Expression of human papillomavirus oncoproteins E6 and E7 inhibits invadopodia activity but promotes cell migration in HPV-positive head and neck squamous cell carcinoma cells

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Abstract
Background: The rapid increase in the incidence of head and neck squamous cell carcinoma (HNSCC) is caused by high-risk human papillomavirus (HPV) infections. The HPV oncogenes E6 and E7 promote carcinogenesis by disrupting signaling pathways that control survival and proliferation. Although these cancers are often diagnosed with metastases, the mechanisms that regulate their dissemination are unknown.

Aims: The aim of this study was to determine whether the HPV-16 E6 and E7 oncogenes affected the invasive and migratory properties of HNSCC cells which promote their spread and metastasis.

Methods and results: Invasiveness was determined using invadopodia assays which allow for quantitation of extracellular matrix (ECM) degradation by invadopodia which are proteolytic membrane protrusions that facilitate invasion. Using cell lines and genetic manipulations, we found that HPV inhibited invadopodia activity in aggressive cell lines which was mediated by the E6 and E7 oncogenes. Given these findings, we also tested whether HPV caused differences in the migratory ability of HNSCC cells using Transwell assays. In contrast to our invadopodia results, we found no correlation between HPV status and cell migration; however, blocking the expression of the E6 and E7 oncoproteins in a HPV-positive (HPV+) HNSCC cell line resulted in decreased migration.

Conclusions: Our data suggest that the E6 and E7 oncoproteins are negative regulators of invadopodia activity but may promote migration in HPV+ HNSCC cells. Despite the need for ECM proteolysis to penetrate most tissues, the unique structure of the head and neck tissues in which these cancers arise may facilitate the spread of migratory cancer cells without significant proteolytic ability.

KEYWORDS
head and neck cancer, human papillomavirus, invadopodia, invasion, migration

1 | INTRODUCTION

While the overall incidence of head and neck squamous cell carcinoma (HNSCC) has been declining, there has been an alarming rise in human
papillomavirus-positive (HPV+) HNSCC cases over the last 30 years which are projected to eclipse all other types of HNSCC by 2030.\textsuperscript{1-4} Despite these potentially "epidemic" levels given the link to a prevalent infectious agent,\textsuperscript{5-7} HPV+ HNSCC does not have unique treatment options despite its distinct biology.\textsuperscript{8-10} HPV+ tumors develop in the oropharyngeal region of the head and neck with localization to the tonsils and base of the tongue.\textsuperscript{11-13} The majority of HPV infections leading to HNSCC arise from the HPV-16 strain.\textsuperscript{14,15} Carcinogenesis caused by HPV-16 as well as the other high-risk strains is driven by the E6 and E7 oncoproteins which target a variety of proteins crucial for several cellular processes including apoptosis and the cell cycle.\textsuperscript{8,10,16-20}

Clinically, HPV+ HNSCC is more frequently diagnosed with metastatic disease than human papillomavirus-negative (HPV-) HNSCC.\textsuperscript{1,2,21} To metastasize, migrating cancer cells must breach cross-linked and dense tissues that typically require ECM degradation.\textsuperscript{22-24} To invade these tissues, cancer cells use actin-rich adhesive protrusions called invadopodia that localize proteinases for ECM proteolysis.\textsuperscript{25-27} Invadopodia formation correlates well with in vitro and in vivo invasive behavior of cancer cells and has thus been implicated in tumor progression.\textsuperscript{23,28,29} Therefore, the goal of this study was to determine whether HPV promotes invadopodia activity and migration in HNSCC cells given the frequency of metastases in HPV+ HNSCCs.

