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Molecular Biology of Oral Cavity Squamous Cell Carcinoma

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Summary

Oral cavity squamous cell carcinoma (OCSCC) is a heterogeneous and complex disease that arises due to dysfunction of multiple molecular signaling pathways. Recent advances in high-throughput genetic sequencing technologies coupled with innovative analytical techniques have begun to characterize the molecular determinants driving OCSCC. An understanding of the key molecular signaling networks underlying the initiation and progression of is essential for informing treatment of the disease. In this chapter, we discuss recent findings of key genes altered in OCSCC and potential treatments targeting these genes.

Introduction

All malignancies are endowed with specific capabilities that allow for continued proliferation, invasion and metastasis. In a hallmark review by Hanahan and Weinberg, it is suggested that there are six essential fundamental processes involved in the maintenance of any malignancy, including the resistance of cell death, continued proliferation, evasion of growth suppressors, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis [1]. These processes are driven by a complex network of molecular signaling pathways that are daunting to scientists and physicians alike.

The discovery of human papillomavirus (HPV) as a key driver of a subset of oropharyngeal squamous cell carcinomas has recently sparked a significant amount of research and interest.

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Conflict of interest

None declared

HPV status informs treatment, as overall survival and prognosis are greater for HPV-positive oropharyngeal squamous cell carcinomas compared to those that are HPV-negative. However, whereas 70.1% of oropharyngeal squamous cell carcinomas are caused by HPV [2], oral cavity squamous cell carcinoma (OCSCC) has proven itself to be a separate disease in that it is almost always negative for HPV. As such, designing treatments are difficult as there is no single predominant pattern or molecular driver of the OCSCC progression. However, the same key processes described by Hanahan and Weinberg are in play in oral cavity squamous cell carcinomas. Recent research has begun to reveal the ways in which the molecular players controlling these processes are dysregulated in OCSCC. For instance, loss of function of p16 and p53 is associated with proliferation and evasion of growth suppressors, dysregulation of the Notch and WNT pathways may assist in enabling replicative immortality and avoiding cell differentiation, and the AKT pathway plays key roles in invasion and metastasis. In this review, we provide an overview of our current understanding of these and other molecular players involved in OCSCC tumorigenesis, both at the genetic and epigenetic levels. We also discuss factors that contribute to invasion and metastasis of established oral malignancies, as well as recent advances in targeted therapy and biomarkers predicting response to treatment. It is essential for any practicing clinician who takes care of oral cancer patients to have a basic understanding of these pathways, as these will likely form the basis for therapies down the road.

Molecular Genetics

p16/Cyclin D1/pRb/p53

Two major tumor suppressor proteins, p16 and p53, are frequently inactivated in head and neck cancer (Figure 1) [3–6]. p16, which is encoded by the *CDKN2A* gene located on chromosome 9p21, is a tumor suppressor protein that is involved in cell growth and cell cycle control. Specifically, it blocks progression from the G1 to S phase of the cell cycle by inhibiting another protein called Cyclin D1. Thus, disruption of p16 activity results in a loss of cell senescence, subsequently leading to dysplasia. Similar to other types of H&N malignancies, OCSCC is often negative for p16 [7,8]. Moreover, OCSCC patients with p16 inactivation have significantly lower survival than patients with normal or amplified levels of p16 [9].

CCND1 is a gene which encodes Cyclin D1 protein, whose activity is inhibited by p16. CCND1 is amplified in 25–43% of OCSCCs [10–12], whereas its product, Cyclin D1, was found to be overexpressed at early stages of oral carcinogenesis and is associated with tumorigenic proliferation [13]. Specifically, the cytoplasmic Cyclin D1 expression was increased in OCSCC tumors with advanced stages, poor differentiation, increased mitosis, and invasive cell morphology, suggesting that elevated cytoplasmic cyclin D1 levels may promote cell migration and invasion [14]. Moreover, several studies showed that CCND1 amplification and cyclin D1 expression were associated with decreased survival and poor prognosis in OCSCC patients [10,14–16].

Inhibition of Cyclin D1 suppresses activation of the cyclin dependent kinases CDK4 and CDK6, which drive cell cycle progression by preventing the phosphorylation and inactivation of pRb. pRb is a tumor suppressor protein encoded by *RB1*; in its

hypophosphorylated state, pRb prevents cell cycle progression from the G1 to S phase [17,18]. Dysregulation of pRb occurs early in oral epithelial dysplasia and is associated with higher likelihood of transformation to malignant carcinoma [19].

