homology domains 1 to 7 (JH1-7). The JH1 domain is located at the C-terminal end of the protein and has tyrosine kinase activity containing conserved tyrosines (e.g., Y1038/Y1039 in JAK1, Y1007/Y1008 in JAK2, Y980/Y981 in JAK3, and Y1054/Y1055 in TYK2). The JH2 has tyrosine kinase structure but does not have the enzymatic activity (pseudokinase domain) and it regulates JH1 activity. The JH3–JH4 domains share homology with Src-homology-2 (SH2) domains. The JH4-JH7 domains are assumed kinase domains with JH6 and JH7 domains (N-terminal of JAK) being associated with cytokine receptor binding [110–112].

The STAT proteins are a family of transcription factors with seven identified members, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 which each has six conserved domains including oligomerisation domain at the N-terminal of protein, coiled coil domain, DNA binding domain, linker domain, SH2 domain and transactivation domain at the C-terminal. Additionally, all STATs have an essential tyrosine near SH2 domain which is critical for their dimerisation, nuclear translocation and DNA binding. Moreover, STAT1, 3 and 5 can also be phosphorylated at a C-terminal serine (Y727 in STAT3) to achieve their maximum transcriptional activity [113]. Extracellular binding of cytokines to their receptors induces activation of the intracellular JAK activation to recruit and phosphorylate STAT proteins resulting in STAT dimerisation via their SH2 domain and then translocation to the nucleus to activate transcription of their target genes. JAKs can also be phosphorylated directly by RTKs such as EGFR and also G-protein-coupled receptors (GCPRs) to activate downstream signal cascades including RAS/MAPK and PI3K/AKT pathways [114,115]. STATs can also be activated independently of JAKs by growth factor receptors such as activation of STAT1, 3 and 5 by EGFR [116–118] and STAT5 by the platelet-derived growth factor receptor (PDGFR) [119]. Additionally, STAT3 can be activated by non-receptor tyrosine kinases such as c-Src and Abl [120,121].

Among all STATs, STAT1, 3 and 5 have been most implicated in tumorigenesis including in head and neck cancers [122]. Activation of STAT3 stimulates tumour-associated angiogenesis via modulating the stability and activity of hypoxia-inducible factor-1α (HIF-1α) as well as enhancing VEGF expression by binding to the VEGF promoter with HIF-1α [123,124]. STAT3 also promotes cell invasion/metastasis by activating the transcription of matrix metalloproteinase 1 (MMP1), MMP2, MMP9, and MMP10 [117,125–128]. In addition, STAT3 can suppress p53 expression [129] and expression of pro-inflammatory cytokines and chemokines necessary for immune-mediated tumour rejection including IL-12, TNF, IFN-γ, and IFN-β [130,131].

The SOCS1-7 protein family, also known as STAT-induced STAT inhibitors (SSIs), are members of cytokine-inducible negative regulators of cytokine signalling affecting JAK/STAT pathway by JAK activation and constitutive signalling [132]. In lung cancer, SOCS-3 acts as a growth suppressor and apoptosis inducer [133]. Methylation of SOCS-1 has been shown in 65% of hepatocellular carcinoma (HCC) cases (Yoshikawa et al., 2001). Hyper-methylation of SOCS-1 and SOCS-3 promoters was shown to be correlated with SOCS-1 and SOCS-3 gene silencing in HNSCC samples [134,135]. Increased EGFR signalling and STAT3 activation and overexpression has been detected in HNSCC [136]. STAT3 has also been found to be a Src-dependent mediator of EGFR-stimulated growth of HNSCC in vitro and decreased apoptosis and increased tumour growth in vivo [136,137]. Src family kinases can contribute to gastrin-releasing (GRP)-mediated EGFR growth and invasion pathways by facilitating cleavage and release of TGF-α and amphiregulin in HNSCC [138,139]. However, another study showed that after durable Src inhibition in HNSCC cell lines, STAT3 was reactivated through a compensatory pathway via altered JAK-STAT3 binding and JAK kinase activity [140].

