S079 CELL MEDIATED IMMUNE RESPONSE IN HPV ASSOCIATED OROPHARYNGEAL SQUAMOUS CELL CARCINOMA (UKCRN11945 TRIAL)

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Background: Immunological responses to viral infection during the development and progression of HPV16 positive oropharyngeal cancer are poorly understood but may provide important insights for type and timing of new therapeutic strategies.

Methods: Twenty-four patients with primary oropharyngeal squamous cell carcinoma (4 with stage III and 20 with stage IV disease) were included in this prospective clinical trial (UKCRN11945). We examined innate immunity and specific cell-mediated immune responses to HPV16 E2, E6 and E7 in peripheral blood using IFN-γ enzyme-linked (ELISPOT) assay pre- and post-treatment. CD4+, CD8+ and CD56+ lymphocyte cell counts were measured by flow cytometry (Figure 1). Study participants were recruited at baseline before HPV infection was sought using DNA / RNA extraction plus PCR with sequencing and p16 immunohistochemistry. Controls were those undergoing tonsillectomy for benign disease. All patients were treated with concurrent chemoradiation (cisplatin). Clinical response (primary outcome) was assessed 12 weeks post treatment by surface measurements at direct laryngoscopy, supplemented by radiographic imaging (MRI / PET-CT). A complete response was defined as no evidence of primary or nodal disease; partial response defined as >50% reduction; no response defined as <50% reduction.

Results: Correlations of subsets (HPV positive / negative) with tumor size, nodal status, physiological performance score, smoking, neck dissection, histology grade, age, sex and objective response to chemoradiotherapy were determined. Within the HPV cohort of patients, the CD4+ T-cell response to HPV16 E7 showed a significant increase from baseline (p<0.02; Figure 2). HPV16 positive status (p<0.03) and a low CD4+/CD8+ ratio (p<0.01) were both associated with complete tumor response after chemoradiation. The remaining variables showed no correlation with objective clinical response (HPV16 IFN-γ response / nodal status / tumor size / smoking / sex / age / performance status / p16 status / SCC differentiation).

Conclusion: This is the first study to prospectively evaluate cell-mediated immune response to HPV16 peptides in oropharyngeal carcinoma. Enhanced response of CD4+ T-lymphocytes to the E7 antigen may support a potential role for immunotherapy in future clinical trials.

Figure 1: Lymphocyte selection as verified by a fluorescence activated cell sorter (FACS) using relevant antibodies to cell surface markers (e.g. anti-human CD8 / RPE-Cy5)
Figure 2: IFN-γ ELISPOT HPV16 E6/E7 response among HPV positive OSCC patients before and after treatment. CD4+ response to HPV16 E7 was significantly elevated after treatment (P<0.02; CRT, chemoradiotherapy).
Carcinogenesis related to high-risk human papillomaviruses (HPV) is a significant prognostic factor for oropharyngeal squamous cell carcinomas (OSCC). The CD56 antigen is expressed by natural killer (NK) cells, which can trigger cell death of virus infected or tumor cells. NK cells can be classified into regulatory (CD56 high / CD16 low) and cytotoxic (CD56 low / CD16 high) cell populations. The serine protease Granzyme B is produced and released by activated NK cells to induce apoptosis within virus-infected tumor cells. In our study, the presence and activity of NK cells in HPV-related and unrelated OSCC was investigated within the tumors and in the tumor microenvironment. Further the prognostic impact of these findings was analyzed.

FFPE samples from patients with OSCC (n=140) were analyzed by immunohistochemistry. HPV status was assessed by staining for p16INK4a and HPV-DNA detection by PCR. Presence and number of CD56 positive cells was determined and evaluated statistically in regard to clinical parameters and HPV status. Activity status of NK cells was analyzed by double staining for Granzyme B and NK cell marker proteins (CD56 / CD16 / CD3) and immunofluorescence microscopy.