Inactivation of the p53 protein, which is encoded by the gene *TP53* (located on chromosome 17p12), plays a prominent role in the pathogenesis of various solid malignancies including head and neck cancers. p53 regulates the cell cycle; cellular stress such as DNA damage causes translocation of p53 to the nucleus, where it plays role in multiple cellular processes including cell growth arrest or apoptosis [5,20]. Mutations in exon 4 or intron 6 of the *TP53* [21,22] are commonly detected in the majority (84%) of H&N cancers, including OCSCC [5],

p53 is a useful prognosticator of OCSCC. Its expression is correlated with tumor stage and grade, and also predicts the presence of dysplastic surgical margins in early OCSCC [23,24]. However, *TP53* expression is not correlated with lymph node metastasis [23]. p53 interacts with a complex network of proteins to regulate the cell cycle progression, apoptosis and differentiation. It was recently found to be co-expressed with platelet-derived growth factor receptor A (PDGFRα), a stimulator of cell growth, in many poorly differentiated OCSCCs, suggesting that the two proteins may cooperate to increase tumor aggressiveness [25]. Thus, potential treatments restoring the activity of p53 are currently being explored. Operculina turpathum extract has been found to upregulate p53, thus inducing apoptosis, and also downregulates cyclin D1, causing cell cycle arrest [26]. *Cis*-3-*O*-p-hydroxycinnamoyl ursolic acid (HCUA) also causes p53-mediated apoptosis in oral cancer cells, and thus may have an antitumor effect [27].

Notch

Notch signaling is a highly conserved pathway of communication between neighboring cells, regulating cell proliferation and fate [28]. Activating mutations in NOTCH1 are present in some cancers, particularly in human T cell acute lymphoblastic leukemia [29,30]. On the other hand, inactivating mutations in NOTCH1 are present in 11–19% of HNSCC tumors, suggesting that NOTCH1 may act as a tumor suppressor in HNSCC, in contrast with its proto-oncogenic role in other cancers [5,20,31].

NOTCH1 is physiologically expressed in basal cells of the oral squamous epithelium, and its expression is inhibited in oral cancer and oral epithelial dysplasia [32]. Pickering and colleagues found that 9% of patients with OCSCC harbor inactivating mutations in NOTCH1 and that OCSCC proliferation *in vitro* was inhibited by functional NOTCH1 signaling [33]. In their study, 66% of OCSCC patients had defective signaling in at least one member of the Notch signaling pathway. Higher rates of NOTCH1 mutation (43%) were found in Chinese patients with OSC [34], whereas NOTCH1 mutations were uncommon in Singaporean patients with oral tongue squamous cell carcinoma [35], suggesting that NOTCH1 inactivation may have complex genetic interactions in the pathogenesis of OCSCC.

Interestingly, activation of the Notch signaling pathway may also promote OCSCC progression in some cases. Two groups found that NOTCH1 expression was increased in

OCSCCs [36,37]. In these studies, NOTCH1 expression was correlated with T-stage and clinical stage, and depletion of Notch1 caused decreased cell proliferation. Additionally, overexpression of Nrf2 (which activates the Notch signaling pathway among its other activities) in OCSCC cells was found to promote a cancer phenotype [38]. Thus, tumorigenesis may arise from multiple forms of dysregulation of the Notch signaling pathway, suggesting that its careful regulation is essential for proper cell function.

Wnt/β-catenin

The Wnt/β-catenin signaling pathway is a conserved pathway that regulates cell fate determination, proliferation, and differentiation. Upregulation of the pathway signaling leads to oncogenesis in OCSCC, often through multiple mechanisms stemming from aberrant activation of β-catenin. Mutations in *CTNNB1*, which encodes β-catenin, are rare in HNSCC; however, inactivating mutations in NOTCH1 and FAT1 signaling prevent their inhibition of *CTNNB1* expression [5].

Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that, when activated, upregulates several downstream signaling pathways, including MAPK, AKT, ERK, and Jak/STAT, which are essential for tumor growth, proliferation, apoptosis, survival, angiogenesis, invasion, and metastasis [39–43]. EGFR levels are elevated in more than 90% of patients with HNSCC [44]. High expression of EGFR is associated with tumor aggressiveness and poor survival of patients with OCSCC [45–47], including specifically oral tongue cancers [48,49]. Additionally, EGFR expression in the nucleus of more than 5% of tumor cells was found in 24.3% of OCSCC patients, and was associated with poor survival [50].

