**cGAS-STING responses are dampened in high-risk HPV type 16 positive
head and neck squamous cell carcinoma cells**

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Abstract

Head and neck cancers (HNCs) are a major health problem and a leading cause of morbidity and mortality worldwide. More than 90% of these tumours are head and neck squamous cell carcinomas (HNSCCs). Amongst the common risk factors for HNCs (tobacco and alcohol use), there is a strong association of human papillomavirus (HPV) with HNSCCs. HPV type 16 (HPV 16), the major high-risk HPV type, is most commonly associated with HPV-driven HNSCCs. The promiscuous nature of the major HPV oncogene, E7, allows its interaction with a myriad of host proteins including STING, a component of the viral DNA-sensing cyclic GMP-AMP synthase (cGAS) - stimulator of interferon genes (STING) machinery. Sensing of viral DNA by the cGAS-STING machinery results in a type I interferon (IFN)-mediated anti-viral response. Amelioration of IFN responses resulting from the direct blockade of STING by E7 was first demonstrated in high-risk HPV type 18 (HPV 18) positive (+) cervical squamous cell carcinoma (CESC) cells. However, the role of E7 from HPV 16 (HPV 16E7) in antagonising cGAS-STING responses have not been investigated, let alone in the context of HNSCCs. Here, we show that HPV 16E7+, but not HPV 16E7 negative (-), HNSCC cells respond poorly to cGAS-STING activation stimulus. We further confirm that this inhibition occurred via the highly conserved LXCXE motif in 16E7. This finding contributes to the better understanding of role of high-risk HPV E7 in blocking cGAS-STING pathway, especially in the context of HNSCCs.

Keywords

HNSCC; cGAS; STING; oral cancer; HPV; E7

Introduction

Head and neck squamous cell carcinomas (HNSCCs) has one of the fastest growing incidence rates among all cancers [1]. Although the prognosis of HPV positive (+) HNSCC is better than that of the tobacco and alcohol-related HPV negative (–) HNSCC [2], currently, the treatment of these two entities is identical [3]. While platinum-based chemotherapy and radiation therapy (alone or combined) are effective in reducing the bulk of the tumour, they have some significant drawbacks, including low specificity for tumour, toxicity, and severe adverse effects. Though current therapies for HPV+ HNSCCs are effective, severe late toxicity associated with current treatments contributes to the deterioration of patient quality of life [4]. Furthermore, given that current therapy fails in 15% of HPV+ HNSCCs with locoregional recurrence or distant metastatic disease, treatment deintensification is not appropriate for all patients [5]. New therapies for HPV+ HNSCCs are therefore needed. HNSCC is now the most common HPV-associated cancer and is now established as being mostly due to persistent infection with high-risk HPV type 16 [6]. Although HPV has been suggested as a risk factor for HNSCC, the recent dramatic increase in HPV-associated HNSCC has elicited considerable attention and these now outnumber cervical disease in the USA [1]. HPV-driven malignant transformation in cervical cancer is correlated with molecular events mediated by the major HPV oncogene, E7, which bind and down-regulate the tumour suppressor protein, retinoblastoma (Rb) [7]. We recently showed that HPV E7 is essential for survival of HPV+ HNSCCs [8].

DNA tumour virus oncogenes have been shown to antagonise an important anti-viral DNA sensing innate immune machinery known as the Cyclic GMP-AMP synthase (cGAS) - stimulator of interferon genes (STING) pathway [9], a well-established cytosolic DNA-

