Genetic Alterations in TRAF3 and CYLD That Regulate Nuclear Factor κB and Interferon Signaling Define Head and Neck Cancer Subsets Harboring Human Papillomavirus

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INTRODUCTION

In virally induced cancers, nuclear factor κB (NF-κB) transcription factors that promote neoplastic transformation are aberrantly activated, and antiviral innate and adaptive immune responses often are deregulated. However, to our knowledge, the nature and role of the genetic changes required for sustained infection and transformation and their clinical consequences are less clear. Studies from The Cancer Genome Atlas (TCGA) and other sequencing projects recently provided important clues underlying the development and pathogenesis of a subset of head and neck squamous cell carcinomas (HNSCCs) harboring human papillomavirus (HPV) infection. Deletions or mutations were uncovered in the tumor necrosis factor receptor-associated factor 3 (TRAF3) and cylindromatosis lysine 63 deubiquitinase (CYLD) genes, which previously were implicated in the regulation of NF-κB and antiviral interferon (IFN) signaling in response to other DNA viruses. In this issue of Cancer, Hajek et al provide evidence that deletions or mutations in TRAF3 and CYLD define distinct subsets of HPV-positive (HPV+) HNSCC with associated activation of transcription factor NF-κB, episomal HPV infection of tumors, and improved patient survival.1

Aberrant Activation of NF-κB and Deregulation of Antiviral Immunity in Patients With HNSCC

The NF-κB transcription factors promote cell survival and induce innate and adaptive immunity to pathogens, and previously have been reported to be aberrantly activated during cancer development and progression.2,3 Various stimuli have been implicated in this activation, including viral or bacterial pathogens, carcinogens, growth factors, and inflammatory cytokines. Previous studies have identified 2 NF-κB pathways: the canonical pathway and the alternative (or noncanonical) pathway. The canonical pathway is activated by tumor necrosis factor receptor (TNFR) family members, as well as interleukin 1 and certain viral and bacterial components that induce Toll-like receptor (TLR) family activation. These receptors promote the activation of TGF-β-activating kinase (TAK1) and the tripartite inhibitor κB kinase (IKK) protein complex, which phosphorylates and marks inhibitor κB for ubiquitylation and proteasomal degradation, liberating the p50/RELA and p50/c-REL heterodimers to translocate to the nucleus and regulate NF-κB gene transcription. The alternative pathway is activated by alternative TNFR family members (eg, CD40 and lymphotoxin β receptor) via NF-κB-inducing kinase, which mediates IKK-ζ-mediated phosphorylation and proteasome processing of p100 necessary to form p52/RELB heterodimers that translocate to the nucleus and activate transcription. Both pathways can modulate overlapping and distinct gene programs promoting cell survival and immunity. Allen et al4 found evidence of the nuclear coactivation of these canonical and alternative pathway subunits in oropharyngeal HNSCCs that often harbor HPV, but to the best of our knowledge, the roles of HPV and genetic alterations in the activation of these pathways appear to be incompletely understood.

The first evidence that viral pathogen activation of NF-κB can contribute to cancer development followed the identification of the reticuloendotheliosis virus strain T (REV-T) viral oncogene v-rel, which causes reticuloendothelial lymphomatosis in chickens. v-rel shares a REL homology DNA-binding domain with all 5 human NF-κB/REL subunits.2,3 Aberrant activation of NF-κB in viral-associated cancers has been previously linked to viral oncoproteins. These include HPV types E6 and E7, which are implicated in cervical cancer and HNSCCs of the oropharynx and larynx, and Epstein-Barr virus latent membrane protein 1, which is associated with nasopharyngeal cancers. HPV E6 and EBV LMP1 have
both been implicated in commandeering activation of the alternative NF-κB pathway to sustain cell proliferation and survival to promote viral replication. However, the exact contribution of cellular genetic changes permissive for sustained viral infection and aberrant activation of the NF-κB pathways in tumor progression of viral-associated HNSCCs to our knowledge still are not completely understood.

