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ORIGINAL PAPER

Comparison of Interleukin-1 Ligand Expression by Human Papilloma Virus Status in HNSCCs

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Abstract

Interleukin-1 alpha (IL-1α) is a cytokine involved in the acute phase immune response and its expression is upregulated in a variety of solid tumors including head and neck squamous cell carcinomas (HNSCCs). Tumor expression of IL-1α is associated with increased tumor aggressiveness in HNSCCs, but this has yet to be studied in the context of human papilloma virus (HPV) status. This study is aimed at determining differences in tumor expression and subcellular localization of IL-1α in HPV-positive (HPV+) and HPV-negative (HPV−) HNSCC tumors. Tissue microarrays (TMAs) containing HPV+ (n = 31) and HPV− (n = 47) primary and metastatic HNSCCs were analyzed for IL-1α expression using immunohistochemical (IHC) staining. HPV status was confirmed using p16 IHC staining and RNA in situ hybridization (RNA ISH). Differences in IL-1α protein expression and secretion in HPV+ and HPV− HNSCC cell lines were determined by western blot and ELISA respectively. Associations between tumor *IL1A* expression and survival outcomes were assessed in HPV+ and HPV− HNSCC patients from publicly available gene expression datasets. Tumor expression of IL-1α was significantly increased in HPV− tumors and cell lines (as detected by IHC and western blot respectively) compared to HPV+ tumors and cell lines. There was no difference in IL-1α release between HPV+ and HPV− cell lines. IL-1α was expressed in both nuclear and cytoplasmic compartments, with predominant expression in the nucleus. Gene expression of *IL1A* was significantly increased in HPV-tumors/cell lines compared to HPV+ tumors/cell lines. Lastly, increased *IL1A* gene expression was significantly associated with worse survival in HPV- tumors but not in HPV+ tumors. Overall IL-1α expression particularly in the nucleus may possess more prognostic significance in HPV− tumors rather than HPV+ tumors. This work warrants further investigation into the role of intracellular IL-1α ligand expression in HNSCCs and may have important implications in IL-1 pathway blockade as therapeutic strategy.

Keywords Head and neck squamous cell carcinoma · IL-1α · IL-1β · HPV · Tissue microarray

Introduction

The IL-1 pathway plays a critical role in the regulation of immune and inflammatory responses to infections and sterile insults [1, 2]. Dysregulation of this pathway is involved in a number of autoinflammatory disorders (e.g. fever, rashes, arthritis, and organ-specific inflammation) [2]. This pathway is triggered when the ligands IL-1α and IL-1β bind to the IL-1 receptor type 1 (IL-1R1). Upon ligand binding, the receptor forms a heterodimer with the IL-1 receptor accessory protein (IL-1RAP). This receptor complex leads to the recruitment of myeloid differentiation primary response protein 88 (MyD88), followed by recruitment of IL-1 receptor-associated kinases (IRAKs) and TRAF6. These signaling events are important for the assembly of a complex containing mitogen-activated protein kinase kinase

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kinase 7 (MAP3K7/Tak1) which can prompt nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinase (MAPK) signaling leading to the expression of IL-1 target genes including IL-6 and IL-8 [1, 2].

The role of IL-1 signaling in tumor biology is controversial [3] given reports of both pro-survival and anti-tumor outcomes in response to IL-1 ligands [4–6]. However, tumor overexpression of IL-1 activating ligands has been well known to be associated with an aggressive/malignant phenotype and therefore associated with poor outcomes in several disease models [7–14]. IL-1 signaling induces expression of metastatic genes such as matrix metalloproteinases (MMPs), angiogenic factors (IL-8, VEGF) and growth factors including IL-6, TNFα, and TGFβ [14]. Preclinical studies have shown that IL-1 pathway blockade using exogenously administered interleukin-1 receptor antagonist (IL-1RA/anakinra), decreased tumor proliferation rate, metastases, and angiogenesis in several xenograft mouse models [15–20]. Additionally, a phase 3 clinical trial (CANTOS [NCT01327846]) surprisingly showed that an IL-1β antagonist reduced the incidence of lung cancer in patients with prior myocardial infarction [21]. However, clinical application of IL-1 blockade as monotherapy in cancer treatment is still unclear given the failure of a human monoclonal IL-1α antibody in advanced colon cancer patients (NCT01767857), the suspension of patient accrual in a phase I/II clinical trial for anakinra in melanoma patients (NCT02492750), and the lack of results for a phase 1 trial for anakinra in metastatic cancer patients (NCT00072111). Therefore, it remains unclear how IL-1 blockade can be appropriately utilized to effectively treat cancer patients.