sensing machinery and responds to viral DNA during an infection [10]. cGAS is a DNA sensor protein, which, upon binding dsDNA independently of DNA sequence, and catalyzes the synthesis of 2'-3'-cyclic GMP-AMP (cGAMP). cGAMP functions as a second messenger that, in turn, engages the endoplasmic reticulum (ER)-membrane adaptor protein STING initiating a well-defined downstream signalling cascade culminating in the induction of type I interferons (IFNs). Among other viral oncogenes, the E7 oncogene from HPV 18 (HPV 18E7) was shown to be a potent inhibitor of the STING pathway [9]. Importantly, this was due to direct E7 binding to STING via its LXCXE motif, a motif which is essential for blockade of Rb. Indeed, CRISPR/Cas editing of E7 from HeLa cells, a HPV 18E7+ cervical squamous cell carcinoma (CESC) cell line, restored the cGAS-STING pathway. However, work to date have only shown the blockade of cGAS-STING responses by HPV 18E7. HPV 16 accounts for over 80% of HPV-related head and neck cancers [6]. Although, the LXCXE motif is highly conserved, the mechanism of action on known biological targets such as, pRB [11] and p130 [12] have been shown to be distinct between different HPV E7 types. The role of HPV 16E7 in antagonising cGAS-STING responses have yet to be addressed, let alone in the context of HNSCCs. Interestingly, STING is highly expressed in advanced HPV+, not in HPV-, HNSCC tumours [13-15]. Importantly, STING is expressed by basal cells in the oral mucosa, which are a key target for HPV infection, and expression is maintained in these cells through HPV+ dysplastic progression [13]. We therefore explored whether cGAS-STING antagonism by HPV 16E7 occurs in HNSCCs.

Materials and Methods

Cell culture

HNSCC cell lines used in this study include UDSCC2 (SCC2), UMSCC104 (SCC104), immortalized normal oral keratinocytes (NOKs) [16], HPV 16-transformed NOKs (NOK_HPV16) [16], UPCI:SCC90 (SCC90) and UMSCC1 (SCC1) (Kindly provided Prof.

Paul Lambert, University of Wisconsin). With the exception of SCC1, cells were obtained from various sources [8] and cultured as previously described [8]. SCC1 cells were grown in complete DMEM (Gibco-Invitrogen, Waltham, MA) supplemented with 10% heat inactivated foetal bovine serum (FBS) (Gibco-Invitrogen, Waltham, MA) and 1% of antibiotic/glutamine preparation (100 U/ml penicillin G, 100 U/ml streptomycin sulphate, and 2.9 mg/ml of L-glutamine) (Gibco-Invitrogen, Waltham, MA) supplemented with 0.4ug/ml of hydrocortisone (Gibco-Invitrogen, Waltham, MA).

Nucleic acids

UltraPure sheared salmon sperm DNA was obtained from Thermo Scientific (Waltham, MA). Calf thymus DNA was obtained from Sigma-Aldrich (St Louis, MI) and was subjected to four rounds of Triton X-114 purification to remove endotoxin contamination as previously described [17]. 2'3'-cGAMP and 5' ppp-dsRNA were obtained from InvivoGen (San Diego, CA).

SCC1 stable cell line generation

Cells were forward-transfected with plasmids bearing wild type and LXCXE mutant forms of the HPV 16E7 gene [18] using Fugene 6 (Promega, Madison, WI) in OptiMEM (Gibco-Invitrogen, Waltham, MA) followed by stable selection in 500µg/ml of G418 (Sigma-Aldrich, St Louis, MI).

Nucleic acid transfection

Cells were seeded overnight in a 12-well plate to 70-90% confluency before transfecting nucleic acids with Lipofectamine 2000 (Invitrogen, California USA) at a 1:1 ratio (1µg DNA:1 µl Lipofectamine 2000) in Opti-MEM (Gibco-Invitrogen, Waltham, MA).

Immunoblotting

Protein from cells were extracted in RIPA buffer (Thermo Scientific, Waltham, MA) containing 1x Halt Protease Inhibitor (Thermo Scientific, Waltham, MA). Immunoblots were

probed with antibodies against HPV 16E7 (ED-17) (Santa Cruz Technologies Biotechnologies, Dallas, TX), cGAS (D1D3G) (Cell Signaling Technologies, Danvers, MA), STING (D2P2F) (Cell Signaling Technologies, Danvers, MA), s6 ribosome (Cell Signaling Technologies, Danvers, MA) and β -tubulin (Sigma-Aldrich, St Louis, MI). Rabbit and mouse secondary antibodies (Cell Signaling Technologies, Danvers, MA) and ECL was used to detect protein signals on a Chemidoc XRS Visualiser (BioRad, Hercules, CA).