Furthermore, activation of STAT5 has been shown to enhance tumour growth, invasion and epithelial-mesenchymal transition in HNSCC [141]. It was demonstrated that HNSCC cell invasion requires STAT-5A, but not STAT-5B. The activation of STAT-5A can be activated independent from JAK2, but with EGFR activity [142]. Conversely, HNSCC proliferation in vitro and in vivo requires STAT-5B but not STAT-5A [143,144]. Additionally, erythropoietin has been found to mediate activation of STAT5 through JAK2 and contribute to cellular invasion in HNSCC [145]. Moreover, it was shown that after Src inhibition which diminishes SOCS2 expression, STAT5A-mediated SOCS2 expression regulates JAK2 /STAT3 activity and survival signals [146].

2.7. TP53/RB and HPV-Mediated Pathogenesis

The proliferation of mammalian cells is a strictly regulated event and genetic aberrations in each part of this multistep process may confer tumorigenic effects. P53 transcription

factor and retinoblastoma protein (RB) are two prominent tumour suppressors which are also assumed as cell cycle regulators [147]. The TP53 gene (*TP53*) is located on the short arm of chromosome 17 (17p13.1) and is involved in many events including glucose metabolism in cancer cells, and DNA-repair and apoptosis [148,149]. The level of p53 is regulated by mouse double minute 2 homolog (MDM2) which binds to the N-terminal of protein and after its ubiquitination degrades p53 as E3 ubiquitin protein ligase [150]. Conversely, *CDKN2A* encodes for p14 (also known as ARF and INK4B) which inhibits MDM2 and protects p53 from degradation [147,151]. Moreover, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) pathways detect DNA damage and activate p53 via phosphorylation of the cell cycle checkpoint kinases CHK1 and CHK2 [152,153]. Activation of p53 results in induction of other cell cycle regulatory and apoptotic genes and proteins including *BAX* and *PUMA* [151,154,155] and p21 (cyclin-dependent kinase inhibitor 1 (CDKN1)) [156,157], p63 and p73 [158,159].

The RB protein is encoded by the *RB1* gene which is located at 13q14.1-q14.2 and prevents the cell division with damaged DNA through G1 restriction by preventing its progression from G1 (first gap phase) to S (synthesis phase) [160]. RB binds to and inhibits E2F transcription factor family from processing through the cell cycle. The mitogenic signals activate cyclin and cyclin-dependent kinases (CDK) including cyclin D1/CDK4/CDK6 complex which results in inactivation of RB via phosphorylation to pRB, followed by additional phosphorylation by cyclin E/CDK2 complex. Cyclin D1-CDK4/6 complex is normally inhibited by p21 (CDKN1) and p16 (also known as INK4A, MTS1 and CDKN2) which is encoded by *CDKN2A*. RB phosphorylation results in release of E2F and cell cycle transition to S, G2 and M phases [147,161].

TP53 mutations are the most common genetic alterations in HNSCC (50–80%), mostly in exons 5 to 8 which encode the DNA binding domain of the protein (L1-L2 region) [15,162,163]. Mutations can also be detected as an early event in premalignant dysplastic lesions and histopathologically negative tumour surgical margins [164–166]. p53 has been associated with increased risk of progression from mild dysplasia to invasive carcinoma [165,167,168]. These mutations can confer either loss of tumour suppressor function or gain of function as a dominant oncogene to promote tumorigenesis via different pathways [169,170]. Genome sequencing data of HNSCC cases have revealed that 50–63% of *TP53* mutations are missense with the remainder (16% nonsense, 16% insertion/deletion, and 8% splice site mutations) predicted to be inactivating mutations [15,16]. According to TCGA, 69.8% of HNSCC showed *TP53* mutations, which makes HNSCC the third most mutated p53 carrier after ovarian cancer and lung squamous cell carcinoma [171]. In 2020, a computational analysis of TP53 mutational landscape using TCGA database showed that 286 HNSCC patients exhibited 129 different kinds of *TP53* mutations [172].

