Cleaved NOTCH1 Expression Pattern in Head and Neck Squamous Cell Carcinoma Is Associated with NOTCH1 Mutation, HPV Status, and High-Risk Features

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Abstract

The Notch pathway is frequently altered in head and neck squamous cell carcinomas (HNSCC); however, the clinical significance of NOTCH1 dysregulation is poorly understood. This study was designed to characterize expression of the transcriptionally active NOTCH1 intracellular domain (NICD1) in HNSCCs and evaluate its association with NOTCH1 mutation status and clinical parameters. IHC for NICD1 was performed on 79 previously sequenced archival HNSCCs with known NOTCH1 mutation status. Three distinct immunohistochemical staining patterns were identified: positive/peripheral (47%), positive/nonperipheral (34%), and negative (19%). NICD1 expression was associated with NOTCH1 mutation status ($P < 0.001$). Most NOTCH1–wild-type tumors were peripheral (55%), whereas mutated NOTCH1 tumors were most commonly negative (47%). Nonperipheral tumors were more likely than peripheral tumors to have extracapsular spread [adjusted odds ratio (aOR), 16.01; 95% confidence interval (CI), 1.92–133.46; $P = 0.010$] and poor differentiation (aOR, 5.27; 95% CI, 0.90–30.86; $P = 0.066$). Negative staining tumors tended to be poorly differentiated (aOR, 24.71; 95% CI, 1.53–399.33; $P = 0.024$) and were less likely to be human papillomavirus ( HPV) positive (aOR, 0.043; 95% CI, 0.001–1.59; $P = 0.087$). NOTCH1 mutagenesis was significantly associated with HPV status, with NOTCH1–wild-type tumors more likely to be HPV positive than NOTCH1–mutated tumors (aOR, 19.06; 95% CI, 1.31–276.15; $P = 0.031$). TP53 disruptive mutations were not associated with NICD1 expression or NOTCH1 mutation. In conclusion, NICD1 is expressed in three distinct patterns in HNSCC that are significantly associated with high-risk features. These findings further support a dual role for NOTCH1 as both tumor suppressor and oncogene in HNSCC. Further research is necessary to clarify the role of NOTCH1 in HNSCC and understand the clinical and therapeutic implications therein. Cancer Prev Res; 8(4); 287–95. ©2015 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common malignancy worldwide (1). Tobacco, alcohol, and human papillomavirus (HPV) are responsible for the majority of HNSCCs (2, 3). Genetic alterations in HNSCC are highly heterogeneous, and distinct for HPV-negative and HPV-positive tumors (4–6). The diversity of genetic alterations and tumor-suppressor predominance in HNSCC has underscored the importance of identifying molecular targets for tailored therapeutic regimens specific to the unique characteristics of individual tumors (7, 8).

Recently, NOTCH1 was identified as a frequently mutated gene in HNSCC, with 10% to 15% prevalence of inactivating mutations (4, 5, 9, 10). The NOTCH1 protein is one of four Notch transmembrane signaling protein paralogs with key roles in the regulation of cell differentiation, proliferation, and survival (11, 12). NOTCH1 is activated in a juxtacrine fashion when bound by ligands on neighboring cells. Following ligand binding, stepwise proteolytic cleavage releases the effector domain of the NOTCH1 protein, the Notch1 intracellular domain (NICD1), for translocation to the nucleus. The NICD1 binds transcriptional coactivators and initiates transcription of various target genes involved in cell differentiation and proliferation. The downstream effects of NOTCH1 activation are highly context dependent, and vary with cell lineage, pathology, and stage of differentiation (11, 12). In normal keratinocytes, the cell type from which HNSCCs are derived, NICD1 signaling promotes cell differentiation (13).
NOTCH1 acts as a tumor suppressor or as an oncogene in hematopoietic and solid organ malignancies, depending on the cancer type (11). In HNSCC, whole-exome sequencing revealed loss-of-function mutations consistent with a tumor-suppressor role (4, 5). Although subsequent studies confirmed the NOTCH1 inactivating mutations (9, 10) and demonstrated NOTCH1 tumor-suppressor activity in oral squamous cell carcinoma cell lines (9), NOTCH1 dysregulation appears to be more complex than simple loss-of-function (10). Indeed, approximately one third of HNSCCs displayed evidence of increased NOTCH1 pathway activation as compared with normal mucosa (10).

