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Notch Signaling and Human Papillomavirus–Associated Oral Tumorigenesis

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Abstract

The NOTCH pathway is critical for the development of many cell types including the squamous epithelium lining of cutaneous and mucosal surfaces. In genetically engineered mouse models, Notch1 acts as one of the first steps to commit basal keratinocytes to terminally differentiate. Similarly, in human head and neck squamous cell cancers (HNSCCs), NOTCH1 is often lost consistent with its essential tumor-suppressive role for initiating keratinocyte differentiation. However, constitutive NOTCH1 activity in the epithelium results in expansion of the spinous keratinocyte layers and impaired terminal differentiation is consistent with the role of NOTCH1 as an oncogene in other cancers, especially in T-cell acute lymphoblastic leukemia. We have previously observed that NOTCH1 plays a dual role as both a tumor suppressor and oncogene, depending on the mutational context of the tumor. Namely, gain or loss of NOTCH1 activity promotes the development of human papillomavirus (HPV)–associated cancers. The additional HPV oncogenes likely disrupt the tumor-suppressive activities of NOTCH and enable the oncogenic pathways activated by NOTCH to promote tumor growth. In this review, we detail the role of NOTCH pathway in head and neck cancers with a focus on HPV-associated cancers.

Keywords

Head and Neck Cancers; NOTCH; Human papillomavirus 16

Introduction

Head and neck squamous cell carcinomas (HNSCCs) develop from the squamous mucosal lining of the aerodigestive tract primarily encompassing the nasopharynx, oral cavity, oropharynx, larynx, and hypopharynx (Argiris et al. 2008). HNSCC is the seventh most

common type of cancer, constituting 5% of the all the cancers worldwide (Siegel et al. 2019), and causing more than 50,000 new cases and 12,000 deaths each year in the United States (Argiris et al. 2008). According to the Surveillance Epidemiology and End Results (SEER) database, the 5-year survival rate of patients with HNSCCs approximates 50–60% (Siegel et al. 2019). Despite substantial progress in surgical and radiotherapeutic techniques as well as the introduction of chemotherapy and immunotherapy agents alone or with radiotherapy, survival has only been modestly improved over the past 30 years (Rampias et al. 2014).

HNSCCs are divided into two categories: human papillomavirus (HPV)–associated cancers and HPV-negative cancers. Currently, HPV-associated cancers primarily arise in the oropharynx, and account for 70% of cancers in this site driving the recent increase in oropharyngeal cancer incidence (Chaturvedi et al. 2011). The HPV-associated oropharyngeal cancers occur more often in younger, nonsmoking patients and are associated with better survival outcomes compared to HPV-negative patients (Ang et al. 2010). Although HPV-positive patients have substantially better prognosis, approximately 20% of patients still fail to therapy, indicating the unmet need to understand the biology underlying HPV carcinogenesis and metastasis.

HPV HNSCCs also comprise a subset of virally induced cancers, many of which regulate members of the Notch pathway (Vazquez-Ulloa et al. 2018). In fact, HPV oncogenes disrupt NOTCH transcription of differentiation-related genes, which may promote carcinogenesis. Here, we will discuss the intersecting roles of HPV and the NOTCH pathway during epithelial differentiation and carcinogenesis.

HPV Life Cycle Promotes Viral Replication in Differentiating Keratinocytes

All papillomaviruses genomes exist as circular double-stranded DNA episomes of approximately 8000 base pairs in the nucleus of host cells. The HPV genome contains eight open reading frames falling into three major regions: (1) an early gene region, (2) a late gene region, or (3) a long control region. These regions are separated by two polyadenylation sites (Stoler 2000; Doorbar et al. 2015). The six early genes, E1, E2, E4, E5, E6, and E7, encode proteins necessary for viral replication and, as an unintended consequence, cellular transformation. The two late genes, L1 and L2, encode structural proteins of the virus necessary for viral capsid formation (Graham 2010). The 1000–base pair noncoding region is essential viral DNA replication by containing elements that regulate the spatiotemporal differences in early and late gene expression.

HPV replication is intertwined with keratinocyte differentiation and characterized by three distinct phases of replication (Fig. 8.1). First, HPV establishes infection by gaining access to the proliferating stem cells in the basal keratinocyte layer via wounds or microabrasions that disrupt the epithelial layer. After infection of basal keratinocytes, HPV initiates “establishment replication” to generate 50 to 100 viral DNA copies in an episomal form that reside in the undifferentiated basal cell reservoir.

Of the HPV genes, the E6 and E7 genes are best known to contribute to oncogenesis (Fig. 8.2). The E6 protein binds the tumor suppressor protein p53 along with the cellular E3-ubiquitin ligase E6-AP in order to target p53 for ubiquitination and subsequent proteasomal degradation. E7 binds to retinoblastoma (Rb), also a tumor suppressor, to facilitate the release of E2F family of transcription factors.