p53, RB, p16 and p14 inactivation through mutation, deletion or epigenetic silencing as well as cyclin D1 (*CCND1* gene), MDM2 and CDK4 overexpression have been associated with tumorigenesis and/or reduced survival in HNSCC [147,151,162,173,174]. Loss of heterozygosity (LOH) of p16 located at 9p21 has been reported in 30% and up to 80% of premalignant and malignant lesions, respectively [175]. While LOH in chromosome 3p has also been shown to be involved in HNSCC [176], co-occurrence of LOH in 3p and 9p can promote differentiation of dysplastic and hyperplastic lesions that are likely to progress to carcinoma [175,177,178]. Moreover, sequencing data have shown that inactivation of *CDKN2A* is associated with mutations and copy number loss found in 7–9% and 20–30% of HNSCC cases, respectively [15,16]. However, it has been shown that *CDKN2A* inactivation via mutation is less common than deletions or epigenetic inactivation, which together cause up to 75% of gene inactivation in HNSCC [179,180].

Activation of wild type p53 and RB in HNSCC can be inhibited by other mechanisms including human papillomavirus (HPV) infection. HPV is a small non-enveloped DNA virus which has been associated with carcinogenesis in both male and female sexual organs [181]. HPV infection has also been identified as a major cause of oropharyngeal squamous cell carcinoma (OPSCC) [182]. The HPV genome consists of eight open reading

frames which encode two late (L1 and L2) capsids and six early (E1–E7) proteins [183,184]. Among more than 100 identified HPV genotypes, types 16, 18, 31, 33 and 45 (high risk HPV) have been most frequently associated with epithelial cell malignant transformation, with type 16 implicated in over 90% of HNSCC cases [181,185]. HPV E6 and E7 oncoproteins are able to engage with p53 and RB protein selectively. HPV E6 interacts with E6-associated protein (E6-AP) and promotes p53 ubiquitin proteasome degradation [186,187]. HPV E7 protein competes with E2F for binding to RB, and since RB acts as a negative regulator for the cyclin-dependent kinase inhibitor p16, overexpression of p16 has been found to be of great clinical value for determination of HPV-positive status using immunohistochemistry (IHC) [188,189]. It is noteworthy that HPV-negative samples may also reveal p16 expression through *RB1* mutation [190,191] which makes p16 staining a less reliable marker [192]. In addition to HPV E6/7 contribution to tumorigenesis in HPV-positive patients, recent sequencing data have shown that HPV-positive and negative oropharyngeal carcinomas cluster into two different subsets of genetic alterations [15,16,190]. HPV-negative HNSCC show *TP53* mutations in almost all samples as well as *CCND1* amplification and *CDKN2A/B* deletion in approximately 50% of tested cases. HPV-positive HNSCC however, show more frequent *PIK3CA*, *PTEN*, *FBXW7* and *KRAS* gene alterations but almost no *TP53* mutations [32,190].

2.8. Cell Cycle Pathway

The eukaryotic cell cycle leads to cell growth and division and consists of two major steps, interphase and M phase. Interphase is a series of events in the cell cytoplasm and nucleus in preparation for cell division and consists of G1, S and G2 phases. Interphase lasts for 91% of the cell cycle. M phase includes mitosis and cytokinesis. Cell chromosomal separation occurs in mitosis, which is immediately followed by cytokinesis, which divides the nuclei, cytoplasm, organelles and cell membrane into two cells with an equal share of cellular components. M phase accounts for approximately 10% of the cell cycle. Two important classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs) are key regulators of the cell cycle.

The G1 phase includes D- and E-type cyclin elevation, activation of cyclin-dependent kinases (CDKs), phosphorylation of the retinoblastoma protein (pRb), and activation of the E2F transcription factor family [32]. In G2 phase, additional cyclin/CDK complexes are activated including cyclin B1/CDC2 complex which stimulates mitotic entry [193].

