

Hypermethylation of *nc886* in HPV-positive oropharyngeal cancer and its clinical implications: An epigenome-wide association study

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The incidence of oropharyngeal squamous cell carcinoma (OPSCC) has increased rapidly in the United States, driven by rising human papillomavirus (HPV) infections in the U.S. population. HPV-positive OPSCC patients have a better prognosis than HPV-negative patients. To gain insights into the unique biology of HPV(+) OPSCC that may contribute to its clinical behaviors, we performed a multi-stage epigenome-wide methylation profiling of leukocyte and tumor DNA in OPSCC patients and compared the methylation levels of CpG sites between HPV(+) and HPV(−) OPSCC patients. We identified and validated a significantly differentially methylated region (DMR) of 1,355 bp encompassing non-coding RNA 886 (*nc886*) gene and its promoter region. *Nc886* is hypermethylated in both leukocytes and tumor DNA of HPV(+) OPSCC patients. Homozygous knockout of *nc886* by CRISPR-Cas9 in head and neck cell lines was lethal, but *nc886* could be knocked out on the background of protein kinase R (*PKR*) knockout. Our data suggest that HPV induces *nc886* hypermethylation, and *nc886* acts as both a viral sensor and a tumor sensor in OPSCC patients and contribute to the better prognosis of HPV(+) OPSCC patients. *Nc886* may become a therapeutic target in OPSCC.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), a group of cancers in the upper aerodigestive tract including the oral cavity, larynx, oropharynx, hypopharynx, and nasopharynx, has traditionally been strongly associated with tobacco and alcohol use.^{1–3} In the United States and some Western countries, the incidence rate of overall HNSCC has been steadily decreasing in the past four decades because of decreased tobacco use; however, the incidence of OPSCC, a distinct subtype of HNSCC, has been increasing by about 2.5% per year.⁴ This increase in OPSCC incidence was driven by the rising HPV infections in the general population in these countries.^{4–6} The percentage of HPV-positive cases among all newly diagnosed OPSCC patients increased from approximately 20% in the 1980s to more than 70% currently, and OPSCC has surpassed cervical cancer as the most

frequently diagnosed cancer caused by HPV.⁷ Although HPV vaccines are highly efficacious for the prevention of anogenital HPV infections and associated malignancies,⁸ the population-level effect of HPV vaccination on the burden of oral HPV infections in U.S. young adults was estimated to be only 25% in women and 7% in men because of low vaccine uptake.⁹ Given the high prevalence of HPV in U.S. population, poor HPV vaccine uptake in men, the male predominance of OPSCC, and the long latency of developing OPSCC following HPV exposure, it was estimated that the incidence of HPV(+) OPSCC will continue to rise until 2060.⁵

HPV(+) and HPV-negative OPSCC are distinct entities with unique epidemiological, clinical, and biological characteristics.¹⁰ Clinically, HPV(+) OPSCC patients have a significantly better therapeutic response and survival than HPV(−) patients.^{11,12} Biologically, although HPV(+) and HPV(−) OPSCC tumors have similar overall mutation rate and mutational burden,^{13,14} HPV(+) tumors have a significantly higher number of aberrant DNA methylations than HPV(−) OPSCC tumors.^{15,16} DNA methylation at CpG dinucleotides plays important roles in cancer development and progression.¹⁷ Hypermethylation in the promoter regions of tumor suppressor genes leads to their transcriptional silencing, and global hypomethylation causes chromosomal instability and/or activate the expression of proto-oncogenes.¹⁷ HPV oncoproteins E6 and E7 can directly target methyltransferases and modulate DNA methylation.^{18,19} Many studies have compared DNA methylations between HPV(+) and HPV(−) HNSCC tumors and identified potential differentially

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Table 1. Clinical characteristics of study populations

| | Discovery stage, N (%) | | Internal validation, N (%) | | External validation, N (%) | |
|----------------|------------------------|------------|----------------------------|-------------|----------------------------|------------|
| | HPV(+) | HPV(-) | HPV(+) | HPV(-) | HPV(+) | HPV(-) |
| N | 5 (50.0) | 5 (50.0) | 15 (53.6) | 13 (46.4) | 79 (50.3) | 78 (49.7) |
| Age (years) | | | | | | |
| Mean (SD) | 58 (6.6) | 58.8 (5.2) | 58.1 (10.6) | 61.6 (10.6) | 55 (8.6) | 58 (10.12) |
| Median (range) | 55 (51–66) | 58 (52–64) | 54 (46–83) | 59 (48–83) | 56 (39–78) | 58 (42–86) |
| Gender | | | | | | |
| Female | 1 (20.0) | 1 (20.0) | 0 | 7 (53.8) | 12 (15.2) | 14 (20.6) |
| Male | 4 (80.0) | 4 (80.0) | 15 (100.0) | 6 (46.2) | 67 (84.8) | 64 (79.4) |
| Smoking status | | | | | | |
| Never | 0 | 0 | 5 (33.3) | 0 | 24 (30.8) | 23 (30.6) |
| Former | 3 (60.0) | 3 (60.0) | 5 (33.3) | 4 (30.8) | 50 (64.1) | 35 (46.7) |
| Current | 2 (40.0) | 2 (40.0) | 5 (33.3) | 9 (69.2) | 4 (5.1) | 17 (22.7) |