The high expression of EGFR in oral cancers makes it an attractive molecular target for treatment. Strategies to target EGFR have been developed, including specific tyrosine kinase inhibitors such as gefitinib and erlotinib; monoclonal antibodies such as cetuximab and panitumumab; and siRNAs, which inhibit mRNA expression [51]. Cetuximab, a structural inhibitor of EGFR signaling [52], has been highly studied, and is the only approved targeted therapy for HNSCCs, including advanced OCSCCs. The combination of cetuximab with platinum-based chemotherapy is approved for first-line use to treat recurrent and metastatic HNSCCs, including OCSCC, and cetuximab alone is a second-line treatment for platinum-resistant HNSCCs [53–55].

Several inhibitors of the tyrosine kinase domain of EGFR have been developed; however, none have shown survival benefit for HNSCCs. Gefitinib has not shown survival benefit for HNSCCs, whether given alone or with chemoradiotherapy [56–58]. Similarly, lapatinib does not show a survival benefit [59,60], and cisplatin and radiotherapy with or without erlotinib showed no difference in progression-free survival or complete response rate [61].

PI3K/AKT/mTOR

The PI3K/AKT/mTOR pathway is a well characterized signaling axis that is known to be involved in proliferation, growth, survival, and drug resistance of multiple cancer types [62].

In the pathway, activation of the PI3 family of protein kinases by tyrosine kinase receptors leads to the formation of second messenger PIP3, which in turn activates AKT and results in activation of multiple downstream targets including mammalian target of rapamycin (mTOR).

Studies have clearly implicated a role of AKT signaling activation in OCSCC. For example, immunohistochemistry analyses have identified staining of AKT, pAKT and pmTOR in a substantial number of OCSCC tumors [63,64], and gene expression profiling has identified PI3K-AKT as one of the pathways commonly enhanced in OCSCC malignancies [65]. Furthermore, somatic copy number alterations in genes encoding the members of the PI3K/AKT/mTOR signaling network are also more commonly seen in patients with OCSCC [66].

Multiple other tumor associated pathways that have been shown to promote growth, survival, invasion or drug resistance via activation of AKT signaling have also been studied in OCSCC. Recently identified contributors activating AKT include ZNF703 [67], FoxM1 [68], PDGF-D [69], Nox1 [70], RACK1 [71], CCL18 [72], and Muc1 [73]. These observations demonstrate that many players impinge on the AKT pathway, which must be tightly controlled to prevent tumorigenesis.

Finally, investigators have demonstrated efficacy of inhibitors of the PI3K/AKT/mTOR pathway in preclinical studies [74–76]. Rapamycin, the canonical inhibitor of mTOR from which the molecule owes its name, has been shown to inhibit oral cavity cancer growth [77]. Conversely, activation of pAKT predicts poorer responses to cetuximab chemotherapy [78].

Epigenetics

Epigenetic modifications to the genome provide one of the major mechanisms underlying gene expression regulation. Processes such as methylation or demethylation of the gene promoter regions play a crucial role in gene silencing or gene expression, respectively [79].The silencing of tumor suppressor genes due to methylation is one of the hallmarks of carcinogenesis [80,81].

Importantly, p16 is frequently inactivated due to promoter hypermethylation in OCSCC, with values between 22–76% of patients with OCSCC having been reported [82–89]. In contrast, the p16 promotor region was found to be methylated in 5.4% of normal mucosa samples, emphasizing the prominence of methylation in the pathogenesis of OCSCC [82]. Methylation of the p16 promoter, as well as other tumor suppressor genes, is likely due to the upregulated expression of the DNA methyltransferases as a result of continuous tobacco use.[90,91]. In tobacco users, 28–58% of precancerous oral tissues demonstrated genome-wide DNA methylation, with levels of methylation increasing throughout the progression to oral cancers [82,92–95].

Many other genes are also hypermethylated at promoter regions in OCSCC, including p14, p15, RASSF1A, RASSF2A, DAPK, SFRP1, CDH1, and MLH1. These genes play important roles in various cancer-promoting signaling networks, such as those including cell cycle arrest and apoptosis, Wnt signaling, cell adhesion, and DNA repair. [85,95–104].