Cyclin D1 (CD1) is a member of cyclin family, which includes CD1, CD2 and CD3 (also known as CDK4). CD1 is a 45-kDa protein encoded by the *CCND1* gene on chromosome 11q13. Amplification of *CCND1* and overexpression of cyclin D1 have been reported in almost 50% and 80% of OSCCs, respectively [194]. Overexpression of cyclin D1 leads to shortening of the G1 phase and increases independence of growth factors and abnormal cell proliferation, which fosters the occurrence of additional genetic alterations [33]. *CCND1* amplification and cyclin D1 overexpression in HNSCCs have been reported to be associated with a high rate of relapse, lymph node metastasis and shorter patient survival [194].

Cell division protein kinase 6 (CDK6) is an enzyme encoded by *CDK6* gene and is regulated by cyclins, more specifically by Cyclin D proteins and Cyclin-dependent kinase inhibitor proteins. This kinase is a catalytic subunit of the protein kinase complex, important for G1 phase progression and G1/S cell cycle transition. CDK6 and CDK4, phosphorylate and thus regulate the activity of tumour suppressor Retinoblastoma protein, making CDK6 an important protein in cancer development [1].

Aberrant expression of CDK6 protein has been observed in oesophageal and oral SCC [195]. Overexpression of CDK6 was observed in OSCC cell lines as well as OSCC tissue compared with normal oral mucosa and oral leukoplakia tissues with or without dysplasia [195]. CDK6 overexpression in HNSCC was significantly correlated with T classification and resulted in tumour progression [31].

The tumour suppressor *CDKN2A* was identified as the second most commonly altered gene in HNSCC in TCGA [63]. *CDKN2A* (also known as P16, INK4, p16INK4A, and MTS1)

is allelic to chromosome 9p21 and encodes a CDK4/CDK6 kinase inhibitor that constrains cells from progressing through the G1 restriction point. It is thought to be involved in the early stages of HNSCC development. It is affected in up to 80% of HNSCC; often deleted, hyper-methylated, or, more rarely, mutated [196]. *CDKN2A* mutation is considered "noncoding mutation", "inactivation", or "loss of function" [197]. It is associated with worse overall survival in patients with recurrent and metastatic HNSCC [63]. Most mutations in *CDKN2A* are found in exon 2 [196]; however, these are likely insufficient to drive tumorigenesis in their own right [26]. Therapeutic targeting of *CDKN2A* presents a challenge of restoring tumour suppressor activity or inhibiting downstream targets that have been rendered overactive [198].

2.9. DNA Repair Pathway

DNA repair is a series of events by which a cell identifies and corrects DNA damage. Cell cycle checkpoints are activated following DNA damage. Checkpoint activation pauses the cell cycle and gives the cell time to repair the damage before continuing with division. DNA damage checkpoints occur at the G1/S and G2/M boundaries. Checkpoint activation is controlled by two master kinases, ATM (ataxia–telangiectasia mutated) and ATR (ataxia–telangiectasia and Rad3 related) alongside BRCA1, BRCA2, MDC1, and 53BP1 [199]. These proteins are required for transmitting the checkpoint activation signal to a downstream signal transduction cascade and result in cell cycle arrest [200]. Additionally, PALB2 (partner and localiser of BRCA2) interacts with BRCA1 and BRCA2 and plays pivotal roles in DNA repair [201].

Although *BRCA1* and *BRCA2* are among the top 30 of 236 most commonly altered genes in HPV-negative HNSCC [202], somatic mutations in other genes involved in DNA damage in HNSCC are seen at varying frequencies [202]. The frequency of somatic mutations in *BRCA1* (6%), *BRCA2* (7%), *ATR* (4–10%) and *ATM* (1–16%) support the rationale for targeting components of the DNA repair pathway as druggable targets in HNSCC [203].