**TRAF3 and CYLD Are Negative Regulators of NF-κB Pathway Activation**

It is interesting to note that recent genomic studies of HNSCC by TCGA as well as other groups identified deletions or mutations in TRAF3 and CYLD (Turban tumor syndrome)⁵ that previously were suggested to be negative regulators of NF-κB activation.⁷⁻⁹ TRAF3 is a member of the TRAF family and has multiple cellular functions in normal cells. Certain TRAF proteins were identified as adaptor proteins in TNFR and TLR signaling that bind to receptors at the time of activation and recruit other proteins to form an intracellular complex for IKK-NF-κB signaling. TRAF3 is implicated in TLR-induced canonical and alternative NF-κB pathway signaling. TRAF3 is an E3 ubiquitin ligase, which ubiquitylate target proteins and either can alter their function or target them for proteasomal degradation.⁹ With regard to these functions, TLR activation can recruit TRAF3 to ubiquitylate a scaffold to activate canonical IKK-NF-κB signaling and proinflammatory cytokines, whereas alternative pathway LTβR and CD40 receptors displace TRAF3 to stabilize NF-κB-inducing kinase to activate alternative IKK-α-mediated NF-κB pathway signaling. Both these functions are critical to regulating canonical and alternative NF-κB pathway activation and can act simultaneously. In response to viral pathogens, TRAF3 also serves a third function with RIG-I-like receptors to signal and activate type I IFN genes. Thus, TRAF3’s multifunctionality confers versatility when responding to receptor activation and promoting downstream signaling. Reflecting these critical functions, loss-of-function mutations in TRAF3 have been linked to decreased IFN production and antiviral immunity in pediatric herpes simplex encephalitis, and alternative NF-κB pathway activation in multiple myeloma.⁹ Recent studies have identified loss-of-function mutations and deletions in TRAF3 in HNSCC, specifically in patients with HPV+ HNSCCs.⁵,⁶ Taken together, these data suggest that TRAF3 could act as a tumor suppressor during pathogenesis of viral infections and tumor progression.

CYLD is a deubiquitinating enzyme that removes ubiquitin chains from proteins and subsequently alters their cellular functions. Its primary cellular targets are components of the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways. The primary target of CYLD is NEMO/IKK-γ, the noncatalytic subunit of the IKK complex that mediates canonical NF-κB signaling. CYLD deubiquitinitates NEMO, thus decreasing its stability and preventing the IKK complex from phosphorylating IkB and NF-κB activation. Loss of function in CYLD primarily leads to aberrant NF-κB activation and cylindromatosis, a disease in which benign tumors grow out of sweat glands around the head, face, and neck. Furthermore, loss of function of CYLD is linked to NF-κB-mediated inflammation that enhances the progression of various cancers.

**TRAF3 and CYLD Define a Distinct Subset of HPV+ HNSCC Cases with Associated Activation of Transcriptional Factor NF-κB, Episomal HPV Infection of Tumors, and Improved Patient Survival.**

In this issue of Cancer, the objective of the study by Hajek et al was to identify novel molecular characteristics and elucidate potentially novel and/or uncharacterized HPV-related mechanisms of HNSCC carcinogenesis.¹ Several key relationships between genomic alterations of TRAF3 and CYLD, HPV status, and NF-κB gene activation were revealed by bioinformatic analyses of TCGA data sets for HPV+/HPV-negative (HPV-) HNSCC and HPV+ cervical squamous cell carcinoma. By comparing cervical and HPV+ or HPV- HNSCC, the authors identified 2 unique and distinct HPV+ HNSCC tumor groups marked by the presence or absence of genomic alterations in the CYLD or TRAF3 genes.¹ Closer evaluation localized approximately one-half of the CYLD gene alterations to its ubiquitin hydrolase domain, close to the TRAF2 binding site, which is of potential importance in modulating canonical IKK-NF-κB signaling. It is interesting to note that the CYLD and TRAF3 genetic alterations affected mutually exclusive HPV+ HNSCC tumor groups, thereby suggesting that alterations in either TRAF3 or CYLD may function independently and sufficiently to deregulate the downstream NF-κB and IFN responses. Although the antiviral RIG-I-like receptors-TRAF3-IFN pathway has been characterized, to the best of our knowledge the molecular partners and mechanisms by which CYLD alterations could affect IFN responses remain to be defined. However, further analysis revealed that the majority of CYLD-mutated HPV+ HNSCC tumors...
(85%) lacked genomic HPV integration but were associated with the subset of HPV+ HNSCC cases that harbored episomes. Based on these results, Hajek et al have proposed that the TRAF3 or CYLD genomic alterations can enhance the presence of episomal HPV DNA.\(^1\)

Hajek et al evaluated the association of genetic TRAF3/CYLD alterations in episome-containing HPV+ HNSCC versus wild-type tumors, and found evidence of the activation of NF-κB, cooperating transcription factors (TF), and their target genes.\(^1\) Significantly enriched genes in TRAF3/CYLD-altered HPV+ HNSCC tumors were those that harbored NF-κB or epidermal growth factor 1 TF-binding sites, which are associated with increased NF-κB target transcription, including genes known to promote cell survival and proliferation in several cancers. Based on the above observations, Hajek et al proposed that NF-κB activation may contribute to episomal maintenance in these HPV+ HNSCC tumor subsets.\(^1\)

Furthermore, analysis in episome-containing TRAF3/CYLD-altered HPV+ HNSCC tumors also demonstrated an enrichment of gene clusters related to adhesion, motility, proliferation, differentiation, and DNA replication, gene profiles that clearly are distinct from integrated HPV+ or HPV- HNSCC tumors. They suggest that the unique enriched transcriptome profile in HPV+ tumors demonstrating altered TRAF3/CYLD and episomal DNA may include genes that help to maintain replicating viral DNA and promote encapsidation in well-differentiated squamous cells.