\section{METHODS}

\subsection{Cell culture and reagents}

HPV+ (SCC-25, SCC-61, and SCC-1) and HPV- (SCC-47 and SCC-104) HNSCC cell lines were used in this study. SCC-61 was originally obtained from the Yarbrough laboratory at our institution. The HPV status of these cells was previously verified.\textsuperscript{30} SCC-25 and SCC-1 with confirmed HPV+ status were purchased from ATCC and Millipore Sigma, respectively. SCC-47 and SCC-104 with verified HPV+ status were purchased from Millipore Sigma. SCC-61 and SCC-25 were originally derived from aggressive tumors that had metastasized to lymph nodes.\textsuperscript{31,32} The HPV+ HNSCC cell lines were also derived from aggressive tumors that had spread to lymph nodes.\textsuperscript{32} SCC-61 cells were cultured as previously described using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FBS) (both from ThermoFisher).\textsuperscript{33,34} SCC-25 cells were cultured in DMEM/Ham’s nutrient mixture F-12 (DMEM/F12) (ThermoFisher) supplemented with 20% FBS and 0.4 \mu g/mL hydrocortisone (Millipore Sigma).\textsuperscript{31,35-37} SCC-1 and SCC-47 cells were both cultured in the same medium as SCC-25 cells but with an additional supplement of 1:100 minimum essential media nonessential amino acids (MEM NEAA) and 0.4 \mu g/mL hydrocortisone (both from Millipore Sigma). SCC-104 cells were cultured in the same medium as SCC-1 and SCC-47 cells but supplemented with 1:100 penicillin-streptomycin (10 000 U/mL penicillin-10 000 \mu g/mL streptomycin mixture) (ThermoFisher). SCC-25 cells were treated with lentiviruses engineered for the simultaneous expression of the HPV-16 E6 and E7 genes according to the manufacturer’s instructions (ABM). SCC-47 cells were treated with separate lentiviral particles for the simultaneous knockdown of the HPV-16 E6 and E7 genes using different shRNA based on published siRNA sequences according to the manufacturer’s protocol (Santa Cruz).\textsuperscript{38,39} Transduced cells were cultured in puromycin-containing growth medium for selection. Blank vectors were used as controls in both cell lines.

\subsection{Quantitative real-time PCR}

Gene expression was measured as previously described\textsuperscript{40} using previously validated primers and the \textit{ΔΔCt} method for quantitation\textsuperscript{41} with GAPDH as the internal control. In this case, mRNA was isolated with the RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer’s instructions. The expression of E6 and E7 was measured using the SsoAdvanced Universal SYBR Green Supermix and CFX Connect Real-Time PCR Detection System (both from BioRad). Because E6 and E7 are undetectable in SCC-25 cells which are HPV+, we chose a cutoff value for Ct of 40 for these samples which were used as controls in the \textit{ΔΔCt} calculations.

\subsection{Western blotting}

Western blotting was performed as previously described\textsuperscript{34} using an anti-E6 mouse monoclonal antibody (Euromedex) and a 1:1 combination of anti-E7 mouse monoclonal antibodies (Santa Cruz and ThermoFisher).\textsuperscript{41} In this case, 12% SDS-PAGE gels were used, and Super Block T20 (TBS) blocking buffer (ThermoFisher) was used for blocking.\textsuperscript{41} Blots were detected using a horseradish peroxidase-conjugated secondary antibody (PerkinElmer) which was visualized with chemiluminescence.

\subsection{Invadopodia assays}

Invadopodia assays were performed as previously described.\textsuperscript{34} Briefly, glass-bottom dishes were overlaid with a 1:5 ratio of FITC-labeled gelatin (ThermoFisher) and unlabeled gelatin (Polysciences) cross-linked with 0.05% glutaraldehyde. Twenty-five thousand cells were incubated for 4 or 18 hours in invadopodia medium which contains DMEM/Roswell Park Memorial Institute (RPMI) 1640, 5% NuSerum, 10% FBS, and 20 ng/mL epidermal growth factor (all from ThermoFisher).\textsuperscript{33,34,40,42,43}

\subsection{Immunofluorescence}

Invadopodia were identified as previously described.\textsuperscript{34} Briefly, the punctate colocalization of F-actin and cortactin was used as a marker and were identified with Alexa Fluor 546 phalloidin (ThermoFisher) and an anti-cortactin mouse primary monoclonal antibody (clone 4F11 from Millipore Sigma), respectively. Fluorescent images were captured on a Nikon Ti-E inverted microscope with a Plan Fluor 40× oil immersion objective. Invadopodia were manually counted, and ECM degradation was quantitated by thresholding the loss of FITC signal underneath the cells in Nikon Elements software (Nikon) and/or MetaMorph software (Molecular Devices).

\subsection{Transwell assays}

Transwell migration and invasion assays were performed as previously described.\textsuperscript{34} Briefly, 75 000 cells in serum-free invadopodia medium were plated in uncoated and Matrigel-coated Transwell inserts (ThermoFisher) with 5 and 8-\mu m pore sizes, respectively, and
incubated for 18 hours with complete invadopodia medium in the lower chamber. Cells that migrated or invaded through the permeable supports were quantitated using Hoechst (ThermoFisher) for nuclear staining in Nikon Elements and MetaMorph from fluorescent images captured using a Plan Fluor 20× objective.