The second phase of HPV replication, the early phase, begins upon epithelial differentiation where viral genomes are amplified in the more differentiated suprabasal layers. Viral replication is regulated by the six early genes. E1 and E2 promote viral genome replication as E1, a virus-specific helicase, aids the unwinding of viral DNA, and E2 regulates the expression of viral and cellular genes necessary for replication. E6 and E7 interact with p53 and pRb, respectively, to control apoptosis, differentiation, and cell cycle which is necessary for viral replication in differentiating cells (Blanpain et al. 2006). E5 is primarily expressed in high-risk HPV subtypes and plays an important but less recognized role in cellular transformation and immune escape.

The final phase of HPV replication, the late phase, involves virion packaging, assembly, and release. E4 plays a crucial role in packaging the viral genome, and the two late genes (L1 and L2) comprise the virus capsid required for the production of mature viruses. This occurs in terminally differentiating keratinocytes which release HPV virions for further infection. Thus, the HPV genome is designed to disrupt keratinocyte differentiation in order to facilitate viral replication and release.

NOTCH Pathway Initiates Spinous Differentiation of Basal Keratinocytes

The stratified squamous epithelium consists of keratinocyte cells layered upon a basement membrane. The keratinocyte cells most proximal to the basement membrane comprise the basal layer. The basal layer, along with the hair follicles in cutaneous epithelium, contains the stem cell compartment from which keratinocytes differentiate. The initial steps of epithelial differentiation involve the keratinocytes leaving the basal layer to the suprabasal layers, traversing from the spinous layer to the granulosum layer, to the lucidum layer, and finally to the corneal layer. As keratinocytes traverse each layer, they further differentiate, acquiring expression of different keratins and other cytoskeletal and other structural proteins as well as undergoing nuclear involution and loss of reproductive capacity.

Notch was initially described in the fruit fly *Drosophila melanogaster* in the early twentieth century as a phenotype where female flies developed “notches” in their wings (Mohr 1919). The Notch pathway is highly conserved signaling pathway regulated by cell–cell communication. In humans, Notch family members include NOTCH1, NOTCH2, NOTCH3, and NOTCH4. The NOTCH proteins are Type I transmembrane receptors composed of a large extracellular domain, a single transmembrane domain, and an intracellular domain that executes the NOTCH transcriptional program (Fig. 8.3). The NOTCH receptor recognizes five ligands: the Delta-like ligands DLL1, DLL3, and DLL4 and the Serrate-like ligands Jagged1 and Jagged2. NOTCH signaling is initiated when cell surface ligands, including Delta-like 4 and Jagged family members, on one cell bind to NOTCH receptors on an

adjacent cell resulting in transmembrane cleavage of NOTCH and the release of the intracellular (NICD) domain (Mumm and Kopan 2000).

The Notch family functions in a cell and context-specific manner to regulate cell-fate determination and differentiation (Bray 2016). In many tissues, such as the hematopoietic and pancreatic organs, Notch activation has been shown to maintain stem cell potential and inhibit differentiation. However, in other contexts, including squamous epithelium, Notch activity induces the exit of keratinocytes from the stem cell compartment via two ways. First, localized NOTCH expression commits cells to a “transient amplifying” phenotype where cells retain limited proliferative potential but are committed toward a terminal differentiation program (Lefort and Dotto 2004; Lowell et al. 2000). Alternatively, the Fuchs group demonstrated that mice overexpressing the constitutively activated NICD1, the constitutively active truncated C-terminal domain of Notch1, led to expansion of the spinous layer and induction of differentiation-related genes specific for cells in the spinous layer (Blanpain et al. 2006). The expression of these spinous-related genes was dependent on the expression of the Notch1 target gene *Hes1*. Notch differentiation programs are likely dependent on the polarity of the mitotic spindle as cells unable to initiate asymmetric cell division, defined by the orientation of mitotic spindles to the basal layer, and displayed impaired Notch1 signaling and spinous differentiation (Williams et al. 2011). Thus, in contrast to other organ systems where the NOTCH pathway preserves the stem cell compartment, in the skin and mucosal surfaces, the NOTCH pathway promotes epithelial differentiation via distinct mechanisms.

NOTCH signaling programs are activated by trans-receptor–ligand interactions on adjacent cells, resulting in the successive cleavage of NOTCH proteins. NOTCH is first cleaved by TNF α converting enzyme (TACE), a member of the ADAM-17 family of metalloprotease (van Tetering et al. 2009). The subsequent cleavage results from a γ -secretase–presenilin complex (Meng et al. 2009), resulting in the release of the Notch intracellular domain (NICD), a functional, active C-terminal NOTCH fragment. The NICD translocates to the nucleus and binds via its RAM and ankyrin domains to the DNA-binding transcription factors CBF or RBP-JK recruiting coactivators such as Mastermind like-1 (MAML1) and p300/CBP (Hansson et al. 2009; Wu et al. 2000; Wu and Griffin 2004). In the absence of NICD, CBF-1 represses gene expression by binding the histone deacetylase complex SMRT–sin3 and HDAC-1. Binding of NICD to CBF-1 displaces the repressor complex and recruits nuclear coactivators, such as MAML1 and histone acetyltransferases (Hansson et al. 2009; Wu et al. 2000; Wu and Griffin 2004; Guruharsha et al. 2012). The conversion of CBF-1-NICD from a transcriptional repressor to a transcriptional activator results in the expression of target genes including hairy/enhancer of split (HES) gene family and HEY subfamily members (Rettig et al. 2015). In addition, CBF-1-NICD also induces the expression of cell cycle–related genes, p21, p27, E2F, and transcription factors NF- κ B and peroxisome-proliferator-activated receptor transcription factors that subsequently execute pro-survival functions toward carcinogenesis (Brimer et al. 2012; Rangarajan et al. 2001; Suman et al. 2014).