Additionally, in OCSCC, certain microRNAs undergo hypermethylation (i.e. miR-200ab-429, miR-200c-141, miR-205, miR-137) or hypomethylation (i.e. miR-127), which may change their expression and play a role in tumorigenesis [105]. The field of microRNA research is still relatively young; the advent of new technologies for profiling RNA will likely bring about greater understanding of the contributions of microRNA hypermethylation to tumor growth.

The prominence of epigenetic alterations in OCSCC suggests that treatments targeting methyltransferases may prove to be effective. Indeed, the DNA methyltransferase inhibitor azacitidine is approved for myelodysplastic syndrome and acute myeloid leukemia [106]. *In vitro* studies using decitabine (5-aza-2'-deoxycytidine), the deoxy derivative of azacitidine, have shown increased expression of tumor suppressor genes, subsequently decreasing OCSCC growth; thus, potential translational applications may be possible [107,108]. Recent research has also explored the use of the epigenetic modification 5-hydroxymethylcytosine, the oxidative product of methylated cytosine (methylcytosine), as a diagnostic biomarker of HNSCC. For example, circulating tumor DNA of esophageal squamous cell carcinoma, which can be isolated from plasma, has been shown to have a signature genomic pattern of 5-hydroxymethylcytosine on certain genes, including NXN, KIAA0040, and LRRC3B [109], supporting its potential as an avenue to diagnose the disease [109]. Further research on epigenetic alterations in circulating tumor DNA in patients with OCSCC and other HNSCCs may allow early and non-invasive diagnosis of the disease using plasma samples.

Invasion/Metastasis

Metastasis is a complex process where selected malignant cells gain the ability to survive in a distant environment. Its main steps include angiogenesis, or the recruitment of disorganized vasculature, and the epithelial-mesenchymal transition (EMT), the process by which epithelial cells lose cell adhesive properties and gain migratory and invasive properties, thus allowing for seeding of metastatic foci. An understanding of how genomic markers predict risk of both nodal and distant metastasis can be used to inform the approach to treatment.

Angiogenesis is a process that centers around the activation of a protein called vascular endothelial growth factor (VEGF). VEGFA, a member of the VEGF protein family, has shown to be a useful prognosticator for oral tongue SCC [110]. Its overexpression has been shown to be associated with poor survival [111]. In addition, Bevacizumab, a monoclonal antibody against VEGF, shows efficacy in preclinical studies in oral cancer [112,113]. Its concomitant use with irradiation dramatically decreases tumor growth and reduces the number of metastatic nodes. Mechanistically, Bevacizumab has been shown to lower cell migration *in vitro*, in accordance with its role inhibiting VEGF.

EMT specifically involves the reduction in molecules that govern cell adhesion, such as E-cadherin, and the upregulation of mesenchymal proteins such as N-cadherin and cytoskeletal proteins such as Vimentin. In immunohistochemical studies, oral cavity cancers have been shown to have decreased E-cadherin and increased Vimentin expression [114,115], thus suggesting that cancers utilize EMT to invade and metastasize. EMT is also prominently driven by the expression of transforming growth factor beta (TGF-β), which activates cell

spreading and separation of cell borders. Overexpression of TGF-β1 increases the migration and invasiveness of OCSCC cells and promotes malignancy [116,117].

Processes such as EMT, angiogenesis, invasion and metastasis are frequently driven by a combination of tumor cell centric factors and the tumor microenvironment. Most notably, hypoxia is well documented in HNSCC and likely to be central to the metastatic process by induction of EMT [118]. Hif1alpha is a key protein whose expression is promoted by hypoxia, and its expression is associated with lower patient survival [119]. In addition, its activation is correlated with EMT as assessed by *in-vitro* assays for invasion [120].

Invasion and metastasis are driven by complex molecular signaling networks, with multiple interacting pathways that interplay in the process. The PI3K/AKT/mTOR pathway is increasingly being studied as one of the major pathways involved. Overexpression of many of the molecules activating AKT described earlier cause an increase in invasion and migration as demonstrated by EMT marker expression and *in vitro* invasion assays. These include ZNF703, FoxM1, PDGF-D, and CXCL9 [67–69,121].

Although inactivation of the Notch pathway is often seen in OCSCC, activation of the Notch pathway may promote the migration and invasion of OCSCC cell lines and squamous cell carcinoma of the tongue. Weaver and colleagues found that activation of Notch signaling in OCSCC correlates with increased expression of FGF-1, which causes increased cell migration and invasion, as well as increased patient mortality [122]. Notch activity also leads to increased angiogenesis of OCSCCs, contributing to invasion [123]. Moreover, hypoxia upregulates the expression of Notch receptors, whereas inhibition of Notch by γ-secretase inhibitor prevents the invasive effects of hypoxia [124]. Similarly, several other groups found that inhibition of Notch activity due to depletion of genes such as HNF1A-AS1 and Glutaredoxin 3 consequently decreases cell migration and invasion [125,126].