2.7 | Statistics

Statistics were performed as previously described using SPSS Statistics (IBM) with a P value less than .05 considered significant. Briefly, all data were evaluated for normality using the Shapiro-Wilk or Kolmogorov-Smirnov test. Normal data were analyzed with a Student’s t test for comparisons between 2 datasets or a 1-way ANOVA with multiple t tests and Bonferroni correction for group and pairwise comparisons, respectively. Non-normal data were analyzed with a Mann-Whitney test for comparisons between 2 datasets or Kruskal-Wallis test with a Tamhane post-hoc test or Mann-Whitney test with Bonferroni correction for group and pairwise comparisons, respectively.

3 | RESULTS

Because HPV+ HNSCC has higher rates of metastasis, we first evaluated invadopodia activity in established HPV+ and HPV− HNSCC cell lines because the ability to degrade the ECM is an important component of invasion. HPV+ SCC-25, SCC-61, and SCC-1 cells are frequently used as cell line models for studying invadopodia. HPV+ SCC-47 and SCC-104 cells are common cell line models for studying the effects of HPV. We performed invadopodia assays on these cell lines and quantitated invadopodia numbers and ECM degradation of a fluorescently labeled ECM (Figure 1B-D). Contrary to our expectations, HPV+ HNSCC cell lines degraded significantly less ECM and formed fewer actively degrading (colocalized with ECM degradation) and total (degrading and nondegrading) invadopodia when compared to HPV+ HNSCC cell lines (Figure 1B-D). However, we observed considerable variation among cell lines within each group which led us to perform individual comparisons between the HPV+ and HPV− HNSCC cell lines. SCC-25 and SCC-61 cells degraded more ECM and formed more degrading and total invadopodia than the SCC-47 and SCC-104 cells, while SCC-1 cells were only significantly greater when compared to SCC-104 cells (Figure 1B-D). These differences are consistent with previous reports of invadopodia activity by these cell lines (except SCC-104 cells which have not previously been reported to form invadopodia to our knowledge).

To determine whether the differences in invadopodia activity were dependent on HPV status, we selected a representative cell line from the HPV+ group, SCC-25, and stably knocked down the E6 and E7 oncogenes (Figure 3A). In this case, Western blots were used for confirmation because preliminary PCR results indicated that the knockdown (KD) of E6 and E7 occurred through translational repression (Figure 3A). E6 and E7 KD (E6/E7 KD) in SCC-47 cells had the inverse effect and increased ECM degradation and the number of actively degrading but not total invadopodia when compared to the blank vector control (Figure 3B-E).

Because our data indicated that E6 and E7 inhibit invadopodia activity, we determined whether HPV affected the migration and invasion of HNSCC cells which is necessary for dissemination and metastasis. We first performed Transwell migration assays with the HPV− and HPV+ HNSCC cell lines and found no significant correlation between migratory potential and HPV status (Figure 4A, B). Similar results were observed in Transwell invasion assays wherein no significant trend was observed in HPV− versus HPV+ HNSCC cell lines (Figure 4C, D). However, compared to the empty vector control, knockdown of the E6 and E7 oncoproteins (E6/E7 KD) significantly decreased cell migration and invasion in SCC-47 cells (Figure 4E-H). Similar assays could not be performed with SCC-25 cells overexpressing E6 and E7 because these cells did not reliably attach to the Transwell inserts.

4 | DISCUSSION

High-risk HPV drives tumorigenesis by altering the signaling pathways that control transcription, differentiation, DNA repair, apoptosis, and the cell cycle. The E6 and E7 oncoproteins target various molecules for ubiquitin-mediated proteasomal degradation relevant for these processes thus destabilizing important regulatory mechanisms. Although E6 and E7 have different targets that contribute to carcinogenesis, they are expressed together from polycistronic mRNA and may cooperate to amplify their individual effects. Therefore, we chose to simultaneously manipulate E6 and E7 to first understand the general effects of HPV-16 on the invasive and migratory properties of HNSCC cells in this study. In this study, we demonstrate that HPV-infected HNSCC cell lines have lower invadopodia activity than HPV− HNSCC cells. HPV status does not correlate with the migratory potential of a cell line; however, cell migration is compromised in HPV+ HNSCC cells with reduced expression of the E6 and E7 oncoproteins.

