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Cleaved NOTCH1 expression pattern in head and neck squamous cell carcinoma is associated with NOTCH1 mutation, HPV status and high-risk features

Eleni M Rettig¹, Christine H Chung^{1,2,3}, Justin A Bishop⁴, Jason D Howard³, Rajni Sharma⁴, Ryan J Li¹, Christopher Douville⁵, Rachel Karchin^{3,5}, Evgeny Izumchenko¹, David Sidransky¹, Wayne Koch¹, Joseph Califano^{1,6}, Nishant Agrawal^{1,7}, and Carole Fakhry^{1,6,8}

¹Johns Hopkins University School of Medicine, Department of Otolaryngology-Head and Neck Surgery, Baltimore, MD, U.S.A.

²Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD, U.S.A.

³Johns Hopkins University School of Medicine, Department of Oncology, Baltimore, MD, U.S.A.

⁴Johns Hopkins School of Medicine, Pathology, Johns Hopkins Medicine, Baltimore, MD, U.S.A.

⁵Johns Hopkins Institute for Computational Medicine and Department of Biomedical Engineering, Baltimore, MD, U.S.A.

⁶Milton J. Dance Jr Head and Neck Center, Greater Baltimore Medical Center, Baltimore, MD, U.S.A.

⁷Johns Hopkins University School of Medicine Ludwig Center for Cancer Genetics and Therapeutics, Baltimore, MD, U.S.A.

⁸Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, U.S.A.

Abstract

The Notch pathway is frequently altered in HNSCCs, 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. Immunohistochemistry 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/non-peripheral (34%) and negative (19%). NICD1 expression was associated with *NOTCH1* mutation status ($p < 0.001$). Most *NOTCH1*-wild type tumors were peripheral (55%), while mutated *NOTCH1* tumors were most commonly negative (47%). Non-peripheral tumors were more likely than peripheral tumors to have extracapsular spread (aOR

Corresponding authors: Carole Fakhry, 601 N. Caroline St. 6th Floor, Baltimore MD 21287; cfakhry1@jhmi.edu; phone 410-287-2024, fax 410-955-6526 and Nishant Agrawal, 601 N. Caroline St. 6th Floor, Baltimore MD 21287; nagrawal@jhmi.edu; phone 410-502-9827, fax 510-955-6526.

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16.01, 95% 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 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.

Keywords

NOTCH1; squamous cell carcinoma of the head and neck; HPV; extracapsular spread; differentiation

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 10–15% 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 co-activators 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) While 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 under-expression have been observed in tumors compared with normal tissue.(10, 14) Increased NOTCH1 expression by immunohistochemistry (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 was 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 immunohistochemistry 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 immunohistochemistry was available for 23 of 29 cases.

NICD1 Immunohistochemistry

Paraffin-embedded archival tumor tissue was used to prepare slides with standard 4µm 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, Beverly, MA) using a previously described immunohistochemistry (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, Tucson, AZ).

Stained specimens were reviewed and categorized into patterns of staining by a pathologist with expertise in tumors of the head and neck (JAB). Images were captured using an Olympus BX41 microscope, Olympus DP71 camera and Olympus cellSens® Standard

software (Center Valley, PA). 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. Each cell line was authenticated on July 31, 2011 using a short tandem repeat (STR) analysis kit, Identifiler (Applied Biosystems, Foster City, CA), 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 6–8 week old 20–22g Hsd:Athymic Nude-*FOXn1^{nu}* females purchased from Harlan Laboratories, Inc (Indianapolis, IN).

Characterization of *TP53* mutations

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

Analysis

Descriptive variables were summarized with frequencies and proportions for categorical variables, and medians and interquartile ranges (IQRs) for continuous variables. NICD1 IHC staining pattern, *NOTCH1* mutation status, clinicopathologic characteristics and *TP53* disruptive mutation status were considered categorical variables and compared using chi-squared 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 (College Station, TX, 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 to 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 (Supplementary Table 1).