methylated CpG sites (DMCs) and differentially methylated regions (DMRs).^{15,16,20–24} Differential DNA methylations contribute to the clinical and biological differences between HPV(+) and HPV(-) tumors.

Besides inducing methylation changes in tumor tissues, HPV infection also induces extensive systemic DNA methylation alterations, particularly in the immune system. CpG methylation in leukocyte DNA has profound, long-standing effects on inflammation and immune responses.²⁵ Leukocyte DNA methylation profile has been used to derive systemic inflammation indices^{26–29} and immune cell lineages.²⁵ This may be particularly relevant to HPV-associated OPSCC, as HPV-induced immune activation has been shown to be a critical driver of OPSCC prognosis.^{30–33} No study has specifically compared DNA methylations in leukocytes from HPV(+) OPSCC patients and HPV(-) patients.

In this study, we performed a multi-stage epigenome-wide methylation profiling to leukocytes and tumor tissues from HPV(+) and HPV(-) OPSCC patients to identify DMCs and DMRs between HPV(+) and HPV(-) OPSCC patients. The most notable observation was that *nc886* was specifically hypermethylated in both leukocyte and tumor DNA of HPV(+) compared with HPV(-) patients. Moreover, functional studies suggest that hypermethylation of *nc886*, leading to down-regulation of *nc886*, may contribute to the better prognosis of HPV(+) OPSCC patients.

RESULTS

Hypermethylation of *nc886* gene in leukocyte DNA from HPV(+) OPSCC patients compared with HPV(-) patients

We conducted a pilot epigenome-wide association analysis (EWAS) of DNA methylation with the HPV status of OPSCC patients. We used a three-phase case-control design: the discovery phase included 5 pairs of HPV(+) and HPV(-) OPSCC patients, the internal validation phase consisted of 15 HPV(+) and 13 HPV(-) OPSCC patients, and the external validation phase had 79 HPV(+) and 78 HPV(-)

OPSCC patients (Table 1). A total of 790,462 CpG sites passed quality control and were analyzed for their differential methylation between HPV(+) and HPV(-) OPSCC. In individual CpG site analysis, 1,107 CpG sites reached a false discovery rate (FDR) *q* value of <0.05, among which 25 sites had test statistic scores (*d*) of >5 and were defined as highly significant DMCs (Table S1). Most of the DMCs are in open seas, although some are located in potential enhancer regions and DNase I hypersensitivity sites, and two CpG sites are located in CpG islands (Table S1). Gene Ontology (GO) enrichment analyses using genes containing these 1,107 DMCs found the top enriched biological processes included cytoskeleton organization, negative regulation of transcription by polymerase II, cell cycle process, cellular localization, and organelle organization, and the top enriched molecular functions were kinase binding, enzyme binding, ion binding, and protein binding (data not shown). Reactome pathway enrichment analysis revealed that three pathways, chromatin-modifying enzymes, chromatin organization, and olfactory signaling pathway, were enriched, with an FDR < 0.05 (Table S2).

Because DMRs, particularly those within or encompassing CpG islands, are more likely to be functionally important, we focused on analyses on DMRs rather than individual CpG sites. We used the Bumphunter method³⁴ to identify DMRs between HPV(+) and HPV(-) patients. At the discovery phase, there were 86 DMRs at *p* value area < 0.05; at the internal validation stage, there were 32 DMRs with the same testing criteria. Four DMRs were consistent in both the discovery and internal validation phases (Table 2). Each DMR contained at least one CpG island or was part of a CpG island. All the four replicated DMRs are located near genes and in the DNase I hypersensitivity region on the basis of University of California, Santa Cruz (UCSC), annotation. The most significant one was located on chromosome 5 covering the gene of *nc886* and its promoter region (1,500 bp of transcription start site [TSS1500]). The methylation levels of all the 15 CpG sites in the *nc886* DMR were consistently higher in HPV(+) OPSCC patients than in HPV(-) patients (Figure 1A).