HPV Carcinogenesis Occurs via Disruption of p53 and pRB

Approximately 200 serotypes of HPV exist and are classified into five genera (α , β , γ , μ , ν) based on DNA sequence similarity and tissue tropism (Van Doorslaer et al. 2013; Moody 2017). Alpha-papillomaviruses (α -HPV) infect mucosal tissues, whereas β -, γ -, ν -, and μ -papillomaviruses infect cutaneous tissues (Rautava and Syrjanen 2012). Overall, mucosal tropic HPVs infect the anogenital tract, upper digestive tract causing HNSCC, cervix cancer, vaginal and vulvar cancer, and anal cancer. Mucosal types can be subdivided into low-risk and high-risk serotypes that are associated with differing degrees of oncogenic potential. Low-risk serotypes are associated benign genital lesions and include HPV 6 and 11 serotypes. High-risk serotypes are associated with cancers and include HPV16, 18, 31, 33, 45, 52, and 58 serotypes. Of these high-risk subtypes, HPV 16 and 18 are most commonly associated with cervical cancer and HPV 16 is almost exclusively associated with oropharyngeal carcinomas (Stoler 2000; Kreimer et al. 2005; Miralles-Guri et al. 2009). Unlike α -HPVs, most of the β -HPV and γ -HPVs infections are asymptomatic in immunocompetent individuals without any clinical manifestations of disease (Gottschling et al. 2009; Nindl et al. 2007).

Classically, the initial steps of HPV carcinogenesis occur via the integration of high-risk HPV episomal DNA into the host-cell genome. While HPV-associated cancers can arise without integration of the viral genome, this is usually less frequent. Analysis of The Cancer Genome Atlas (TCGA) demonstrated that more than 80% of cervical cancers displayed integrated viral genomes (Cancer Genome Atlas Research N, Albert Einstein College of M, Analytical Biological S, Barretos Cancer H, Baylor College of M, Beckman Research Institute of City of H, et al. 2017). By contrast, the HPV genome is integrated in oropharyngeal cancers at slightly lower rates, approximating 70% (Parfenov et al. 2014; Vojtechova et al. 2016). Although several groups have suggested differences in outcomes in patients with episomal versus integrated HPV genomes, the data is overall conflicting where one cannot draw a consensus opinion. The integration of the HPV genome usually disrupts the E2 protein, which regulates the transition of early gene expression to late gene expression in differentiating keratinocytes. This results in a loss of the negative feedback controlling E6 and E7 expression and, consequently, the persistent expression of E6 and E7 and the resulting disruption of the tumor suppressors p53 and pRB, respectively (Collins et al. 2009).

Dysfunction and inactivation of p53 and pRb are the classical, initial steps for the development of cancers in multiple tissue types (Fig. 8.2). In HPV-associated cancers, E6 disrupts p53 by forming a trimeric complex with p53 and the cellular ubiquitin ligase E6-AP protein (Huibregtse et al. 1995) leading to the ubiquitination and rapid proteasomal degradation of p53 (Talis et al. 1998; Munger et al. 2004). The targeting of p53 by E6 prevents the activation of cell death pathways that would normally be activated by abnormally proliferating cells. By contrast, E7 canonically disrupts the Rb family of proteins including Rb, p107, and p130, to induce abnormal cellular proliferation. E7 contains an LXCXE motif that binds to the pocket of Rb to disrupt the sequestration of E2F family members from the nucleus. Once E7 binds to Rb, E2F is freed to migrate to the nucleus and

to induce expression gene regulating cell cycle progression and, as a consequence, genomic instability (Ghittoni et al. 2010).

HPV and NOTCH in HNSCCs

The TCGA and other massively paralleled sequencing efforts to elucidate the mutational changes in HNSCCs and other squamous cell cancers have identified both cellular and viral drivers of carcinogenesis (Cancer Genome Atlas Research N, Albert Einstein College of M, Analytical Biological S, Barretos Cancer H, Baylor College of M, Beckman Research Institute of City of H, et al. 2017; Cancer Genome Atlas 2015; Gillison et al. 2019; Seiwert et al. 2015) (Table 8.1). Previous studies have shown that mutation of genes *TP53*, *CDKN2A*, *PIK3CA*, *EGFR*, *CCND1*, *PTEN*, and *HRAS*, either by gain or loss of function together with *FBXW7*, *NOTCH1*, *IRF6*, and *TP63*, causes dysregulation of signaling pathways and chromosomal abnormalities that are responsible for pathogenesis of HNSCC (Leemans et al. 2011; Stransky et al. 2011; Pickering et al. 2013; Agrawal et al. 2011). Of note, HPV-associated HNSCCs have a genetic landscape that is distinct from HPV-negative HNSCCs. Globally, HPV-negative HNSCCs display approximately a two-fold greater mutational burden than HPV-positive tumors, which was independent of smoking status (Stransky et al. 2011). Furthermore, the presence of mutations in TP53 was inversely associated with HPV tumor status as no HPV-associated cancers had TP53 mutations while 78% of HPV-negative cancers contained a TP53 mutation (Stransky et al. 2011). Furthermore, HPV-negative HNSCCs are more likely to have higher expression of EGFR and chromosomal aberrations in 3p, 9p, and 17p (Munger et al. 2004; Benson et al. 2014; Braakhuis et al. 2004; Kumar et al. 2008). In addition, we have shown HPV oncogene expressing HPV-positive autochthonous oral tumors grew faster and gained expression of MCM7 as compared to HPV-negative tumors (Zhong et al. 2014).

