Journal of Cancer Clinical Trials

Issaeva, et al, J Cancer Clin Trials 2018, 3:1

Review article Open Access

TRAF3 and *CYLD* Gene Defects in HPV-Associated Head and Neck Cancer: Biomarker of Response and Indicator of Targets for New Therapies

Natalia Issaeva[1,2], **Barbara Burtness**[1,3] **and Wendell G. Yarbrough**[1,2,4*]

[1]*Yale Cancer Center New Haven, USA*

[2]*Division of Otolaryngology, Yale Department of Surgery, Yale Cancer Center, New Haven, USA*

[3]*Section of Medical Oncology, Yale Department of Medicine, Yale Cancer Center, New Haven, USA*

[4]*Yale Department of Pathology, Yale Cancer Center, New Haven, USA*

[*]**Corresponding author:** Wendell GY, Division of Otolaryngology, Department of Surgery, Yale Cancer Center, New Haven, USA, E-mail: wendell.yarbrough@yale.edu

Received date: September 13, 2017; **Accepted date:** October 7, 2017; **Published date:** October 12, 2017

Abstract

The incidence of HPV-associated (HPV+) Head and Neck Squamous Cell Carcinomas (HNSCC) has dramatically increased over the last 2 decades and continues to rise. These tumors are distinct from tobacco-associated HNSCC and have improved response to therapy and survival. Despite molecular, demographic and response differences between HPV+ and HPV-negative tumors, they are each treated with aggressive multi-modality therapy that can result in lifelong morbidity. To minimize long term morbidity, there are ongoing efforts to de-escalate therapy for HPV + HNSCC, but tools for selection of appropriate low risk patients has been limited without available molecular markers of tumor response. In addition, more targeted and less morbid therapies for HPV+ are not currently available. Recently, inactivating defects of the TNF Receptor-Associated Factor 3 (TRAF3) and cylindromatosis (CYLD) genes were identified in approximately 30% of HPV+ HNSCC and associated with improved survival, possibly accounting for the entire survival advantage of HPV association in HNSCC. In addition, gene expression analysis revealed that loss of TRAF3/CYLD was associated with increased NF-kB and decreased type I interferon signaling, suggesting that reversing these changes in signaling could offer new treatment strategies. Here we review the potential to use TRAF3/CYLD status of HPV+ HNSCC as a marker of selection for therapeutic de-escalation, or as an indication of new therapies that could be used to target this subset of tumors.

Keywords: Head and neck cancer; Biomarker; NF-κB; Prognostic subsets

Introduction

Landmark studies found that HPV is a causative agent in Head and Neck Squamous Cell Carcinoma (HNSCC) [1-3], and since this discovery, the annual rate of HPV-associated (HPV+) HNSCC has been rapidly increasing [4,5]. In 2012, HNSCC surpassed uterine cervical cancer to become the most frequently diagnosed HPV-associated cancer in the United States [6] surpassing uterine cervical cancer for the first time. Epidemiologic studies and post hoc analyses of clinical trials found that patients with HPV-associated HNSCC have dramatically improved survival and response to therapy compared to HNSCC lacking HPV (HPV- HNSCC) [7]. Demographics of patients with HNSCC differ based on HPV status with HPV+ tumors being found in younger patients with less tobacco use [8]. Likewise, gene expression, mutation, methylation and protein expression analyses identified vast differences between HPV+ and HPV- HNSCC [9-11]. A plethora of data suggests that HPV+ and HPV- HNSCCs are distinct clinical and molecular entities.

Although patients afflicted with HPV-associated HNSCC have improved outcome relative to those without HPV, 20-30% of patients with locally advanced HPV+ cancer will have their tumors recur [7,12]. Examination of clinical trial data correlated smoking history with poor outcome, but the basis for this association is not well understood. Molecular markers to distinguish those patients with HPV-related cancers who are at higher risk of disease recurrence have previously not been identified. Here, we review reports that TRAF3 and CYLD gene defects occur in a substantial portion of HPV+, but not HPV- HNSCC [13,14]. Mutations or deletions of these two genes were recently associated with maintenance of episomal HPV, with activation of NF-kB, with inhibition of immune response, lower likelihood of tobacco exposure and with improved patient survival [14]. Implications of these findings to identify patients with good prognosis and for identifying new therapeutic targets are explored.