Table 2. Differentially methylated regions in leukocyte DNA between HPV(+) and HPV(-) OPSCC patients

| Chromosome | Gene | Discovery | | Internal validation | | External validation | |
|------------|-----------------|----------------------------------|---------|----------------------------------|---------|----------------------------------|---------|
| | | $\Delta\beta$ value ^a | p value | $\Delta\beta$ value ^a | p value | $\Delta\beta$ value ^a | p value |
| Chr5 | <i>Nc886</i> | 0.130 | 0.003 | 0.100 | 0.001 | 0.046 | 0.042 |
| Chr11 | <i>CAT</i> | -0.091 | 0.006 | -0.033 | 0.020 | -0.012 | 0.020 |
| Chr12 | <i>GLIPR1L2</i> | -0.120 | 0.010 | -0.062 | 0.009 | 0.008 | 0.321 |
| Chr6 | <i>PTCHD4</i> | -0.054 | 0.049 | -0.034 | 0.032 | -0.002 | 0.759 |

^a $\Delta\beta$ value is the difference of the mean β of all the CpG sites in the DMR between HPV(+) and HPV(-) patients.

We then used a large independent external validation population to validate these four DMRs. Two of the four DMRs were validated, *nc886* region and *CAT* gene on chromosome 11, which was hypomethylated in HPV(+) OPSCC patients (Table 2). Figure 2 and Table 3 show the detailed distribution of the 15 CpG sites within this DMR and their differential methylation in the validation population. One CpG site, cg04481923, sits in the gene body of *nc886*. This CpG site and its close neighboring CpG sites exhibited >20% higher methylation in HPV(+) OPSCC patients than HPV(-) patients.

nc886 DMR is also hypermethylated in HPV(+) compared with HPV(-) OPSCC tumors

To determine whether *nc886* DMR is also hypermethylated in HPV(+) OPSCC tumors, we performed the same MethylationEPIC methylation array in tumor DNA from the 5 pairs of HPV(+) and HPV(-) OPSCC patients in the discovery phase. Like leukocyte DNA, *nc886* region was hypermethylated in HPV(+) tumors compared with HPV(-) tumors (mean β value 0.613 vs. 0.525, $p = 0.0387$). We further confirmed *nc886* hypermethylation in HPV(+) OPSCC tumors using a Cancer Genome Atlas (TCGA) methylation dataset of 28 HPV(+) tumors and 40 HPV(-) tumors.³⁵ Because TCGA used Illumina Methylation450K arrays, there were slightly fewer CpG sites covered in this region than

MethylationEPIC (12 vs. 15 sites). Again, the mean β values of all CpG sites in *nc886* were significantly higher in HPV(+) tumors than HPV(-) tumors (0.632 vs. 0.540, $p < 0.001$) in the TCGA dataset. All the 12 CpGs showed significantly higher methylation in HPV(+) tumors than in HPV(-) tumors (Figure 1B), similar to the observations in leukocytes. These data from independent patient cohorts provided compelling evidence that HPV infection induces *nc886* promoter CpG island hypermethylation in both leukocytes and tumor DNA.

Functional consequence of *nc886* knockout on HNSCC cell growth and apoptosis

We next determined the functional impact of *nc886* knockout (KO) on HNSCC cell proliferation and apoptosis. We used the CRISPR-Cas9 HDR method to replace the *nc886* with GFP in FaDu cells. GFP-positive cells were sorted using flow cytometry and cultured in 96-well plates before moving to 6-well plates for expansion. The DNA modification of *nc886* sequence area was verified using Sanger sequencing (Figure 3A). After moving to 6-well plates, all the selected GFP(+) cell colonies died during expansion (Figure 3B). The inability to establish stable *nc886* KO cells suggests that *nc886* is essential for cell growth and knocking out *nc886* leads to growth inhibition and cell death. We could not produce *nc886* KO in another HNSCC cell line, SCC-47, either (data not shown).

