Spring 5-2-2018

Hypoxia induced centrosome amplification underlies aggressive disease course in HPV-negative oropharyngeal squamous cell carcinoma

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HYPOXIA-INDUCED CENTROSOME AMPLIFICATION UNDERLIES AGGRESSIVE DISEASE COURSE IN HPV-NEGATIVE OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

by

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Under the Direction of Ritu Aneja, PhD

ABSTRACT

Human papillomavirus (HPV)-negative (-ve) oropharyngeal squamous cell carcinomas (OPSCCs) are associated with poorer overall survival (OS) compared to HPV-positive (+ve) OPSCCs. The major obstacle to improving outcomes of HPV -ve patients is the absence of good biomarkers. Herein, we investigated the role of centrosome amplification (CA) as a prognostic marker in HPV –ve OPSCCs. We first quantitatively assessed CA in OPSCC tumor samples and found that HPV -ve OPSCCs exhibit higher CA compared to HPV +ve OPSCCs, and was associated with poor OS even after adjusting for potentially confounding variables. Further, the expression of genes associated with hypoxia and CA was significantly higher in HPV -ve OPSCCs than in HPV +ve OPSCCs. We further uncovered a mechanism by which hypoxia-induced HIF-1α downregulates miR-34a resulting in cyclin D1 overexpression and rampant CA in HPV -ve OPSCCs. Our findings demonstrate that assessment of CA may aid in therapeutic decision-making for these patients.

INDEX WORDS: Head and neck cancers, Oropharyngeal squamous cell carcinomas, HPV-negative, HPV-positive, Centrosome amplification, Hypoxia

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2018

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May 2018

ACKNOWLEDGEMENTS

I would like to acknowledge Karuna Mittal, who has been instrumental in mentoring me on this research project and her significant contributions in the formation of this manuscript cannot go unnoticed.

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LIST OF ABBREVIATIONS

CA – Centrosome Amplification

CIN – Chromosomal Instability

HNSCC – Head and Neck Squamous Cell Carcinomas

HPV – Human Papillomavirus

OPSCC – Oropharyngeal Squamous Cell Carcinomas

1 INTRODUCTION

1.1 Purpose of Study

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide[1]. Although the incidence and mortality rate of HNSCC are declining globally, there is a gradual increase in incidence rate of oropharyngeal squamous cell carcinomas (OPSCCs) in recent years[2,3]. OPSSC is a type of HNSCC, which includes cancer of tonsils, base of tongue, back of the roof of the mouth and the side and back walls of the throat. A major contributing factor to the increase in incidence rate of OPSSCs is human papillomavirus (HPV) infection[4-9]. Studies have shown that HPV positive (+ve) OPSCC patients respond better to treatment when compared with HPV negative (-ve) OPSCCs. A retrospective study by *Ang et al*. showed that in a randomized trial comparing accelerated-fractionation with standard-fractionation radiotherapy, each combined with cisplatin therapy, HPV +ve OPSCC patients had better rates of three year overall survival (82.4%) compared to that of their HPV -ve counterparts (57.1%) (overall survival was taken from time of randomization till the death)[11]. The relatively more favorable prognosis within the HPV +ve group has led clinicians to focus on treatment de-escalation in these patients in an effort to achieve similar treatment efficacy with reduced toxicity [12,13]. Little success has, however, been achieved with improving survival rates for HPV -ve patients despite the use of novel treatment strategies. For example, the Eastern Cooperative Oncology group observed a 2-year overall survival of 95% for HPV +ve patients as compared to 62% in HPV -ve patients treated with induction chemotherapy followed by concurrent chemoradiotherapy[14]. This drastic survival difference between the two groups was again observed in a separate study of patients treated with concurrent radiotherapy and cisplatin using either conventional fractionation or accelerated fractionation[15]. In the absence of a good therapeutic target, conventional chemotherapy is unfortunately still the mainstay of treatment for HPV -ve OPSCC patients whose prognosis remains grim.

Treatment decisions for OPSCC patients were made based on disease stage and tumor location, without taking into account the tumor's HPV status.[10] Recent studies have suggested that HPV +ve and HPV -ve OPSCCs are biologically unique entities,[11,16] with differing tumor biology and characteristics. In recognition of the unique character and prognosis of the latter, a new stage classification has been introduced for HPV +ve OPSCC in the 8th edition TNM and p16 staining serves as a surrogate for HPV status and choice of staging system. Studies have also reported that HNSCC is a heterogeneous disease with higher chromosomal instability (CIN) reported in HPV -ve (50% more mutational load) compared to the HPV +ve HNSCCs[17,18]. Inactivation of tumor suppressor genes such TP53 and CDKNA2 and the oncogenic activation of the *CCND1* have been shown to be crucial for pathogenesis and disease progression in HPV -ve HNSCCs[19]. By contrast, inactivation of the tumor suppressor genes and activation of oncoproteins in HPV +ve tumors has been linked to the viral E6 and E7 oncoproteins[20]. A key feature of E6 and E7 oncoproteins is that they both work to induce centrosome amplification (CA),[21-23] which is hailed as a hallmark of cancer and a critical driver of chromosomal instability that fuels tumorigenesis, tumor progression, drug resistance and, as a result, poor prognosis[24]. CA can be numerical (increase in the number of centrosomes) as well as structural (increase in size of centrosomes) and can arise in multiple ways, including failure of the cell to undergo cytokinesis, inappropriate replication of centrosomes, and de novo generation[25]. In cancer cells, excess centrosomes cluster into two polar groups during mitosis, giving rise to pseudo-bipolar spindles. Merotelic attachment of individual kinetochores to more than one spindle pole is a frequent occurrence[26]. Such inappropriate attachments can cause missegregation of whole chromosomes and/or chromosomal breakage. Furthermore, clustered supernumerary centrosomes are inherited by progeny cells, leading to a perpetuation of CIN in the cell lineage[27].

Past genomic analyses of HNSCCs have described CIN to be a more prominent feature in HPV -ve tumors than in HPV +ve tumors[28,29]. However, due to the absence of the E6 and E7 oncoproteins that normally drive CA in HPV +ve HNSCCs, the origin and potential involvement of CA in driving the CIN observed in HPV -ve HNSCCs has been overlooked. Instead, attention has focused on upregulation of DNA damage response (DDR) proteins such as Aurora A kinase and PLK1 as major factors contributing to CIN in HPV -ve HNSCCs[30]. Both Aurora A and PLK1 promote CIN by deregulating the spindle assembly checkpoint, resulting in chromosome missegregation and amplification of centrosomes[31,32]. Aurora A kinase inhibitors, when used in combination with Wee1 inhibitors or cetuximab, (directed against EGFR) have shown promise in treating HPV -ve OPSCC[33,34]. Furthermore, the tumor suppressor, p53, that has been implicated in the regulation of centrosome duplication and CA is often mutated in HNSCCs[35-37]. Therefore, we reasoned that CA may be a readily quantifiable prognostic marker and druggable target for HPV -ve tumors.

Another major contributing factor underlying poorer prognosis and survival outcomes in HPV -ve cancers is tumor hypoxia. Tumor hypoxia has long been known to be associated with poor responses to radiotherapy and chemotherapy[38]. A recent study has shown that HPV -ve oropharyngeal tumors display higher tumor hypoxia[39]. Additionally, reduced partial pressure of oxygen inside tumors plays a significant role in overexpression of Aurora-A/STK15[40], and this overexpression results in CA[41], chromosomal instability, and aneuploidy. Also, there are literature evidences that support hypoxia-mediated overexpression of PLK4, which has been well documented to induce CA.[42] Recently, we have shown that hypoxic tumor microenvironment can induce CA via the stabilization of the transcriptional factor HIF-1α in breast cancer, facilitating an aggressive disease course.[43] Thus, there is mounting evidence that hypoxia-associated CA may underlie the aggressive disease course and treatment resistance of HPV -ve OPSCCs.

No studies to date have reported quantitation of centrosomal aberrations in OPSCCs with inherently different HPV status. Herein, we performed a thorough quantitative analysis of centrosomal aberrations in OPSCC tumors to establish differences in incidence and severity of CA between HPV +ve and HPV -ve OPSCC patients. Interestingly, we found that HPV -ve OPSSCs exhibited significantly higher CA when compared with the HPV +ve OPSCCs, and CA was associated with the poor overall survival in HPV -ve OPSCCs. Furthermore, we also established a strong association between CA and HIF-1α expression in HPV -ve OPSCCs. Our results indicated HPV -ve tumors show higher expression of HIF-1α and was correlated with the higher CA percentage. In addition, we found that HPV -ve tumors exhibited higher expression of CA-associated protein cyclin D1. To confirm the molecular association of HIF-1α and CA in OPSCC, we performed a series of *in silico* analyses. Firstly, we used publicly available GEO database to show strong correlation between the 26-gene hypoxia signature and a novel 7-gene CA signature. With emerging new data for the role of miRNAs in OPSCCs, we uncovered the possible function of HIF-1α regulated miRNA-34a in driving CA by inducing expression of cyclin D1 in HNSCCs. Taken together, these results shed new light on the drivers of tumor biology in HPV -ve tumors and emphasize the role of CA as new prognostic marker and actionable target to improve outcomes in HPV -ve OPSCCs.

2 MATERIALS AND METHODS

2.1 Clinical tissue samples

Formalin-fixed paraffin embedded OPSCC TMA sections of tonsil, base of tongue, and soft palate tumors (ICART4 cohort) were procured from the Poznan Cancer Center in Poland (ICART member). Patients were diagnosed between YEAR 2007 and 2014, and based on the location of tumor (tonsil, base of tongue and soft palate) the patients were selected for the TMA construction. Clinicopathologic characteristics of the patients are provided in Table 1. The Institutional Review Board of Greater Poland Cancer Centre, Poznan, PL approved all aspects of the study. Methods were carried out in "accordance" with approved guidelines stipulated in MTAs and DUAs between Greater Poland Cancer Centre, Poznan, PL and Georgia State University. Informed consent was obtained from all subjects.

2.2 HPV analysis

The detection of high risk HPV was performed using GP5+/GP6+ HPV DNA PCR with enzyme-immunoassay (EIA). For genotyping of the viral DNA, the Luminex platform was used for bead-based array. The EIA detected 14 HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68. globin PCR was used to test for sample quality post-DNA extraction[77].