Profiling of HPV+ Cancers to Identify Prognostic Subsets

DNA methylation, gene expression differences, reverse phase protein array, and chromosomal copy number differences each identified two subtypes of HPV+ HNSCC that correlated with HPV integration status [15-17], but these analyses did not explore differences in survival of identified subsets. Efforts to characterize subsets of HPV+ HNSCC that predict outcome have explored integrated analyses of omics data from tumors and adaptive immune response to these cancers. Combining gene expression data from multiple platforms, HPV+ cancers classified into two subtypes named inflamed/mesenchymal and classical [18]. The inflamed/mesenchymal group contained markers suggestive of CD8+ lymphocyte infiltration, and the inflamed/mesenchymal subtype had a trend toward improved survival. Examination of HPV-positive HNSCC by immunostaining revealed that tumors with more PD-1 expression on tumor infiltrating lymphocytes (TILs) had improved survival [19]. This seemingly paradoxical finding was further explored revealing that roughly half of

the PD-1 cells were active based on absence of Tim-3 expression suggesting that they may be active.

An additional study also suggested that adaptive immune response may predict survival in HPV+ HNSCC. Oropharyngeal cancer treated with induction therapy before definitive surgery for non-responders or concurrent chemo/radiation therapy revealed that higher counts of tumor infiltrating CD4 + CD8 lymphocytes and lower CD4:CD8 ratio was associated with improved survival in HPV+ tumors [20]. Interestingly, T lymphocyte infiltration was not associated with improved response to induction chemotherapy, even though response to induction chemotherapy was used in this study to determine definitive therapy [20].

TRAF3/CYLD as Biomarkers for Personalized Therapy

Improved prognosis for HPV+ oropharynx cancer has been demonstrated for patients managed with a number of approaches, including sequential multimodality therapy, radiation concurrent either with cisplatin or with cetuximab, or transoral resection with risk-based post-operative therapy [7,21-24]. Local and regional recurrence, as well as distant metastases, sometimes with late presentation, remains barriers to cure in a subset of HPV+ cancers [6]. Because current treatment paradigms were developed over several decades in trials of patients with the more treatment-resistant tobacco-associated and HPV-negative head and neck cancer, treatment was pushed to maximal intensity with high radiation dose, induction chemotherapy, altered fractionation, and concurrent radiation sensitization, with progressive increase in toxicity burden. Current clinical trials in HPV+ patients seek to develop new approaches which will cure the same number of patients, but lower the risk and severity of acute toxicities such as mucositis, dermatitis, rash, dysphagia and weight loss, ototoxicity and renal failure, and chronic toxicities such as disordered swallowing, xerostomia, osteoradionecrosis, hypothyroidism, accelerated cardiopulmonary decline and increased non-cancer mortality. To succeed in demonstrating improved functional outcome and non-cancer survival, without increasing the risk of cancer recurrence and cancer-specific mortality, it is important to conduct such de-escalation trials in patients with cancers that are truly favorable risk and treatment responsive. The highest cure rates (2 year survivals of 92-96%) are observed among patients with non-bulky disease, a lifetime smoking exposure of 10 pack years or fewer, and those whose tumors are responsive to induction chemotherapy. A recent trial of de-escalated therapy for HPV+ HNSCC, ECOG 3311, stratified patients into low risk, intermediate risk or high risk based on pathological examination after tumor excision and neck dissection. Margin status, five or fewer metastatic lymph nodes and extracapsular extension were used for risk assessment to determine post-operative therapy. The results of this trial have not been reported, but accrual is complete. To date, only pathologic and clinical predictors of outcome have been validated for these trials [25,26].