Phosphorylation of β-catenin leads to its dissociation from a complex with E-cadherin, resulting in loss of cell adhesion and subsequent tumor cell invasion, while translocation of β-catenin to the nucleus allows it to act as an oncogenic transcription factor [127,128].

EGFR also plays important roles in tumor invasion, demonstrating overexpression in over 80% of invasive HNSCCs [129,130]. Both EGFR gene copy number and elevated protein levels are associated with tumor stage, depth of invasion, lymph node metastasis, bone invasion, and perineural invasion [47]. Mechanistically, EGFR orchestrates various processes involved in angiogenesis and invasion via several pathways, including Ras/Raf/MAPK, PI3K/AKT/mTOR, and JAK/STAT. EGFR may also cause invasion by transforming neighboring epithelial cells; EGFR-containing extracellular vesicles derived from OCSCCs transform neighboring epithelial cells into mesenchymal cells, an effect that can be targeted and inhibited by the anti-EGFR antibody cetuximab [131].

Immunotherapy

The immune system protects against cancer by identifying and eliminating premalignant tumor cells. Thus, to avoid detection by the immune system, tumor cells may upregulate expression of inhibitory checkpoint receptors such as cytotoxic T lymphocyte antigen 4

(CTLA4), programmed death 1 (PD1), and its ligand PD-L1 [132]. Like other cancers, HNSCCs demonstrate suppression of the immune system, marked by low numbers of white blood cells in the periphery and a suppressive population of tumor infiltrating lymphocytes [133–136]. Thus, recent research has focused on the effectiveness of immunotherapies targeting inhibitory checkpoint receptors in treating HNSCCs [137].

Among the most promising immunotherapies are inhibitors of PD-1 and PD-L1, which cause a durable anti-tumor response and disease stabilization [138]. Pembrolizumab and nivolumab are anti-PD-1 receptor antibodies approved in 2016 by the Food and Drug Administration for treatment of recurrent or metastatic HNSCC that did not respond to treatment with a platinum-based systemic agent. Pembrolizumab demonstrates a modest increase in overall survival and response duration, as well as a less severe side effect profile, compared to standard therapy [139,140]. Similarly, patients with recurrent HNSCC refractory to platinum who were treated with nivolumab had longer overall survival compared to patients treated with standard, single-agent therapy [141]. In addition, the combination of nivolumab and ipilimumab, an antibody against CTLA4, which has been approved by the FDA for treatment of patients with unresectable or metastatic melanoma, has been shown to successfully treat a case of refractory oral tongue SCC [142].

As the majority of cancers continue to progress on PD-1 and PD-L1 inhibitors, the identification of predictive biomarkers is essential for improved treatment. Several prognostic biomarkers that have been extensively studied in HNSCC and other malignancies include PD-L1 expression, tumor mutational burden, and immune gene signatures.

PD-L1 expression has been shown to moderately predict response to treatment across several solid tumors [143], and multiple trials have evaluated its predictive value specifically in HNSCC. Checkmate 141 showed that treatment with nivolumab caused a greater reduction in risk of death in HNSCC patients with ≥1% of tumor cells expressing PD-L1 compared to patients with PD-L1 negative tumors [141]. Importantly, expression of PD-L1 on tumor-associated immune cells was a better predictor of benefit than tumor cell PD-L1 expression alone [144]. Similarly, KEYNOTE-40 showed that HNSCC patients with ≥50% of tumor cells expressing PD-L1 who were treated with pembrolizumab, but not those with <50% of tumor cells expressing PD-L1, had increased overall survival and progression free survival compared to standard of care [139]. KEYNOTE-012 also demonstrated that pembrolizumab treatment showed greater overall response rate in PD-L1-positive versus -negative patients [145].

Tumor mutational burden, a measure of the total number of mutations per coding area of a tumor genome, is also emerging as a prominent biomarker of the response to immunotherapy with PD-1 inhibitors [146,147]. Cancers with greater mutational burden demonstrate increased response to immunotherapy with PD-1 inhibitors, perhaps due to augmented formation of tumor-specific antigens (neoantigens), which may provide targets recognized by the immune system[148–152]. Overall, HNSCCs contain high mutational burden; although the effect of tumor mutational burden on treatment of HNSCCs is still unclear, immunotherapies such as anti-PD-1 or anti-PD-L1 inhibitors may serve as promising approaches [146,147,153].