HPV-association as indicated by p16INK4a and HPV-DNA positivity was found in 29/140 (21%) cases. Overall survival of these patients was significantly increased in contrary to HPV-unrelated OSCC (Log Rank test, p<0.001). Additionally, the presence of CD56+ cells was increased in tumor and tumor microenvironment of HPV-associated OSCC (x2, p<0.001). Overall survival was significantly increased (6.2 ±0.5 vs. 4.9 ±0.6 years) for patients with detectable CD56+ cells in tumor and tumor microenvironment compared to patients with no detectable CD56+ cells (Log Rank test, p<0.05). Preliminary results demonstrate a higher proportion of activated NK cells in HPV associated samples as indicated be colocalization of Granzyme B and NK cell markers.

Presence and activity of NK cells is significantly increased in HPV-associated OSCC. Beside higher sensitivity of HPV-associated OSCC to radiation, this could be an additional reason for better prognosis of these patients compared to those with HPV-unrelated OSCC. Furthermore strategies activating NK cells might be a therapeutic option for these cancers in the future.
Objective: to determine whether HPV 16 infection, tumor-associated immune cells, and peripheral blood neutrophils and lymphocytes may predict therapy response and survival in HNSCC patients.

Methods: This unplanned analysis of data of a phase II single center prospective clinical trial investigated the HPV16 status determined by p16 immunohistochemistry, and pretreatment levels of NK cells, cytotoxic T lymphocytes, regulatory T (Treg) cells and mature dendritic cells (DCs) in paraffin-embedded biopsy samples by immunohistochemistry of NKp46, CD8, FOXP3 and DC-LAMP markers, respectively.

Study population: 47 patients with untreated stage III-IV resectable cancers of the oral cavity (OC), oropharynx (OPH), hypopharynx (HPH) and larynx (L): 9, 19, 13 and 6 patients, respectively, were evaluable for response to two cycles of TPF plus cetuximab induction chemotherapy (ICT) followed by radiotherapy plus cetuximab in the responders. All but one nonresponders received active combined modality anticancer treatment: either surgery followed by radiotherapy, or radiotherapy with or without cisplatin or cetuximab. Samples of 42/47 cases were available for HPV examination and 39/47 for immune cell analysis. Pretreatment peripheral blood neutrophil and lymphocyte counts were analyzed in all 47 patients.

Results: Response rate to ICT was 33/47 (70.2%), which was lowest (1/9, 11%) in OC cancer patients. After 47-month median follow-up there was no difference in survival according to treatment response. p16 positivity was observed in 14/42 (33%) samples in the whole population, 9/16 OPC (56%) 4/12 HPC (33%), 1/8 OC (12%), while all 6 laryngeal tumors were p16-negative. p16 status did not show correlation with response to ICT or with survival. However, most p16+ cases (8/14) belonged to the group of regular smokers and alcohol drinkers with less than 2 year survival in 5/8 cases compared to 5/17 in p16-negative smoker-drinkers. Among the tumor-infiltrating immune cell types studied, we found significant association of high density (>160 cells/mm²) of DC-LAMP+ mature DCs with response to ICT: 20/26 (77%) of samples from responders vs. 3/10 (30%) of those from nonresponders contained high amount of these cells (p<0.01). 11/13 (85%) p16+ cases were characterized by high density of mature DCs, compared to 12/23 (52%) of p16- ones. The majority of tumors of the oropharynx (10/13, 77%) and hypopharynx (9/11, 82%) contained high number of DC-LAMP+ DCs compared to 1/7 (14%) of oral cavity cancers. The same tendency for difference according to tumor site was seen in the case of tumor-associated NK cells. Similarly to DC-LAMP+ DCs, infiltration by CD8+ T cells also showed association with p16 positivity. NK cell, CD8+ T lymphocyte and Treg numbers did not correlate with treatment response, and none of the cell types studied showed association with survival. Cases with elevated pretreatment blood neutrophil count (>6.5 x 10⁹/L) showed tendency for poor outcome (p=0.07).