2.3 Immunohistochemistry of Cyclin D1 and HIF-1α and scoring.

The initial steps from deparaffinization to antigen retrieval are the same as those for immunofluorescence. The tissues were then blocked with Ultravision Protein Block (ThermoFisher, Waltham, MA) for 30 minutes followed by hydrogen peroxide block with Ultravision Hydrogen Peroxide Block (ThermoFisher) for 10 minutes. The tissues were then immunolabeled with HIF-1α antibody (Abcam, Cambridge, MA) or cyclin D1 (ThermoFisher)

for 1 hour. After 3X washes in TBST, the slides were subjected to secondary antibody incubation using Anti-Rabbit HRP (Biocare, Pacheco, CA) for 1 hour. Enzymatic detection was performed using DAB Chromogen Kit (Biocare). Nuclear HIF-1α and cyclin D1 staining was categorized as 0=none, 1=low, 2=moderate, and 3=high. The percentage of positive cells, defined to be 1+ staining intensity, from around 500 cells was determined. The weighted index (WI) for each sample was calculated as the product of percent cell positivity and staining intensity.

2.4 Immunofluorescence staining

TMA slides were deparaffinized in the oven at 60°C for 30 minutes followed by 3 washes of in xylene and rehydration in series of washes in 100%, 95%, 70%, and 50% ethanol. Antigen retrieval was done by heating in a pressure cooker with citrate buffer (pH 6.0). The tissues were blocked with 5% BSA in PBS solution with 0.1% Triton X for 30 minutes. After blocking, primary antibody incubation with γ-tubulin (Sigma, St. Louis, MO) at a dilution of 1:1000 was performed for 1 hour at room temperature. The tissues were then washed 3X with PBS after which incubation with secondary antibody (Alexa-488 anti-mouse) was done at room temperature for 1 hour at a dilution of 1:2000. After 3X washes in PBS, coverslips were mounted with Prolong-Gold Antifade with DAPI (Invitrogen, Waltham, MA).

2.5 Quantification of both structural and numerical centrosome amplification

We define CA as both structural and numerical aberration of centrosomes. The TMA's were immunostained for γ-tubulin were observed with Zeiss LSM 700 confocal microscope (Ziess, Oberkochen, Germany) for centrosome amplification. At least 10 fields were imaged at 63X to capture enough nuclei and centrosomes. Analyses of images were performed using Imaris software that provided the capability to visualize the images in 3D. Interphase nuclei with more than two associated centrosomes were considered to exhibit numerical CA. Also, interphase nuclei associated with centrosomes whose 3-dimensional volume

exceeded the defined cut-off value of 0.7um^3 were deemed to exhibit structural CA. A total of at least 250 nuclei were counted for each tissue. Percentage of CA was obtained by adding percentage of cells containing either structurally amplified centrosomes or numerically amplified centrosomes, or both.

2.6 Drug Sensitivity Test

We obtained the drug sensitivity data of CDK4/6 inhbitor palbociclib and Aurora A inhibitor ZM447439, in HNSSC cancer cell lines from www.cancerrxgene.org which contains drug sensitivity information of anticancer drugs in multiple cancer cell lines. Cell lines were divided into HPV +ve and HPV -ve based on the HPV status as per literature and IC-50 values were plotted against cell lines for each drug. Students' t-test was used to see if the two groups differ in sensitivity to Palbociclib and ZM447439.

2.7 Statistical analyses

For clinical data as well as the in silico data analysis patient's overall survival was used as the endpoint for survival analysis. Log-rank test was applied to test the differences among Kaplan Meier survival curves. The cutoff points that we found for CA and HIF1-a were those which maximized survival differences between high- and low-risk subgroups. The test of group mean differences shown in Box-Whisker Plots are based on Mann–Whitney U test. In cases with more than two groups, the difference was evaluated by the Kruskal–Wallis test. Statistical analysis was performed using SAS software 9.4(SAS Institute Inc., Cary, NC). For the survival model used in in silco analysis with individual genes, each gene was split into a high and low expression group by optimizing the log-rank test statistic and the hazard ratio parameter estimate for the high expression group was determined. High expression gene groups with negative impact on survival had a positive parameter estimate while genes that correlated positively with good prognosis had a negative parameter estimate. The total weighted sum, for each patient, was generated by adding the parameter estimates

for each gene, which had above threshold expression (if they were in the low expression group they were given a 0 for that gene weight). The cutoff between high and low weighted scores was similarly derived by finding the optimal log rank statistic.

3 RESULTS

3.1 HPV -ve OPSCCs exhibit significantly higher CA than HPV +ve OPSCCs and CA is associated with poor overall survival in HPV -ve OPSCCs

Previous studies in our lab have shown that CA can serve as a poor prognosis biomarker in multiple malignancies including breast cancer, pancreatic cancer, and serous ovarian adenocarcinoma[44-46]. Given the higher expression of DDR genes in HPV -ve OPSCC, we postulated that HPV -ve tumors will exhibit significant CA. Since no rigorous and quantitative studies to date have compared CA in HPV +ve and -ve OPSCCs, we visualized centrosomes in resection samples from 98 OPSCC patients (n=47 HPV +ve and n=51 HPV -ve samples) (ICART4 cohort). In order to measure the degree of structural and numerical CA, we performed fluorescent immunostaining of our tissue samples. The centrosomes (red) were stained with γ-tubulin and the nuclei (blue) with DAPI. We then imaged the tissues to capture images 10 visual fields at 20x using Zeiss LSM 700 confocal microscope (Oberkochen, Germany). Within each field, we selected regions containing distinguishable nuclei and quantified the number and volume of g-tubulin foci for each nucleus to assess numerical and structural CA, respectively. 250 nuclei in total were counted at random using Imaris software that allowed us to visualize the nuclei and centrosomes in 3D. We defined “numerical” amplification as interphase nuclei associated with more than two centrosomes, and “structural” amplification was defined as nuclei associated with at least one centrosome that had a volume greater than 0.7 μm^3. This threshold value was determined after past studies in our lab that defined the normal volumetric range of centrosomes across different cancer types, including breast, pancreas, bladder and OPSCCs, in which 500 centrosomes for each cancer type were counted from tissues of adjacent uninvolved cancer patients and normal tissues. Then an average normal range of volumes was determined, above which was considered to be abnormal. Then, we

independently counted cells (nuclei) that harbored numerical amplification of centrosomes and cells that harbored structural amplification from our total of 250. A percentage was calculated for each type of amplification and the values were added to determine a CA value for each sample. HPV status for the samples was determined by the DNA PCR utilizing the enzyme immunoassay (details added in the Materials and Methods). Patient cohort details are shown in Table 1. Surprisingly, Wilcoxon distribution scores revealed that the HPV -ve samples exhibited significantly higher CA (numerical and structural) when compared with the HPV +ve samples (p=0.034; Fig. 2A). In line with previous studies, we observed that HPV -ve OPSCCs were associated with poorer overall survival when compared with HPV +ve OPSCCs. (p=0.0062; HR=4.629) (Fig. 1B). Interestingly, when we stratified all the patients into low- and high-CA groups (threshold used was the one that minimized log-rank p-value) within HPV +ve and HPV -ve subgroups (Fig. 1C), we observed that high-CA HPV -ve OPSCCs were associated with poorer overall survival than the high-CA HPV +ve OPSCCs (p=0.02; HR=7.7). Furthermore, within HPV -ve OPSCCs, the high CA group was associated with poorer overall survival when compared with the low CA HPV -ve OPSCCs. This association stayed significant (p=0.03; HR=5.4) in our multivariable analysis when potentially confounding factors like smoking, alcohol consumption, grade, and tumor stage were included (Table 2). No significant differences were found between overall survival of low-CA HPV +ve and HPV -ve subgroups. These findings contrast with the long-held belief that CA is higher in HPV +ve tumors that harbor the E6 and E7 viral oncoproteins. To further test the clinical significance of CA, we evaluated associations between CA and other clinical parameters. In line with the understanding that CA drives tumor progression, our data (Fig. 2B) showed that among HPV -ve tumors, higher CA was associated with higher disease stage (p=0.1013). Moreover, HPV -ve tumors displayed higher CA compared to that of stage-matched (stage III and stage IV; p=0.1225 and p=0.0551, respectively) HPV +ve tumors (Fig. 2C, 2D). In

sum, these findings suggest that HPV -ve OPSCCs exhibit higher CA when compared with HPV +ve OPSCCs and higher CA in the HPV -ve OPSCCs is associated with poorer overall survival.