Finally, the extent to which immune cells in the tumor microenvironment are activated has been characterized as a biomarker for response to treatment with PD-1 and PD-L1 inhibitors. KEYNOTE-012 demonstrated that in HNSCC patients treated with pembrolizumab, expression of six immune genes (interferon gamma and the related genes CXCL9, CXCL10, IDO1, HLA-DRA, and STAT1) showed correlation with response rate and progression free survival [154]. Supporting these observations, an analysis of patients treated with pembrolizumab in KEYNOTE-012 and −055 demonstrated that expression of 18 immune genes correlated with increased response, progression free survival, and overall survival [155].

Conclusion

Here, we have reviewed some of the current understanding and recent findings of molecular pathways prominent in OCSCC. As OCSCC is a complex and heterogeneous disease process (Figure 1), treatment must be guided by identification of the patient-specific aberrations that underlie the disease. Recent advances in high-throughput genomic sequencing technologies and molecular research techniques have shown great potential, both for directing current treatments and identifying new etiologies of the disease. Moreover, new techniques to detect changes at the genomic and epigenetic level early on in the disease may allow more robust diagnosis, further contributing to improved treatment options.

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Highlights

We review the current understanding and recent findings of molecular pathways prominent in OCSCC. As OCSCC is a heterogeneous and complex disease, treatment must be guided by identification of the patient-specific aberrations that underlie the disease. Recent advances in high-throughput genomic sequencing technologies and molecular research techniques have shown great potential, both for directing current treatments and identifying new etiologies of the disease. Moreover, new techniques to detect changes at the genomic and epigenetic level early on in the disease may allow more robust diagnosis, further contributing to improved treatment options.

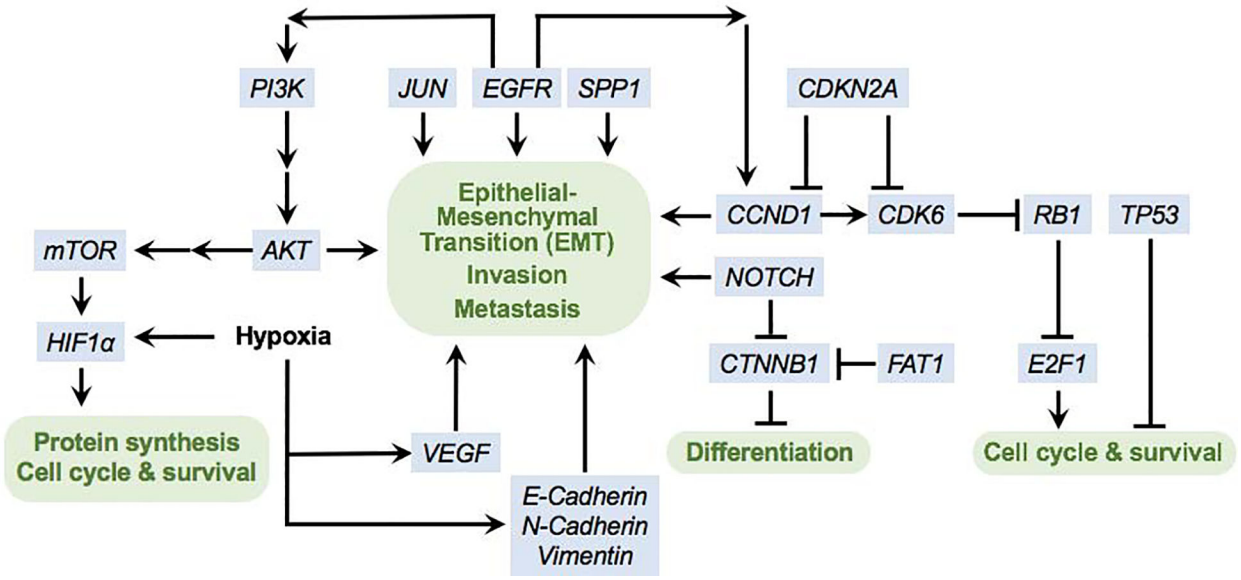

Figure 1. Signaling pathways in oral cavity squamous cell carcinoma.
Partial list of key affected pathways.