The TCGA along with other sequencing efforts identified disrupting mutations or loss of NOTCH1 as a frequent event in head and neck cancers (Cancer Genome Atlas 2015; Agrawal et al. 2011). Agarwal et al. demonstrated that NOTCH1 was frequently mutated in HNSCCs in which 40% of these mutations were predicted to truncate and inactivate the NOTCH1 gene product (Agrawal et al. 2011). Stransky et al. identified mutations in *NOTCH1*, *IRF6*, and *TP63* genes in 30% of HNSCC patients (Stransky et al. 2011). Similarly, the TCGA demonstrated frequent mutations in differentiation-related genes including NOTCH1, TP63, FAT1, and AJUBA (Cancer Genome Atlas 2015). Similarly, we observed Notch1 was frequently mutated using a transposon-based insertional mutagenesis as a functional screen to identify cellular genes responsible for autochthonous HPV-positive tumors (Zhong et al. 2015).

The Interaction of NOTCH Pathway During Viral Carcinogenesis

HPV oncogenes likely modulate the NOTCH pathway and vice versa in HPV-associated cancers. Several reports have shown that several HPV serotypes, including HPV5, HPV8, and β -HPVs impair NOTCH activity by manipulating the NOTCH-associated transcriptional machinery (Fig. 8.4). The E6 of β -HPVs directly binds to the MAML1 and interferes with the interaction of MAML and NICD, resulting in the loss of expression of NOTCH target

genes HEY and HES (Rampias et al. 2014; Brimer et al. 2012; Meyers et al. 2013). HPV cancers have also been shown to directly downregulate NOTCH expression in order to inhibit the NOTCH pathway.

Although β -papillomaviruses but not α -papillomaviruses have been shown to directly inhibit Notch signaling, the NOTCH pathway can also be disrupted in high-risk HPV cancers, as evidenced by Talora et al., demonstrating that cervical cancers expressing high-risk HPV subtypes downregulated NOTCH1 expression (Talora et al. 2002). High-risk α -papillomaviruses may inhibit NOTCH signaling indirectly through degradation of the TP53 tumor suppressor that, when upregulated, induces NOTCH expression (Dotto 2009). Furthermore, HPV16 E6 inhibited NOTCH cleavage and decreases NOTCH transcription which depended on TP53 degradation. Conversely, increasing NOTCH1 activity may downmodulate E6 expression via inhibition of the AP1 transcription factor complex (Wang et al. 2007). Similarly, Kranjec et al. demonstrated that the expression of HPV16 E6 disrupted NOTCH expression, which was dependent on p53 (Kranjec et al. 2017). Finally, high-risk HPVs may also target other p53 family members such as Tap63 β and indirectly inhibit NOTCH1 expression (Ben Khalifa et al. 2011). Thus, both oncogenic and non-oncogenic HPV subtypes have evolved mechanisms to inhibit NOTCH signaling.

HNSCCs Display Both Inactivating and Activating NOTCH1 Mutations

The role of Notch signaling during tumor development is likely context dependent, as Notch has been shown to promote to tumorigenesis in some models while suppressing tumorigenesis in other models. NOTCH was initially described as an oncogene in hematopoietic cancers but has been shown to function primarily as a tumor suppressor in epithelial cancers. In T-cell acute lymphoblastic leukemia (T-cell ALL), activating NOTCH mutations promoted proliferation via activation of the Myc pathway (Sanchez-Martin and Ferrando 2017). By contrast, many HSNCC displayed nonsense mutations in NOTCH, resulting in truncated proteins lacking a portion of the C-terminal intracellular domains which inactivate Notch by making it incapable of transcribing downstream genes (Agrawal et al. 2011). In mouse models, loss of Notch1 drove cutaneous and oral carcinogenesis (Nicolas et al. 2003; Nyman et al. 2018). Furthermore, Retting et al. demonstrated that NOTCH1-inactivating mutations were more likely in HPV-negative cancers than in HPV-positive cancers (Rettig et al. 2015). By contrast, Izumchenko et al. demonstrated that activating NOTCH mutations, similar to those found in T-cell ALL, were also present in premalignant lesions of oral cavity cancers (Izumchenko et al. 2015). Similarly, we observed that in HPV tumor models the constitutively active Ncd1 and loss of Notch1 promoted oral tumorigenesis via distinct mechanisms. Thus, several lines of evidence define a bifunctional role for the NOTCH pathway in cancers that is likely dependent on the mutational and tissue context.