Tissues have long been thought to represent formidable barriers to migrating cancer cells thus necessitating ECM proteolysis for invasion which is facilitated by invadopodia. However, more recent work suggests that migrating cancer cells can invade in a nonproteolytic manner by taking advantage of variations and defects in epithelial and stromal tissues as well as physically deforming and pushing through porous ECMs. While Matrigel is routinely used for assessing proteolytic invasion, it lacks the native cross-links of a true basement membrane and is readily traversed by many cell types exhibiting a nonproteolytic mode of migration. Thus, Matrigel may not represent a formidable structural barrier that necessitates the use of proteolytic machinery for invasion by some types of migrating cancer cells which would be consistent with our findings that showed similar results for Transwell migration and invasion, particularly for the HPV+ HNSCC cells which exhibited significantly less invadopodia activity. Furthermore, we also found a decrease in
Transwell invasion with E6 and E7 inhibition despite an increase in invadopodia formation and activity. However, the reduction in migration likely accounts for this discrepancy which would limit the ability of cells to move through the Matrigel. Alternatively, cells may use other mechanisms to mediate ECM degradation such as the release of extracellular vesicles containing proteolytic enzymes. However, the secretion of exosomes is enhanced by invadopodia, and microvesicle secretion is promoted by compliant matrices with thicknesses far greater than the thin coating found in Transwells.

Although the molecular mechanisms that regulate the spread of HPV+ HNSCC remain to be explored, E6 and E7 have also been shown to increase the migration of cervical cancer cells which is thought to
FIGURE 2  Expression of HPV E6 and E7 inhibits invadopodia activity. (A) Relative expression (fold change) in mRNA levels of E6 and E7 in HPV− SCC-25 cells transduced with an E6/E7 lentiviral expression vector was evaluated with quantitative real-time PCR which was performed in triplicate for each experiment and overall for 3 independent experiments. Representative wide-field fluorescence images of (B) control and E6/E7 OE SCC-25 cells in invadopodia assays after 4-hour incubation. Invadopodia puncta (magnified inlays of invadopodia in white boxes) were identified by colocalization of actin (orange) and cortactin (purple). Active invadopodia (ie, actively degrading) were identified by further colocalization with black degraded areas of ECM lacking FITC signal (green), while total invadopodia included active and nondegrading puncta. Quantitation of (C) the degradation area per cell, (D) active invadopodia per cell, and (E) total invadopodia per cell for control and E6/E7 OE SCC-25 cells. Imaging data were quantitated for n = 118 to 125 cells from 4 independent experiments. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating \( P < .05 \). Scale bar represents 5 μm

FIGURE 3  Knockdown of HPV E6 and E7 promotes invadopodia activity. (A) Representative western blots of E6 (16 kDa) and E7 (20 kDa) in HPV+ SCC-47 cells simultaneously transduced with E6 and E7 lentiviral vectors with different shRNA from 2 independent experiments which resulted in a 76% decrease for both E6 and E7, when normalized to β-actin. Representative wide-field fluorescence images of (B) control and E6/E7 KD SCC-47 cells in invadopodia assays after 4-hour incubation. Invadopodia puncta (magnified inlays of invadopodia in white boxes) were identified by colocalization of actin (orange) and cortactin (purple). Active invadopodia (ie, actively degrading) were identified by further colocalization with black degraded areas of ECM lacking FITC signal (green) while total invadopodia included active and nondegrading puncta. Quantitation of (C) the degradation area per cell, (D) active invadopodia per cell, and (E) total invadopodia per cell for control and E6/E7 KD SCC-47 cells. Imaging data were quantitated for n = 60 cells from 3 independent experiments. All data are presented as box and whisker plots with the black lines indicating the medians, the whiskers representing the 10th and 90th percentiles, and * indicating \( P < .05 \). Scale bar represents 10 μm
occur by decreasing RhoA activity. Rho GTPases are overexpressed in HNSCC, and RhoA controls actomyosin contractility to mediate cell migration. Inhibition of the Rho pathway can promote cell migration in some cell types by reducing substrate adhesion, which is normally reinforced by large contractile forces. Furthermore, RhoA signaling can promote invadopodia activity. While E6 can deregulate miRNA expression to affect migration, these mechanisms are mediated by p53, which is inactivated in SCC-25 cells. Therefore, HPV may exert its effects on Rho through additional mechanisms which alter downstream signaling important for migration and invadopodia activity.