Studies of NOTCH1 protein expression in HNSCC are similarly conflicting. Both NOTCH1 over- and underexpression have been observed in tumors compared with normal tissue (10, 14). Increased NOTCH1 expression by IHC has been correlated with poor prognosis (15, 16), and high-risk clinical features, including cervical lymph node metastasis (15, 17), advanced stage (15), higher histologic grade (15), greater depth of invasion (17), and cisplatin resistance (18, 19). These findings appear to be at odds with the putative tumor-suppressor role of this protein. To our knowledge, IHC studies to date have only evaluated full-length NOTCH1, which is not transcriptionally active. Here, we explored the expression patterns of the transcriptionally active NICD1 in HNSCC tumor samples, and their association with NOTCH1 mutation status and clinicopathologic parameters.

Materials and Methods

Subjects

This study was approved by the Johns Hopkins Hospital Institutional Review Board (Protocol NA_00036235) and informed consent was obtained. Patients treated for HNSCC at the Johns Hopkins Hospital from 1995 to 2010 and for whom tumor whole-exome sequencing data were available were eligible for analysis. Whole-exome sequencing methods and sequencing data for all of the specimens included in this study were previously reported (4). Retrospective medical record abstraction was performed to determine clinicopathologic variables of interest.

HPV tumor status

HPV status for oropharyngeal tumors was based upon p16 IHC and/or DNA in situ hybridization (ISH) results, as available clinically (20) and/or from previous report (4). ISH for high-risk HPV DNA results were available for all 29 oropharyngeal cases and p16 IHC was available for 23 of 29 cases.

NICD1 immunohistochemistry

Paraffin-embedded archival tumor tissue was used to prepare slides with standard 4-μm tissue sections for eligible tumors with sufficient tissue available. Specimens were stained with anti-NICD1 rabbit monoclonal antibody (clone D3B8, catalog #4147, Cell Signaling Technology) using a previously described IHC protocol (21) with slight modifications as follows: slides were immuno-stained on the Ultra Benchmark autostainer, applying 64 minutes of heat-induced epitope retrieval (CC1 buffer) and 44 minutes of antibody incubation (room temperature) followed by an amplification step. The reaction was then developed with ultra-view detection (Ventana-Roche Medical Systems).

Stained specimens were reviewed and categorized into patterns of staining by a pathologist with expertise in tumors of the head and neck (J.A. Bishop). Images were captured using an Olympus BX41 microscope, Olympus DP71 camera and Olympus cellSens Standard software. Cell line–derived mouse xenografts from the HaCaT cell line (22) with known wild-type Notch1 expression (23) and normal human tonsil tissue were used for positive controls. Xenografts from the SCC47-E545K cell line (9) with a known NOTCH1 deletion and lack of NOTCH1 expression were used for negative controls. Cell lines were authenticated on August 18, 2014 (SCC47) or January 9, 2015 (HaCaT), using a short tandem repeat (STR) analysis kit, Identifier (Applied Biosystems), as directed at the Johns Hopkins Genetic Resources Core Facility. SCC47 was obtained from the University of Michigan (Ann Arbor, MI) and modified to introduce a PI3KA E545K activating mutation to facilitate in vivo tumor growth (24). SCC47 STR was matched with published cell line genotyping (25). HaCaT was purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany). Mice were 6- to 8-week-old, 20 to 22 g Hsd:Athymic Nude-FOXn1™ females purchased from Harlan Laboratories, Inc.

Characterization of TP53 mutations

TP53 mutation data were obtained from whole-exome sequencing as previously described (4). Potential functional significance of the mutations was assessed and categorized as “disruptive” or “nondisruptive” as previously described (26).