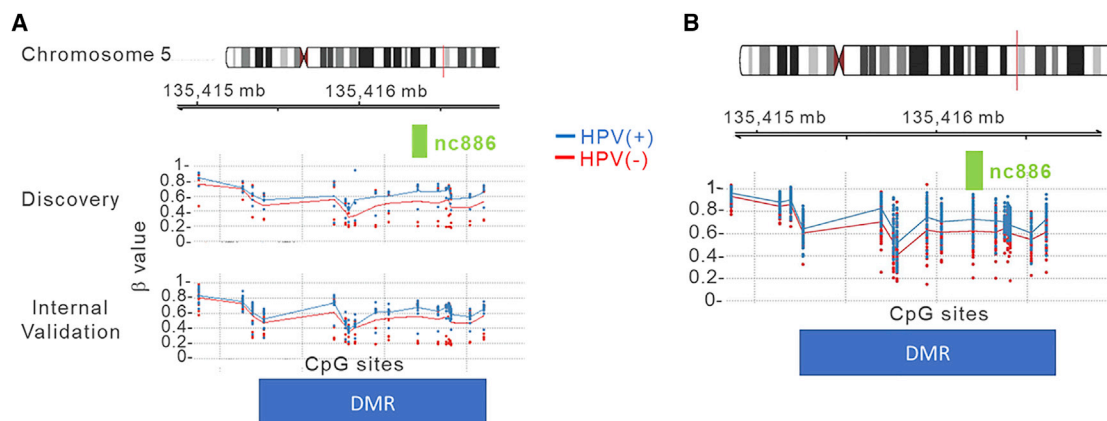


Figure 1. CpG sites in the *Nc886* genomic region are significantly hypermethylated in HPV(+) OPSCC patients compared with HPV(-) patients
(A) Leukocytes (discovery and interval validation). (B) Tumor tissues.

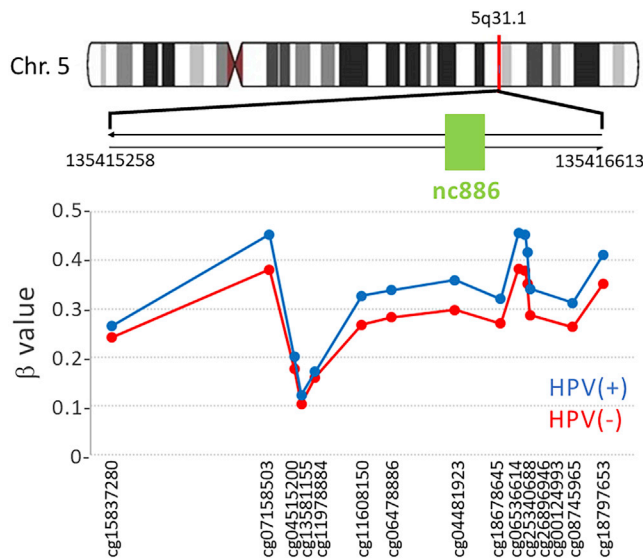


Figure 2. Detailed distribution of the 15 differentially methylated CpG sites in the DMR of *nc886* gene and vicinity in leukocytes of the external validation patient population

Previous studies have shown that *nc886* binds and inhibits PKR activity.³⁶ *Nc886* KO is believed to induce apoptosis mainly through PKR activation, resulting in blockade of translation.^{37–39} We then tested whether we could generate *nc886* KO in *PKR* KO FaDu cells. We first generated *PKR* KO cells (Figure 4A) and then introduced *GFP* into *PKR*^{KO} cells to replace *nc886*. We were able to amplify *GFP*-positive colonies (Figure 4B). Sequencing confirmed *nc886* sequence modifi-

cation and real-time reverse transcription PCR showed dramatic reduction of *nc886* expression (Figure 4C). Interestingly, the *nc886*^{KO}/*PKR*^{KO} cells grew slowly than the *PKR*^{KO} cells (Figure 4D), suggesting that there might be additional pathway(s) that mediate *nc886*'s effect on cell proliferation and apoptosis besides PKR.

DISCUSSION

In this study, we applied epigenome-wide DNA methylation profiling to leukocytes and tumor tissues of OPSCC patients and identified a large number of DMCs and four DMRs between HPV(+) and HPV(-) patients. Most interestingly, we found that the methylation levels of *nc886* were significantly higher in both leukocytes and tumors tissues in HPV(+) than HPV(-) OPSCC patients. Furthermore, we found that knockout of *nc886* resulted in growth inhibition and apoptosis, suggesting that hypermethylation of *nc886*, leading to down-regulation of *nc886*, may contribute to the better prognosis of HPV(+) OPSCC patients. Methylation is generally tissue specific and rarely is there common methylation changes between immune cells and tumor cells. To our knowledge, this is the first report linking *nc886* hypermethylation to HPV infection and the first report of a molecule that is hypermethylated in both leukocytes and tumors of cancer patients.