NOTCH as Tumor Suppressor in HNSCC

In head and neck cancers, loss-of-function mutations in Notch family members are among the most recurrent mutations in HNSCC (Table 8.2). The mutations identified include missense mutations, splice site mutations, and nonsense mutations that result in truncated

proteins lacking the C-terminal trans-activating domain (Cancer Genome Atlas 2015). These aberrations occur predominantly in NOTCH1, but are also found in NOTCH2 and NOTCH3 (Pickering et al. 2013). Genetic alterations have also been detected that lead to reduced Notch activity by altering other Notch pathway cofactors, such as mastermind-like 1 (MAML1) (Arruga et al. 2018). In the mouse, conditional deletion of Notch signaling in epithelial progenitor cells through the expression of a dominant negative form of the Notch coactivator Maml1 promoted the expansion of pre-neoplastic clones harboring inactivating Tp53 mutations. Thus, loss of Notch signaling may even be an early event during head and neck carcinogenesis (Natsuizaka et al. 2017).

In vitro and in vivo, Notch1 has been shown to negatively regulate keratinocyte proliferation and initiate the process of epithelial differentiation (Blanpain et al. 2006; Rangarajan et al. 2001). In mice, with conditional deletion of Notch1 in the skin, they developed epidermal hyperplasia leading the basal cell line carcinomas with increased Gli2 expression consistent with activation of the beta-catenin pathway (Nicolas et al. 2003). Loss of the Notch signaling pathway in adjacent nonepithelial tissues may also promote epithelial tumorigenesis. To this end, Hu et al. demonstrated that mice with conditional deletion in Notch1 co-factor Rbp-J κ in the dermis induced keratosis followed by squamous cell carcinoma formation in the epidermis of mice. In other words, with the loss of Notch signaling in the stroma facilitated the development of premalignant epithelial lesions (Hu et al. 2012). Stromal cells with Notch1 loss promoted epithelial carcinogenesis by expressing higher levels of growth factors, cytokines, and matrix-metalloproteinases that promoted the proliferation and invasion of adjacent epithelial cells. Similarly, Demehri et al. demonstrated that Notch1-expressing keratinocytes can also form carcinomas when adjacent Notch1-deficient keratinocytes primed a wound-like microenvironment to promote tumor growth (Demehri et al. 2009). In chemical carcinogenesis models, mice with Notch1-deficient epithelial tissues developed dramatically more benign papillomas and squamous cell carcinomas. Namely, wild-type mice treated with 7,12-Dimethylbenz[a] anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) developed cutaneous papillomas with frequent missense and nonsense mutations in Notch1 (Nicolas et al. 2003; Rizzo et al. 2015). Confirming NOTCH1 as a tumor suppressor in HNSCCs, Pickering et al. overexpressed NOTCH1 in various head and neck cancer cell lines, which inhibited proliferation and induced senescence (Pickering et al. 2013). Consistent with these preclinical observations, a clinical trial studying γ -secretase inhibitor in Alzheimer's disease reported an unexpected increase in nonmelanoma skin cancers further supporting the role of the Notch pathway as a tumor suppressor in epithelial cancers (Extance 2010). Thus, genetic, functional, and clinical observations support a tumor suppressive role for the Notch pathway in viral and nonviral epithelial cancers.

The NOTCH pathway may suppress tumor growth is through inhibition of the beta-catenin pathway, Np63 and/or the cell cycle. Nicolas et al. demonstrated that Notch1 inactivation in the epidermis restored beta-catenin signaling in cells differentiating epithelium and keratinocytes (Fuchs and Raghavan 2002). Enhanced beta-catenin signaling was reversed by expressing a dominant active form of the Notch1 receptor, which was associated with a reduction in the signaling-competent pool of beta-catenin. In the epidermis, Np63 expression, which enforces the stem cell phenotype, is often inversely correlated with Notch

activity and may inhibit the pro-differentiation effect of Notch signaling. Conversely, Notch has been shown to inhibit the expression of p63 in epidermal progenitor cells as they differentiate. In HNSCCs and other squamous cancers, p63 expression is upregulated and, consequently, a potentially important mechanism to inhibit NOTCH signaling in cancers without non-mutated NOTCH family members. Finally, Notch may function as a tumor suppressor by inhibiting the cell cycle. The NOTCH pathway has been shown to induce p21WAF1/Cip1, which disrupts cell cycle progression (Roy et al. 2007). Thus, loss of NOTCH may inhibit differentiation and promote cell cycle progression.

Conversely, activation of the NOTCH pathway has also been shown to inhibit proliferation of head and neck cancer models. Overexpression of the active NICD in oral squamous cell cancer line Tca8113 inhibited cell proliferation in vitro and in vivo accompanied by G0/G1 cell cycle arrest and apoptosis (Duan et al. 2006). In laryngeal cancer cell line Hep-2, overexpression of NOTCH1 also inhibited proliferation, causing cell cycle arrest in the G0/G1 phase and inducing apoptosis (Jiao et al. 2009). Finally, Pickering et al. demonstrated that expression of cleaved NICD or full-length NOTCH1 in HNSCC cell lines inhibited in vitro proliferation and tumorigenic growth in mice (Pickering et al. 2013).