Analysis

Descriptive variables were summarized with frequencies and proportions for categorical variables, and medians and interquartile ranges for continuous variables. NICD1 IHC staining pattern, NOTCH1 mutation status, clinicopathologic characteristics, and TP53 disruptive mutation status were considered categorical variables and compared using 2 tests. Clinicopathologic characteristics were also considered as binary outcome variables and analyzed using logistic regression. Odds ratios (ORs) were reported with 95% confidence intervals (CIs). Two tailed P values less than 0.05 were considered statistically significant. Data analysis was performed using STATA 11.2 (2012).

Results

Study population

Tumors were available for 79 of 105 (75.2%) previously sequenced HNSCCs (4). Patients with available archival specimens were more likely to be female (P = 0.044), have higher T stage tumors (P = 0.028), and harbor a NOTCH1 mutation (P = 0.038) as compared with patients with unavailable tissue. There were no differences in terms of age, race, tobacco and alcohol use, tumor site, HPV status, differentiation, and extracapsular spread (ECS; Supplementary Table S1).

Tumor sites included oropharynx (N = 23, 29%), oral cavity (N = 38, 48%), larynx (N = 10, 13%), hypopharynx (N = 7, 9%), and an unknown primary (N = 1, 1%). Most tumors were incident disease (N = 57, 72%) and had not received prior radiation (N = 62, 78%).

NICD1 IHC patterns

Normal tonsil tissue was used as a control to determine expected NICD1 staining in nonpathologic tissue. Nuclear
NICD1 staining was observed in the suprabasal layer of the stratified squamous epithelium, both on the tonsil surface and in the crypts (Fig. 1A and B) in 5 of 5 (100%) controls, which was consistent with previously published results (21). In tumor samples, 64 (81%) stained positive for NICD1 staining. Three distinct patterns of NICD1 IHC staining were observed (Fig. 1C–H). The positive, peripheral pattern consisted of nuclear staining only at the tumor periphery, sparing the outermost cell layer. This was the most common (N = 37, 47%) pattern of staining. The positive, nonperipheral pattern exhibited nuclear staining of tumor cells, but in a more diffuse distribution. The nonperipheral pattern was observed in 34% of samples (N = 27). Nuclear staining was absent in negative pattern tumors (N = 15, 19%).

Association of NICD1 IHC pattern with NOTCH1 mutation status

NOTCH1 mutations were previously described in 17 (22%) tumors in this study and were predicted to be predominantly inactivating (Table 1 and Fig. 2; ref. 4). Most wild-type NOTCH1 tumors had positive staining (55/62, 89%), and the majority of these demonstrated a peripheral pattern (34/55, 62%; Table 2). Approximately half of the mutated NOTCH1 tumors had a negative pattern (8/17, 47%), including 6 of the 9 (67%) tumors with truncating mutations. Of the 9 tumors with mutated NOTCH1 that stained positive, the majority (6/9, 67%) were in a nonperipheral pattern (Table 1 and Table 2). Overall, NICD1 IHC staining patterns differed significantly by NOTCH1 mutation status (P < 0.001).

Clinicopathologic characteristics and TP53 disruptive mutations compared by NICD1 IHC pattern

Clinicopathologic characteristics were compared by NICD1 IHC staining pattern (Table 3). NICD1 IHC staining pattern was significantly different by HPV tumor status (P = 0.024). The majority of positive staining tumors were HPV positive (19/22, 86%). Among the positive staining tumors, similar proportions of peripheral (10/20, 50%) and nonperipheral (9/11, 45%) pattern tumors were HPV positive. By contrast, only 3 of 22 (14%) positive staining tumors were HPV negative.

NICD1 staining pattern was also associated with tumor differentiation (P = 0.012). The majority of poorly differentiated tumors were nonperipheral (44%) or negative pattern (39%). Nonperipheral pattern was associated with a 6-fold increase in odds of poor differentiation, as compared with a peripheral pattern (OR, 6.16; 95% CI, 1.18–22.57). Negative pattern was also associated with increased odds of poor differentiation (OR, 8.46; 95% CI, 1.75–40.81; P = 0.029). These associations remained even after adjustment for HPV tumor status and overall stage of disease (adjusted odds ratio [aOR], 5.27; 95% CI, 0.90–20.86; P = 0.066 for nonperipheral tumors; and aOR, 24.71; 95% CI, 1.53–399.33; P = 0.024 for negative tumors as compared with peripheral tumors).