Conclusion: Our results support the potential role of immune mechanisms in the biology of HNSCC. Furthermore, they suggest that in addition to smoking, heavy drinking may further deteriorate the prognosis of HPV-positive patients.

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**S082 TRANSIENT IMMUNOMODULATORY EFFECT OF INDUCTION CHEMOTHERAPY IN HPV-POSITIVE HEAD AND NECK CANCER**

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**Introduction:** HPV-related OPC is clinically and epidemiologically distinct from environmentally-related OPC with better survival outcomes. The presence of viral antigens and a subsequent HPV-specific response likely plays a role and suggests immune-based therapy could be a viable option to integrate with standard of care surgical and chemoradiation regimens. Induction chemotherapy with taxane-platinum-5FU (TPF) prior to chemoradiation therapy has improved outcomes in OPC. Yet it is still unclear how this initial therapy interacts with the endogenous immune response to HPV and affects the tumor microenvironment. Of particular interest are the changes in tumor-mediated immunosuppression through mechanisms such as the release of inflammatory mediators like VEGF and the regulation of suppressive/regulatory immunocytes like myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg). Understanding these early changes in the immune profile will guide both timing and specific targets of immunotherapy.

**Methods:** The immune responses in the peripheral blood of 11 stage II-IV HPV+ OPC patients receiving taxol-platinum-5FU (TPF) induction chemotherapy followed by concomitant chemotherapy (CCRT) were evaluated by serial blood sampling before and after each treatment. Circulating immunocytes including immunosuppressive MDSCs and Treg cells, and effector CD4+ and CD8+ T cells were measured by flow cytometry, and levels of serum cytokines were assessed by Luminex multiplex array. HPV antigen-specific T cell responses were measured by Luminex analysis of IFN-γ production after stimulation of PBMC with HPV16 E6 and E7 peptide pools.

**Results:** Following induction chemotherapy there was no change in CD4+ and CD8+ T cell numbers, a marked decrease in MDSC (1.8 fold), small increase in Treg, and an increase in VEGF (1.8 fold) and VEGFR (1.7 fold). After CCRT there was a decline in CD4+ (1.5 fold) and CD8+ (2.0 fold) T cell numbers from baseline, an increase in MDSC (1.9 fold), decline in T reg (1.6 fold), and further increase in VEGFR (2.8 fold). HPV-specific T cell responses were present in 7/11 patients at baseline, 8/10 patients following induction chemotherapy, and only 4/11 patients after CCRT. 3 patients had dramatic increases in these responses following induction therapy, including 2 that had no detectable baseline response.

**Discussion:** We observed a generally immuno-stimulatory response following induction chemotherapy with some patients developing strong HPV-specific responses, decrease in MDSC levels, and unchanged effector cell populations. Yet these levels quickly reversed following CCRT, which had an overall immunosuppressive effect. The rising VEGFR levels provide one possible mechanism for driving MDSC expansion and immunosuppression. These data suggest that different standard-of-care treatments can have divergent effects on immune response. Future studies to better characterize these immune responses to existing therapy will help determine the appropriate timing of concurrent immunotherapy.
ABERRANT DNA METHYLATION CHANGES ASSOCIATED WITH HPV-POSITIVE OROPHARYNGEAL SQUAMOUS CELL CARCINOMAS TARGET THE CDKN2A LOCUS
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Background: Human papillomavirus (HPV) positive oropharyngeal squamous cell carcinoma (OPSCC) is increasingly recognized as a distinct disease associated with improved survival. HPV oncoproteins, E6 and E7, are known to interfere with the activity of tumor suppressor proteins p53 and pRb, respectively, and may affect the expression and transcription of other tumor related proteins that influence prognosis including CDKN2A(p16). However, infection of HPV alone is not sufficient to induce malignant changes, and new evidence now suggests that HPV-induced epigenetic changes in host cell DNA may be important for cancer progression.

Objective: Given that gene expression and DNA hypermethylation profiles differ significantly by HPV status in OPSCC, we wanted to identify a specific subset of CpG loci that altered their DNA methylation in response to HPV infection in OPSCC.