Our previous work has shown that intracellular IL-1α expression, specifically nuclear IL-1α expression, is associated with rapid disease recurrence in epidermal growth factor receptor (EGFR)-positive oral squamous cell carcinomas (OSCC) patients. Nuclear localization of IL-1α is believed to be functionally important, due to the ability to bind DNA, although the nature of intranuclear IL-1α remains unclear [22]. Therefore, it is possible that strategies that target secreted IL-1 ligands or membrane-associated IL-1R1 are limited by the presence and function of intracellular IL-1 ligands. Given that our previous work with OSCCs are a subset of a larger group of HNSCCs, the current work seeks to analyze the intracellular distribution of IL-1α in a variety of HNSCC subsites. Additionally, since an increasing number of HNSCCs develop due to persistent infection with high-risk HPVs (HPV16/18) and HPV infection is associated with an inflamed tumor microenvironment [23], differences in IL-1α expression between HPV+ and HPV− HNSCCs will be evaluated. Here we find that expression of nuclear IL-1α is significantly increased in HPV− HNSCC tumors compared to HPV+ tumors and that high IL-1α gene expression is significant correlated with poor survival outcomes in HPV− but not HPV+ tumors.

Methods

HNSCC Tumor Microarrays (TMAs)

HNSCC TMAs were designed and donated by a study pathologist (JDC), constructed by the University of Virginia Biorepository and Tissue Research Facility from formalin-fixed, paraffin-embedded, and de-identified human specimens from the archives of the Department of Pathology at the University of Virginia (IRB #13310). TMAs consisted of four replicate cylindrical punches (0.6 mm) from morphologically representative regions of each of the included 78 HNSCCs, 50 HPV- cases and 28 HPV+ cases, including sampling of the tumor periphery and center. Ten 0.6 mm punches each of normal spleen, liver, kidney, and placenta served as control tissue. The HPV− cases consisted of oral tongue, floor of mouth, buccal, pharyngeal, laryngeal, anterior tonsillar pillar, tonsillar, tongue base and soft palate primary tumors, and regional neck lymph nodes harboring metastatic disease. The HPV+ cases consisted of tonsillar and tongue base primary tumors and regional neck lymph nodes harboring metastatic disease. HPV status was established using p16 immunohistochemistry (IHC) and RNA in situ hybridization (RNA ISH) as previously described by Coppock et al. [24]. All tumor samples derived from surgical resection specimens and were chosen based on the quality and availability of sufficient non-frozen tumor material for research purposes, spanning 10 years of time (2008–2017). Patients with included samples ranged in age from 17 to 94 years, with a median age of 61 years, and 81% were male, 92.3% Caucasian, 5.1% African American, and 2.6% Middle Eastern. Four μM sections from the TMAs were obtained on poly-L-lysine-coated glass slides. Routine hematoxylin-and eosin (H&E) sections were reviewed to confirm the original diagnosis.

IL-1 Ligand IHC

IL-1α and IL-1β expression were determined by KGC by evaluating the percentage of positive tumor cells by IHC using human specific IL-1α (ab9614, Abcam) and IL-1β (ab2105, Abcam) antibodies at 1:250 for 2 h and 1:160 for 1 h respectively in Dako diluent followed by rabbit Envision (Dako) and counterstained with hematoxylin. After completion of IHC, slides were stored at room temperature and a virtual scanned copy of the TMA slides were kept for further reference. IL-1α/IL-1β intensity (of each entire core) was scored on a scale from 0 to 2, with 0 representing no staining, 1 low/weak staining, and 2 representing strong/

intense staining. IL-1α expression was scored separately for the nuclear and cytoplasmic compartments.

HNSCC Cell Lines

SCC90, SCC152, FaDu and Cal-27 HNSCC cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SCC47 was obtained from Millipore Sigma, Burlington, MA). SQ20B cells were previously gifted to our lab from Dr. Anjali Gupta (University of Iowa, IA). The cell lines SCC47, SCC90, SCC152 are HPV+ and FaDu, Cal27, SQ20B are HPV- HNSCC cells. All cell lines were passaged no more than 20 times. SCC47, SCC90 and SCC152 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and 0.1% gentamycin. FaDu, Cal27 and SQ20B cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 0.1% gentamycin. All the HNSCC cells are adherent and were cultured in humidified incubator, maintaining 37 °C temperature and 5% CO_2 in vented flasks.

Western Blots

Whole protein lysates and cytoplasmic, nuclear soluble and chromatin bound fractions (Subcellular Protein Fractionation Kit [Thermo Fisher Scientific]) from indicated HNSCC cells were standardized for protein content, resolved on 4–12% SDS polyacrylamide gels, and blotted onto nitrocellulose membranes. Membranes were probed with rabbit anti-IL-1α antibody (ab9614, 1:1000, Abcam), rabbit anti-alpha tubulin antibody (#2144, 1:2500, Cell Signaling Technologies), rabbit anti-HP-1α (#2616, 1:1500, Cell Signaling Technologies) and rabbit anti-lamin B1 (#12586, 1:1500, Cell Signaling Technologies) followed by horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (#7074, 1:5000, Cell Signaling Technologies). Immunological bands were visualized with SuperSignalTM West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged with the Chemi-Doc™ Touch Imaging System (Bio-Rad Laboratories Inc.). Volumetric analysis of bands was determined using Image Lab Software (Bio-Rad Laboratories Inc.).