Strategies used in clinical trials exploring treatment deintensification variably omit chemotherapy, reduce radiation dose, or utilize minimally invasive surgery to reduce or eliminate disease burden and incorporate pathologic findings into selection of risk-based adjuvant therapy. It is likely that each of these approaches will be appropriate for a subset of patients, depending on underlying biologic factors such as risk of distant disease dissemination, and personalized prediction of toxicity, for which we do not currently have validated biomarkers. Thus, the advent of a mutational signature associated with higher survival, particularly if this can be demonstrated in clinical trials to augment or supersede current clinical or pathological predictors would permit stronger clinical trial designs as a stratification factor or potentially as eligibility criterion. As noted, the ECOG-ACRIN cooperative group recently demonstrated that for patients with non-bulky p16-positive head and neck cancer and a lifetime history of <10 pack years tobacco exposure who are responsive to induction chemotherapy, a reduced dose of radiation to 54Gy, and the substitution of cetuximab for cisplatin, results in a 2 year survival of 96% (disease-specific survival 100%) with statistically significant decreases in 1-year swallowing symptoms [25,26]. These findings led to the proposal for a randomized phase III trial in clinically favorable risk patients, comparing induction chemotherapy and selective radiation deintensification with the standard approach of 70Gy radiation with cisplatin or cetuximab. Incorporation of the TRAF3/CYLD signature is proposed as a stratification factor, with planned subset analysis in TRAF3/CYLD mutated vs. wild-type tumors. If TRAF3/CYLD gene alterations correlate with improved survival following de-escalation of therapy, it would be feasible to design de-escalation trials based on defects of TRAF3/CYLD to avoid induction chemotherapy or to include patients whose smoking status would indicate higher risk.

Identification of Subsets of HPV+ Cancers to Accelerate New Targeted Therapies

HPV+ HNSCC with defective TRAF3/CYLD are distinguished from HPV-driven cancers lacking these defects [14]. Tumors harboring TRAF3/CYLD mutations lack HPV integration, differentially express HPV genes, and can be distinguished based on human gene expression and methylation profiles. In fact, tumors lacking TRAF3/CYLD mutations cluster with HPV-negative tumors based on gene expression and methylation patterns [11,14]. These correlations suggest that a subset of oropharyngeal HNSCC is driven by the combination of episomal HPV and specific alterations in human genome. Here we will present rationale suggesting that gene defects in a subset of HNSCC may allow maintenance of HPV episomes leading to an alternative mechanism of HPV tumorigenesis or maintenance and that this subset of tumors may have vulnerabilities that can be targeted.

To begin exploring differences between tumors with episomal HPV and other HPV-driven tumors, we summarize the current understanding of how HPV drives cellular transformation. The classic or canonical HPV tumorigenesis model is based on study of uterine cervical cancer and pre-cancerous lesions. This canonical model shows that tumorigenesis following HPV infection relies on a period of HPV maintenance as episomes; however, transformation, marked as progression to CIN3 or cancer, relies on increased expression of HPV oncogenes E6 and E7 that occurs upon HPV genome integration [27,28]. Direct analyses to determine steps of carcinogenesis by HPV in HNSCC is impossible, since there are no identified pre-cancerous lesions; however accumulating data suggest that integration of the HPV genome is not the primary or necessary driver of HNSCC tumorigenesis. Several reports identify a substantial fraction (~30-40%) of head and neck cancers lacking HPV integration [11,29,30]. Although no reported studies focused on mechanisms through which episomal HPV drives head and neck cancer development, we postulate that a subset of HPV-associated HNSCC is driven by an alternative mechanism, distinct from the canonical HPV tumorigenesis, and that tumor driven by this alternative HPV carcinogenesis mechanism can be identified by molecular changes unique to this subset.

TRAF3 or CYLD mutations were found in tumors lacking HPV integration, and this correlation combined with known activities of TRAF3 and CYLD suggest that defects in these genes may allow HPV episomal maintenance and cell survival in HPV infected cells. Improved understanding of this subset of tumors and their dependency on TRAF3/CYLD mutations could be used to design targeted therapies. Gene expression analyses revealed that NF-kB was activated and interferon signaling was decreased in TRAF3/CYLD defective tumors [14]. In many cancer types including HNSCC, NF-kB signaling is oncogenic supporting cancer cell survival and therapeutic resistance [31]. On the other hand, interferon signaling inhibits viral replication and limits infection for many viruses. Currently there are few effective therapies for the estimated 20% of patients whose HPV+ tumors harboring TRAF3/CYLD mutations recur [14]. Immune modulatory therapy targeting PD-1 or PD-L1 has recently been approved for head and neck cancers and may be effective for these tumors; however, response in unselected HPV-associated tumors is limited at 20-30%, and no biomarkers are approved to improve patient selection [32]. The substantial rate of recurrence and lack of therapies for these patients, as well as expected morbidities of current therapy suggest that new, effective, and less morbid therapeutic options would be welcomed. Reactivation of interferon signaling and or inhibition of NF-κB as potential therapies targeting TRAF3/CYLD defective tumors will be discussed below.