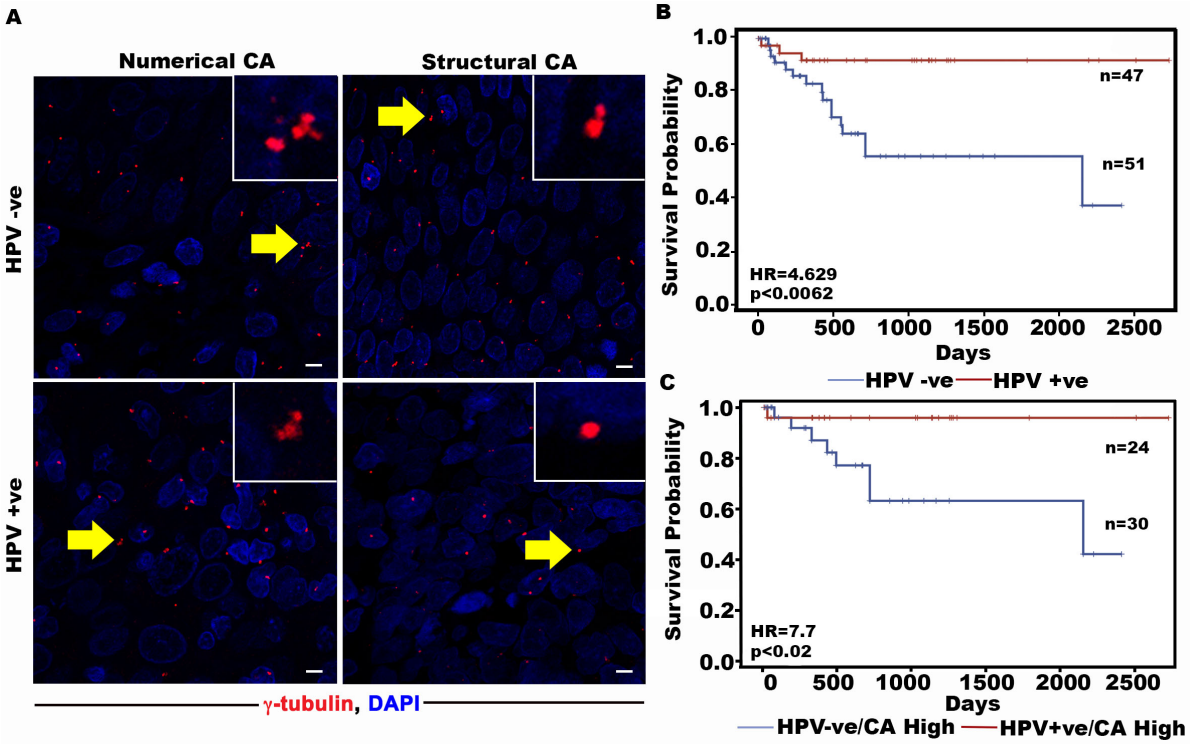

Figure 1. HPV -ve tumors show higher CA and poorer prognosis than HPV +ve tumors
(A) Confocal micrographs showing numerical and structural CA in HPV +ve and HPV -ve tumor sections OPSCC tissue sections were immunostained for centrosomes (γ-tubulin, red) and counterstained with DAPI (blue). Scale bar (white), 20μm. (B) and (C) Kaplan Meier survival curves representing the survival probabilities of HPV -ve (n=51) and HPV +ve (n=47) OPSCC patients (HR=4.629, p=0.0062) and the survival probabilities of high-CA HPV -ve (n=30) and HPV +ve (n=24) OPSCC patients (HR=7.7; p=0.02), respectively.

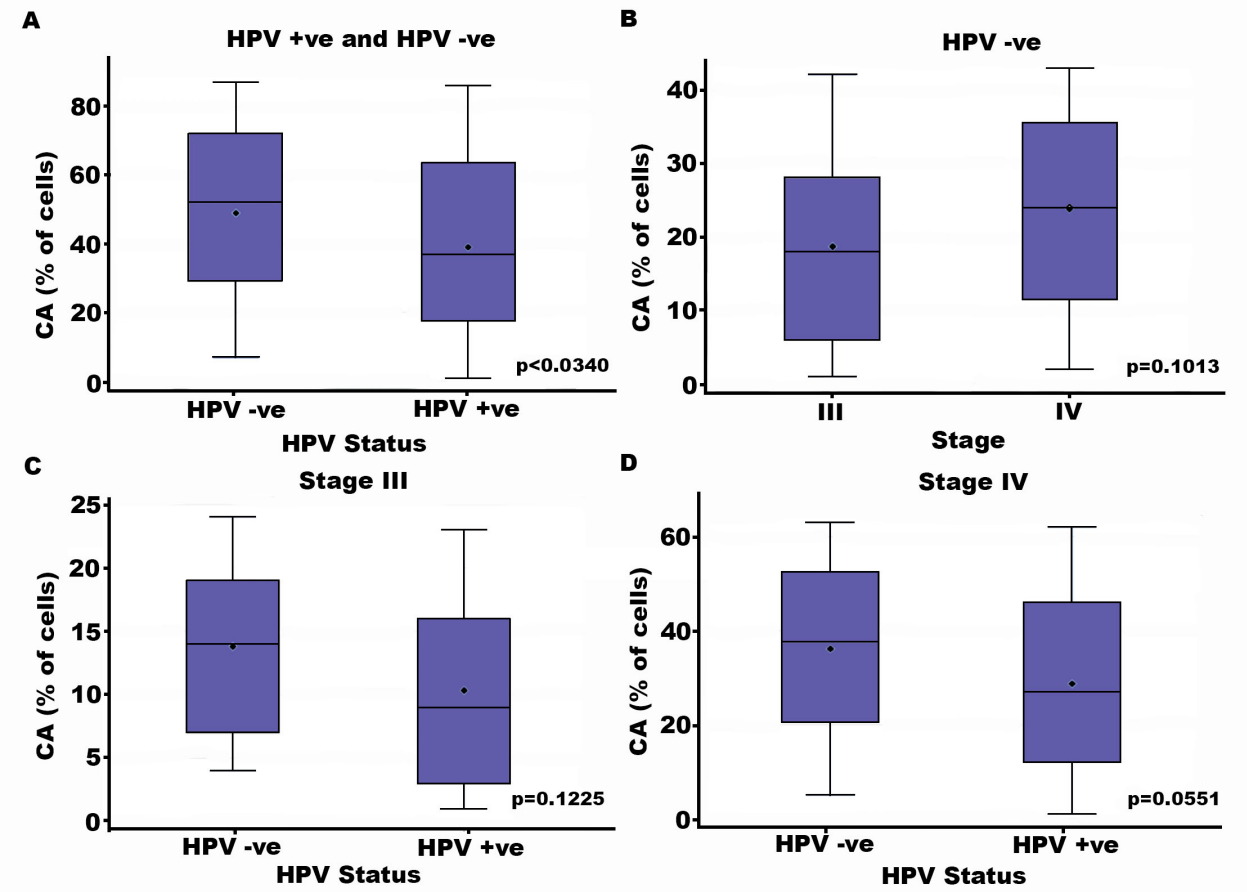

Figure 2. Analysis of CA in OPSCC clinical samples
(A) Distribution of % cells with CA (structural and numerical) in HPV -ve (n=51) and HPV +ve (n=47) patients (p=0.0340). (B) Distribution of CA between stage III (n=15) and stage IV (n=28) in HPV -ve patients (p=0.1013). (C) Distribution of CA between HPV -ve (n=15) and HPV +ve (n=9) stage III patients (p=0.1225). (D) Distribution of CA between HPV -ve (n=28) and HPV +ve (n=35) stage IV patients (p=0.0551). Distributions for stage I (n=0) and II (n=1) were not depicted.

Table 1. Descriptive statistics of clinicopathological characteristics for OPSCC patients (ICART4 cohort) in the analysis of centrosome amplification (clinical samples).

Variable	Level	Number	Percentage
Gender	Male	68	79.4
	Female	30	30.6
HPV Status	+ve	47	48
	-ve	51	52
Grade	1	6	6.1
	2	49	50
	3	31	31.6
	N/A	12	12.2
Stage	I	0	0

	II	1	1
	III	24	24.5
	IV	63	64.3
	N/A	10	10.2
Tumor Site	Tonsil	67	68.4
	Base of Tongue	13	13.3
	Soft Palate	13	13.3
	Tonsil+Base of Tongue	1	1
	N/A	4	4
Alcohol	No alcohol/Occasional	90	91.8
	Alcohol Abuse	8	8.2
Smoking	Never	32	32.7
	Previous	10	10.2
	Current	45	45.9
	N/A	11	11.2
CA (%)	Low (<23%)	38	38.8
	High (>23%)	60	61.2
HPV +ve CA(%)	Low (<23%)	21	44.7
	High (>23%)	26	55.3
HPV -ve CA(%)	Low (<23%)	17	33.3
	High (>23%)	34	66.7
Chemotherapy Type	None	50	51
	Concomitant	38	38.8
	Neoadjuvant	2	2
	Concomitant & Neoadjuvant	0	0
	Adjunctive	2	2
	N/A	6	6.1
Radiotherapy Type	None	7	7.1
	Primary	25	25.5
	Adjuvant	55	56.1
	Palliative	7	7.1
	N/A	4	4.1

Table 2. Multivariate analysis for high and low CA groups in HPV -ve OPSCCs

Parameter	HR	p-value
CA	5.480	0.0324
Gender	1.263	0.7820
Age At Diagnosis	1.080	0.1910
Overall Stage	1.242	0.7671
Alcohol	4.088	0.2723
Radiotherapy	0	0.9930
Smoking	3.108	0.2923

3.2 Novel CA7 score based on CA-associated genes has prognostic value in HPV –ve HNSCCs

Given the association of CA with poor overall survival in HPV -ve OPSCCs, we wanted to evaluate whether centrosomal abnormalities in HPV -ve OPSCCs were accompanied by dysregulation of the genes associated with centrosome biogenesis/duplication. To this end, we used publicly available TCGA microarray data of HNSCC patients to evaluate gene expression levels for seven genes associated with CA[43,47]. By adding the log-transformed values, normalized gene expression for CCND1, NEK2, PIN1, TUBG1, PLK1, BIRC5 and AURKA were calculated into a cumulative score (CA7). First, we evaluated the CA7 score in all (n=521) HNSCCs regardless of subtypes and HPV status. Patients were stratified into high and low CA subgroups using the optimal CA7 score cut-point (based on the log-rank test). Our findings demonstrated that (Fig. 3A) high CA7 score HNSCCs (n=420) were associated with poorer survival (p=0.0389; HR 1.497) when compared with the low CA7 HNSCCs, (n=101). Interestingly, among OPSCC patients (n=80; HPV -ve=26 and HPV +ve=54) we found that high CA7 score was

associated with poor overall survival (Fig. 3B) regardless of HPV status ($p<0.0001$; HR=11.369).

When we looked at the HPV -ve HNSCCs, we observed that CA7 score was not able to stratify this group into high- and low-risk subgroups significantly. Since HPV +ve and -ve tumors are distinct disease entities, we designed a subtype-specific weighted gene expression signature based on the appropriately weighted expression of the CA7 genes in each subgroup. To develop this signature, the expression for each CA7 gene was split into a high versus low expression subgroups through optimization of the log-rank test statistic, and then the Hazard Ratio parameter estimate for the high expression group was determined. High expression gene groups that had a negative impact on survival had a positive parameter estimate while genes that correlated positively with good prognosis had a negative parameter estimate. The total weighted sum, for each patient, was generated by adding the parameter estimates for each gene that had above threshold expression (if they were in the low expression group they were given a 0 for that gene weight). The cutoff between high and low weighted CA7 scores was also performed by finding the optimal log-rank statistics. Interestingly, we observed that this new model was able to stratify HPV -ve HNSSCs with higher significance (HR=1.867; $p<0.001$). Among HPV -ve OPSCCs, high CA7 group (n=6) showed a strong trend towards poorer overall survival (HR=2.242; $p=0.113$) when compared to low CA7 group (n=20) (data not shown), but owing to the small sample size, we were not able to achieve statistical significance.