HPV(+) OPSCC differs from HPV(-) OPSCC in etiology, treatment, prognosis, and biology.⁴⁰ HPV(+) OPSCC patients are more sensitive to radiotherapy and have significantly better survival than HPV(-) patients.¹¹ The biological mechanisms underlying the better therapeutic response and survival of HPV(+) OPSCC patients are not fully understood. Although the mutational spectrums of HPV(+) and HPV(-) tumors are understandably different because of the different

Table 3. Differentially methylated region of *nc886* on 5q31.1 and its encompassing CpG sites

| CpG ID | β value | | Δβ value ^a | Chromosome positions | Gene locations | p value |
|------------|---------|---------|-----------------------|----------------------|----------------|---------|
| | HPV (-) | HPV (+) | | | | |
| cg15837280 | 0.242 | 0.265 | 0.024 | 135415258 | | 0.280 |
| cg07158503 | 0.381 | 0.453 | 0.072 | 135415693 | | 0.042 |
| cg04515200 | 0.177 | 0.202 | 0.025 | 135415762 | | 0.113 |
| cg13581155 | 0.104 | 0.123 | 0.019 | 135415781 | | 0.074 |
| cg11978884 | 0.159 | 0.171 | 0.012 | 135415819 | | 0.558 |
| cg11608150 | 0.267 | 0.327 | 0.060 | 135415948 | | 0.021 |
| cg06478886 | 0.283 | 0.339 | 0.056 | 135416029 | | 0.018 |
| cg04481923 | 0.298 | 0.359 | 0.062 | 135416205 | Gene body | 0.038 |
| cg18678645 | 0.270 | 0.321 | 0.051 | 135416331 | TSS200 | 0.061 |
| cg06536614 | 0.384 | 0.456 | 0.072 | 135416381 | TSS200 | 0.036 |
| cg25340688 | 0.379 | 0.454 | 0.075 | 135416398 | TSS200 | 0.035 |
| cg26896946 | 0.353 | 0.417 | 0.065 | 135416405 | TSS200 | 0.042 |
| cg00124993 | 0.288 | 0.342 | 0.054 | 135416412 | TSS200 | 0.049 |
| cg08745965 | 0.263 | 0.313 | 0.050 | 135416529 | TSS1500 | 0.052 |
| cg18797653 | 0.352 | 0.411 | 0.059 | 135416613 | TSS1500 | 0.060 |

^aΔβ value is the difference of the mean β of all the CpG sites in the DMR between HPV(+) and HPV(-) patients.

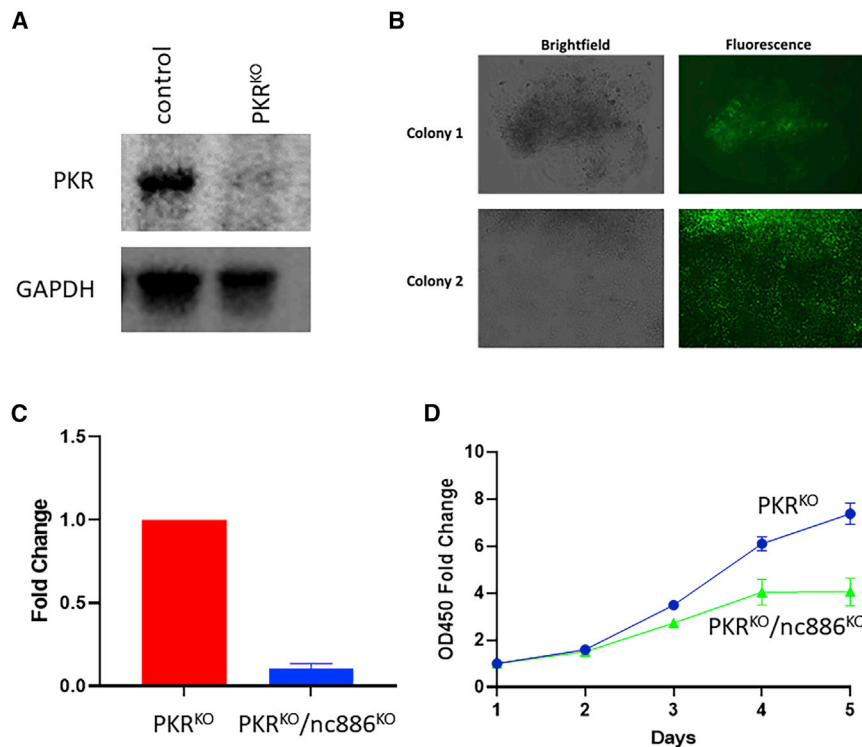


Figure 4. Double knockout of *PKR* and *nc886* in FaDu cells

(A) Confirmation of *PKR*^{KO} by western blot. (B) Representative images of two *PKR*^{KO}/*nc886*^{KO} colonies. (C) Confirmation of *nc886*^{KO} by real-time quantitative reverse transcription PCR. (D) CCK-8 cell proliferation assays of *PKR*^{KO} and *PKR*^{KO}/*nc886*^{KO} cells.