Real-time (RT) and digital PCR

RT PCR of human IFN β (F- 5' AAA CTC ATG AGC AGT CTG CA 3', R-5' AGG AGA TCT TCA GTT TCG GAG G 3') was done on the Rotor-Gene (Gibco-Invitrogen, Waltham, MA) using the Rotor-Gene SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Human GAPDH (F- 5' CGAGATCCCTCCAAAATCAA 3', R- 5' TTCACACCCATGACGAACAT 3') was used as the housekeeping gene. Absolute quantification of the HPV 16 E7 gene copy numbers was performed on the QuantStudio 3D Digital PCR System (Thermo Scientific, Waltham, MA) as previously described [8].

Results and discussion

Firstly, we wanted to verify HPV+ HNSCCs are indeed HPV 16E7+. We confirmed that HPV 16E7 were expressed in HPV+ HNSCC cell lines SCC2, SCC104 and SCC90 at comparable levels, both at a protein and mRNA level (Fig. 1a). This is in contrast to findings in a previous study where lower 16E7 protein expression was reported in SCC2 and SCC104 cells compared to SCC90 cells [19]. Furthermore, their 16E7 relative expression data using real-time PCR was incongruent to their protein expression data. Our absolute quantification data using digital PCR reflects a more accurate 16E7 expression pattern across the tested HPV+ HNSCC lines as this method measures the exact 16E7 gene copy numbers expressed in each cell. Indeed, a range of HNSCC cell lines and immortalized normal oral keratinocytes (NOKs) express cGAS and STING proteins (Fig. 1b). Consistent with previous findings [15],

we also demonstrate that SCC90 cells express little to no STING protein. It is interesting to note that NOKs express lower levels of the cGAS protein than seen in HNSCCs. This could possibly be a characteristic of non-SCC oral keratinocytes, though further work is needed to address this. HPV associated-HNSCC have a distinct morphological tropism in the oropharynx [20]. As a matter of fact, are increasingly found to be the causative agent for a subset of HNSCC, oropharyngeal squamous cell carcinomas (OPSCCs) [21]. Although the preference of HPV for the oropharynx is not clear, a plausible explanation is the unique presence of transitional mucosa in the oropharynx (histologically similar to the cervical mucosa). Other than NOKs, all the other tested HNSCC cells used in this study have non-gingival origins [22]. This could explain why the cGAS-STING machinery is intact in these cells and therefore not surprising to find STING highly expressed in HPV+ HNSCC patient tumours from this anatomical site [13-15].

To test the ability of HNSCC cells to respond to cGAS-STING stimulation, cells were transfected with salmon sperm DNA (S.S. DNA), a known cGAS-STING activator [23]. Only transfection of HPV- SCC1 cells resulted in a robust type I IFN induction as early as 6h (Fig. 1c). All tested HPV+ HNSCC cell failed to mount an IFN response suggesting that the presence of HPV 16E7 is dampening cGAS-STING responses. This was further confirmed where transfection of 2'3'-cGAMP, a direct STING activator, failed to mount an appropriate type I IFN response in HPV+ HNSCC cells, but not SCC1 cells (Fig. 1c). Consistent with previous observations [15], SCC90 cells responded poorly to 2'3'-cGAMP (Fig. 1c). Transfection with 5'ppp-dsRNA, a ligand that activates the cytoplasmic dsRNA sensing receptor retinoic acid-inducible gene I (RIG-I) [24], resulted in a comparable robust type I IFN response in all tested HNSCC lines implying that the presence of HPV 16E7 had no effect on cytosolic dsRNA-mediated responses. It is important to note that 5'ppp-dsRNA was only able to induce IFN β induction at 24h, but not 6h, post-transfection (Fig. 1c). The ability

for 5'ppp-dsRNA to induce RIG-I dependent type I IFN induction is expected as RIG-I is shown to be highly expressed in HNSCCs [25]. Parallel to previous findings in HPV 18E7+ HeLa cells [9], insensitivity of SCC2 and SCC104 cells to cGAS-STING stimulation correlated with HPV 16E7 expression (Fig. 1c). For the first time, we demonstrate that HPV+ HNSCCs responded poorly to cGAS-STING stimulation *in vitro*. To definitively show whether loss of 16E7 would recover the cGAS-STING pathway in HPV+ HNSCC cells, a gene targeting approach to knockdown 16E7 (e.g. CRISPR/Cas9 gene editing) could be done to address this in future studies.