In addition to NOTCH1, other NOTCH family members also inhibited virally induced head and neck cancers and other cancers. NOTCH3 overexpression in EBV-driven nasopharyngeal carcinoma cells inhibited cell proliferation and induced apoptosis. These tumor suppressor properties were associated with the downregulation of cell cycle and anti-apoptotic genes including CCND1, C-MYC, NFKB1, BCL2, BCL-XL, and SURVIVIN. Furthermore, xenograft spheroid formation was remarkably decreased by inhibiting NICD3, the constitutively active form of NOTCH3 (Man et al. 2012). Similarly, Lobry et al. demonstrated that loss of NOTCH signaling through the conditional deletion of Nicastrin (NCSTN), an essential component of the γ -secretase complex, or compound deletion of NOTCH1 and NOTCH2, resulted in chronic myelomonocytic leukemia. Furthermore, sequencing of Notch pathway genes revealed that ~12% of CMML patients harbored inactivating mutations in NOTCH2, NCSTN, and MAML1. Overall, these studies implicate that Notch cofactors and pathways prevented uncontrolled proliferation and transformation of myeloid cells during hematopoietic development (Lobry et al. 2011). Thus, several NOTCH family members likely have tumor suppressive functions.

NOTCH as an Oncogene in HNSCC

Initially, NOTCH was identified as an oncogene due to the identification of rare chromosomal translocations involving the NOTCH1 locus in T-cell ALLs. Namely, the NOTCH1 locus was disrupted by the t(7; 9)(q34; q34.3) chromosomal translocation placing the C-terminal region of NOTCH1 next to the TCR β locus and allowing for constitutive expression of an activated NOTCH gene product (Yoshida et al. 2017; Sakamoto 2016). Of note, more than 50% of T-cell ALLs display this activating truncation of NOTCH1 (Weng et al. 2004; Ellisen et al. 1991).

Activation of the NOTCH pathway may also promote the growth of epithelial cancers (Table 8.3). As previously mentioned, Izumchenko et al. sequenced 95 oral cavity cancers and

identified NOTCH1 mutations in 54% of invasive and 60% of preinvasive lesions (Izumchenko et al. 2015). Furthermore, oral cavity cancers from Chinese patients were frequently mutated in the HD domain, transactivation domain, and PEST domain, which are locations where the majority of activating mutations in T-cell ALL reside. Constitutive activation of the Notch signaling initiated by the direct interaction between JAG1 and NOTCH1 in HNSCC cell lines also resulting in cells with increased migration and metastatic phenotypes (Egloff and Grandis 2012; Lin et al. 2010). Abnormal expression of JAG1 triggered Notch1 activation in the HNSCC cell lines (Lin et al. 2010; Tohda and Nara 2001) as well as in adjacent endothelial cells to promote angiogenesis (Zeng et al. 2005). In addition, Lin and others have shown that HNSCCs overexpressing JAG1 or NOTCH1 displayed accelerated tumor growth and angiogenesis in vivo (Zeng et al. 2005; Joo et al. 2009). In a TCGA analysis, HNSCC significantly upregulated HEY1 compared with normal tissues. Furthermore, the expression of NOTCH pathway members NOTCH1, NICD, JAG1, and HES1 was upregulated during the progression of normal tissues to dysplasia and malignancy. Immunohistochemical examination of oral tongue cancers showed increased staining of Notch1 and Notch3 protein in malignant cells compared to adjacent normal tissues. Furthermore, a positive correlation between JAG2 and NOTCH3 were found in tongue carcinoma (Zhang et al. 2011, 2013). The transcriptional alterations of NOTCH signaling pathways genes in HNSCC tumors revealed that 11 genes, including JAG1, JAG2, NOTCH3, NCSTN, DTX3L, ADAM17, DVL3, HES1, HDAC2, NCOR2, and NUMBL, were significantly upregulated, and 4, including KAT2B, MAML3, DTX1, and MFNG, downregulated (Sun et al. 2014). Finally, mutations in FBXW7, a member of the SCF ubiquitin ligase complex which regulates NOTCH1 by targeting it for proteasomal degradation, may result in increased NOTCH (O'Neil et al. 2007). Thus, HNSCCs demonstrate genetic and transcriptional evidence for the activation of the NOTCH pathway as an oncogenic mediator of tumor growth.

The first proof confirming increased NOTCH signaling promotes solid tumor development was observed by integration of the mouse mammary tumor virus into the Notch4 gene. This integration resulted in mammary tumorigenesis via LTR-driven expression of the truncated, constitutively active form of the Notch4 gene (Uyttendaele et al. 1996). Similarly, we observed that increasing the expression of NICD1 in primary HPV oral tumors promoted tumor growth via upregulation of gene expression pathways encompassing MYC and other genes that promote cell proliferation. Furthermore, compared to other invasive SCCs, NOTCH1 was overexpressed in verrucous carcinomas, a rare variant of oral cancer with pushing borders rather than deeply invasive (Zhong et al. 2015). NOTCH1 is also significantly related to cervical lymph node metastasis in oral tongue cancers (Joo et al. 2009; Zhang et al. 2011). Similarly, Leethanakul et al. demonstrated that NOTCH2 expression was associated with lymph node metastasis in HNSCCs (Leethanakul et al. 2000). Finally, the NOTCH pathway may play an important role in cell renewal and survival as upregulation of NOTCH1-mediated chemoresistance by promoting self-renewal and stemness in HNSCC cell lines (Zhao et al. 2016).