Real-Time Quantitative PCR

Total RNA was extracted from cells after indicated time points using RNeasy Plus mini kit (Qiagen). Conversion of RNA into cDNA was accomplished with the iScript cDNA Synthesis Kit (Bio-Rad) prior to RT-PCR analysis. RT-PCR was performed on ABI PRISM Sequence Detection System (model 7000, Applied Biosystems) using the following 5′–3′ primer sets from Integrated DNA Technologies (Coralville, IA): IL1A—5′CCGTGAGTTTCCCAGAAG AA3′ and 5′ACTGCCCAAGATGAAGACCA3′; GAPDH—5′AATGAAGGGG TCATTGATGG3′ and 5′AAGGTGAAG GTCGGAGTCAA3′. Threshold cycle (CT) values for analyzed genes (in triplicate) were normalized as compared to GAPDH CT values. Relative abundance was calculated as $0.5^{(\Delta CT)}$, with ΔCT being the CT value of the analyzed gene minus the CT value of the reference gene (GAPDH).

ELISA

Levels of released/secreted IL1α from HNSCC cells was determined by ELISA. The culture media of the treated cells at each indicated time point was collected and each cytokine was detected according to the manufacturer's protocol using an IL-1α Human Quantikine ELISA Kit (R&D Systems).

Analysis of IL-1 Ligand Gene Expression

The cBioportal for Cancer Genomics was used to download a HNSCC dataset (Head and Neck Squamous Cell Carcinoma [TCGA, Firehose Legacy], n = 530) along with corresponding clinical data (i.e. HPV status and overall survival). Only HNSCC patients (n = 124) in the database where HPV status was reported, were included in these analyses. Patients were separated into HPV+ (n = 40) and HPV− (n = 84) and ranked according to *IL1A* gene expression, with the upper quartile denoted as "high" and the lower quartile as "low" gene expression. Kaplan–Meier survival curves were plotted comparing the overall survival of patients with high or low tumor expression of *IL1A*.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism version 7 for Mac (GraphPad Software, La Jolla, CA, USA). Differences in means between two groups were determined by unpaired t-test. Associations between HPV status and available pathological features were tested using the chi-square test. Kaplan–Meier survival curves were generated to illustrate the different survival rates over time. Differences in survival were determined by Log-rank (Mantel-Cox) test. For all experiments, differences were considered significant if $p < 0.05$.

Results

HNSCC TMA Characteristics

Of the 78 HNSCC cases analyzed, 50 were HPV− and 28 were HPV+ (Table 1). HPV status at diagnosis was determined by p16 IHC (Fig. 1A, B) as reported here [24] and

Table 1 Tumor sites of HPV + and HPV − tumors

	Total (n)	HPV− n (%)	HPV+* n (%)	p value
Cases (n)	78	50	28	
Primary	53	36 (72)	17 (61)	0.1
Metastases	25	14 (28)	11 (39)	
Tumor sites				
Oral tongue	17	17 (34)		< 0.001
Floor of mouth	3	3 (6)		
Buccal	1	1 (2)		
Pharyngeal	1	1 (2)		
Laryngeal	6	6 (12)		
Anterior tonsillar pillar	2	2 (4)		
Tonsillar	15	1 (2)	14 (50)	
Tongue base	6	3 (6)	3 (11)	
Soft palate	2	2 (4)		
Regional neck lymph node**	25	14 (28)	11 (39)	

*Confirmed by RNA ISH

**Metastatic tumors

confirmed by RNA ISH (Fig. 1C, D). While there was good agreement between p16 expression and HPV RNA ISH positivity, 3 tumors that were initially found to be p16 positive were confirmed as HPV− by RNA ISH. The majority of HPV− primary tumors were from oral tongue (34%), followed by larynx (12%) (Table 1). The majority of HPV+ tumors were tonsillar (50%) followed by base of the tongue (11%). Metastatic tumors comprised 28% and 39% of the HPV− and HPV+ tumors, respectively, derived from regional neck lymph node dissection (Table 1). Of the HPV− tumors, 56% were moderately differentiated, 22% were poorly differentiated, 11% were well-differentiated, with the remainder being moderate-poor, well-moderate, and papillary (data not shown).

IL-1 Ligand Expression in HNSCC Tumors

IL-1α was expressed in all HNSCC cases analyzed with exception of 1 HPV− case. IL-1α was identified in both nuclear and cytoplasmic compartments (Fig. 2A) and was therefore scored separately for the nuclear and cytoplasmic compartments on a scale from 0 to 2, with 0 representing no staining, 1 low/weak staining, and 2 representing strong/intense staining. Examples of IHC images of IL-1α