Design and Methods: We performed genome-wide DNA methylation profiling using the Illumina HumanMethylation27 beadchip on primary tumor samples and corresponding adjacent mucosa from 46 OPSCC patients tested for HPV DNA and RNA and p16 protein expression. Formalin fixed tumor specimens were tested for p16 protein expression by immuno-histochemistry. HPV type-16 DNA and RNA was detected by MY09/11-PCR and E6/E7 RT-PCR on matched frozen tissue. Validation of the beadchip measurements was carried out using mass spectrometry and real-time reverse-transcriptase PCR. We also created OPSCC cell lines expressing HPV16 E6/E7 oncogenes by transfection with the pLXSN16-E6/E7 vector, and assessed changes in DNA methylation in response to HPV infection.

Results: We identified 22 CpG loci showing a statistically significant difference in methylation when comparing HPV-positive to HPV-negative OPSCC patients, including four loci located downstream of the CDKN2A(p16) transcription start site. The identified region may play a role in the transcription of multiple splice variants including p14(ARF), p12, p16 gamma and INK4A. DNA hypermethylation of this region correlated with an increase in expression of ARF, but not to any of the other transcript variants of the CDKN2A locus. We are now completing in vitro experiments to determine whether or not transfection of HPV16 E6/E7 oncogenes are sufficient to recapitulate this effect.

Conclusion: HPV infection in OPSCC is associated with novel epigenetic events that may regulate key effector proteins, including for four panel loci downstream of the transcriptional start site of CDKN2A that, despite being hypermethylated in tumor compared to normal tissues, are associated with increased expression of the p14(ARF) splice variant of the cellular gene.
DISRUPTION OF THE VIRAL E2 GENE IN HPV-ASSOCIATED HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Background: Human papillomavirus (HPV)-16, a high-risk strain, is the predominant genotype in HPV+ head and neck squamous cell carcinoma (SCC). The HPV genome can exist in the keratinocyte host cell as a circular episome, or it can linearize and integrate into the human host genome. Integration of the HPV genome into human DNA often results in disruption of the HPV E2 oncogene. This is significant because the HPV E2 protein has been shown to regulate the transcription of the E6 and E7 HPV oncoproteins. In cervical cancer, where HPV is the major etiological agent, disruption of the E2 oncogene is associated with malignant progression and studies have suggested that the physical state of the E2 oncogene may influence HPV-induced radiosensitivity. It is therefore important to assess the integrity of the E2 oncogene in HPV positive HNSCC and determine if there is a relationship between E2 disruption, E6/E7 expression, prognosis and treatment response of these tumors.

Objective: In HPV positive HNSCC, disruption of the E2 oncogene and its impact on expression of HPV oncoproteins is not well studied. We first hypothesized that disruption of the E2 oncogene would result in deregulated transcription of the HPV oncoproteins, E6 & E7, and that the deregulation of transcription would result in overexpression of the oncogenes. Secondly, the physical state of the E2 oncogene may influence radiosensitivity for HPV positive oropharyngeal cancer.

Methods: In this study, expression of HPV oncoproteins in head and neck SCC specimens was assessed by qRT-PCR with probes for HPV-16 E6, E7 & E2 oncoproteins. The physical state of the E2 oncogene in HNSCC specimens was determined using a PCR-based method that amplifies sequential sequences of the gene with several different probes. Then, the E2 disruption data was combined with HPV E6 & E7 oncogene expression data from the same HNSCC samples. We also attempted to identify correlations between the E2 disruption, E6 & E7 expression and various clino-pathologic and molecular biomarker data collected as part of an ongoing prospective array study of HNSCC tumors conducted at Montefiore Medical Center.