Given that the seven CA-associated genes were associated with poor prognosis in HPV -ve HNSCCs, we suspected that inhibitors of these genes should be effective on HPV -ve HNSCCs. To this end, utilizing publicly available CancerRxgene database we looked at the effect of Aurora A inhibitor in HPV -ve and HPV +ve HNSCC cell lines. As we expected, HPV -ve HNSCC cell lines were more susceptible to Aurora A inhibitor compared to HPV +ve, HNSCC cell lines (Fig. 4A)**.** Thus, collectively these findings suggest that CA is

associated with high-risk HPV -ve OPSCCs can serve as a novel therapeutic target for HPV -ve subtype.

Table 3. Descriptive statistics of clinicopathological characteristics for HNSCC patients in the cohort used for in silico analysis of the prognostic value of CA7 signature

Variable	Level	Number	Percentage
Gender	Male	385	73.9
	Female	136	26.1
HPV status	HPV +ve	97	18.6
	HPV -ve	422	81
	N/A	2	0.4
Grade	1	62	11.9
	2	305	58.5
	3	125	24
	4	7	1.3
	X	18	3.5
	N/A	4	0.76
Stage	I	20	3.8
	II	98	18.8
	III	106	20.3
	IV	283	54.3
	N/A	14	2.9
Tumor Site	Alveolar	18	3.5
	Base of the tongue	28	5.4
	Buccal mucosa	22	4.22
	Floor of mouth	60	11.5
	Hypopharynx	10	1.9
	Larynx, nos	116	22.3
	Lip	3	0.6
	Oral cavity, nos	73	14
	Oral tongue	82	15.7
	Oropharynx	9	1.7
	Palate, hard	7	1.3
	Tongue, nos	47	9
	Tonsil	43	8.3
	N/A	3	0.6
Smoking	Never	117	22.5
	Previous	215	41.3
	Current	177	34
	N/A	12	2.3

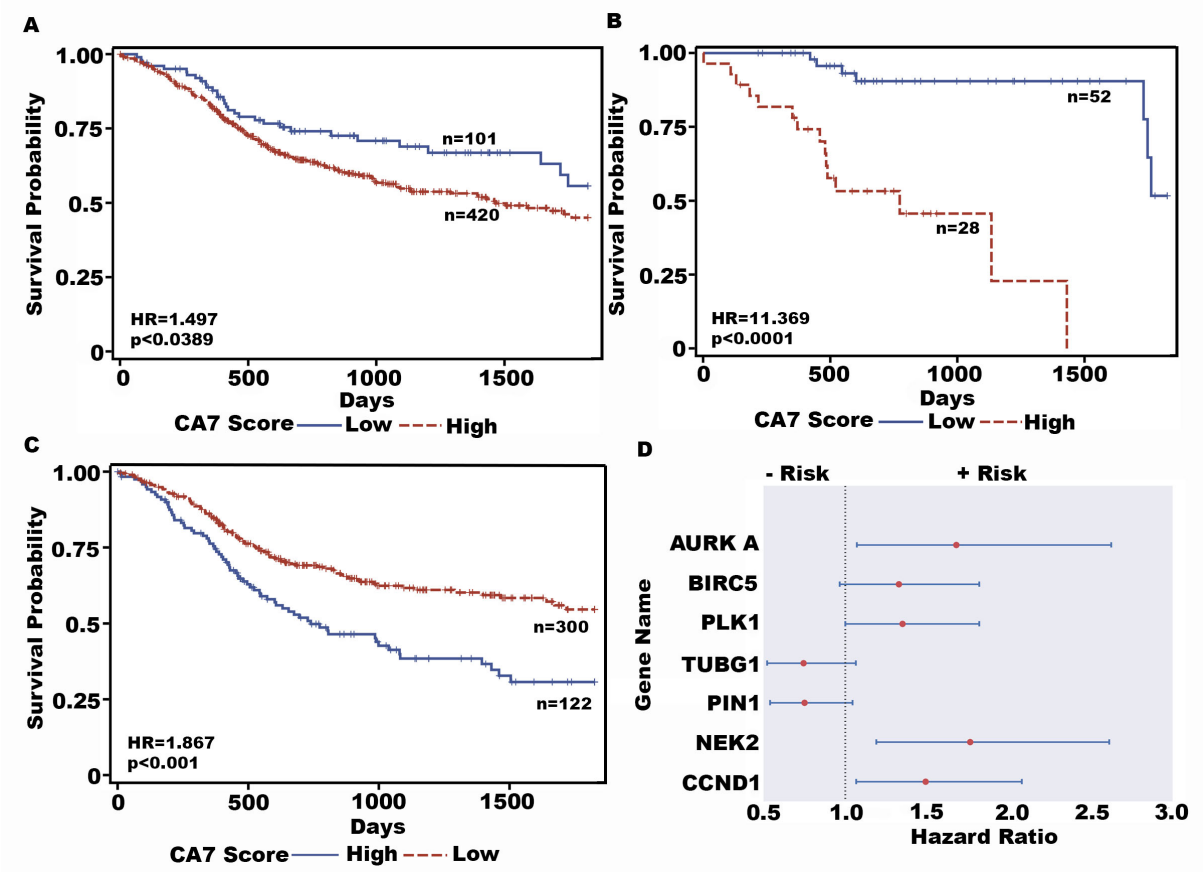

Figure 3. Upregulation of CA7 genes is associated with poor overall survival in HNSCCs. (A) Kaplan Meier survival curves representing the survival probability of HNSCC patients stratified according to CA7 high (n=420) and CA7 low (n=101) expression levels (HR=1.497; p=0.0389). (B) Kaplan Meier survival curves representing the survival probability of OPSCC patients stratified according to CA7 high (n=28) and CA7 low (n=52) expression levels (HR=11.369; p<0.0001). (C) Kaplan Meier Survival curve of HPV -ve HNSCC patients stratified according to the weighted CA7 high (n=122) and CA7 low (n=300) expression levels (HR=1.867; p<0.001). (D) Forest plot of hazard ratios for each of the CA7 genes in HPV -ve HNSCCs. The blue lines represent 95% confidence intervals. The HR for each gene is considered to be significant (p<0.05) if the blue lines do not cross HR of 1.0.

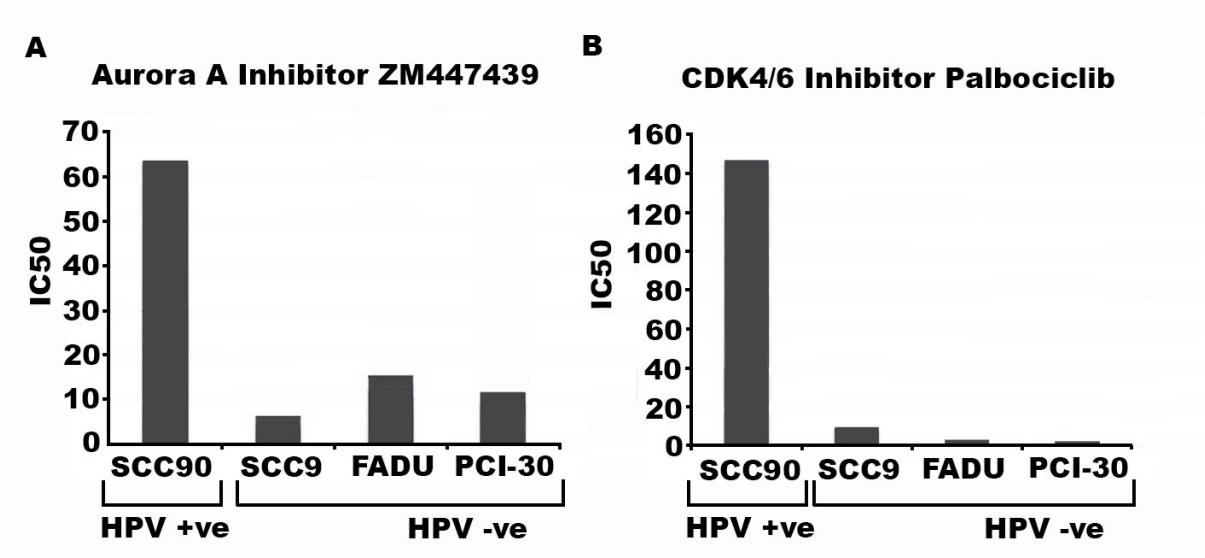

Figure 4. HPV -ve HNSCC cell lines are more susceptible to inhibitos of CA-associated proteins compared to HPV +ve HNSCC cell lines. Bar graphs (derived from analysis of data from CancerRxgenome database) showing IC50 values of (A) ZM447439, Aurora A inhibitor and (B) Palbociclib, CDK4/6 inhibitor in HPV -ve and HPV +ve HNSCC cell lines.

3.3 CA status correlates with HIF-1α expression and hypoxia gene score in HPV -ve OPSCC tumors

Tumor hypoxia has been consistently linked to worse response to radiotherapy and chemotherapy across many types of cancers leading to poor prognoses.[48-50] Previous clinical studies determined HIF-1α as a poor prognostic factor in radiotherapy-treated HPV -ve HNSCC[51] and also suggested that HIF-1α high, HPV -ve OPSCC patients show worse prognosis when compared to those with HIF-1α high, HPV +ve OPSCCs.[52] Based on our lab's finding that hypoxia induces CA in breast tumors via HIF-1α, and given that HPV -ve tumors exhibit higher CA in clinical samples, we suspected that this subset of patients might have higher expression of HIF-1α. To test our hypothesis, we immunohistochemically stained the adjacent sections of the 87 OPSCC samples (used to quantify CA; Fig. 1) for HIF-1α. Nuclear HIF-1α weighted index (WI) was calculated as indicated in Materials and Methods. Patients were stratified into high- and low- HIF-1α expressing subgroups using the optimal HIF-1α expression cut-point (based on the log-rank test). We observed (Fig. 5B)

that high HIF-1α HPV -ve OPSCCs (n=30) showed higher CA (p=0.0479) when compared with low HIF-1α HPV -ve OPSCCs (n=12). The survival analysis (Fig. 5C) demonstrated that the high HIF-1α expressing group was associated with poorer overall survival (HR=3.191; p=0.0826) than the low HIF-1α subgroup.