2.10. Hypoxia and Angiogenesis

In solid tumours, oxygen supply to cells is reduced or abolished due to microvessel structural abnormalities, disturbed microcirculation, and high oxygen consumption associated with high metabolic activities of tumour cells, resulting in tumour hypoxia [204,205]. Tumour hypoxia is a common event in HNSCC and is associated with poor prognosis, treatment resistance and reduced survival in patients [206,207]. Hypoxia-inducible factors (HIFs), including HIF-1α and HIF-2α are ubiquinated and degraded under normoxic conditions by Von Hippel– Lindau protein (VHL). Hypoxia results in HIF stabilisation and heterodimerisation with HIF-2β and translocation into the nucleus act as transcription factors [35,208]. HIF-1α targets genes including phosphoglycerate kinase (PGK), glucose transfer 1 (GLUT1), carbonic anhydrase 9 (CA9), and vascular endothelial growth factor (VEGF) [209,210], while HIF2α mediates EGFR activation [211]. HIFs are partially regulated by mTOR pathway as they show sensitivity to mTOR inhibitors [212].

VEGF (VEGF-A) plays a critical role in angiogenesis and is a member of the platelet-derived growth factor (PDGF) superfamily along with VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PIGF) members [213]. VEGF ligands act through their cell surface tyrosine kinase receptors, VGFR1 (Flt-1), VGFR2 (KDR/Flk-1) and VGFR3; with VGFR2 being the most important receptor involved in proliferation, differentiation and migration of vascular endothelial cells along with VEGF [214]. As with hypoxia, overexpression of VEGF in HNSCC has been associated with more advanced disease, increased resistance to cytotoxic agents and poor prognosis as well as more aggressive phenotype [215].

In addition to VEGF overexpression caused by HIF, hypoxia affects various cellular pathways in different ways which may result in opposing outcomes. For example, hypoxic conditions enhance VEGF mRNA stabilisation by binding proteins to its 3′ untranslated region (UTR) [216] as well as post-transcriptional regulation of VGFR2 while inducing

interaction of VEGF-soluble VGFR1 by overexpression of VGFR1 which inhibits VEGF action [217,218]. Moreover, hypoxia can induce apoptosis in oncogenically transformed cells with HPV E6/E7 or c-myc genes [219,220]. Furthermore, it has been shown that p53 levels increase in hypoxic conditions which induce apoptosis influencing Apaf1 and caspase9 pathways [221]. Hypoxia can also promote apoptosis via a p53-independent pathway, involving HIF-1 and downregulation of expression of the anti-apoptotic BCL-2 family [222,223].

Dysplastic oral tissue displays higher expression of angiogenic cytokines such as VEGF, IL8/CXCL8, FGF2, and HGF compared to normal samples. In HNSCC, there may be two potentially distinct pathways involved in angiogenesis. Samples showed either higher levels of VEGF and FGF2 which were associated with higher angiogenic index, or expressed lower levels of VEGF and FGF2 with higher levels of IL8/CXCL8 and HGF [224]. The multiplicity of HIF induced angiogenesis mechanisms implies that not only is the same angiogenesis phenotype a result of different molecular mechanisms, but that understanding the variability of the angiogenic phenotypes may lead to design of more targeted anti-angiogenic therapies.

2.11. Host Immunity

Cancer immunotherapy is based on the fact that the host immune system detects abnormal cells and activates the cytotoxic potential of immune cells, especially tumour specific cytotoxic T cells, to eliminate cancer cells [225]. T cells target tumour cells by activation via dual signalling pathways. The first signal is through the T cell receptor (TCR) which recognises MHC-antigen on tumour cells. The second signal is induced by interaction of co-stimulatory factors, B7 molecule on the surface of antigen presenting cells (APC) and CD28 on the surface of T cells. Without either of these two signals, T cells cannot be activated [226]. Immune check points regulate the activation of T cells and initiation and termination of host immune responses.