Results: We screened 35 primary oropharyngeal SCC tumors for disruption of the HPV-16 E2 gene. Twenty-three (66%) HPV+ tumors appeared to have an intact E2 oncogene while 12 (34%) have disrupted E2. Additionally, disruption of the E2 oncogene appeared to correlate with decreased E6 & E7 expression. We also observed a positive correlation between the detection of intact E2 and E6/E7 oncogene expression in HPV positive tumors. Correlations between HPV16 physical state and oncogene expression by clino-pathological indicators and molecular biomarker data are being assessed.

Conclusion: Our data suggest that E2 disruption, a surrogate for viral integration into the host genome, is not prevalent in oropharyngeal SCC but is correlated with increased expression of E6 & E7 oncogenes, which have previously been associated with cancer prognosis.
S085  BIONETWORK COUPLING OF METHYLOME DIFFERENCES IN HPV-ASSOCIATED HNSCC

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**Background:** Epidemiological and laboratory evidence indicate the human papilloma virus (HPV) as a causative agent for some squamous head and neck cancer (HNSCC). The biologic significance of HPV as independent risk factor is underscored by the improved prognosis for patients with HPV positive (HPV+) HNSCC relative to HPV negative(HPV-) due in part, to a better therapeutic response to chemoradiotherapy. The mechanism for these improved prognosis outcomes remains underexplored.

**Methods:** To obtain insights into a potential epigenetic role, we profiled the methylome of 4 HPV+ and 4 HPV- HNSCC using the Infinium HumanMethylation450 BeadChip. Degree of methylation was calculated as a β-value (ranging from 0 to 1), and M-values [log (β/ (1- β))] were used for significance tests. Additionally, we evaluated the status of 11 genes previously reported (Lechner et al., 2013) as significantly differentiated between HPV+ and HPV- in our 8 HNSCC sample set and assessed their biologic significance using Ingenuity Pathway Analysis (IPA).

**Results:** In our sample set, of the 11 genes, 7 were significantly differentiated between HPV+ and HPV- (aFDR: adjusted false discovery rate). CDH8, PCDHB11, ELMO1, MSX2, and HTR1E were hypermethylated; MEI1 and C14orf162 (excluded in IPA) were hypomethylated. Methylation status of all 11 genes as either hypo- or hypermethylated was concordant with the Lechner et al., study. IPA connected 7/10 genes in a 35 gene network characterized by the functions: Cellular development, Skeletal and muscular system development and function, and Embryonic development. Significantly ranked molecular and cellular functions included cell to cell signaling and interaction, cellular movement, cell death and survival among others. TGF-β, Gα12/13, and BMP signaling were among the highly ranked 5 canonical pathways. WNT3A was identified as an upstream regulator. TGF-β signaling, identified in IPA’s Toxicity Functions list suggests implication in clinical pathology endpoints.

**Conclusion:** In this independent sample set, confirmation of 7 of the 11 genes as differentially methylated in HPV+ vs HPV- HNSCC with complete concordance of methylation direction (hypermethylated vs hypermethylated), validates their role in differentiating HPV+ and HPV- HNSCC. Cadherins are implicated in tumor progression and metastasis and are targets of the Polycomb group (PcG) proteins, which form chromatin-modifying complexes that are essential for embryonic development and stem cell renewal and are commonly deregulated in cancer. Our study further supports cadherins CDH8 and PCDHB11 together with MEI1, MSX2, ELMO1 and HTR1E as likely biomarkers in HPV-associated HNSCC.

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S086 HIGH PREVALENCE OF DISCORDANT HPV AND P16 OROPHARYNX SQUAMOUS CELL CARCINOMAS IN AN AFRICAN AMERICAN COHORT

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Background: The prevalence of oropharynx squamous cell carcinoma (OPSCC) continues to rise due to the Human Papilloma Virus (HPV), but most of these studies have been performed in whites. A paucity of data on the prevalence and survival of HPV OPSCC in the African American (AA) population has been reported. Our goal was to examine the incidence of HPV in an AA OPSCC cohort and its relationship to survival.

Study type: Retrospective chart and pathological review in a tertiary care hospital.