Reactivation of Interferon Signaling as Therapy for HPV+ HNSCC

TRAF3 is an important component of both innate and acquired responses against viruses [33], including Epstein–Barr Virus (EBV) [34], Human Immunodeficiency Virus (HIV) [35], and HPV [36]. Interestingly, antiviral functions of TRAF3 are unique and cannot be substituted by other members of the TRAF family and seem to require the full-length wild-type protein [37,38], suggesting that truncating mutations of TRAF3 found in HPV(+) HNSCC most likely result in loss of TRAF3 anti-viral activity. CYLD was initially believed to inhibit Interferon (IFN) pathway activation by deubiquitinating the Pattern Recognition Receptors (PRR), and downstream kinases TBK-1 and IKKε [39], but in direct testing, CYLD knockout mice had defective IFN signaling in response to vesicular stomatitis virus [40]. The HPV oncogenic protein, E6 has been shown to bind and inhibit CYLD, but only under hypoxic conditions [41]. Although there is little data related to HPV, these studies demonstrate common functions of TRAF3 and CYLD to activate IFN in response to viral infection.

Gene Set Enrichment Analysis (GSEA) of HPV+ HNSCC revealed that tumors with TRAF3/CYLD mutations were associated with loss of expression of type I interferon target genes [14]. Expression of IFNα or IFNβ within the tumor stimulates not only the epithelial cancer cells, but also stromal and immune cells in the tumor to induce an anti-viral state [42]. After infection, epithelial cells produce and secrete type I IFNs that then activate surrounding cells through binding to the IFN Receptor (IFNAR) resulting in gene expression that regulates cell death, proliferation, differentiation, and immune modulation. Upon HPV infection of epithelial cells, the innate immune system is activated to eliminate infected cells or to protect them and surrounding cells from viral infection; however, HPV has developed strategies to avoid recognition and IFN activation including DNA integration that occurs during transformation. This raises an interesting question of how IFN response is inhibited in tumor cells lacking integrated HPV. Our pathway analyses suggest that somatic mutations in TRAF3 or CYLD genes inhibit interferon response in these cells. Abrogation of IFN signaling through somatic mutations may serve to promote survival of tumor cells containing episomal HPV and suggests that re-activation of IFN pathway may provide a missing link to cell death machinery in TRAF3/CYLD mutant tumors.

After discovery of type I IFNs, composed of 13 IFNα and a single IFNβ, their antiviral activities led to their FDA approval for treatment of chronic viral hepatitis caused by hepatitis B or hepatitis C viruses [43,44]. Interestingly, IFN is also used to treat a benign disease associated with episomal HPV, anogenital warts. Several viral proteins disrupt interferon signaling including the HPV E6 protein that binds interferon response factor-3 (IRF-3) to inhibit transcription of IFN suggesting that this disruption is important for viral lifecycle [44] and reviewed in [45,46]. In addition, loss of the IFNβ gene or the type I Interferon Receptor (IFNAR) in mice renders them extremely sensitive to viral infection [47,48], and mice lacking the IFN receptor are predisposed to develop cancer [49,50]. These anti-viral activities of IFN, as well as their direct anticancer activities to promote cellular apoptosis, inhibit cell cycle, and induce terminal differentiation, made IFNs promising candidates for virally-associated cancers and some cancer types with enhanced responses to innate immunity. More recently, the importance of type I IFN signaling for generalized immune stimulation has emerged as a critical function for protection against cancer [51]. Type I IFNs have been used for the treatment of several types of cancer, including: Kaposi's sarcoma, leukemia, lymphoma, melanoma and renal cell carcinoma [44]. However, despite obvious antitumor effects, IFN therapy has been challenging due to hematological and neurological side effects [52-54]. A better understanding of IFN activities in HPV+ HNSCC, particularly the effect on HPV episomes and cell behavior is needed, but for HNSCCs containing episomal HPV with TRAF3/CYLD mutations, IFN therapy could be considered