One critical effector of Rho is Rho-associated kinase (ROCK) which phosphorylates nonmuscle myosin II (NM II) to regulate actomyosin contractility to mediate cell migration. Inhibition of the ROCK pathway can promote cell migration in some cell types by reducing substrate adhesion, which is normally reinforced by large contractile forces. Furthermore, ROCK expression is also associated with tumor stage and progression and promotes migration and invasion in HNSCC cells. We have shown that the ROCK isoforms differentially regulate invadopodia activity through contractile and noncontractile mechanisms in HNSCC cells. More specifically, we found that actomyosin contractility regulates invadopodia formation and ECM degradation as well as migration through the ROCK1/NM II pathway. Furthermore, we also found that signaling through ROCK2 promotes invadopodia maturation independently of contractile force generation through LIM kinase (LIMK) which is mediated by RhoC. Although further studies are required, E6 and E7 may interfere with these ROCK-dependent signaling pathways by altering the activity of the Rho family of GTPases to affect the migratory and invasive properties of HNSCC cells. Such roles for E6 and/or E7 would be consistent with our findings that these oncoproteins increase migration but inhibit the number of mature invadopodia capable of degradation (i.e., active) but not the total number of invadopodia formed. Thus, these data suggest that HPV does not interfere with invadopodia formation but maturation which requires sustained actin polymerization for stability and ECM degradation.

In addition to their distinct biological characteristics, HPV+ HNSCC tumors also have unique morphological features including little stromal desmoplasia. This ECM normally stiffens tumors which promotes aggressiveness by activating mechanical signaling pathways that drive malignant cellular behavior. Tumor mechanical properties and related signaling pathways are emerging as significant factors in HPV+ HNSCC. For example, these tumors are more rigid than surrounding normal tissues, and their stromal density correlates with tumor stage, poor prognoses, and reduced survival rates. Furthermore, molecules involved in ECM cross-linking, mechanotransduction, and invasion also correlate with cancer risk, tumor progression, and poor survival. In addition, our work has shown that ECM rigidity mimicking tumor mechanical properties promotes invadopodia activity. Despite the lack of tumor-associated ECM, HPV+ HNSCCs are more frequently diagnosed with metastases. While our data indicate that E6 and E7 can alter the relative migratory potential of HNSCC cells, we also found a reduction in proteolytic activity which calls into question the means...
by which these tumors spread given the importance of ECM degradation for invasion of tissue barriers.\textsuperscript{22-24}

HPV+ HNSCC often arises in the lymphoid tissues of the palatine and lingual tonsils which are characterized by tubular and branched crypts composed of discontinuous basement membrane and incomplete connective tissue.\textsuperscript{115-118} While these tissues are designed to facilitate immunological responses between infiltrating immune cells and incoming pathogens, their structure and lack of stromal desmoplasia may also provide little resistance to the spread of cancer cells.\textsuperscript{116-118} Furthermore, tonsillar tissue drains fluids through lymphatic vessels to surrounding lymph nodes in the neck which are the most common sites of metastases for HPV+ HNSCCs.\textsuperscript{141-143} Therefore, these tissues may facilitate the movement of migratory cells to promote tumor progression. This notion would be consistent with the clinical findings that metastases beyond the head and neck occur over much longer time periods for HPV+ versus HPV+ HNSCCs\textsuperscript{144-147} which would require penetration of intact tissues in other bodily regions.

5 | CONCLUSIONS

In this study, we have shown that the HPV-16 oncoproteins E6 and E7 alter the migratory potential of HPV+ HNSCC cells while inhibiting invadopodia activity. Current models describing the effects of high-risk HPV do not address the roles of E6 and E7 on the migration and invasion of HNSCC cells. While the molecular mechanisms still need to be elucidated, our previous studies as well as work by others suggest that these oncoproteins may interfere with mechanical signaling pathways important for force generation and invadopodia dynamics. Inhibition of these pathways may drive a migratory phenotype capable of effective invasion of oropharyngeal tissues that present a permissive environment with little need for significant ECM degradation.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

AUTHORS’ CONTRIBUTIONS

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, C.N.K., A.P.; Methodology, C.N.K., R.J.J., A.P.; Investigation, C.N.K., R.J.J., A.P.; Formal Analysis, C.N.K., R.J.J., A.P.; Resources, C.N.K., R.J.J., A.P.; Writing - Original Draft, C.N.K., R.J.J., A.P.; Writing - Review & Editing, C.N.K., A.P.; Visualization, C.N.K., R.J.J., A.P.; Supervision, R.J.J., A.P.; Funding Acquisition, A.P.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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