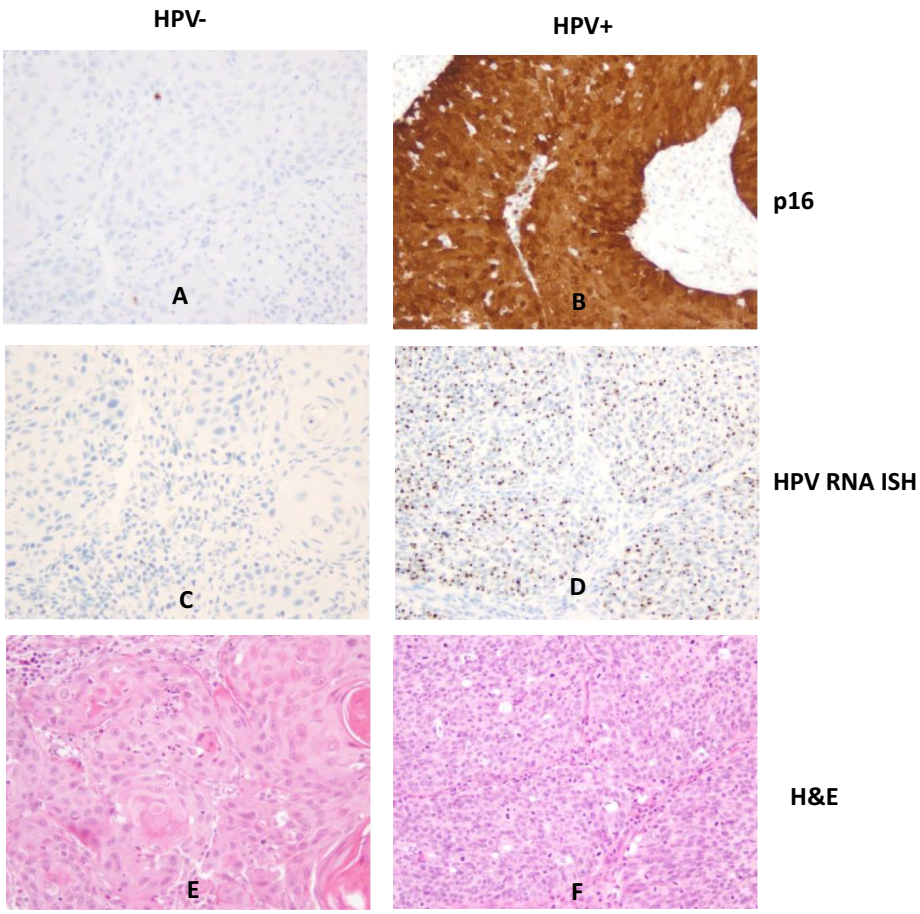

Fig. 1 Representative of positive and negative p16 immunostaining and high-risk HPV RNA ISH in HNSCCs. Shown are images (×200) representing p16 IHC (**A**, **B**), high-risk HPV RNA ISH (**C**, **D**) and H&E (**E**, **F**) in HPV− (**A**, **C**, **E**) and HPV+ (**B**, **D**, **F**) HNSCCs

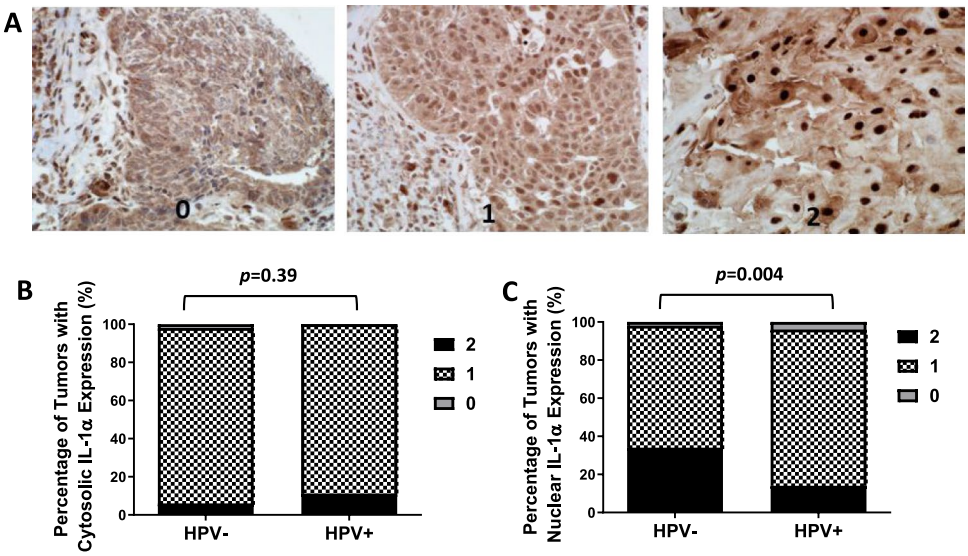

Fig. 2 IL-1α immunostaining and expression scores in HNSCCs. **A** Shown are images (×20) of negative [0], moderate [1], and strong [2] nuclear IL-1α expression. **A**, **C** Percentage of tumors with strong [2], moderate [1] or no [0] cytoplasmic (**B**) and nuclear (**C**) IL-1α expression based on HPV status