Previous work by Lau *et al.*, [9] demonstrated that HPV 18E7 could ameliorate cGAS-STING responses and was dependent on the highly conserved LXCXE motif. To conclusively show that HPV 16E7 is responsible for the lack of DNA-mediated cGAS-STING response seen in HPV+ HNSCCs (Fig. 1c), we generated SCC1 cells stably expressing HPV 16E7. Cells were then transfected with purified un-sheared calf thymus DNA (CT DNA), another commonly used specific activator of the cGAS-STING pathway [26], the same DNA used by Lau *et al.*, [9]. CT DNA-mediated type I IFN induction was significantly reduced in cells that express wild type (WT) 16E7, but not the LXCXE mutant form, suggesting that the LXCXE motif is indispensable for 16E7-mediated cGAS-STING blockade in HNSCCs (Fig. 2a). To rule out the possibility that this effect was not due to high WT 16E7 expression, absolute quantification of E7 for all the SCC1 stable cell lines was done. Indeed, WT 16E7 gene copy numbers were comparable to that of the mutant form (Fig. 2b). A similar blockade was also observed in NOK_16E7, NOKs that contained the episomal form of the entire HPV type 16 genome (Fig. 2c). As NOKs expressed very low levels of cGAS (Fig. 1b), 2'3'-cGAMP was used to induce STING specific-mediated type I IFN response. Overall, we show that the presence of 16E7 dampens cGAS-STING responses in SCC1 cells and NOKs. Whether the level of blockade with observed here with HPV 16E7 is

comparable to that seen previously with HPV 18E7 [9], remains to be seen. Unlike CESC cells, HPV 18+ HNSCC cell lines do not exist. Future work should focus on comparing the level of cGAS-STING blockade between different E7 derived from high and low-risk HPV types in HNSCC cells. This is crucial as it will determine whether E7/cGAS-STING pathway blockade is a unique characteristic of high-risk HPV types.

In conclusion, we show that HPV-, but not HPV+, HNSCC cells responded to cGAS-STING pathway activators. Introduction of 16E7 into HPV- HNSCC cells and NOKs significantly dampened this response. Furthermore, we showed that the LXCXE motif of 16E7 was indispensable for 16E7-mediated STING pathway blockade. By understanding the dynamics of HPV E7/STING interplay in HNSCCs, we can envisage future therapeutic approaches for HNSCCs, which could involve a combination of HPV E7 oncogene targeting and the use of STING agonists. Indeed, tumour regression have been reported in a number of HNSCC *in vivo* models treated with STING agonists [13, 14, 27]. Importantly, the use of STING in HNSCC therapy may find merit in preventing progression of premalignant HNSCC to invasive forms of the disease.