Activation of the NOTCH pathway may also promote the growth of non-squamous head and neck cancers. NOTCH1 is the most frequently altered gene in adenoid cystic carcinoma (ACC), a relatively rare tumor of the head and neck. In this disease, NOTCH1 mutations

were associated with higher NICD expression and poor prognosis. Furthermore, NOTCH1 may be a targetable gene in ACCs as brontictuzumab, a monoclonal antibody to NOTCH1, inhibited NOTCH1-mediated signaling both in patients and xenograft models and was associated with clinical efficacy in patients (Ferrarotto et al. 2018). Similarly, some patients with ACC also responded to γ -secretase inhibitors, which inhibit the cleavage NOTCH1 and activation of the NOTCH pathway (Massard et al. 2018). Finally, the Notch pathway has been shown to promote the growth of other solid tumors including breast cancer, NSCLCs, colorectal cancer, pancreatic cancer, and medulloblastoma (Suman et al. 2014; Brzozowa-Zasada et al. 2017; Du et al. 2018; Kumar et al. 2019). Overall, it confirms that NOTCH is playing an oncogenic role in many epithelial cancers including some HNSCCs.

Activation of the NOTCH pathway may promote head and neck tumor growth via activation of pathways involved in cell proliferation and anti-apoptosis. NOTCH1 has been shown to directly induce c-MYC expression in T-cell acute lymphoblastic leukemia (Weng et al. 2006; Herranz et al. 2014). NOTCH2 also affects cell growth and apoptosis, and knockdown of NOTCH2 inhibited the migration and invasion abilities and decreased the expression levels of its downstream genes such as c-MYC and BCL-2 (Zou et al. 2016). In addition, NOTCH activation increased FGF1 gene expression and cell invasion in oral squamous cell carcinomas (Weaver et al. 2016). Furthermore, inhibition of the NOTCH pathway is associated with decreased phosphorylation of AKT, a serine/threonine-specific protein kinase involved in metabolism, cell proliferation, and migration (Das et al. 2016). In the TCGA data set, cancers with wild-type NOTCH1 exhibited increased expression of the NOTCH1 target genes HEY1 and HES1 as well as an associate with increased BCL-2 expression (Fukusumi and Califano 2018).

The Mutational Context May Determine NOTCH's Role as an Oncogene or Tumor Suppressor

The deciding factors that determine whether NOTCH acts as a tumor suppressor or oncogene remains unresolved. It is unlikely that cell type of origin is the primary factor, given that NOTCH pathway activation or loss is present in cancers of the same tissue type and, in cell and animal models, activation or loss of the NOTCH pathway promotes tumor growth. Rather, it is likely that the mutational context and the timing during which mutations arise help to determine whether the activation or loss of NOTCH pathway promotes tumor growth. As we have described, NOTCH activates pro-tumorigenic pathways including activation of AKT and c-MYC pathways as well as tumor suppressive signals comprising the commitment of differentiation programs and the inhibition of Np63 and the beta-catenin pathway. During carcinogenesis, cells that acquire mutations that block differentiation program and bypass cell cycle arrest may benefit from NOTCH activation which further stimulates proliferation pathways. To this end, we observed that in HPV oral tumors, where E7 can partially block squamous differentiation, NOTCH can function as a tumor suppressor. In this circumstance, loss of NOTCH may also promote tumor growth by helping to enforce a stem cell phenotype and promote the expression of genes involved in cell migration (Zhong et al. 2015). Conversely, in early premalignant lesions, loss of NOTCH may be necessary for transformation. Thus, the role of NOTCH as a tumor

suppressor or oncogene likely depends on the time and context of other mutations during carcinogenesis.

Conclusions

Notch pathway is critical in HSNCCs as 66% of cancers carry some sort of genetic alteration in either of the NOTCH 1–4 signaling proteins (Agrawal et al. 2011). NOTCH has been reported to have both oncogenic and tumor suppressive roles in cancer, which are likely dependent on the cellular context. A variety of both activating and inactivating NOTCH mutations have now been observed in various HNSCC patients. NOTCH activation has been demonstrated in multiple cancers including T-cell ALL, pancreatic cancer, breast cancer, prostate cancer, liver cancer, cervical cancer, and HNSCC among others (Sun et al. 2014). By contrast, NOTCH inactivation is also frequently observed in many epithelial cancers including HNSCCs. The complexity of the opposing roles for the NOTCH pathway in HNSCC and other cancers makes it difficult to implement novel therapeutic approaches. Rather, one must look at the NOTCH mutations specific to individual cancers as well as the context of other mutated and/or altered gene in order to rationally target the NOTCH pathway. Unraveling the molecular decisions that determine the oncogenic or tumor suppressive role of NOTCH will unravel new strategies for a targeted therapy for HPV-associated and HPV-negative HNSCC (Fig. 8.5).