Activators of IFN signaling in tumor cells may be more localized and limit side effects. Interestingly, we recently found that HPV-positive head and neck cancer cell lines and primary cells were more sensitive than HPV-negative cells to the demethylating agent 5-azacytidine [55]. Cancer-related pathways that we found to be altered in HPV-positive, but not in HPV-negative head and neck cancer cells, after demethylation included activation of cytotoxic type I interferon response. Genomic demethylation using cytidine analogs did not change the IFN pathway in normal human keratinocytes, suggesting that IFN-associated toxicity related to demethylation is specific to HPV-positive head and neck cancer cells. Cellular effects of demethylating therapy are currently being tested in a clinical trial designed to compare cancer tissues before and after treatment.

Inactivation of NF-Kb as Therapy for HPV+ HNSCC

NF-κB signaling serves many roles including immune activation, proliferation and anti-apoptosis and its activation has been described in HNSCC and other tumors [56]. NF-kB signaling is particularly involved in B cell activities including their development, activation, and maturation [57]. NF-κB is also connected to IFN signaling, but it is not required for IFN expression late in viral infection, where instead it promotes survival of infected cells [58-60]. We observed that Diffuse Large B Cell Lymphoma (DLBCL) was one of the few tumor types with a high level of TRAF3/CYLD mutations, and previous work described activation of NF-κB through mutation of TRAF3/CYLD in B cell malignancies, multiple myeloma, and Hodgkin lymphoma [57]. Interestingly, a portion of both DLBCL and Hodgkin lymphoma are

associated with Epstein Barr Virus (EBV) infection, once again stressing the potential importance of TRAF3 and CYLD in cellular anti-viral response. In addition, mutations in these two genes have been found in EBV-associated nasopharyngeal cancer indicating that EBV-driven nasopharyngeal cancers may rely on NF-κB activity [61]. Finding TRAF3 and CYLD mutations in a subset of HPV+ HNSCC suggest that these tumors may similarly depend on NF-κB for survival and growth.

NF-κB signaling is described through two pathways: canonical and non-canonical. Inhibitor of kappa B kinase-gamma (IKK-γ= NEMO) is a gatekeeper for the canonical pathway, and after stimulation it along with inhibitor of kappa B kinase-α and -β (IKK-α, IKK-β) phosphorylate inhibitors of kappa B (e.g. IkBα) resulting in their rapid elimination by the ubiquitin proteasome pathway. Degradation of IkBs releases NF-kB dimers, primarily RelA/p^{50} or RelC/p^{50}, which enter the nucleus and directly transcribe target genes. Non-canonical NF-κB is initiated by phosphorylation and activation of the NF-κB-inducing kinase, NIK, which phosphorylates a dimer of inhibitor of kappa B kinase-alpha (IKKα). IKKα dimers then phosphorylate NF-κB2/p100 resulting in its processing by the proteasome into p^{52} enabling nuclear localization of the RelB/p^{52} dimer to initiate transcription. These pathways are differentially activated dependent on the stimulus in normal cells and dependent on mutations that drive cancers.

TRAF3 is a ubiquitin ligase that targets NIK for degradation, and loss of TRAF3 in tumors or in knockout mice results in constitutive activation of NIK and the non-canonical NF-κB pathway [62] On the other hand, CYLD is a de-ubiquitinase, but nonetheless also results in NF-κB activation. CYLD binds to and deubiquitinates IKKγ/NEMO and TRAF2 to inhibit canonical NF-κB signaling [63]. Regardless of which pathway activates NF-κB, it supports survival through transcription of inhibitors of apoptosis such as cIAP1/2 and Bcl-2. NF-κB is activated by cell stress including ionizing radiation and some chemotherapeutic agents and supports survival of cells during treatment, suggesting that its activation may contribute to therapeutic resistance [64]. The identification of inactivating mutations of TRAF3/CYLD in HNSCCs lacking HPV integration suggests that active NF-κB may support maintenance of HPV episomes or survival of cells containing episomes. Inactivation of NF-κB in these tumors may result in elimination of HPV episomes or of cells containing episomes. Understanding which pathway is primarily driving abnormal NF-κB activity will be a starting point to determine precision therapies may best reverse abnormal NF-κB activity. Given that NF-κB supports therapeutic resistance, targeting aberrant NF-κB signaling in combination with chemotherapy is reasonable to decrease survival signals.