Next, we evaluated if there was any difference in expression of hypoxia-associated genes in HPV -ve and HPV +ve HNSCCs. Herein, we used the same publicly available dataset (TCGA dataset) used in result section 1 to probe the 26-gene hypoxic signature.[53,54] Our results indicated significantly ($p=3.77x10^{-7}$) higher expression of the total 26 hypoxia-associated genes including HIF-1α in HPV -ve (n=422) head and neck tumors when compared to those of the HPV +ves (n=97) (Fig. 6A). When we further looked at the subset of only OPSCCs (based on the location of tumor- the base of tongue, tonsils, and oropharynx) among the whole cohort, we found similar results wherein, HPV -ve OPSCCs (n=26) showed significantly ($p<0.001$) higher expression of 26-gene hypoxia signature when compared with the HPV +ve OPSCCs (n=54) (Fig. 6B). Also, the hypoxia score was able to stratify the OPSCCs into high and low-risk groups. The high-hypoxia group was associated with significantly poorer overall survival when compared with lower hypoxia group (HR=3.297; p=0.0127). Interestingly, among the HPV -ve OPSCCs, high hypoxia HPV -ve OPSCCs exhibited poorer overall survival (HR=2.197; p=0.205) than the low hypoxia HPV -ve OPSCCs. We also observed a positive correlation between the CA7 and Hypoxia 26 gene scores in HPV -ve OPSCCs (R=0.34760; p=0.0819).

Collectively, these findings confirm that there is a correlation between HIF-1α and CA in OPSCCs with higher significance in the HPV -ve tumors. These results strongly suggest that the CA observed in HPV -ve OPSCCs may be hypoxia-induced and may underlie their poor prognoses.

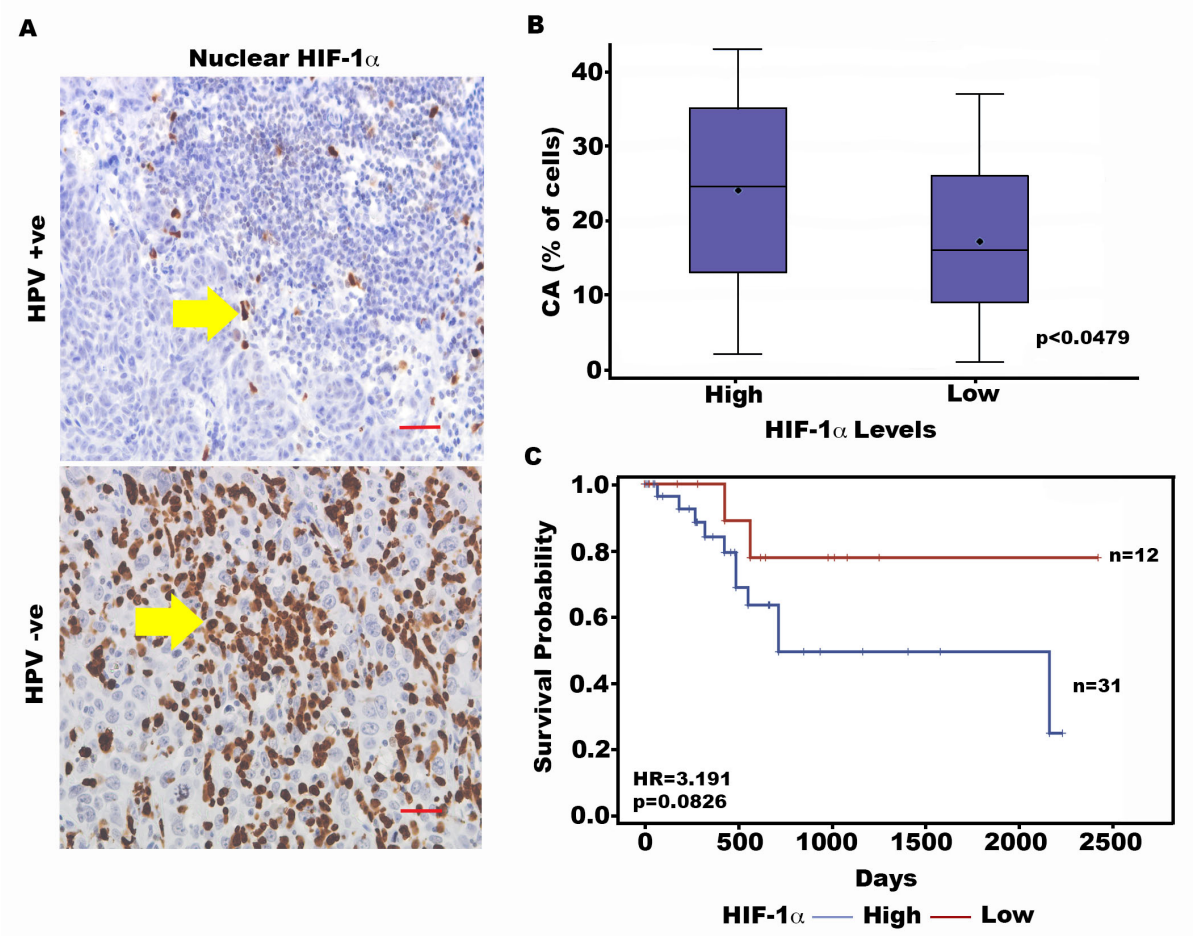

Figure 5. HPV -ve OPSCC tumors show a high association between CA and HIF-α expression. (A) Representative immunohistochemical micrographs of HPV +ve and HPV -ve OPSCC tumors stained for HIF-1α. (B) Box plot depicting the distribution of CA in HIF-1α-high (n=30) and low (n=12) HPV -ve tumors (p=0.0479). (C) Kaplan Meier survival analysis representing overall survival in HPV -ve OPSCCs stratified based on HIF-1α scores (HR=3.191; p=0.0826).

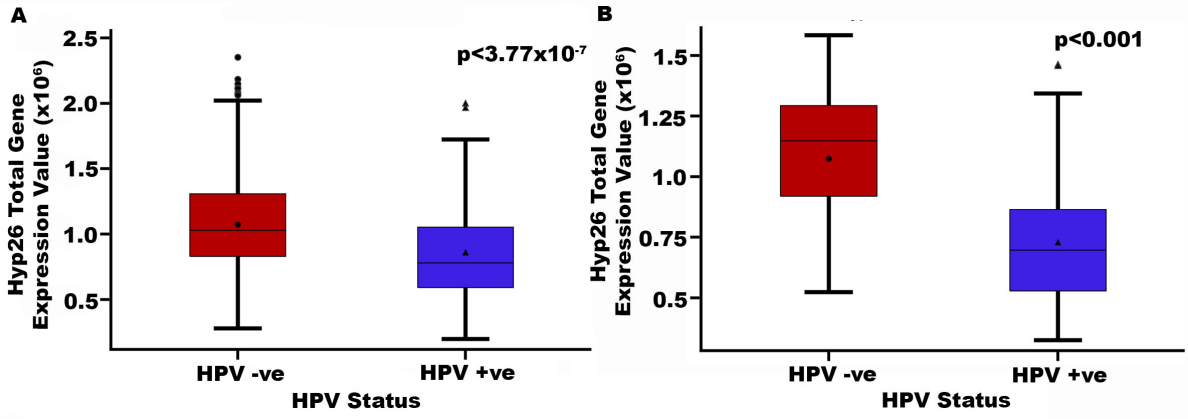

Figure 6. Comparison of 26-gene hypoxia gene signatures of HNSCC tumors in TCGA. (A) Box whisker plot showing expression of the 26-gene hypoxia signature in HPV -ve (n=422) and HPV +ve (n=97) HNSCC patients ($p=3.77x10^{-7}$). (B) Box whisker plot showing expression

of the 26-gene hypoxia signature in HPV -ve (n=26) and HPV +ve (n=54) OPSCC patients ($p<0.001$).

3.4 HIF-1α downregulates miRNA-34a to induce CA via CCND1 overexpression

Having established the relationship between hypoxia and CA in HPV -ve OPSCCs we next sought to delineate the possible role of HIF-1α in the induction of CA in OPSCC. Hypoxia mediates its function through a transcription factor hypoxia-inducible factor-1 alpha (HIF-1α), and changes in gene expression under hypoxia are due to the transcriptional regulation of hypoxia-regulated genes by HIF-1α. Studies have also shown that hypoxia and HIF-1α can regulate a panel of miRNAs[55,56] and these miRNAs regulate the expression of genes involved in many vital events related to angiogenesis, tumorigenesis and even CA in multiple malignancies including head and neck cancer[57]. Therefore, we rationally hypothesized that hypoxia may induce expression of CA-associated genes through regulation of miRNAs in HPV -ve OPSCCs. To test this hypothesis, we used publicly available TCGA miRNA-seq data from 497 HNSCC patients. We analyzed expression of top 19 CA-associated miRNAs (list and expression reported in Table 3). Among these 19 miRNAs, 12 miRNAs were upregulated in HPV +ve and 7 were upregulated in the HPV -ve OPSCCs. Interestingly, we observed significant overexpression of miR-34a in HPV +ve tumors compared to the HPV -ve tumors ($p=0.000248$). CCND1 mRNA is the known target of miR-34a which has been shown to downregulate cyclin D1 expression[58]. In line with this, we observed the expression levels of the CCND1 gene to be significantly downregulated in HPV +ve tumors ($p=9.88 \times 10^{-9}$) and there was a negative correlation between miR-34a and CCND1 (Fig. 7). Furthermore, HIF-1α, which is more highly expressed in HPV -ve tumors, represses the expression of miR-34a.[59] Intriguingly, as mentioned above in the same dataset we observed significantly higher expression of HIF-1α in HPV -ve OPSCCs (Fig. 6). These findings further strengthen our model that CCND1 expression in HPV -ve OPSCC tumors is upregulated by HIF-1α-mediated downregulation of miR-34a, and upregulated CCND1 then drives CA in these tumors.

Table 4. List of CA associated miRNAs sorted by logFC values. FC values represent a ratio of HPV +ve and HPV -ve values.