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Fig. 8.1.

The HPV life cycle. (Left panel) HPV infection occurs as epithelial disruptions enable the virus access to the basal keratinocyte layer. Here, early genes are expressed with disrupt differentiation to enable viral replication. Viral particles are release at the epithelial surface where terminal differentiation occurs. (Right panel) HPV infection and aberrant differentiation and proliferation lead to HPV-associated cancers

Fig. 8.2.

Function of the HPV oncogenes E6 and E7. **(a)** HPV E6 oncogene impairs the function of p53 by shuttling p53 towards degradation. **(b)** HPV E7 oncogene impairs the E2F/pRb complex and allowing free E2F to enter continuous cell cycle progression to promote aberrant cellular proliferation. **(c)** pRB regulates p16 expression and inhibitory E7 viral protein relieves the repression by pRB

Fig. 8.3.

Notch signaling pathway. Notch ligand on one cell induces a series of proteolytic cleavage events in the Notch receptor on an adjacent cell. These cleavage events release the Notch intracellular domain (NICD), which translocates to the nucleus to activate the transcription of Notch target genes together with CSL and Mastermind-like protein (MAML)

Fig. 8.4.
Different mechanisms to regulate Notch signaling by HPV oncoproteins in keratinocyte cells

Fig. 8.5.

Dual role of Notch as oncogene and tumor suppressor in various cancers including HNSCCs

Table 8.1

Molecular identification of genes and proteins that implicates a role in HPV-positive and HPV-negative head and neck squamous cell carcinoma

Gene	Role	Outcome
TP53	Tumor suppressor gene	60–80% of HNSCC has mutated p53
PIK3CA	A catalytic subunit of PI3K effects on metabolism, proliferation, and cell survival	PIK3CA mutations in 8% HNSCC samples, 21% of HNSCC samples
EGFR	Transmembrane receptor and cellular homeostasis	90% of HNSCC showed overexpression Negative prognostic factor after radiotherapy
FGFR	Role in cellular differentiation, migration, and angiogenesis	FGFR1 amplification or mutation is seen in 10% of HPV-negative HNSCC and FGFR3 mutations or fusions occur in 11% of HPV-positive HNSCC
Cyclin D1	Protooncogenes, regulates cell cycle progression G1-S	TCGA study showed 28% of HNSCC has CCND1 amplification, with 32% (77/243 in HPV-negative and 6% (2/26) in HPV-positive samples Resistance to Cisplatin
PTEN	Tumor suppressor genes play a role in apoptosis	11% of HPV-positive HNSCC and 5% of HPV-negative HNSCC
C-MET	EMT and invasion	MET–HGF axis as therapeutic target in HNSCC
MMPs	Degrade basement membrane of ECM and helps in cancer progression, invasion	Overexpression of MMPs-2, 8, and 13; involved in lymph node metastasis and chemotherapy resistance
NOTCH	Tumor suppressor gene as well Oncogene	14–15% of HNSCCs has inactivating mutations 32% has activating mutations
P16	Tumor suppressor regulates cell cycle progression	50–80% of HNSCC has loss of p16
HIF-1 α	Involved in angiogenesis and EMT	Roles in chemoresistance, radio-resistance, and poor prognosis
ERBB	Tyrosine kinase	Amplification and mutation are seen in 4% HPV-negative and 3% of HPV-positive HNSCC Afatinib and dacomitinib (irreversible pain inhibitors are on clinical trial)

Table 8.2

Summary of NOTCH role as tumor suppressor in HNSCC

References	Tumor sample (n)	Sample	Method	Observed effect	Prediction/ Implication
Agrawal et al. (2011)	120	HNSCC tissues	Exome Sequencing	Mutated NOTCH1	Inactive protein
Stransky et al. (2011)	74	HNSCC tissues	Exome Sequencing	Mutated NOTCH1 NOTCH2 NOTCH3	Inactive protein
Pickering et al. (2013)	44	HNSCC tissues	Integrated genome analysis	Mutated NOTCH1, NOTCH2	Inactive protein
Kandoth et al. (2013)	306	HNSCC tissues	Exome sequencing-TGCA	Mutated NOTCH1	Inactive protein
Song et al. (2014)	13	HNSCC cell lines	Single molecule DNA sequencing	Mutated NOTCH	Inactive protein
Fukusumi et al. (2018)	520	HNSCC tissues	TCGA	Mutated NOTCH	Inactive protein
Pickering et al. (2013)	In vitro		Overexpression of NICD	Inhibition of tumor growth	Tumor suppressor

Table 8.3

Summary of NOTCH role as oncogene in HNSCC

References	Experiment	Findings and Implications
Zeng et al. (2005)	Immunohistochemistry of HNSCC tissue microarray	JAG1 was overexpressed indicating that NOTCH1 activity in HNSCCs promotes angiogenesis
Gu et al. (2010)	HNSCC cell line in collagen matrix	NOTCH1 protein positively associates with cisplatin resistance
Sun et al. (2014)	HNSCC cell lines transduced with siRNA against NOTCH1 and HEY1	Inhibition of NOTCH1 inhibited proliferation indicating NOTCH1 important for tumor growth