It was interesting to note that the study by Hajek et al also revealed a unique, strong correlation between TRAF3/CYLD genomic alterations and improved overall survival, with the authors suggesting the use of these alterations and survival data in the selection of subsets of patients with HPV+ HNSCC with improved prognosis. Tumors with these alterations also appear to be distinct from those in another major subset of patients with HPV+ disease harboring mutations or chromosome 3q amplification of the PI3 kinase gene phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA), as defined by TCGA.\(^5\)

**Conclusions and Future Directions**

It is becoming increasingly clear that HPV+ HNSCCs demonstrate molecular and clinical heterogeneity, and respond differently to currently available chemotherapies and/or radiotherapies. Although overall the population of patients with HPV+ HNSCC appears to respond better to treatment, subsets differ with regard to prognosis, and there are lifelong associated side effects and few treatment options for patients with recurrent metastatic HNSCC. Currently, patients are selected for clinical trials based on p16 immunopositivity, tumor staging and smoking history associated with prognosis.

In the study by Hajek et al, the authors identified a distinct subset of HNSCCs with loss-of-function alterations in TRAF3 and CYLD, and episomal HPV levels.\(^1\) The above data suggest that CYLD gene-altered and TRAF3 gene-altered HNSCCs may follow alternative mechanisms in the establishment of HPV infection and malignant transformation. Currently, additional studies are warranted to further elucidate the dual functional role of TRAF3 in HPV+ HNSCC viral status, the antiviral type I IFN response, and function in alternative NF-κB signaling. Identifying which of the functions of TRAF3 is the primary driver of TRAF3-mediated HPV+ HNSCCs could help to elucidate further future approaches for prevention and treatment.

The findings by Hajek et al may be of value, particularly in establishing new genomic markers for early HPV+ HNSCC detection, improved prognosis, and selection for de-escalation or novel alternatives in place of current chemoradiotherapy, which is highly toxic. First, the data in their study demonstrate that TRAF3/CYLD alterations, which are associated with episomal HPV, constitutively active NF-κB, and improved survival, may be integrated as biomarkers to select patients with more sensitive HPV+ HNSCC tumor subtypes for potentially less toxic or personalized treatment. Second, the identification of NF-κB activation and target genes in HPV+ HNSCC tumor subtypes containing episome HPV with altered TRAF3/CYLD could open a new area of research for promising novel cancer treatment options targeting the NF-κB pathways or target genes.

In support of this, a recent study by our laboratory identified a subset of HPV+ HNSCC tumors, with a notably strong association noted between TRAF3 deletion and elevated downstream alternative NF-κB pathway activity.\(^10\) TRAF3 deletion and rescue studies in HPV+ HNSCC-derived cell lines were found to demonstrate an important link between deficient TRAF3 and alternative NF-κB pathway activation, as well as increased tumor cell migration and drug resistance in HPV+ HNSCC. These findings suggest the TRAF3 alteration-mediated alternative NF-κB pathway could provide potential targets for therapy.

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REFERENCES
1. Hajek M, Sewell A, Kaech S, Burtness B, Yarbrough WG, Issaeva N. TRAF3/CYLD mutations identify a distinct subset of human Papillomavirus-associated head and neck squamous cell carcinoma. Cancer. 2017;123:1778-1790.
2. Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. Clin Cancer Res. 2007;13:1076-1082.
3. DiDonato JA, Mercurio F, Karin M. NF-kB and the link between inflammation and cancer. Immuno Rev. 2012;246:379-400.
4. Allen C, Saigal K, Nottingham L, Arun P, Chen Z, Van Waes C. Bortezomib-induced apoptosis with limited clinical response is accompanied by inhibition of canonical but not alternative nuclear factor-κB subunits in head and neck cancer. Clin Cancer Res. 2008;14:4175-4185.
5. Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015;517:576-582.
6. Seiwert TY, Zuo Z, Keck MK, et al. Integrative and comparative genomic analysis of HPV-positive and HPV-negative head and neck squamous cell carcinomas. Clin Cancer Res. 2015;21:632-641.
7. Mathis BJ, Lai Y, Qu C, Janicki JS, Cui T. CYLD-mediated signaling and diseases. Curr Drug Targets. 2015;16:284-294.
8. Font-Burgada J, Seki E, Karin M. CYLD and HCC: when being too sensitive to your dirty neighbors results in self-destruction. Cancer Cell. 2012;21:711-712.
9. Hacker H, Tseng PH, Karin M. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. Nat Rev Immunol. 2011;11:457-468.
10. Zhang J, Chen H, Yang X, et al. Defective TRAF3 modulates alternative NF-κB signaling and cytokine expression to promote cancer cell survival in HPV positive head and neck cancer (TUM10P.1049). J Immunol. 2015;194:211.30.