	Normalized HPV +ve	Normalized HPV -ve	Fold Change (FC)	P-Value
hsa-mir-490	1.353	0.225	6.0258	0.300
hsa-mir-449	15.589	4.767	3.270	0.204
hsa-mir-124	0.416	0.150	2.773	0.335
hsa-mir-30a	75518.415	52389.493	1.441	0.281
hsa-mir-24	231.0938	169.0763	1.367	0.000117
hsa-mir-34a	782.462	591.0943	1.324	0.000248
hsa-mir-769	164.319	127.123	1.293	7.67×10^{-7}
hsa-mir-32	225.810	182.773	1.235	0.000586
hsa-mir-128	736.136	623.156	1.181	0.0165
hsa-mir-195	158.353	154.902	1.022	0.860
hsa-mir-182	107714.647	107089.437	1.006	0.970
hsa-mir-339	259.434	258.0265	1.005	0.933
hsa-mir-140	5075.168	5181.0928	0.980	0.753
hsa-mir-218	184.885	195.486	0.946	0.668
hsa-mir-296	21.716	27.538	0.789	0.111
hsa-mir-144	754.152	969.679	0.778	0.0764
hsa-mir-409	125.684	162.474	0.774	0.00348
hsa-mir-1827	0.00560	0.00924	0.606	0.689
hsa-mir-520a	3.568	23.456	0.152	0.298

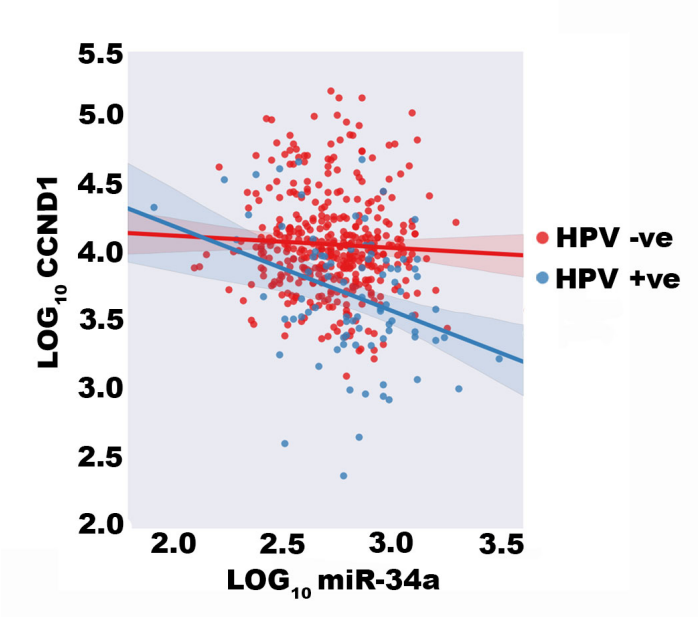

Figure 7. HPV +ve HNSCC samples exhibit greater downregulation of CCND1 by miR-34a. Scatter plot representing the correlation between CCND1 gene expression and miR-34a expression in HPV +ve and HPV -ve HNSCCs. The red line indicates correlation for HPV -ve and blue line indicates correlation for HPV +ve.

To further bolster our *in silico* findings, we also examined the relationship of HIF-1α and cyclin D1 in clinical samples. To this end, we immunohistochemically stained the adjacent sections of the 87 OPSCCs samples used in result section 1 and 2. Nuclear cyclin D1 WI was calculated as indicated in Materials and Methods. We found that cyclin D1 expression was significantly ($p<0.0001$) higher in the HPV -ve (n=43) OPSCCs when compared with the HPV +ve (n=44) OPSCCs (Fig. 8A, B) and high cyclin D1 expression was associated with poorer overall survival in OPSCCs (HR=3.409; p=0.0646) (Fig. 8C). Also, cyclin D1 was further able to stratify HPV -ve OPSCCs in high- and low-risk groups (HR=3.62; p=0.0152) (Fig. 8D). Furthermore, we observed a strong positive correlation between HIF-1α and cyclin D1 scores in HPV -ve OPSCCs (Spearman's rho p=0.642; $p<0.001$). Past studies have confirmed the overexpression of cyclin D1 in promoting CA, aneuploidy, and tumorigenesis.[60,61], In line with this we also observed that in HPV-ve OPSCCs the percent CA in tumor samples was positively correlated with the Cyclin D1 expression (Spearman's rho $p = 0.637$; $p <0.001$). Furthermore, utilizing publicly available

CancerRxgenome database we looked at the effect of CDK inhibitors (with correspondence to cyclin D1), in HPV -ve and HPV +ve HNSCC cell lines. As we expected, HPV -ve HNSCC cell lines were more susceptible to CDK inhibitor (Palbociclib) compared to HPV +ve, HNSCC cell lines (Fig. 4B). As a result, these findings substantiate the paradigm that hypoxia induces CA in HPV -ve OPSCCs, at least in part, by overexpression of cyclin D1.

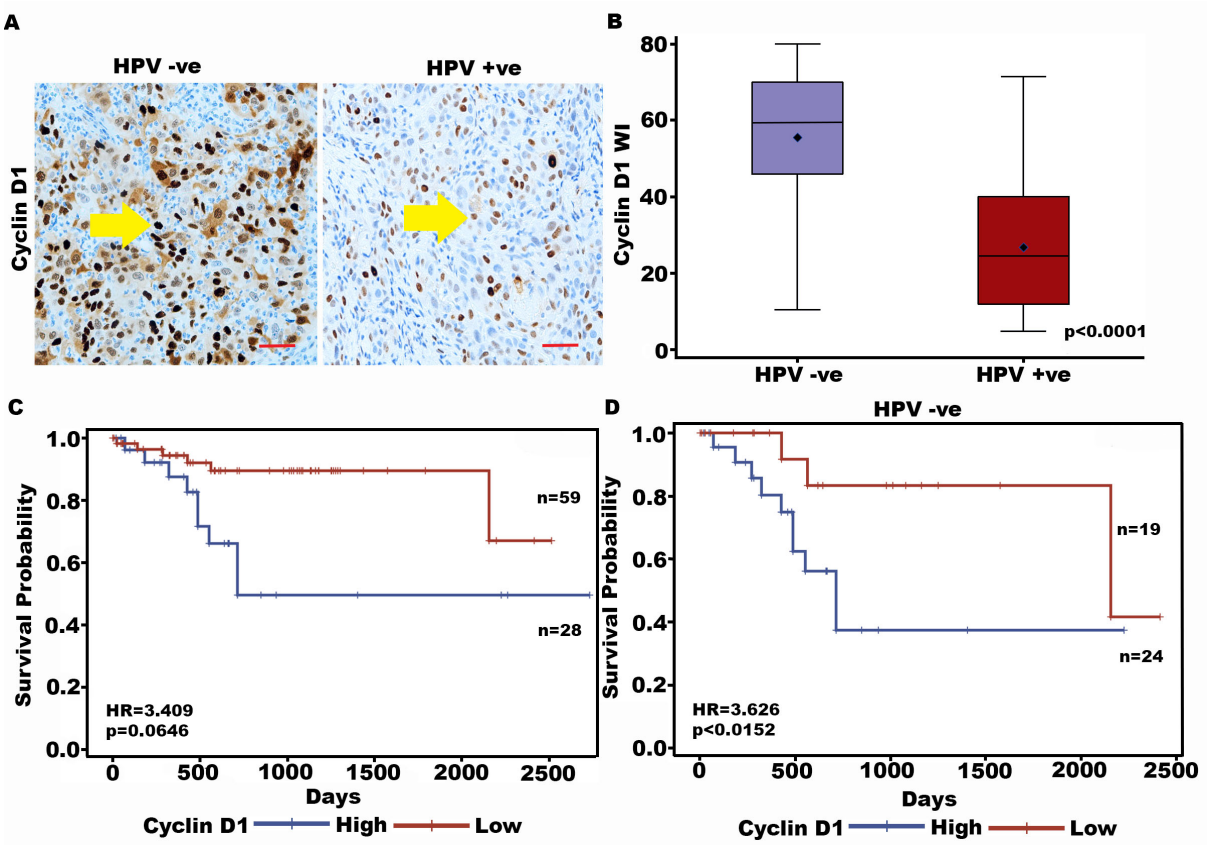

Figure 8. Cyclin D1 expression is upregulated in HPV -ve OPSCCs and is correlated with poor overall survival.
(A) Immunohistochemical micrographs of HPV +ve and HPV -ve OPSCC tumors labeled with nuclear cyclin D1. (B) Box plot representing the distribution of cyclin D1 WI in HPV +ve (n=44) and HPV –ve (n=43) tumors (p<0.0001). (C) Kaplan Meier survival curves representing the overall survival of cyclin D1 high (n=28) and low (n=59) groups in HPV -ve and HPV +ve OPSCC patients (HR=3.409; p=0.0646). (D) Kaplan Meier survival curve representing the overall survival of cyclin D1 high (n=24) and low (n=19) groups in HPV -ve OPSCC patients (HR=3.626; p=0.0152).

Finally, we wanted to see which marker (CA, HIF-1α, or cyclin D1) was best able to stratify HPV -ve OPSCCs into high and low-risk groups and would thus be most clinically informative. First, we performed a multivariate analysis, and noted that only CA showed a association with poor overall survival when other confounding factors like stage, therapy, gender and alcohol consumption and expression levels of HIF-1α and cyclin D1 were taken in account (HR=4.43; p=0.062) (Table 5A). Next, to measure the performance of prognostic models we used a measure of model fit, 2 Log Likelihood (-2LogL) (the model that minimized the -2LogL was considered superior). The results from this statistical test indicated that CA is the best-fit model (Table 5B). Similarly, when we performed the same test for our *in silico* findings, it also indicated the weighted CA7 score was stratifying the HPV -ve HNSCCs in high and low-risk groups better than the hypoxia score (Table 5C). Thus, collectively these findings, suggest that CA can serve as a clinically informative phenotypic biomarker for identification of high-risk HPV -ve OPSCC patients and can potentially also serve as a novel therapeutic target for these patients.

Table 5. Multivariate analysis for HPV -ve OPSCCs comparing the high- and low-CA groups. (A) and (B) -2log L model fit test for clinical samples. (C) -2log L model fit test for *in silico* TCGA dataset.