Finally, NICD1 staining pattern was associated with ECS of lymph node metastases (P = 0.034), and the predominant pattern among tumors with ECS was nonperipheral (58%). As compared with tumors with a peripheral pattern, tumors with a nonperipheral pattern were significantly more likely to have ECS (OR, 9.90; 95% CI, 1.50–65.55; P = 0.017). This association remained robust after adjustment for HPV tumor status and overall stage of disease (aOR, 16.01; 95% CI, 1.92–133.46; P = 0.010). Negative staining pattern, conversely, was not associated with ECS (OR, 1.80; 95% CI, 0.25–12.88; P = 0.56).

TP53 is frequently mutated in HNSCC; therefore, the relationship between disruptive TP53 mutations and NICD1 IHC pattern was explored (Table 3). Disruptive TP53 mutation was not associated with NICD1 IHC staining pattern (P = 0.90), and the proportion of disruptive TP53 mutations was similar for each NICD1 IHC pattern.

Clinicopathologic characteristics and NICD1 IHC pattern in NOTCH1–wild-type tumors

NOTCH1 mutation status was associated with NICD1 IHC staining pattern (Table 2); therefore, clinicopathologic characteristics were compared by NICD1 IHC pattern for NOTCH1–wild-type (WT) tumors only (Supplementary Table S2). Among NOTCH1-WT tumors, oropharyngeal site was significantly associated with IHC pattern (P = 0.0089). All oropharyngeal tumors stained positively (18/18, 100%) and most had a nonperipheral pattern (11/18, 61%). The majority (90%) of oropharyngeal tumors with NOTCH1-WT were HPV positive.
There were limited HPV-negative tumors with NOTCH1-WT; therefore, analysis of IHC pattern by HPV tumor status was not feasible. When restricting to NOTCH-WT tumors, nonperipheral NICD1 IHC pattern was associated with ECS (OR, 14.40; 95% CI, 1.32–157.45; \( P = 0.029 \)) and poor differentiation (OR, 4.73; 95% CI, 0.98–22.71; \( P = 0.052 \)).

TP53 disruptive mutation status was not significantly associated with NICD1 IHC patterns among NOTCH1-WT tumors (\( P = 0.54 \), Supplementary Table S2).

Clinicopathologic characteristics associated with NOTCH1 mutation status

Clinicopathologic characteristics were then compared by NOTCH1 mutation status. Clinicopathologic characteristics and the proportion of disruptive TP53 mutations were largely similar for Notch1–wild-type and mutated tumors (Table 4), except for HPV tumor status, which was significantly associated with NOTCH1 mutation status (\( P = 0.006 \)). NOTCH1–wild-type tumors were more commonly HPV-positive than NOTCH1-mutated tumors both in univariate analysis (OR, 13.33; 95% CI, 1.59–111.47; \( P = 0.017 \)) and after adjustment for overall stage and gender (aOR, 19.06; 95% CI, 1.31–276.15; \( P = 0.031 \)).

Discussion

The Notch signaling pathway is a critical component of normal keratinocyte development, promoting differentiation and cell-cycle withdrawal. Here, we demonstrate that expression of the transcriptionally active NICD1 in HNSCC occurs in three distinct

<table>
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<th>Tumor sample ID</th>
<th>Mutation type</th>
<th>Nucleotide (cDNA)</th>
<th>Amino acid (protein)</th>
<th>Domain of Notch1 protein</th>
<th>Exon #</th>
<th>NICD1 IHC pattern</th>
</tr>
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<tr>
<td>HN12PT</td>
<td>Nonsense</td>
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<td>Nonperipheral</td>
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<td>p.P391S</td>
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<td>p.E450K</td>
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<td>Nonperipheral</td>
</tr>
<tr>
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<td>Missense</td>
<td>c.2434G&gt;T</td>
<td>p.G812W</td>
<td>EGF-like domain (21)</td>
<td>15</td>
<td>Nonperipheral</td>
</tr>
</tbody>
</table>