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 non-pathologic tissue. Nuclear NICD1 staining was observed in the suprabasal layer of the

stratified squamous epithelium, both on the tonsil surface and in the crypts (Figure 1A–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 (Figure 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, non-peripheral pattern exhibited nuclear staining of tumor cells but in a more diffuse distribution. The non-peripheral 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 Figure 2).(4) Most wild-type *NOTCH1* tumors had positive staining (55 of 62, 89%), and the majority of these demonstrated a peripheral pattern (34 of 55, 62%; Table 2). Approximately half of the mutated *NOTCH1* tumors had a negative pattern (8 of 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 of 9, 67%) were in a non-peripheral pattern (Table 1 and Table 2). Overall, NICD1 IHC staining patterns differed significantly by *NOTCH1* mutation status ($p<0.001$).

Clinicopathological characteristics and *TP53* disruptive mutations compared by NICD1 IHC pattern

Clinicopathological 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 of 22, 86%). Among the positive staining tumors, similar proportions of peripheral (10 of 20, 50%) and non-peripheral (9 of 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 non-peripheral (44%) or negative pattern (39%). Non-peripheral 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 (aOR 5.27, 95% CI=0.90–20.86, $p=0.066$ for non-peripheral 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 extracapsular spread (ECS) of lymph node metastases ($p=0.034$), and the predominant pattern among tumors with ECS was non-peripheral (58%). As compared to tumors with a peripheral pattern, tumors with a non-peripheral 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.

Clinicopathological characteristics and NICD1 IHC pattern in *NOTCH1*-wild type tumors

NOTCH1 mutation status was associated with NICD1 IHC staining pattern (Table 2), therefore clinicopathological characteristics were compared by NICD1 IHC pattern for *NOTCH1*-wild-type (WT) tumors only (Supplementary Table 2). Among *NOTCH1*-WT tumors, oropharyngeal site was significantly associated with IHC pattern ($p=0.0089$). All oropharyngeal tumors stained positively (18 of 18, 100%) and most had a non-peripheral pattern (11 of 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, non-peripheral NICD1 IHC pattern was associated with extracapsular spread (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 2).

Clinicopathological characteristics associated with *NOTCH1* mutation status

Clinicopathological 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 immunohistochemical patterns. In addition, NICD1 expression pattern is associated with *NOTCH1* mutation and the high-risk clinical features of extracapsular spread and poor differentiation.

Three distinct NICD1 expression patterns

The three NICD1 expression patterns observed by IHC were (1) positive, peripheral, (2) positive, non-peripheral, and (3) 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 non-pathologic keratinocyte differentiation. The peripheral pattern was also significantly more common in the more differentiated tumors, substantiating this hypothesis.

In the non-peripheral pattern, NICD1 expression appeared disorganized, with more diffuse staining among tumor cells and no distinct peripheral pattern. Tumors with non-peripheral NICD1 pattern were more likely to have the high-risk clinical features of poor differentiation and extracapsular spread 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 non-peripheral 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 of 15, 53%) contained putative inactivating(4) *NOTCH1* mutations, and most of these (6 of 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 HSNCC 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 non-peripheral 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 NSCLC.(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 10–15% 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 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 of 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.

NICD1 is dysregulated independent of *NOTCH1* mutation

Although NICD1 expression was significantly associated with *NOTCH1* mutation status, there was an evident discordance. Abnormal (non-peripheral 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 *JAGGED1* and *JAGGED2*,(10, 27, 41) epigenetic modifications,(42) and altered transcriptional co-activation.(43) Down-regulation or inhibition of *NOTCH1* cleavage by gamma-secretase *NOTCH1*, 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 non-peripheral 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, e.g. the NICD1, it is conceivable that increased NOTCH1 expression as reported corresponds to the non-peripheral 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 to 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 impacted 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 oncogene 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. NICD1 immunohistochemical staining in normal and tumor tissue

A–B, Normal tonsil tissue with nuclear staining in suprabasal epithelial layer. C–D, Tumor tissue with nuclear staining in peripheral pattern, sparing outermost layer. E–F, Tumor tissue with nuclear staining in non-peripheral pattern. G–H, Tumor tissue with negative staining in tumor cells. Magnification: B, 10X; A, C–H, 20X.