immunostaining are shown in Fig. 2A where differences in nuclear immunostaining and assigned scores are shown. An IL-1α cytoplasmic score of 1 represented the majority of HPV− (92%) and HPV+ (89%) tumors with no differences in cytoplasmic IL-1α expression scores observed between HPV− vs HPV+ tumors (p = 0.39, Fig. 2B). However, significant variation in nuclear immunostaining was observed between HPV− and HPV+ tumors (Fig. 2C). Strong/intense nuclear IL-1α staining was significantly increased in HPV− tumors (34%) compared to HPV+ tumors (14%) (p = 0.004, Fig. 2C). In contrast to IL-1α, IL-1β expression was observed solely in the cytoplasm (Fig. 3A). There were significantly more HPV- tumors with strong/intense IL-1β staining (40% HPV− vs 29% HPV+) and more HPV+ tumors with no staining (2% HPV− vs 14% HPV+, p = 0.005, Fig. 3B). Together, these results suggest that strong/intense IL-1α and IL-1β expression is more likely to be observed in HPV− tumors compared to HPV+ tumors and that IL-1α (but not IL-1β) is expressed predominantly in the nucleus.

IL-1α Ligand Expression in HNSCC Cell Lines

To confirm the IL-1α immunostaining results in HNSCC tumors (Fig. 2), IL-1α expression was examined in HNSCC cell lines by western blot. IL-1α can exist as the full-length/precursor (pre-IL-1α, 31-kDa, AA:1–271), the cleaved C-terminal component (17-kDa, AA:113–271) and the N-terminal pro-piece (16-kDa, AA:1–112). Of note, the C-terminal and N-terminal components are difficult to resolve by standard western blot on whole cell lysates unless specific antibodies against the C-terminal and N-terminal residues are used [25]. The IL-1α antibody used here was raised against pre-IL-1α and therefore recognizes the full-length, C-terminal and N-terminal components. Western blot revealed that expression of the pre-IL-1α was observed in whole cell lysates from all the cell lines tested but was notably increased in the HPV- cell lines (FaDu, Cal-27, SQ20B) compared to HPV+ cell lines (SCC47, SCC90, SCC152) (Fig. 4A, B). The smaller band at ~ 16/17 kDa, which likely represents the overlapping N and C-terminal components,

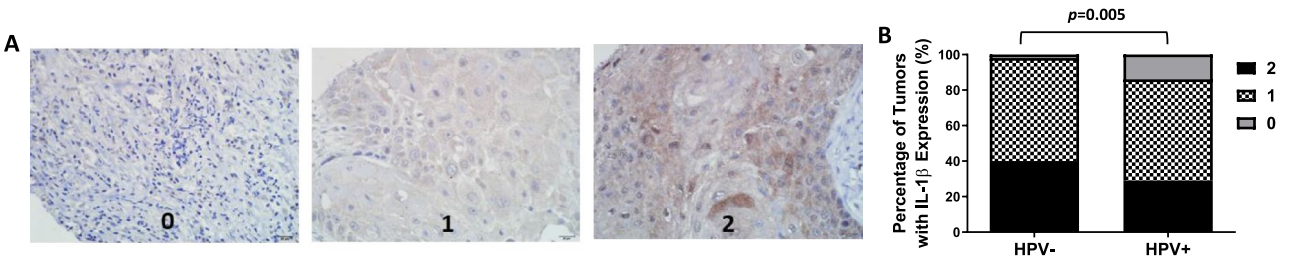

Fig. 3 Representative examples of IL-1β immunostaining and expression scores in HNSCCs. **A** Shown are images (×20) of negative [0], moderate [1], and strong [2] IL-1β expression. **B** Shown are the percentage of tumors with strong [2], moderate [1] or no [0] IL-1β expression based on HPV status