Methods: The tumor registry of two large tertiary care hospitals was used to identify AA OPSCC patients from 1998-2010. Tumor blocks were obtained from pathology archives. HPV16 testing was performed by PCR from DNA extracted from tumor blocks. Additional testing was performed using INNO-LiPA testing. P16 staining was performed using standard immunohistochemistry.

Results: 44 patients were identified for analysis. 73% of the cohort were either current or former smokers. 68% of patients were advanced stage OPSCC. 73% of patients (n=32) were HPV positive, of which all but one HPV16 positive. HPV associated OPSCC was noted throughout the examined time period without a clear increasing trend. Only 37% of HPV+ patients were also P16+. HPV+/P16- patients had intermediate overall survival, with survival outcome between HPV+/P16+ and HPV-/P16- patients. (p=0.08). P16 status was a marker for better overall survival on univariate analysis (OR 0.35, CI 0.13-0.92)

Conclusions: HPV associated OPSCC is strongly present in this AA cohort, with survival outcomes that stratify by HPV/P16 status, trending to significance. A significant discordance between HPV and P16 status was noted with approximately two-thirds of HPV positive patients being P16 negative. Greater study is needed to explain the high P16 negativity amongst HPV+ OPSCC in this AA cohort.
**Background:** The incidence of HPV-associated oropharyngeal carcinomas has increased rapidly over the last thirty years. These tumors behave as a distinct biological entity when compared to classical smoking- and alcohol-related disease. To gain more information regarding the pathway through premalignancy in HPV positive / HPV negative oropharyngeal carcinoma (OSCC), we investigated genomewide expression profiles in histopathologically confirmed tumor samples with site-matched normal epithelial controls.

**Methods:** Twenty-four patients with primary oropharyngeal squamous cell carcinoma (4 with stage III and 20 with stage IV disease) were included in this prospective clinical trial (UKCRN11945). The tumor tissues were assessed by histopathology and p16 immunohistochemistry with HPV status and typing by consensus PGMY PCR, type-specific HPV16 DNA & RNA PCR and DNA sequencing. Fresh tissue samples were subjected to whole transcriptome analysis using the Illumina Bead Array (~47,000 transcripts) and the results validated with quantitative Reverse Transcriptase-PCR. In a separate cohort of twelve OSCC patients, laser capture micro-dissection of FFPE tissue allowed RNA extraction precisely from regions of in situ malignant change (with matched normal and invasive SCC samples).

**Results:** The majority of OSCC patients (28/36) displayed evidence of high risk HPV positivity. Predictable fold changes of RNA expression in HPV-associated disease included multiple transcripts within the p53 oncogenic pathway (e.g. CDKN2A / CCND1). Other candidate transcripts found to have altered levels of expression in this study have not previously been reported in oropharyngeal cancers. These were involved in cell differentiation, proliferation and invasion and demonstrated considerable overlap with expression analysis data in anogenital carcinoma (SFRP1 / CRCT1 / DLG2 / SYCP2 / CRNN). SYCP2 showed the highest consistent fold change from baseline in both fresh frozen and FFPE tissue (Fresh frozen [P=0.046]; FFPE pre-invasive [P=0.012]; FFPE invasive [P=0.024]. Aberrant expression of this meiosis-specific protein may contribute to genetic instability during HPV-associated cancer development.

**Conclusion:** Investigation of differentially expressed genes in HPV-positive tumors may reveal unique pathways that can explain their different natural history and biological properties. The data from this study reveal SYCP2 (amongst others) as a potential biomarker in HPV positive oropharyngeal carcinoma, and if corroborated on a larger scale, may facilitate the development of a non-invasive screening tool.

**Figure 1:** qRT-PCR validation graph
- qRT-PCR (Log2 fold change for fresh frozen biopsy [invasive SCC versus normal])
- qRT-PCR (Log2 fold change LCM FFPE tissue [in situ versus normal])
- qRT-PCR (Log2 fold change LCM FFPE tissue [invasive versus normal])
- Illumina platform (Arithmetic fold change for fresh frozen tissue [invasive SCC versus normal])