hypermethylated in leukocytes of HPV(+) OPSCC patients. Golec et al.⁵⁵ recently reported that *nc886* controls *PKR* and *NF-κB* signaling during T cell activation and regulates cytokine production in human cells. A more recent study showed that *nc886* suppresses the activation of interferon regulatory factor 3 (*IRF3*) and inhibits *NF-κB* and *AP-1* via *PKR* pathway, leading to reduced expression of *IFN-β* and its downstream genes.⁵⁶ Interferons, especially *IFN-β*, are essential players in the innate immune response against pathogens and also have strong anti-tumor activities.⁵⁷ Hypermethylation of *nc886* in leukocytes may therefore attenuate the inhibitory effect of *nc886* on *IFN-β* production and enhance immune response. Figure 5 shows a simplified model of *PKR*-dependent *nc886* activity in HPV(+) OPSCC. HPV infection increases *nc886* gene methylation, leading to reduced *nc886* expression and lessening of its inhibition on *PKR* activity. Increased *PKR* activity results in increased phosphorylation of translation initiation factor *eIF2* and blockage of cellular and viral protein translation, thus exhibiting anti-tumor and anti-viral effects. In immune cells, increased *PKR* activity can increase *IFN-β* production and enhance immune response. The anti-tumor activity of *nc886* hypermethylation in local epithelial cells and systemic immune cells both contribute to the better prognosis of HPV(+) compared with HPV(-) OPSCC patients. *Nc886* hypermethylation acts as both a viral and a tumor sensor.

The second consistent DMR observed in our study encompasses *CAT* gene and its promoter region on chromosome 11p13. *CAT* gene encodes catalase, a key antioxidant enzyme that converts reactive oxygen species hydrogen peroxide to water and oxygen, thus prevent-

ing the accumulation of oxygen species and impedes tumor initiation and progression.⁵⁸ *CAT* gene promoter contains several CpG islands and previous reports have implicated DNA methylation as a regulatory mechanism of *CAT* gene expression.⁵⁸⁻⁶⁰ We found lower promoter methylation level of *CAT* gene in HPV(+) than HPV(-) OPSCC patients, indicating increased expression of catalase and elevated antioxidant activity, likely acting as a host defense mechanism against exogenous HPV infection and tumor progression.

The major strengths of this study include the multi-stage design of discovery, internal and external validation, the integration of blood, tumor tissues, and cell line studies, and the novel, consistent, biologically plausible, and clinically significant observation of *nc886* hypermethylation in HPV(+) OPSCC patients. This is the first report linking *nc886* hypermethylation to HPV infection and the first report of a molecule that is hypermethylated in both blood and tumors. The major weakness of this study is the small sample size, which limits our ability to identify individual DMCs and DMRs with smaller effect sizes between HPV(+) and HPV(-) patients. Further larger studies are needed to validate individual DMCs and additional DMRs.

In summary, we performed an epigenome-wide CpG methylation profiling of leukocyte DNA in OPSCC patients and compared the methylation levels CpG sites between HPV(+) and HPV(-) patients. We found that *nc886* exhibited consistent hypermethylation in both leukocyte and tumor DNA in HPV(+) OPSCC patients. Knockout of *nc886* causes growth inhibition and apoptosis, and *PKR* pathway at least partially mediates the apoptotic effect of *nc886* inhibition. Our study suggests that HPV caused *nc886* hypermethylation, which acts as a viral and tumor sensor in OPSCC patients and may also partially contribute to the better survival of HPV(+) patients.

MATERIALS AND METHODS

Study patients and data collection

All patients in the discovery and internal validation were newly diagnosed, histopathologically confirmed, and untreated OPSCC patients recruited from the University of Texas MD Anderson Cancer Center (MDACC) as part of a case control study of HNSCC.^{61,62} All patients completed an epidemiological questionnaire to provide information