Figure 2. Schematic representation of *NOTCH1* mutations

A. Depiction of *NOTCH1* mutations within corresponding Notch1 exons. B. Depiction of Notch1 protein domains corresponding to exons. The high number of mutations that are likely inactivating, e.g. truncating and/or located N-terminal to the transmembrane domain, has been described as evidence for a tumor-suppressor role of *NOTCH1* in HNSCC.⁶ Abbreviations: EGF, epidermal growth factor; LNR, Lin12/Notch repeat; HD, heterodimerization; TM, transmembrane; RAM, RBP-jkappa-associated module; TAD, transcriptional activation domain; PEST, proline (P), glutamic acid (E), serine (S), and threonine (T)-rich domain.

Table 1

NOTCH1 mutations in individual tumors and corresponding NICD1 IHC staining patterns

Tumor Sample ID	Mutation type	Nucleotide (cDNA)	Amino Acid (protein)	Domain of Notch1 protein	Exon #	NICD1 IHC pattern
HN12PT	Nonsense	c.5529G>A	p.W1843X	RAM	30	Non-peripheral
HN14PT	Missense	c.1171C>T	p.P391S	EGF-like domain (10)	7	Peripheral
HN102PT	Missense	c.1348G>A	p.E450K	EGF-like domain (11)	8	Non-peripheral
HN105PT	Missense Indel	c.2434G>T c.2436_2455delGGGT ACAAAGTGCAACTGCC	p.G812W fs	EGF-like domain (21) EGF-like domain (21)	15 15	Non-peripheral
HN107PT	Nonsense	c.1205C>A	p.S402X	EGF-like domain (10)	7	Negative
HN115PT	Indel Missense	c.1932_1931delGT c.4019G>C	fs p.G1340A	EGF-like domain (17) EGF-like domain (34)	12 25	Peripheral
HN117PT	Missense Missense	c.928G>A c.1366T>C	p.G310R p.C456R	EGF-like domain (8) EGF-like domain (12)	6 8	Negative
HN130PT	Nonsense	c.2845G>T	p.E949X	EGF-like domain (25)	18	Negative
HN139PT	Nonsense	c.1662C>A	p.C554X	EGF-like domain (14)	10	Negative
HN142PT	Missense Missense	c.1058G> c.6032T>G	p.R353H p.M2011R	EGF-like domain (9) Ankyrin repeats Ank_4	6 32	Non-peripheral
HN183PT	Nonsense	c.5872C>T	p.Q1958X	Ankyrin repeats Ank_3	31	Negative
HN194PT	Indel	c.4665delC	fs	LNR-3	26	Negative
HN208PT	Missense Missense	c.1093C> c.3838C>T	p.R365C p.R1280C	EGF-like domain (9) EGF-like domain (33)	6 23	Non-peripheral
HN227PT	Missense	c.1093C>	p.R365C	EGF-like domain (9)	6	Non-peripheral
HN245PT	Indel	c.1130delT	fs	EGF-like domain (10)	7	Negative
HN251PT	Missense	c.3876C>G	p.F1292L	EGF-like domain (33)	23	Negative

Tumor Sample ID	Mutation type	Nucleotide (cDNA)	Amino Acid (protein)	Domain of Notch1 protein	Exon #	NICD1 IHC pattern
HN25SPT	Missense	c.6115G>T	p.V2039L	Ankyrin repeats Ank_5	33	Peripheral
	Missense	c.6116T>A	p.V2039E	Ankyrin repeats Ank_5	33	

Abbreviations: NICD1, NOTCH1 intracellular domain; IHC, immunohistochemistry; Indel, insertion or deletion; fs, frameshift; RAM, RBP- γ kappa-associated module; LNR, Lin12/Notch repeat