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References

1. Van Dyne, E.A., et al., *Trends in Human Papillomavirus-Associated Cancers - United States, 1999-2015*. MMWR Morb Mortal Wkly Rep, 2018. **67**(33): p. 918-924.
2. Ang, K.K., et al., *Human Papillomavirus and Survival of Patients with Oropharyngeal Cancer*. New England Journal of Medicine, 2010. **363**(1): p. 24-35.
3. Ang, K.K., et al., *Human papillomavirus and survival of patients with oropharyngeal cancer*. N Engl J Med, 2010. **363**(1): p. 24-35.
4. Machtay, M., et al., *Factors Associated With Severe Late Toxicity After Concurrent Chemoradiation for Locally Advanced Head and Neck Cancer: An RTOG Analysis*. Journal of Clinical Oncology, 2008. **26**(21): p. 3582-3589.
5. Masterson, L., et al., *De-escalation treatment protocols for human papillomavirus-associated oropharyngeal squamous cell carcinoma: a systematic review and meta-analysis of current clinical trials*. Eur J Cancer, 2014. **50**(15): p. 2636-48.
6. Bratman, S.V., et al., *Human Papillomavirus Genotype Association With Survival in Head and Neck Squamous Cell Carcinoma*. JAMA Oncol, 2016. **2**(6): p. 823-6.
7. Munger, K. and P.M. Howley, *Human papillomavirus immortalization and transformation functions*. Virus Res, 2002. **89**(2): p. 213-28.
8. Shaikh, M.H., et al., *Aurora kinases are a novel therapeutic target for HPV-positive head and neck cancers*. Oral Oncology, 2018. **86**: p. 105-112.
9. Lau, L., et al., *DNA tumor virus oncogenes antagonize the cGAS-STING DNA-sensing pathway*. Science, 2015. **350**(6260): p. 568-71.
10. Cai, X., Y.H. Chiu, and Z.J. Chen, *The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling*. Mol Cell, 2014. **54**(2): p. 289-96.

11. White, E.A., et al., *Systematic identification of interactions between host cell proteins and E7 oncoproteins from diverse human papillomaviruses*. Proc Natl Acad Sci U S A, 2012. **109**(5): p. E260-7.
12. Barrow-Laing, L., W. Chen, and A. Roman, *Low- and high-risk human papillomavirus E7 proteins regulate p130 differently*. Virology, 2010. **400**(2): p. 233-9.
13. Baird, J.R., et al., *STING expression and response to treatment with STING ligands in premalignant and malignant disease*. PLoS One, 2017. **12**(11): p. e0187532.
14. Liang, D., et al., *Activated STING enhances Tregs infiltration in the HPV-related carcinogenesis of tongue squamous cells via the c-jun/CCL22 signal*. Biochim Biophys Acta, 2015. **1852**(11): p. 2494-503.
15. Lu, S., et al., *STING activation enhances cetuximab-mediated NK cell activation and DC maturation and correlates with HPV(+) status in head and neck cancer*. Oral Oncol, 2018. **78**: p. 186-193.
16. Piboonniyom, S.O., et al., *Abrogation of the retinoblastoma tumor suppressor checkpoint during keratinocyte immortalization is not sufficient for induction of centrosome-mediated genomic instability*. Cancer Res, 2003. **63**(2): p. 476-83.
17. Stacey, K.J., et al., *The molecular basis for the lack of immunostimulatory activity of vertebrate DNA*. J Immunol, 2003. **170**(7): p. 3614-20.
18. Antonsson, A., et al., *The human papillomavirus type 16 E7 protein binds human interferon regulatory factor-9 via a novel PEST domain required for transformation*. J Interferon Cytokine Res, 2006. **26**(7): p. 455-61.
19. Kalu, N.N., et al., *Genomic characterization of human papillomavirus-positive and -negative human squamous cell cancer cell lines*. Oncotarget, 2017. **8**(49): p. 86369-86383.

20. Sturgis, E.M. and K.K. Ang, *The epidemic of HPV-associated oropharyngeal cancer is here: is it time to change our treatment paradigms?* J Natl Compr Canc Netw, 2011. **9**(6): p. 665-73.
21. Husain, N. and A. Neyaz, *Human papillomavirus associated head and neck squamous cell carcinoma: Controversies and new concepts.* J Oral Biol Craniofac Res, 2017. **7**(3): p. 198-205.
22. Lin, C.J., et al., *Head and neck squamous cell carcinoma cell lines: established models and rationale for selection.* Head Neck, 2007. **29**(2): p. 163-88.
23. Li, X., et al., *Cyclic GMP-AMP Synthase is Activated by Double-stranded DNA-Induced Oligomerization.* Immunity, 2013. **39**(6): p. 10.1016/j.immuni.2013.10.019.
24. Saito, T., et al., *Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA.* Nature, 2008. **454**(7203): p. 523-7.
25. Hu, J., et al., *Dose dependent activation of retinoic acid-inducible gene-I promotes both proliferation and apoptosis signals in human head and neck squamous cell carcinoma.* PLoS One, 2013. **8**(3): p. e58273.
26. Stetson, D.B. and R. Medzhitov, *Recognition of cytosolic DNA activates an IRF3-dependent innate immune response.* Immunity, 2006. **24**(1): p. 93-103.
27. Baird, J.R., et al., *Evaluation of explant responses to STING ligands: personalized immunosurgical therapy for head and neck squamous cell carcinoma.* Cancer Res, 2018.