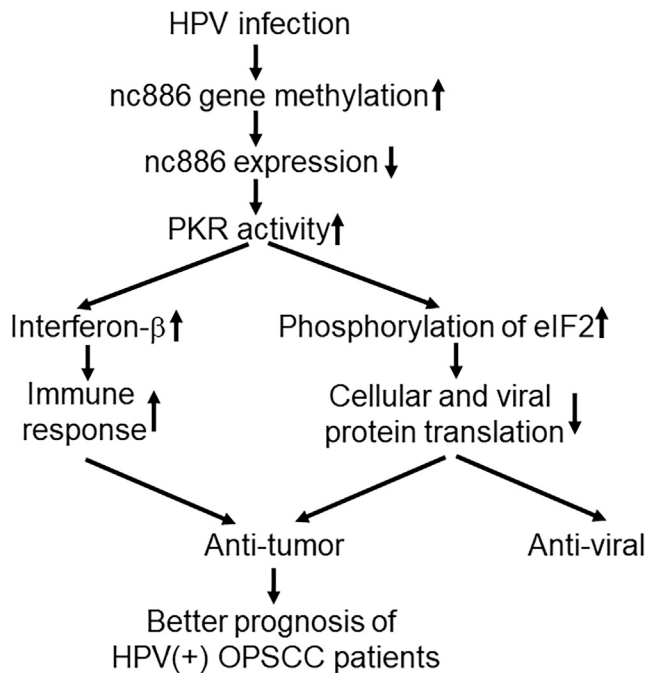


Figure 5. A simplified model of PKR-dependent *nc886* activity in HPV(+) OPSCC

Nc886 hypermethylation acts as both a viral and a tumor sensor. HPV infection increases *nc886* gene methylation, leading to reduced *nc886* expression and lessening of its inhibition on PKR activity. Increased PKR activity results in increased phosphorylation of translation initiation factor eIF2 and blockage of cellular and viral protein translation, thus exhibiting anti-tumor and anti-viral effects. Additionally, in immune cells, increased PKR activity can increase IFN- β production and enhance immune response. The anti-tumor activity of *nc886* hypermethylation in local epithelial cells and systemic immune cells both contribute to the better prognosis of HPV(+) compared with HPV(-) OPSCC patients. There are other PKR-independent activities downstream of *nc886*, which are not depicted in this diagram.

on demographic and risk factors. Clinical and follow-up data were abstracted from the electronic medical record. The details of case recruitment and data collection were described in our previously studies.^{63,64} HPV16 status in tumor tissues was determined by *in situ* hybridization or specific RT-PCR, as well as p16 immunohistochemical analysis as a standard clinical practice at MDACC. The patients in the external validation stage were derived from a case control study of HNSCC in the greater Boston area, and the patient recruitment and data collection process was described in detail previously.^{65,66} This study was approved by the institutional review board of MDACC. All patients provided informed consent.

Epigenome CpG methylation profiling, bioinformatic, and quality control

The whole-epigenome CpG site methylation profiling for both the discovery and the internal and external validation stages was performed using Illumina human MethylationEPIC BeadChip as previously described.^{66,67} The MethylationEPIC BeadChip contains more than 850,000 CpG sites across the human epigenome, which is en-

riched for CpG sites located in gene promoter regions (54%), gene bodies (30%), and CpG islands (19%).⁶⁸ Briefly, genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) and hybridized to the BeadChips. BeadChips were scanned and the fluorescence intensities were extracted using the built-in Genome Studio Methylation Module. Raw fluorescence intensity data (idat files) were processed and normalized using Noob and Functional Normalization from Minfi R package.⁶⁹ The detection p value, an indicative of the quality of the signal, for every CpG in every individual was calculated that compared the total signal (methylated + unmethylated) for each probe to the background signal level. The CpG sites that failed ($p > 0.01$) in more than 10% of the total samples were filtered out. Poor-quality samples on the basis of detection p value (mean $p > 0.05$) were also removed. The probes on the sex chromosomes, the probes for CpGs that overlaps with known SNPs, and the probes that are cross-reactive were excluded. The batch effect was removed using the ComBat function from R package.⁷⁰ The variation in peripheral blood white cell proportions was controlled using cell proportion estimates generated by the estimateCellCounts function in Minfi R package.

Identification of differentially methylation CpG sites and regions

The significantly DMCs between HPV(+) and HPV(-) OPSCC patients were identified using the SAM method.⁷¹ This approach was based on analysis of random fluctuations in the data that accounted for the signal-to-noise ratio. In brief, the statistic was a modified t-test statistic that was based on the ratio of change in methylation level to SD in the data for that probe. The false discovery rate q value was estimated by 2,000 permutations with the modified t-test statistic d , which takes both the effect size and the SD of each CpG site into account. CpG sites with q value < 0.05 and the test score $d > 5$ were regarded as significant DMCs. To identify DMRs between HPV(+) and HPV(-) OPSCC patients, we applied the Bumphunter method.³⁴ Data-driven clusters (methylation regions) were identified with at least 7 CpG sites in each cluster and the largest distance between each CpG site within one cluster being 500 bp. We used the loess curve to smooth the summary statistics of every consecutive CpG sites regressed on the HPV status and the unmeasured confounders. The cluster for which smoothed estimate passed the cutoff threshold (99% of all estimates) was classified as a candidate methylation region. Then each methylation region was assessed by 2,000 permutation tests to assign statistical uncertainty of the candidate region. DMRs with p value area < 0.05 were defined as significant DMRs. All statistical analyses were carried out using R software.