**Table 1. NOTCH1 mutations in individual tumors and corresponding NICD1 IHC staining patterns**

Abbreviations: fs, frameshift; Indel, insertion or deletion; LNR, Lin12/Notch repeat; RAM, RBP-jkappa-associated module.
immunohistochemical patterns. In addition, NICD1 expression pattern is associated with NOTCH1 mutation and the high-risk clinical features of ECS and poor differentiation.

Three distinct NICD1 expression patterns

The three NICD1 expression patterns observed by IHC were (i) positive, peripheral; (ii) positive, nonperipheral; and (iii) negative. The peripheral pattern was most common (n = 37, 47%) and resembled the suprabasal nuclear expression of NICD1 observed in normal human tonsil, oropharyngeal mucosa, and skin (21). Therefore, we considered the peripheral pattern to be the "normal" pattern, with retained features of NICD1 expression in nonpathologic keratinocyte differentiation. The peripheral pattern was also significantly more common in the more differentiated tumors, substantiating this hypothesis.

In the nonperipheral pattern, NICD1 expression appeared disorganized, with more diffuse staining among tumor cells and no distinct peripheral pattern. Tumors with nonperipheral NICD1 pattern were more likely to have the high-risk clinical features of poor differentiation and ECS compared with peripheral staining tumors. These findings may be consistent with Notch pathway escape from normal regulatory mechanisms, widespread NOTCH1 activation, and potentially the promotion of an epithelial–mesenchymal transition phenotype that has been associated with NOTCH1 activation in other malignancies (27–29). Although it is not possible to conclude that NOTCH1 is activated in this subset of tumors without examining its downstream targets, we speculate that NOTCH1 may behave as an oncogene in tumors with nonperipheral staining pattern.

In negative staining tumors, the loss of NICD1 expression and the association with poor differentiation suggest a tumor-suppressor role for NOTCH1, as previously suspected from sequencing studies (4, 5). Over half of the negative staining tumors (8/15, 53%) contained putative inactivating (4) NOTCH1 mutations, and most of these (6/8, 75%) were truncating. Negative tumors were less differentiated than peripheral tumors, but unlike nonperipheral tumors, did not have a greater likelihood of ECS. Loss of NICD1 expression is likely tumorigenic due to disruption of the established Notch1 tumor-suppressive functions in keratinocytes, such as promotion of terminal differentiation and inhibition of cell proliferation (13, 30, 31).

The immunohistochemical pattern of NICD1 staining in HNSCC has not been previously reported. Importantly, clinically significant high-risk markers, ECS, and poor differentiation, were associated with NICD1 IHC pattern but not with NOTCH1 mutation status, which may indicate a role for aberrant Notch signaling in HNSCC independent of NOTCH1 mutation.

The importance of NICD1 expression pattern is perhaps not surprising because canonical Notch signaling is mediated by cell-to-cell interactions, and is, therefore, dependent on tissue architecture (31, 32). The Notch pathway has been shown in other malignancies to interact with various components of the tumor microenvironment, including endothelial cells, stroma, and secreted molecules (31, 33–35). The significant association of nonperipheral NICD1 expression with high-risk features in our study implies that dysregulation of NOTCH1-mediated communication of HNSCC tumors cells with the surrounding microenvironment may be a determinant of disease progression.

Dual role of NOTCH1 in HNSCC

A dual role for NOTCH1 in HNSCC as both a tumor suppressor and oncogene would be consistent with the heterogeneous and context-specific actions of NOTCH1 in normal development and in other cancers. In normal development, Notch signaling promotes a variety of competing cell-cycle regulatory decisions, including terminal differentiation, proliferation and apoptosis, depending on the tissue, developmental stage and other parameters (12, 36). In human malignancies, NOTCH1 behaves as an oncogene in T-ALL (37, 38) and several solid tumors, but as a tumor suppressor in other cancers (32, 39). In fact, there is increasing support for a dual oncogenic and tumor-suppressor role for Notch signaling within the same solid tumor type in several malignancies, including breast, pancreatic, esophageal, and non-small cell lung cancer (32, 40).