Figure legends

Figure 1 HPV16+ HNSCC cells responded poorly to cGAS-STING pathway activators. a) HPV 16 E7 is expressed in a range of HNSCC cell lines. Absolute quantitation of E7 gene copy number on cDNAs from cells was done by digital PCR. Data is

representative of E7 gene copy number/ μl $\pm$ confidence interval (CI). Protein from HPV positive (+ve) and negative (-ve) cells were extracted and immunoblots probed with antibodies against HPV type 16 E7 and s6 as loading control. Data shown are representative of three independent experiments. **b) Normal oral and HNSCC cells express cGAS and STING proteins at varying levels.** Protein from cells were extracted and immunoblots probed with antibodies against cGAS, STING and s6 as loading control. Individual blots shown are representative of three independent experiments. **c) DNA-mediated cGAS-STING activation is not observed in HPV16+ HNSCC cells.** Cells were transfected with or without (Tx alone) 5 μg of salmon sperm DNA (S.S. DNA), 1 μg of 2'3'-cGAMP or 1 μg of 5'ppp-dsRNA (3pRNA) complexed with lipofectamine before extracting RNA at the indicated times. IFN β mRNA expression relative to GAPDH mRNA was determined by RT PCR. Data is representative of the mean $\pm$ SD (n=3); ** p value < 0.01 and **** p value < 0.0001, Student's T test.

Figure 2 HPV type 16E7 blocked DNA-mediated cGAS-STING responses in SCC1 cells. **a) Wild type 16E7, but not the LXCXE mutant form, blocked cGAS-STING responses in SCC1 cells.** Parental (Control) and SCC1 cells stably expressing wild type 16E7 (16E7) or an LXCXE mutant form (16E7^{LXCXE}) were transfected with 5 μg of CT DNA complexed with lipofectamine before extracting RNA at 6h post-transfection. IFN β mRNA expression relative to GAPDH mRNA was determined by RT PCR. Percentage IFN β mRNA expression was compared to that of control cells. Data is representative of the mean $\pm$ SD (n=3) * p value < 0.05, Student's T test. **b) HPV 16E7-mediated blockade of cGAS-STING responses was not due to discrepancies in the absolute expression levels of E7.** Absolute quantitation of E7 gene copy number on cDNAs from cells was done by digital PCR. Data is representative of E7 gene copy number/ μl $\pm$ confidence interval (CI). Protein from cells were extracted and immunoblots probed with antibodies against HPV type 16 E7 and β -tubulin as

324 loading control. c) **Direct STING activation with 2'3'-cGAMP was dampened in NOKs**
325 **retrovirally transduced with HPV type 16 genome.** Parental (NOK) and retrovirally
326 transduced NOKs with HPV type 16 genome (NOK_16E7) were transfected with or without
327 (Tx alone) 5µg of 2'3'-cGAMP complexed with lipofectamine before extracting RNA 24h
328 post-transfection. IFNβ mRNA expression relative to GAPDH mRNA was determined by RT
329 PCR. Data is representative of the mean ± SD.

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Highlights

- HPV-positive HNSCC cells respond poorly to activators of the cGAS-STING pathway
- cGAS-STING blockade was due to the presence of the high-risk HPV 16E7 oncogene
- The highly conserved LXCXE motif of the 16E7 oncogene is responsible for cGAS-STING blockade