Knockout of *nc886* using the CRISPR-Cas9 system

We attempted to knock out *nc886* gene and replace with GFP reporter gene using the CRISPR-Cas9 homology-directed repair (HDR) method. Briefly, two partially complementary oligos containing guide RNA (gRNA) sequences for *nc886* (gRNA-F: CACCGCGGGTCCG GAGTTAGCTCAAG; gRNA-R: AAACCTTGAGCTAACTCCGA CCCGC) were annealed and subcloned into the BsmBI sites of LentiCRISPR V2 (plasmid #52961; Addgene). The GFP-HDR fragment was constructed by connecting the DNA sequences upstream

and downstream of nc886 and the GFP coding sequence using overlap PCR. To generate the overlap PCR constructs, two fragments corresponding to ~500 bp upstream and downstream sequences of nc886 were amplified by PCR from human genomic DNA (nc886-upstream-F: TGCGTAACAGCTCCCTTTTT; nc886-upstream-R: AAGTCCCCTTGATTTTGGTGGCCTGATCAAAGGTGCGTAT; nc886-downstream-F: AAAACCTCCCACACCTCCCTTTTAAGCAAGACAGGCAGACA; nc886-downstream-R: CCTGCTAACGTGTCCTGGAG). The upstream-R and downstream-F primers incorporated 25 bases complementary to the GFP coding sequence. The GFP coding sequence was amplified by PCR using the primers (GFP-F: ATACGCACCTTTGATCAGGCCACCAAATCAACGGGACTT; GFP-R: TGTCTGCCTGTCTTGCTTAAAGGGAGGTGTGGGAGGTTTT). All three PCR amplicons were purified and mixed in a 1:1:1 ratio for a second round PCR with the nc886-up-F and nc886-down-R primers to construct the HDR fragment. The nc886 gRNA expression LentiCRISPR V2 vector and the HDR fragment were co-transfected into FaDu cells using the Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA). At 48 h post-transfection, cells were selected by puromycin (1 µg/mL) for six days. The GFP-positive cells were then sorted using the MoFlo Astrios Cell Sorter (Beckman Coulter). The knockout of nc886 and insertion of GFP was verified by PCR amplification and Sanger sequencing.

Double knockout of protein kinase RNA-activated (*PKR*) and *nc886* in FaDu cells

Because *PKR* acts downstream of nc886 and *nc886* knockout is lethal, we hypothesize that *PKR* knockout may reverse the lethal phenotype of *nc886* knockout. We therefore attempted to knock out *PKR* first followed by *nc886* KO. The *PKR* gene on 2p22.2 was first knocked out using the CRISPR-Cas9 non-homologous end-joining (NHEJ) method. The *PKR* gRNA and Cas9 expression and control plasmids were purchased from Addgene (lentiCas9-Blast #52962; *PKR* gRNA 1 #75637; *PKR* gRNA 2 #75638). The gRNA and Cas9 plasmids were introduced into FaDu cells via lentivirus transduction using the 3rd-generation packaging plasmids pMD2.G (plasmid #12259; Addgene), pMDL/RREg/p (plasmid #12251; Addgene), and pRSV-Rev (plasmid #12253; Addgene). At 48 h post-infection, cells were selected by treating with 10 µg/mL Blasticidin. The knockout of *PKR* was verified by western blot. After we obtained *PKR*-KO cells, we then used the same approach as above to knockout *nc886* and replace with *GFP* in *PKR*-KO background. The *nc886* KO single colonies were verified using Sanger sequencing.

DATA AVAILABILITY

The data presented in this study are available on request from the corresponding author upon signed material transfer form (MTA). The data are not publicly available because of patient privacy information and ethical consideration.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2022.11.012>.

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AUTHOR CONTRIBUTIONS

J.G. and G.L. conceived and planned the experiments. Z.W., P.W., and R.G. analyzed performed bioinformatics analysis and data analysis. Y.X. performed laboratory experiments. K.T.K. and A.G.S. provided study materials and thoughtful discussions. Y.X., Z.W., and J.G. drafted the manuscript. All the authors reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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