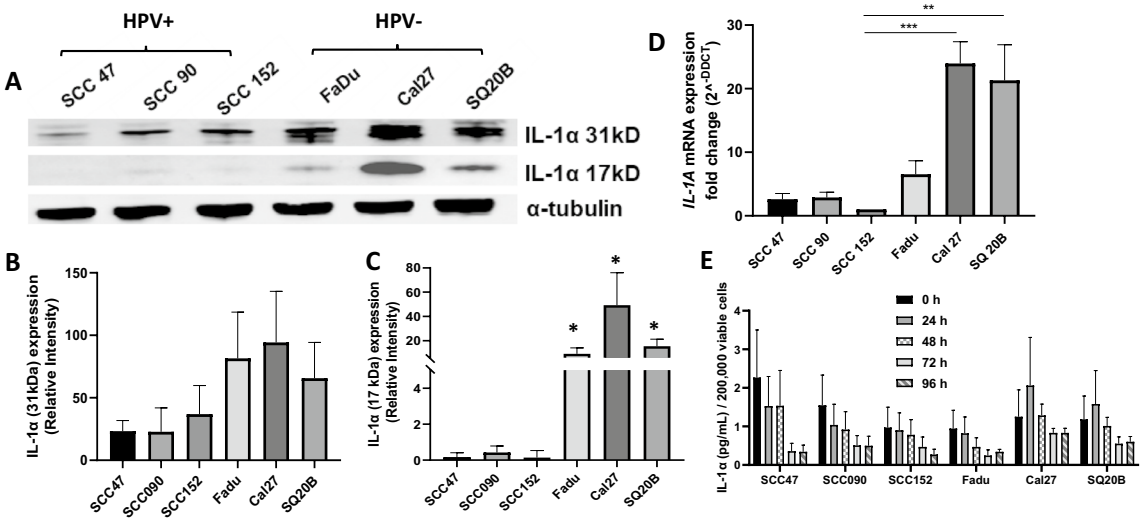

Fig. 4 IL-1α protein expression in HPV+ and HPV− HNSCC cell lines. **A** Lysates isolated from three HPV+ HNSCC cell lines (SCC47, SCC90, SCC152) and three HPV− HNSCC cell lines (FaDu, Cal-27, SQ20B) were analyzed for IL-1α by Western blot. Alpha-tubulin was used as a loading control. **B**, **C** Volumetric analysis was conducted on 31 kDa (**B**) and 16/17 kDa (**C**) IL-1α bands and normalized to α-tubulin. **D** RNA isolated from HNSCC cell lines were analyzed for IL1A expression by RT-PCR. **E** Secreted IL-1α from HNSCC-derived cell culture media at the indicated time points was measured by ELISA. n = 3; errors bars = SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs SCC152

was significantly increased in the HPV− cell lines compared to the HPV+ cell lines with the most expression observed in the Cal-27 cell line (Fig. 4A, C). *IL1A* gene expression was also increased in the HPV− cell lines compared to the HPV+ cell lines with significant expression observed in the Cal-27 and SQ20B lines (Fig. 4D). On the other hand, IL-1β showed only slight increases in the precursor (31-kDa) and cleaved (17-kDa) proteins in HPV− cell lines compared to HPV+ cells (Supplemental Fig. 1A) accompanied by variable *IL1B* gene expression among the cell lines (Supplemental Fig. 1B). There was no difference in IL-1α (Fig. 4E) or IL-1β (Supplemental Fig. 1C) release between the HPV− and HPV+ cell lines at baseline or over a 96 h period.

To visualize intracellular differences in IL-1α (full length, N-terminal and C-terminal components) expression between HPV− and HPV+ cells, subcellular fractionation was performed on the Cal-27 (HPV−) and SCC90 (HPV+) cell lines. Pre-IL-1α was observed in the Cal-27 cytoplasmic, soluble nuclear and chromatin-bound nuclear fractions (Fig. 5A) suggesting that pre-IL-1α is expressed in both nuclear and cytoplasmic compartments in Cal-27 cells. In the cytoplasmic fraction, pre-IL-1α protein was expressed similarly in both Cal-27 and SCC90 cells, but was significantly increased in the soluble and chromatin-bound nuclear fractions in Cal-27 compared to SCC90 (Fig. 5A, B). The C-terminal (17-kDa) component lacks the nuclear localization signal (NLS, AA: 79–86) present in pre-IL-1α and the N-terminal component and is therefore not expressed in the nucleus. Therefore, the proteins (~ 16/17-kDa) observed in the chromatin-bound nuclear fractions likely represent the N-terminal component where similar amounts were observed in the Cal-27 and SCC90 cell lines (Fig. 5A, C). Little to no expression of the N-terminal component was observed in the soluble nuclear fraction of both cell lines (Fig. 5A, C) which supports prior findings that IL-1α is a chromatin-binding protein [26]. Together, these results show that pre-IL-1α can be expressed in both the nuclear and cytoplasmic fractions of HNSCC cell lines. Additionally, the increased expression of IL-1α in the nuclear fractions of HPV− Cal-27 cells (compared to HPV+ SCC90 cells) supports the IHC findings in HNSCC tumors showing that HPV- cells express significantly more nuclear IL-1α that HPV+ cells (Fig. 2C).

Gene Expression of IL-1 Ligands

RNA-sequencing data for HPV− (n = 84) and HPV+ HNSCC (n = 40) tumors was analyzed from the cBioportal for Cancer Genomics for mRNA levels of IL-1α (representing the full length [pre-IL-1α] protein). *IL1A* was found to be significantly increased in HPV− tumors compared to HPV+ tumors (Fig. 6A). The RNA-sequencing data from these HPV− and HPV+ tumors were separated into high and low *IL1A* expression and plotted against associated overall survival data (Fig. 6B, C). In HPV− patients, high gene levels of *IL1A* were significantly associated with worse survival (median survival = 14.2 months) compared to low *IL1A* (median survival = 32.6 months) (Fig. 6B, p = 0.008). On the other hand,