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

NICD1 IHC, pattern of staining				
	<i>Positive peripheral</i>	<i>Positive, non-peripheral</i>	<i>Negative</i>	<i>Total</i>
<i>NOTCH1</i> mutation	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>
Wild type	34 (55)	21 (34)	7 (11)	62 (78)
Missense	2 (25)	4 (50)	2 (25)	8 (10)
Truncating	0 (0)	1 (14)	6 (86)	7 (9)
Missense & Truncating	1 (50)	1 (50)	0 (0)	2 (3)
Total	37 (47)	27 (34)	15 (19)	79 (100)

p<0.001

NICD1 IHC, positive vs. negative			
	<i>Positive</i>	<i>Negative</i>	<i>Total</i>
<i>NOTCH1</i> status	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>
Wild type	55 (89)	7 (11)	62 (78)
Mutated	9 (53)	8 (47)	17 (22)
Total	64 (81)	15 (19)	79 (100)

p=0.002

Abbreviations: NICD1, NOTCH1 intracellular domain; IHC, immunohistochemistry

Table 3

Clinicopathological characteristics associated with NICD1 IHC staining pattern.

Clinicopathological characteristics	NICD1 IHC staining pattern				p-value
	Positive, peripheral N (%)	Positive, non-peripheral N (%)	Negative N (%)	Total N (%)	
	37 (47)	27 (34)	15 (19)	79 (100)	
Age (years)					0.34
55	18 (55)	11 (33)	4 (12)	33 (42)	
> 55	19 (41)	16 (35)	11 (24)	46 (58)	
Sex					0.14
male	24 (44)	22 (41)	8 (15)	54 (68)	
female	13 (52)	5 (20)	7 (28)	25 (32)	
Race					0.77
white	31 (46)	22 (33)	14 (21)	67 (88)	
other	5 (56)	3 (33)	1 (11)	9 (12)	
Tobacco					0.42
no	14 (58)	7 (29)	3 (12)	24 (32)	
yes	22 (42)	20 (38)	10 (19)	52 (68)	
Alcohol					0.19
no	18 (58)	8 (26)	5 (16)	31 (47)	
yes	13 (47)	16 (46)	6 (17)	35 (53)	
Site					0.10
oropharynx	8 (35)	12 (52)	3 (13)	23 (29)	
other	29 (52)	15 (27)	12 (21)	56 (71)	

Clinicopathological characteristics	NICD1 IHC staining pattern				p-value
	Positive, peripheral	Positive, non-peripheral	Negative	Total	
	N (%)	N(%)	N (%)	N (%)	
HPV status					0.024
negative	1 (17)	2 (33)	3 (50)	6 (23)	
positive	10 (50)	9 (45)	1 (5)	20 (77)	
T Stage					0.69
T1-2	18 (44)	14 (34)	9 (22)	41 (59)	
T3-T4	14 (48)	11 (38)	4 (14)	29 (41)	
N Stage					0.54
N0	13 (46)	8 (29)	7 (25)	28 (39)	
N1-N2	19 (44)	17 (40)	7 (16)	43 (61)	
Overall stage					0.29
stage<4	13 (48)	7 (26)	7 (26)	27 (38)	
stage 4	20 (45)	18 (41)	6 (14)	44 (62)	
Differentiation					0.012
moderate or well	29 (56)	15 (29)	8 (15)	52 (74)	
poor	3 (17)	8 (44)	7 (39)	18 (26)	
Angiolymphatic invasion					0.30
no	8 (47)	6 (35)	3 (18)	17 (47)	
yes	7 (47)	11 (58)	1 (5)	19 (53)	
Perineural Invasion					0.57
no	7 (41)	9 (53)	1 (6)	17 (50)	
yes	6 (35)	8 (47)	3 (18)	17 (50)	
Extracapsular spread					0.034