NF-κB has been non-specifically targeted using drugs to inhibit the 26S proteasome in human cancers. Since the proteasome is critical for activation of both the canonical and non-canonical pathways, these drugs alter activity of both pathways. Proteasome inhibitors are approved for therapy of multiple myeloma where they are used singly, as well as in combination with standard therapy [65]. As a single agent, proteasomal inhibition resulted in improved progression free survival compared to the existing standard of care agent. Proteasomal inhibitors have also shown activity in EBV-infected B-lymphoblastoid cell lines and have been approved for therapy of a subtype of B-cell lymphoma, mantle cell carcinoma [66]. In HNSCC, a clinical trial of proteasome inhibition with re-irradiation for recurrent HNSCC enrolled several patients with oropharyngeal cancer [67]. This trial showed that proteasomal inhibition increased apoptosis in tumor biopsies, but had limited clinical effect (sustained partial responses in 28%). Examination of tumor specimens revealed inhibition of canonical NF-κB activation, but did not alter the non-canonical pathway, possibly explaining the limited efficacy observed [67]. Available proteasome inhibitors have a narrow therapeutic range with acceptable side effect profiles, but despite their utility there is a significant portion of intrinsic and acquired resistance [68]. Despite the shortcomings of proteasome inhibitors, they may be useful in HPV-associated HNSCC either alone or in combination.

The efficacy of proteasomal inhibitors led to development of more specific agents for NF-κB inhibition that target upstream or downstream components, but this field is relatively new. The Tumor Necrosis Factor Receptor (TNFR) family is a major upstream activator of NF-κB, and inhibitors of the ligand or receptor are used as anti-inflammatory modulators in autoimmune diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease [69]. Since molecular defects found in HPV+ HNSCC and other cancers do not require TNFR stimulation, these agents are not likely to be effective in cancers harboring gene defects activating NF-κB downstream of receptors.

Inhibitors of the IKK complex targeting the IKKβ subunit are being developed, and these agents should primarily target the canonical pathway since IKKβ is uniquely required for phosphorylation and degradation of IkBs [64]. On the other hand, inhibitors of IKKα will target both the canonical and non-canonical pathways, and pre-clinical work in HNSCC suggests that inhibition of IKKα using siRNAs or heat shock protein 90 (HSP90) inhibitors is more effective than targeting IKKbeta, likely related to IKKbeta restriction to the canonical pathway [70,71]. Inhibitors of IKKs have also shown promising results in preclinical models of breast cancer [72] and prostate cancer [73]. The non-canonical NF-κB pathway can be inhibited by targeting the NF-κb-Inducing Kinase (NIK) which is the upstream activator of this pathway. NIK is targeted for degradation by TRAF3 and is stabilized by loss of TRAF3 [74]; therefore, NIK is a prime candidate for inhibition in HPV+ HNSCC with TRAF3 mutations. NIK inhibition has shown efficacy against Hodgkin's lymphoma lines and in TRAF3 mutant multiple myeloma xenografts [75]. As more inhibitors are developed targeting NIK, they should be tested for efficacy in HPV+ HNSCC with TRAF3 mutations.

Another mechanism for activation of non-canonical NF-κB signaling is through Cellular Inhibitors of Apoptosis (cIAP) antagonists. TRAF3 degradation and NIK activation depends on cIAP activity. Drugs abrogating cIAP activity upregulate TRAF3 and inhibit NIK, and since cIAPs activity is upstream of TRAF3 drugs abrogating cIAP activity were not effective in multiple myeloma cells with TRAF3 inactivating mutations [76-78]. These drugs may be effective to inhibit NF-κB activity in HPV+ HNSCC lacking TRAF3 mutations, but will likely not have activity in TRAF3 mutant tumors.

Conclusion

TRAF3 and CYLD gene defects occur in a subset of HPV+ HNSCC and correlate with lack of HPV genome integration, with activation of NF-κB signaling, inhibition of IFN signaling, and with overall survival [14]. Given these findings, molecular profiling of HPV+ tumors to determine mutation status may be used to select patients for de-escalated therapy trials and suggest that strategies to induce IFN or inhibit NF-κB may be new therapeutic approaches for tumors harboring TRAF3 or CYLD mutations.

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