Specific to HNSCC, a dual role for NOTCH1 may help to explain apparent incongruities in studies reported thus far. Initially, whole-exome sequencing revealed inactivating NOTCH1 mutations in 10% to 15% of HNSCCs, indicative of a tumor-suppressor role (4, 5). An integrative genomic analysis then reported that NICD1 expression in HNSCC cell lines with inactivating NOTCH1 mutations resulted in decreased cell growth and reduced tumor size in mice, again consistent with NOTCH1 functioning as a tumor suppressor (9). However, a subsequent comprehensive characterization of NOTCH1 in HNSCC

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**Table 2. NOTCH1 mutation compared with NICD1 IHC staining pattern**

<table>
<thead>
<tr>
<th>NOTCH1 mutation</th>
<th>Positive, peripheral</th>
<th>Positive, nonperipheral</th>
<th>Negative</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
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<tr>
<td>Wild-type</td>
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<td>21 (34)</td>
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<td>Total</td>
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<td>27 (34)</td>
<td>15 (19)</td>
<td>79 (100)</td>
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**NICD1 IHC, positive vs. negative**

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<tr>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
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<tr>
<td>Wild-type</td>
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<td>Total</td>
<td>64 (81)</td>
<td>15 (19)</td>
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P = 0.002

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demonstrated NOTCH1 pathway activation in a subset of tumors, with increased copy number or gene expression of NOTCH1 ligands such as JAG1 and JAG2. Furthermore, nearly one third of tumors (14/44) exhibited increased expression of NOTCH1 downstream target genes HES1 and/or HEY1 (10), suggesting an oncogenic rather than tumor-suppressor mechanism. Our findings introduce the possibility that the characteristics of Notch
dysregulation may in fact differ from tumor to tumor among HNSCCs, with NOTCH1 functioning as a tumor suppressor in a subset of tumors and as an oncogene in another subset, while retaining normal function in the remainder. This paradigm would aid in interpreting the apparent inconsistencies of existing literature.

Table 3. Clinicopathologic characteristics associated with NICD1 IHC staining pattern

<table>
<thead>
<tr>
<th>Clinicopathologic characteristics</th>
<th>NICD1 IHC staining pattern</th>
<th>Positive, peripheral N (%)</th>
<th>Positive, nonperipheral N (%)</th>
<th>Negative N (%)</th>
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<td>Stage &lt; 4</td>
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<td>6 (14)</td>
<td>44 (62)</td>
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<td>Stage 4</td>
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<td>20 (45)</td>
<td>18 (41)</td>
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<td>Differentiation</td>
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<td>Moderate or well</td>
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<tr>
<td>Poor</td>
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<td>8 (44)</td>
<td>7 (29)</td>
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<td>Angiolymphatic invasion</td>
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<td>3 (18)</td>
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<tr>
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<td></td>
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<td>11 (58)</td>
<td>1 (5)</td>
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<td></td>
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Abnormal (nonperipheral or negative) NICD1 IHC patterns were observed in 45% of wild-type NOTCH1 tumors and a normal (peripheral) pattern was seen in 18% of tumors with mutated NOTCH1. There are several explanations for this discrepancy, including heterogeneous functional consequences of NOTCH1 mutation dependent upon location within the NOTCH1 protein, potential sampling error in some tumors, and additional mechanisms of Notch pathway alterations independent of gene mutation. The latter is likely the driving factor behind our observations given that when analysis was limited to wild-type NOTCH1 tumors, the associations with ECS and differentiation noted in the whole cohort remained robust. There are numerous modalities of Notch signaling perturbations in other diseases, such as chromosomal translocation (38), abnormal activation by Notch ligands such as JAG1 and JAG2 (10, 27, 41), epigenetic modifications (42), and altered transcriptional coactivation (43). Downregulation or inhibition of NOTCH1 cleavage by gamma-secretase, required for release of NICD1, is another possible mechanism to explain loss of NICD1 expression in tumors with wild-type NOTCH1. Pathway-level Notch signaling aberrations in HNSCC have not yet been fully elucidated, but our results support their importance in a significant subset of tumors.