Clinicopathological characteristics	NICD1 IHC staining pattern					p-value
	Positive, peripheral		Positive, non-peripheral		Negative	
	N (%)	N (%)	N (%)	N (%)	N (%)	
no	9 (64)	2 (14)	3 (21)	14 (42)		0.88
yes	5 (36)	11 (58)	3 (16)	19 (58)		
Locoregional recurrence						
no	22 (45)	17 (35)	10 (20)	49 (62)		0.27
yes	15 (50)	10 (33)	5 (17)	30 (38)		
Distant recurrence						
no	34 (50)	21 (31)	13 (19)	68 (86)		0.90
yes	3 (27)	6 (55)	2 (18)	11 (14)		
TP53 disruptive mutation						
No	27 (46)	21 (36)	11 (19)	59 (75)		0.90
Yes	10 (50)	6 (30)	4 (20)	20 (25)		

Abbreviations: NICD1, NOTCH1 intracellular domain; IHC, immunohistochemistry; HPV, human papillomavirus

Table 4Clinicopathological characteristics associated with *NOTCH1* mutation.

Clinicopathological characteristics	<i>NOTCH1</i> mutation status			p-value
	Wild-type	Mutated	Total	
	<i>N</i> (%)	<i>N</i> (%)	<i>N</i> (%)	
	87 (83)	18 (17)	105 (100)	
Age				0.42
55 years	38 (86)	6 (14)	44 (42)	
> 55 years	49 (80)	12 (20)	61 (58)	
Sex				0.061
male	67 (87)	10 (13)	77 (73)	
female	20 (71)	8 (29)	28 (27)	
Race				0.91
white	73 (82)	16 (18)	89 (88)	
other	10 (83)	2 (17)	12 (12)	
Tobacco				0.83
no	27 (84)	5 (16)	32 (32)	
yes	57 (83)	12 (17)	69 (68)	
Alcohol				0.32
no	38 (86)	6 (14)	44 (52)	
yes	32 (78)	9 (22)	41 (48)	
Site				0.77
oropharynx	26 (81)	6 (19)	32 (30)	
other	61 (84)	12 (16)	73 (70)	
HPV status				0.006
negative	3 (43)	4 (57)	7 (24)	
positive	20 (91)	2 (9)	22 (76)	
T Stage				0.43
T1-2	52 (85)	9 (15)	61 (65)	
T3-T4	26 (79)	7 (21)	33 (35)	
N Stage				0.18
N0	28 (76)	9 (24)	37 (39)	
N1-N2	51 (86)	8 (14)	59 (61)	

Clinicopathological characteristics	<i>NOTCH1</i> mutation status			p-value
	Wild-type	Mutated	Total	
	<i>N</i> (%)	<i>N</i> (%)	<i>N</i> (%)	
Overall stage				0.33
stage<4	36 (88)	5 (12)	41 (42)	
stage 4	45 (80)	11 (20)	56 (58)	
Differentiation				0.23
moderate or well	53 (84)	10 (16)	63 (71)	
Poor	19 (73)	7 (27)	26 (29)	
Angiolymphatic invasion				0.58
no	16 (80)	4 (20)	20 (48)	
yes	19 (86)	3 (14)	22 (52)	
Perineural Invasion				0.52
no	20 (83)	4 (17)	24 (55)	
yes	18 (90)	2 (10)	20 (45)	
Extracapsular spread				0.98
no	15 (88)	2 (12)	17 (40)	
yes	22 (88)	3 (12)	25 (60)	
Locoregional recurrence				0.86
no	55 (83)	11 (17)	66 (63)	
yes	32 (82)	7 (18)	39 (37)	
Distant recurrence				0.59
no	73 (82)	16 (18)	89 (85)	
yes	14 (88)	2 (12)	16 (15)	
<i>TP53</i> disruptive mutation				0.66
no	67 (84)	13 (16)	80 (76)	
yes	20 (80)	5 (20)	25 (24)	

Abbreviations: HPV, human papillomavirus