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is mainly expressed on T cells and to a lesser extent in active B cells, monocytes, granulocytes and dendritic cells (DCs). CTLA-4 is also expressed on regulatory T cells (Tregs) and produces the immunosuppressive molecule transforming growth factor-β (TGF-β) when activated with CD28 [227]. CTLA-4 (CD152) binds to B7 protein to induce T cell dysfunction and participate in negative regulation of the immune response. Under normal circumstances, the immunosuppressive effect of CTLA-4 is to stimulate the immune response effectively without excessive damage to normal tissues. However, cancer cells secrete TGF-β, which can stimulate the expression of CTLA-4, leading to T cell exhaustion [228]. T cell exhaustion is a state in which T cells have weak functions and might exert immunosuppression. The affinity of CD28 for CTLA-4 on the surface of T cells exceeds its affinity for the co-stimulatory molecules CD80 and CD86. Thus, T cells are prevented from proliferating and fail to function.

Programmed cell death protein-1 (PD-1) belongs to the CD28 receptor family and is mainly expressed on activated T cells and B cells. It is also found on monocytes and a small fraction of thymocytes. Ligands for PD-1 include programmed death-ligand 1 and 2 (PD-L1, PD-L2). Both are expressed on antigen-presenting cells, endothelial cells and activated lymphocytes [229]. PD-L1 is inducible and constitutively expressed in many malignancies despite limited expression in normal tissue, and its overexpression in malignant cells can promote tumour formation [230]. In melanoma and non-small-cell lung carcinoma (NSCLC), high expression of PD-L1 on tumour cells has been strongly associated with both high tumour grade and poor prognosis [231,232]. HNSCC tissues produce PD-L1 through an abnormal PD-1 signalling pathway, which leads to tumour immunosuppression [233], with the PD-1/PD-L1 pathway being activated in a chronic inflammatory environment. This axis contributes to the formation of HPV-positive HNSCC, whereby HPV-positive HNSCC tissues express more lymphocytes and higher levels of PD-L1 compared to HPV-negative HNSCC, while infiltrating CTLs express more PD-1 [234]. PD-L1 also delivers immunosuppressive signals by binding to T cells via the CD80 receptor. Depending on

the HNSCC tumour immune environment, the PD-1/PD-L1 axis can be blocked by either targeting PD-1, thereby inhibiting its binding to PD-L1/PD-L2, or targeting PD-L1 to inhibit its binding to PD-1/CD80.

The PD-L1 signalling pathway not only downregulates anti-tumour T cell function but also affects cellular interactions between the innate and adaptive immune responses. Interactions between PD-1 and PD-L1 can regulate the tumour microenvironment by modulating the effects of T cells, DCs, myeloid-derived suppressor cells (MDSCs) and Tregs. Blocking PD-1 on Tregs thus inhibits their ability to mediate immune tolerance [235]. PD-1 expression on effector T cells increases sensitivity to the PD-L1 death signalling pathway.

3. Conclusions and Future Directions

Head and neck cancer is a highly heterogeneous tumour type, hence individual management should be based on both patient and tumour characteristics. To date, the optimal treatment for HNSCC patients involves a multidisciplinary approach including coordination of surgery, chemotherapy, radiation therapy and systemic therapies. Exploration of the molecular landscape of head and neck cancers utilising next generation sequencing has confirmed previously known HNSCC genome alterations such as mutations in *TP53*, *CDKN2A*, *PIK3CA*, *PTEN*, and *HRAS*, but also identified novel genes such as *NOTCH1*, *XRCC1*, and *RAD51C*. While *TP53* mutations are the most common genetic alterations in HNSCC, inactivation of p16 continues to be an important target in HPV-positive tumours, while exploration of DNA damage repair genes and synthetic lethality is emerging as a promising approach in combating HPV-negative tumours. Our ever-increasing understanding of etiological factors, tumour and patient characteristics, and the underlying molecular mutations in stratified tumours from different head and neck anatomical locations will continue to expand the development of therapeutic options against druggable targets to improve patient outcomes and control the personal and economic burden of head and neck cancer.

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