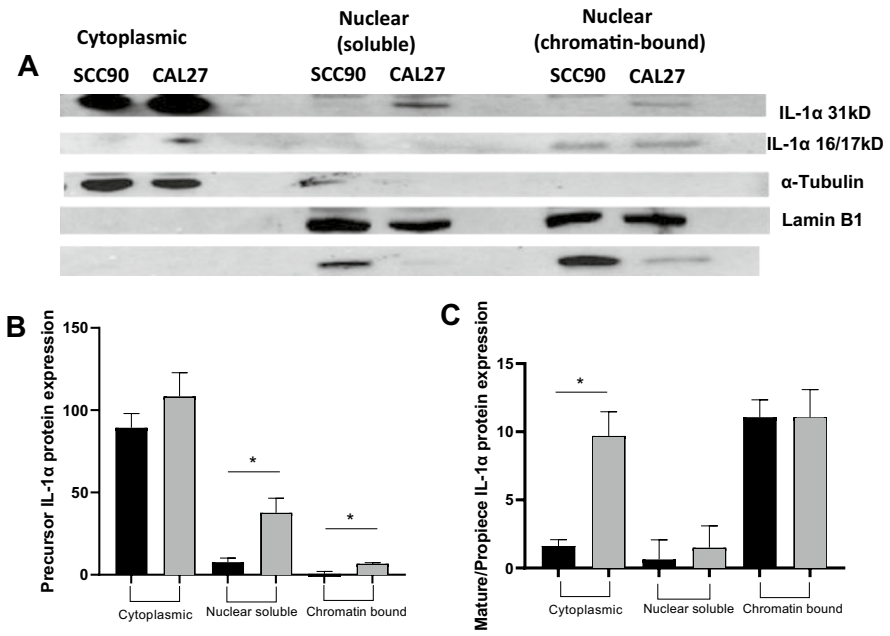

Fig. 5 IL-1α cytoplasmic and nuclear protein expression in SCC90 and CAL27 HNSCC cell lines. **A** Cytoplasmic and nuclear (soluble and chromatin-bound) fractions from lysates isolated from SCC90 (HPV+) and Cal-27 (HPV−) cell lines were analyzed for IL-1α by Western blot. Alpha-tubulin, lamin B1 and HP-1 was used as loading controls for the cytoplasmic, nuclear (soluble) and nuclear (chromatin-bound) fractions respectively. **B**, **C** Volumetric analysis was conducted on 31 kDa (**B**) and 17 kDa (**C**) IL-1α bands and normalized to the respective loading controls. n = 3; errors bars = SEM. *:$p < 0.05$

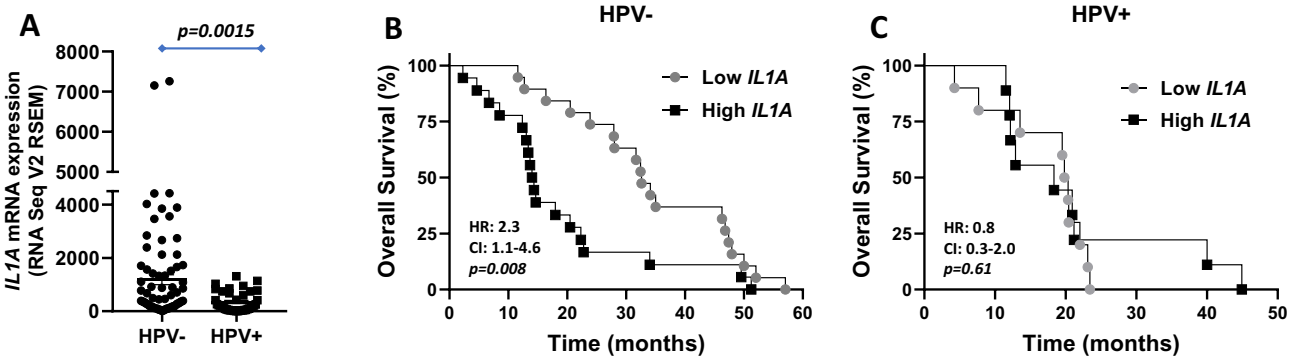

Fig. 6 Association of *IL1A* gene expression in HPV+ and HPV− tumors with clinical outcomes. **A** Gene expression of *IL1A* (**A**) from HPV− (n = 84) and HPV+ HNSCC s (n = 40) was plotted for HPV-tested HNSCC patients from the TCGA database. Error bars represent standard error of the mean. **B**–**C** Shown are Kaplan-Meier estimates of the overall survival of HPV− (**B**) and HPV+ (**C**) HNSCC patients according to Low (lower quartile) and High (upper quartile) *IL1A* tumor gene expression. *HR* hazard ratio, *CI* 95% confidence interval

in HPV+ patients, overall survival was not affected by *IL1A* (Fig. 6C, p = 0.61). *IL1B* was also significantly increased in HPV− tumors compared to HPV+ tumors (p = 0.0075, Supplemental Fig. 2A), however there was only a trend toward association with poor survival (Supplemental Fig. 2B, p = 0.09) in HPV− tumors but not HPV+ tumors. (Supplemental Fig. 2C p = 0.21). Together these results suggest that increased *IL1A* (but not *IL-1B*) may be associated with tumor aggressiveness and poor survival outcomes in HNSCC patients bearing HPV− tumors but not HPV+ tumors.