Therapeutic and clinical implications

The findings reported in this study may have therapeutic implications. If indeed Notch pathway dysregulation in HNSCC is bimodal, there may be an opportunity to tailor Notch-targeted therapy based on biomarkers of oncogenic or tumor-suppressor NOTCH1 activity in specific tumors. Several Notch pathway–specific drugs are currently under study for the treatment of solid tumors, including both inhibitors such as gamma-secretase inhibitors, and activators such as valproic acid (11).

The association of nonperipheral staining tumors with ECS and poor differentiation may be consistent with prior studies correlating increased NOTCH1 expression with poor prognostic parameters in HNSCC such as decreased survival (15, 16), cervical lymph node metastasis (15, 17), advanced stage (15), higher histologic grade (15), and cisplatin resistance (18, 19). Although these studies did not specifically evaluate transcriptionally active NOTCH1, that is, the NICD1, it is conceivable that increased NOTCH1 expression as reported corresponds to the nonperipheral pattern in our study, with its more diffuse NICD1 expression. Additional larger studies are necessary to determine the prognostic significance of NICD1 expression pattern.

HPV status and NOTCH1

The association of HPV-negative tumor status with both negative NICD1 expression and presence of NOTCH1 mutation has not been previously reported to our knowledge. The significance of this finding is unclear. In cervical cancer cell line studies, NOTCH1 interacts with HPV oncoproteins E6 and E7 in a complex manner: NOTCH1 has been shown to inhibit E6/E7 expression and induce growth arrest (44, 45), but E6/E7 have also been reported to upregulate NOTCH1 expression and lead to cell transformation (46). The association observed in our study may be a result of NOTCH1–E6/E7 interaction, but may also be a reflection of fewer genetic alterations in HPV-positive versus HPV-negative tumors (4, 5).

Disruptive TP53 mutations and NOTCH1

TP53 is the most commonly mutated gene in HNSCC and considerable cross-talk is observed between p53 and Notch signaling in keratinocytes (47–50); however, in our study there was no significant correlation between TP53 disruptive mutations and either NICD1 expression or NOTCH1 mutation status. This may indicate that Notch dysregulation is TP53 independent in HNSCCs, although it is difficult to draw conclusions in this relatively small sample size.

Limitations

The findings in this study are largely descriptive, and are, therefore, limited to hypothesis generation with regard to mechanistic reasons for and molecular consequences of the distinct NICD1 expression patterns. Without characterization of upstream regulators and downstream targets associated with the NICD1 expression patterns described here it is not possible to draw conclusions regarding the significance of these patterns in HNSCC. In addition, among the eligible archival tumors specimens, there were significant differences in those with available tissue for NICD1 IHC staining compared with those without tissue available, including higher T stage and higher likelihood of both having a NOTCH1 mutation and being from a female patient. The increased amount of tissue in higher T stage tumors likely improved availability. However, the higher prevalence of NOTCH1 mutation and greater female representation in this group are unexplained, and may have affected the results reported here.

Conclusion

Notch signaling alterations occur in a subset of HNSCCs but are poorly understood. The findings reported in this study indicate that there are three distinct patterns of NICD1 expression in HNSCC, and that these patterns are significantly associated with the presence or absence of clinically high-risk features. Although this may be consistent with a dual tumor suppressor and oncoprotein role for NOTCH1 in HNSCC, further research is necessary to confirm these findings, fully characterize Notch signaling aberrations in HNSCC, and elucidate the clinical importance and therapeutic implications therein.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH.

Authors’ Contributions

Conception and design: E.M. Rettig, C.H. Chung, J.D. Howard, N. Agrawal, C. Fakhry
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.M. Rettig, C.H. Chung, J.D. Howard, R.J. Li, D. Sidransky, W. Koch, N. Agrawal, C. Fakhry


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