A

Parameter	HR	p-value
CA	4.433	0.0627
Gender	1.070	0.9396
Age At Diagnosis	1.076	0.2271
Overall Stage	1.163	0.8518
Alcohol	0.160	0.1656
Radiotherapy	0.000	0.9937
HIF-1α	0.991	0.9943
Cyclin D1	3.343	0.2866

B

Variable	Cyclin D1	HIF-1α	CA
-2 Log L	74.741	76.340	74.547

C

Variable	-2 Log L
Null	1912.9
Weighted Index of CA7	1897.6
Basic Sum CA7	1911.0
Hyp26 Score	1911.9
CCND1	1907.1

4 DISCUSSION

CA, a key driver of CIN and an early driver of intratumoral heterogeneity[62], is a hallmark of cancer and is associated with tumorigenesis and tumor progression in multiple cancers including head and neck. Quantitation of CA in tumor samples can thus capture important risk-predictive information and offer insights into the clinical course of multiple cancer types, including HNSCCs. Studies have shown that higher CA is associated with local recurrence in surgically-resected HNSSCs[63]. Another study in surgically-resected HNSCCs found that structural and numerical CA were able to better predict recurrence than other commonly used parameters like T stage; this study found a non-significant trend among the high-CA HNSSCs towards poor recurrence-free survival in these patients[53,64]. Furthermore, in another study, it has been reported that higher CA was associated with poor overall survival in HNSCCs [65]. However, no studies to date have performed a rigorous comparison of CA in OPSCCs that differed in their HPV status, nor have they investigated the prognostic role of CA in HPV +ve/-ve OPSCCs after accounting for potential confounders like age, gender, stage, grade and HPV status. Previous studies also focused on upregulation of DDR and failure of cytokinesis as causes of CA in HNSCCs[66 67]. Another important mechanism that has been studied at length in generation of CA in HNSCCs is related to HPV infection, where due to the presence of E6 and E7 genes in HPV +ve HNSCCs[18,19,20], it was presumed that this group exhibits higher CA when compared with HPV-ve HNSCCs. To shed light on these questions, we performed rigorous quantitation of CA (structural and numerical centrosomal aberrations) in a large cohort of HPV -ve and HPV +ve OPSCC tumor samples and explored the role of hypoxia - a hitherto overlooked driver of CA- in the generation of CA in HPV -ve tumors.

Our findings from clinical samples uncovered that HPV -ve OPSCCs exhibited higher CA than HPV +ve OPSCCs, and that CA was associated with poorer overall survival in HPV -ve OPSCCs, even when all the other confounding factors were controlled for. In addition, higher CA was associated with poorer overall survival in OPSCCs, regardless of HPV status. These

findings were corroborated by our *in silico* analysis of CA-associated genes in the large, well-annotated TCGA microarray dataset (Fig. 3). Genomic and proteomic research have revealed that HPV -ve HNSCCs exhibit high intratumoral heterogeneity that underlies therapeutic resistance and recurrence[68]. Our findings suggest that the higher CA observed in HPV -ve HNSCCs may drive higher CIN and intratumoral heterogeneity in HPV -ve OPSCC tumors, leading to therapy resistance and worse prognosis. Since CA can be induced by perturbations in the expression of many different genes, we rationally identified a set of 7 CA-associated genes commonly associated with centrosome structure/biogenesis whose dysregulation is known to induce CA. Impressively, this "CA7" gene signature was significantly prognostic in HNSCCs as well as in OPSCCs and was able to stratify HPV -ve HNSCCs into high and low-risk groups.

Our study further showed that the percentage of cells showing CA was strongly correlated with the nuclear WI for HIF-1α in HPV -ve OPSCCs and that a 26-gene hypoxia signature was able to significantly stratify HPV -ve OPSCCs in the TCGA dataset into high- and low-risk subgroups. These data strongly support that idea that hypoxia is likely to be a major driver of the CA. Our study also delineated the molecular mechanism whereby HIF-1α induces CA in HPV -ve OPSCCs - HIF-1α downregulates miR-34a that results in the strong upregulation of cyclin D1 and drives rampant CA in these tumors. The overexpression of cyclin D1 renders these tumors susceptible to the CDK4/6 inhibitor palbociclib as well as to Aurora A inhibitor ZM447439. Thus, our study has uncovered CA as an objectively evaluable and actionable phenotypic biomarker and has yielded novel insights into potential therapeutic targets for HPV -ve OPSCC patients who are in dire need of new treatment approaches that could improve their outcomes.

Among multiple factors, varied tumor microenvironment is one of the major contributing factors for the different biology in HPV -ve and HPV +ve OPSCCs[69]. Oxygen is necessary for radiosensitization, as ionizing radiation leads to a series of chemical reactions that contribute to

DNA damage.[70] Hypoxic tumors are more resistant to treatments and are associated with poor prognosis across a variety of cancers. In line with this, we observed higher expression of HIF-1α in the HPV -ve OPSCCs, and high HIF-1α was strongly correlated with the percentage of cells showing high CA in HPV -ve OPSCCs. However, contradicting reports can be found in the literature with regard to HIF-1α expression in HPV +ve versus HPV -ve tumors; one study reported that HIF-1α expression was higher in HPV +ve HNSCCs[71], while a second study reported higher expression of HIF-1α in HPV -ve head and neck cell lines.[72] Other endogenous hypoxia markers such as CA IX have also not shown definitive results[73]. These individual markers alone may not capture the entire picture of the hypoxic environment in HNSCCs. A more comprehensive approach of using a collection of hypoxia markers may be more pertinent in better accounting for intratumoral heterogeneity and cellular responses to hypoxia. Therefore, in this study, a previously established 26-gene signatures of hypoxia were used for *in silico* analyses on the publicly available dataset, revealing higher expression of hypoxia associated genes in HPV -ve OPSCCs and higher expression of these genes were associated with poor overall survival within this subset. With the understanding that hypoxia and the resulting HIF-1α activation can induce CA, it explains the higher CA observed in our HPV -ve OPSCC clinical samples and the corresponding poorer survival compared to HPV -ve OPSCCs.

The analysis of miRNA expression in OPSCC has provided a possible mechanistic explanation for the link between hypoxia and CA. As key players of post-transcriptional regulation of gene expression, miRNAs are fundamental for normal biological processes by preventing the formation of the gene product. And just like oncogenes, miRNA deregulation can lead to a variety of cancers.[74,75] Although miRNAs have been identified in OPSCC in relation to their prognostic value, there is a lack of characterization of their potential role in oncogenesis and in driving tumor evolution. In this study, we have newly identified miR-34a as a possible player in driving aggressive tumor characteristics in OPSCC through induction of CA. The deregulation of miR-34a has been revealed to be involved in different types of cancers. By

targeting CCND1, miR-34a controls the expression of cyclin D1, a protein whose upregulation triggers CA. Since hypoxia and HIF-1α have been shown to repress the expression of miR-34a[52,76] , miR-34a's downregulation in HPV -ve tumors leads to cyclin D1 overexpression and CA. We observed similar findings in our clinical samples where HPV -ve OPSCCs expressed higher levels of cyclin D1, and its expression was associated with poor overall in these patients. Therefore, our findings indicate that HIF-1α-mediated downregulation of miR-34a in HPV -ve tumors drives its distinct tumor biology, and establish a causative link between two biological phenomena – hypoxia and CA – that co-occur in many solid tumors.

Studies have shown that EGFR inhibitors such as Cetuximab are effective in treating HPV -ve HNSCCs. However, only a modest effect on survival has been shown when cetuximab was co-administered with conventional chemotherapy. Therefore, new molecular targets are required to improve survival in HPV -ve HNSCCs. Our study shows that the HPV -ve HNSCC cell lines are more sensitive to CDK 4/6, and Aurora A inhibitors when compared with HPV +ve HNSCC cells. This finding is in concordance with previous studies showing that HPV -ve HNSCCs are sensitive to treatment with CDK 4/6 inhibitor palbociclib and Aurora A inhibitors ZM447439. Thus, our results from *in silico* and clinical studies indicate that non-toxic centrosome declustering drugs (such as noscapinoids, griseofulvin, KifC1/HSET inhibitors, and PJ34), which selectively target cells with CA and compel them to construct a potentially lethal multipolar spindle during mitosis, might also be promising therapeutic agents for HPV -ve HNSCCs.

Collectively, the data presented support the idea that hypoxia is likely to be a major driver of the CA observed in HPV -ve OPSCCs. Our study also delineated the molecular mechanism whereby HIF-1α induces CA in HPV -ve OPSCCs - HIF-1α downregulates miR-34a, which results in the strong upregulation of cyclinD1 and drives rampant CA in these tumors. Thus, our study has uncovered CA as an objectively evaluable and actionable phenotypic

biomarker and has yielded novel insights into potential therapeutic targets for HPV -ve OPSCCs that are in dire need of new and more effective treatment options. In conclusion, this is the first report to substantiate the previously unrecognized role of HIF-1α-induced CA in HPV -ve OPSSCs, revealing a molecular pathway that may be responsible for the CIN, intratumoral heterogeneity and poor prognosis associated with these tumors. The prognostic potential of CA is especially resounding within HPV -ve OPSSCs, facilitating enhanced identification of higher risk patients, influencing future treatment strategies, and providing a platform for the discovery of effective molecular targets.

REFERENCES

1 Vigneswaran, N. & Williams, M. D. Epidemiological Trends in Head and Neck Cancer and Aids in Diagnosis. *Oral and maxillofacial surgery clinics of North America* **26**, 123-141, doi:10.1016/j.coms.2014.01.001 (2014).

2 van Monsjou, H. S., Balm, A. J., van den Brekel, M. M. & Wreesmann, V. B. Oropharyngeal squamous cell carcinoma: a unique disease on the rise? *Oral oncology* **46**, 780-785, doi:10.1016/j.oraloncology.2010.08.011 (2010).

3 Mignogna, M. D., Fedele, S. & Lo Russo, L. The World Cancer Report and the burden of oral cancer. *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP)* **13**, 139-142 (2004).

4 Viens, L. J. *et al.* Human Papillomavirus-Associated Cancers - United States, 2008-2012. *MMWR. Morbidity and mortality weekly report* **65**, 661-666, doi:10.15585/mmwr.mm6526a1 (2016).