Discussion

Here we show that IL-1α expression is significantly increased in HPV− tumors compared to HPV+ tumors, and that this result supports prior work showing increased expression of IL-1α in HPV− oropharyngeal cancer (OPC) cell lines (FaDu, SCC89, SCC72) compared to HPV+ OPC cell lines (SCC90, SCC2, SCC152) [27]. These findings may however contradict the large body of work showing that HPV infection (in addition to other viral infections) is associated with chronic inflammation and cancer development [28]. For example, HPV infection triggers the production and release of several inflammatory cytokines

(including IL-1α and IL-1β) from keratinocytes, fibroblasts and immune cells that act as the first line of defense against the virus infection by inhibiting the transcription of HPV oncogenes and inhibiting growth of cells that harboring the viral genome [29]. Additionally, increased circulating levels of pro-inflammatory cytokines are observed in HPV+ HNSCC patients compared to HPV− patients [30]. However, it is established that HPV evades the immune response in some cases resulting in persistent infections, which in the case of high-risk oncogenic HPV types (i.e. HPV16/18), result in cancer development [31]. Previous work has shown that the gradual downregulation of IL-1β in HPV-infected keratinocytes by the HPV16 E6 oncoprotein is a crucial step in immune evasion and tumor development [32], which may explain the decreased IL-1 ligand expression observed in HPV+ vs HPV− HNSCC tumors presented here.

More importantly, this work highlights the potential significance of IL-1α localized to the nucleus. IL-1α was observed in the nucleus in the vast majority of HPV+ and HPV− HNSCC tumors tested (Fig. 2) which supports our previous results in OSCCs that IL-1α is predominantly localized in the nucleus [33] unlike IL-1β. As mentioned before, pre-IL-1α contains a NLS that is not present in the cleaved C-terminal component but is retained in the N-terminal component after cleavage of pre-IL-1α. This allows both pre-IL-1α and the N-terminal component to translocate to the nucleus (34). Therefore, the nuclear IL-1α we observe by IHC may be pre-IL-1α and/or the N-terminal component (Fig. 2A) since the limitation here is that we have not used N or C-terminal specific antibodies.

To further probe whether the difference in nuclear IL-1α between HPV− and HPV+ tumors was due to the pre-IL-1α or the N-terminal component, we utilized sub-cellular fractionation of representative HPV+ (SCC90) and HPV− (Cal-27) cell lines prior to western blot. The major difference in the cell lines was the increased pre-IL-1α in both nuclear fractions (soluble and chromatin) in the Cal-27 cell line compared to SCC90 (Fig. 5A, B). There was no difference in the N-terminal component between the cell lines (Fig. 5A, B). Given this evidence, it is possible that pre-IL-1α represents the majority of the increased nuclear IL-1α immunostaining detected in the HNSCC tumors (Fig. 2A) although again—more specific N and/or C-terminal IL-1α antibodies are needed to confirm in additional cell lines.

Nuclear localization of IL-1α is believed to be functionally important, due to the ability to bind DNA, although the nature of intranuclear IL-1α remains unclear [22]. Some studies have reported that overexpression of pre-IL-1α and the N-terminal component can inhibit cell proliferation and trigger apoptosis respectively [34–36]; and in other studies—promote cell proliferation [37–39]. Additionally, the N-terminal component was found to induce malignant transformation in perivascular mesangial cells, pointing to an oncogenic role for the N-terminal component [39]. It is likely that differences in cell models may explain these contradicting reports. However, what these studies do agree on is that the function of nuclear IL-1α is independent of IL-1R1 signaling and may have little association with inflammation and immune responses.

Given that pre-IL-1α gene and protein levels were increased in HPV− HNSCC cells compared to HPV+ cells (Figs. 2, 4, 6), we asked if IL-1α expression was correlated with survival outcomes equally in HPV− and HPV + patients. Clinical outcome data for the tumors in the HNSCC TMA (Table 1) was not available, therefore we analyzed gene expression data (with associated clinical outcome data) from a publicly available dataset. We found that high *IL1A* expression was associated with worse survival in HPV− patients (Fig. 6B) but not HPV+ patients (Fig. 6C). These results were not observed with *IL1B* in HPV+ or HPV− patients (Supplemental Fig. 2). This suggests that the clinical significance of IL-1α may be specific to HPV− patients.

Lastly, given the presence and possible importance of nuclear IL-1α in tumor biology, this raises the question of IL-1 blockade as a cancer therapy. As mentioned previously, current strategies such as antibodies against IL-1 ligands and IL-1RA/anakinra have no effect against nuclear IL-1α and may explain the ineffectiveness of these IL-1 blockade strategies in clinical trials. Future work should examine in detail the specific function(s) and clinical significance of nuclear IL-1α in tumor aggressiveness in order to provide novel strategies for targeting IL-1 signaling.

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Declarations

Conflict of interest No conflicts of interest to disclose.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This work was approved by the Institutional Review Board of the University of Virginia (IRB #13310). This article does not contain any studies with animals performed by any of the authors.

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