5 Pytynia, K. B., Dahlstrom, K. R. & Sturgis, E. M. Epidemiology of HPV-associated oropharyngeal cancer. *Oral oncology* **50**, 380-386, doi:10.1016/j.oraloncology.2013.12.019 (2014).

6 Hammarstedt, L. *et al.* Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer. *Int J Cancer* **119**, 2620-2623, doi:10.1002/ijc.22177 (2006).

7 Shiboski, C. H., Schmidt, B. L. & Jordan, R. C. Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20-44 years. *Cancer* **103**, 1843-1849, doi:10.1002/cncr.20998 (2005).

8 Chaturvedi, A. K. *et al.* Worldwide Trends in Incidence Rates for Oral Cavity and Oropharyngeal Cancers. *Journal of Clinical Oncology* **31**, 4550-4559, doi:10.1200/jco.2013.50.3870 (2013).

9 Forte, T., Niu, J., Lockwood, G. A. & Bryant, H. E. Incidence trends in head and neck cancers and human papillomavirus (HPV)-associated oropharyngeal cancer in Canada, 1992–2009. *Cancer Causes & Control* **23**, 1343-1348, doi:10.1007/s10552-012-0013-z (2012).

10 Lowy, D. R. & Munger, K. Prognostic implications of HPV in oropharyngeal cancer. *The New England journal of medicine* **363**, 82-84, doi:10.1056/NEJMe1003607 (2010).

11 Ang, K. K. *et al.* Human papillomavirus and survival of patients with oropharyngeal cancer. *The New England journal of medicine* **363**, 24-35, doi:10.1056/NEJMoa0912217 (2010).

12 Mirghani, H. & Blanchard, P. Treatment de-escalation for HPV-driven oropharyngeal cancer: Where do we stand? *Clinical and Translational Radiation Oncology* **8**, 4-11, doi:https://doi.org/10.1016/j.ctro.2017.10.005 (2018).

13 Mirghani, H. *et al.* Treatment de-escalation in HPV-positive oropharyngeal carcinoma: ongoing trials, critical issues and perspectives. *Int J Cancer* **136**, 1494-1503, doi:10.1002/ijc.28847 (2015).

14 Fakhry, C. *et al.* Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst* **100**, 261-269, doi:10.1093/jnci/djn011 (2008).

15 Adelstein, D. J. & Rodriguez, C. P. Human papillomavirus: changing paradigms in oropharyngeal cancer. *Current oncology reports* **12**, 115-120, doi:10.1007/s11912-010-0084-5 (2010).

16 Selcuk, O. T. Human papillomavirus positive oropharyngeal cancer: The general information. *Egyptian Journal of Ear, Nose, Throat and Allied Sciences* **17**, 127-132, doi:https://doi.org/10.1016/j.ejenta.2016.07.007 (2016).

17 Stransky, N. *et al.* The mutational landscape of head and neck squamous cell carcinoma. *Science (New York, N.Y.)* **333**, 1157-1160, doi:10.1126/science.1208130 (2011).

18 Kaczmar, J. & Mehra, R. HPV-negative head and neck squamous cell carcinomas: how can we use the tumor protein signature? *Biomarkers in medicine* **9**, 183-185, doi:10.2217/bmm.15.1 (2015).

19 Rothenberg, S. M. & Ellisen, L. W. The molecular pathogenesis of head and neck squamous cell carcinoma. *The Journal of Clinical Investigation* **122**, 1951-1957, doi:10.1172/jci59889 (2012).

20 Munger, K., Scheffner, M., Huibregtse, J. M. & Howley, P. M. Interactions of HPV E6 and E7 oncoproteins with tumour suppressor gene products. *Cancer surveys* **12**, 197-217 (1992).

21 Duensing, S. & Münger, K. Human papillomaviruses and centrosome duplication errors: modeling the origins of genomic instability. *Oncogene* **21**, 6241, doi:10.1038/sj.onc.1205709 (2002).

22 Korzeniewski, N., Treat, B. & Duensing, S. The HPV-16 E7 oncoprotein induces centriole multiplication through deregulation of Polo-like kinase 4 expression. *Molecular Cancer* **10**, 61, doi:10.1186/1476-4598-10-61 (2011).

23 Duensing, S. & Munger, K. The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. *Cancer research* **62**, 7075-7082 (2002).

24 Chan, J. Y. A Clinical Overview of Centrosome Amplification in Human Cancers. *International Journal of Biological Sciences* **7**, 1122-1144 (2011).

25 D'Assoro, A. B., Lingle, W. L. & Salisbury, J. L. Centrosome amplification and the development of cancer. *Oncogene* **21**, 6146, doi:10.1038/sj.onc.1205772 (2002).

26 Godinho, S. A. & Pellman, D. Causes and consequences of centrosome abnormalities in cancer. *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**, doi:10.1098/rstb.2013.0467 (2014).

27 Ogden, A., Rida, P. C. & Aneja, R. Heading off with the herd: how cancer cells might maneuver supernumerary centrosomes for directional migration. *Cancer metastasis reviews* **32**, 269-287, doi:10.1007/s10555-012-9413-5 (2013).

28 Leemans, C. R., Braakhuis, B. J. & Brakenhoff, R. H. The molecular biology of head and neck cancer. *Nature reviews. Cancer* **11**, 9-22, doi:10.1038/nrc2982 (2011).

29 Riaz, N., Morris, L. G., Lee, W. & Chan, T. A. Unraveling the molecular genetics of head and neck cancer through genome-wide approaches. *Genes & Diseases* **1**, 75-86, doi:10.1016/j.gendis.2014.07.002 (2014).

30 Broustas, C. G. & Lieberman, H. B. DNA damage response genes and the development of cancer metastasis. *Radiation research* **181**, 111-130, doi:10.1667/rr13515.1 (2014).

31 van Vugt, M. A. & Medema, R. H. Getting in and out of mitosis with Polo-like kinase-1. *Oncogene* **24**, 2844-2859, doi:10.1038/sj.onc.1208617 (2005).

32 Katayama, H. & Sen, S. Aurora kinase inhibitors as anticancer molecules. *Biochimica et biophysica acta* **1799**, 829-839, doi:10.1016/j.bbagrm.2010.09.004 (2010).

33 Parameswaran, J., Lee, J. W., Sandoval-Schaefer, T., Koo, J. P. & Burtness, B. Combination of WEE1 and AURKA inhibition in HPV negative head and neck squamous cell carcinoma. *Journal of Clinical Oncology* **35**, e14105-e14105, doi:10.1200/JCO.2017.35.15_suppl.e14105 (2017).

34 Mehra, R., Serebriiskii, I. G., Burtness, B., Astsaturov, I. & Golemis, E. A. The role and targeting of Aurora kinases in head and neck cancer. *The Lancet. Oncology* **14**, e425-435, doi:10.1016/s1470-2045(13)70128-1 (2013).

35 Fukasawa, K. P53, cyclin-dependent kinase and abnormal amplification of centrosomes. *Biochimica et biophysica acta* **1786**, 15-23, doi:10.1016/j.bbcan.2008.04.002 (2008).

36 Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S. & Vande Woude, G. F. Abnormal centrosome amplification in the absence of p53. *Science* **271**, 1744-1747 (1996).

37 Tarapore, P. & Fukasawa, K. Loss of p53 and centrosome hyperamplification. *Oncogene* **21**, 6234-6240, doi:10.1038/sj.onc.1205707 (2002).

38 Nurwidya, F., Takahashi, F., Minakata, K., Murakami, A. & Takahashi, K. From tumor hypoxia to cancer progression: the implications of hypoxia-inducible factor-1 expression in cancers. *Anatomy & Cell Biology* **45**, 73-78, doi:10.5115/acb.2012.45.2.73 (2012).

39 Hanns, E. *et al.* Human Papillomavirus-related tumours of the oropharynx display a lower tumour hypoxia signature. *Oral oncology* **51**, 848-856, doi:10.1016/j.oraloncology.2015.06.003 (2015).

40 Klein, A., Flugel, D. & Kietzmann, T. Transcriptional regulation of serine/threonine kinase-15 (STK15) expression by hypoxia and HIF-1. *Molecular biology of the cell* **19**, 3667-3675, doi:10.1091/mbc.E08-01-0042 (2008).

41 Katayama, H., Zhou, H., Li, Q., Tatsuka, M. & Sen, S. Interaction and feedback regulation between STK15/BTAK/Aurora-A kinase and protein phosphatase 1 through mitotic cell division cycle. *The Journal of biological chemistry* **276**, 46219-46224, doi:10.1074/jbc.M107540200 (2001).

42 Rosario, C. & Swallow, C. J. Abstract 4045: Hypoxia induces Plk4 expression and promotes immortalization of proliferating cells. *Cancer Research* **73**, 4045 (2013).

43 Mittal, K. *et al.* Amplified centrosomes and mitotic index display poor concordance between patient tumors and cultured cancer cells. *Scientific Reports* **7**, doi:10.1038/srep43984 (2017).

44 Mittal, K. *et al.* A centrosome clustering protein, KIFC1, predicts aggressive disease course in serous ovarian adenocarcinomas. *Journal of ovarian research* **9**, 17, doi:10.1186/s13048-016-0224-0 (2016).

45 Mittal, K. *et al.* Amplified centrosomes may underlie aggressive disease course in pancreatic ductal adenocarcinoma. *Cell cycle (Georgetown, Tex.)* **14**, 2798-2809, doi:10.1080/15384101.2015.1068478 (2015).

46 Pannu, V. *et al.* Rampant centrosome amplification underlies more aggressive disease course of triple negative breast cancers. *Oncotarget* **6**, 10487-10497, doi:10.18632/oncotarget.3402 (2015).

47 TCGA Releases Head and Neck Cancer Data. *Cancer discovery* **5**, 340-341, doi:10.1158/2159-8290.cd-nb2015-024 (2015).

48 Aebersold, D. M. *et al.* Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. *Cancer research* **61**, 2911-2916 (2001).

49 Bachtiary, B. *et al.* Overexpression of hypoxia-inducible factor 1alpha indicates diminished response to radiotherapy and unfavorable prognosis in patients receiving radical radiotherapy for cervical cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **9**, 2234-2240 (2003).

50 Yasuda, H. Solid tumor physiology and hypoxia-induced chemo/radio-resistance: novel strategy for cancer therapy: nitric oxide donor as a therapeutic enhancer. *Nitric oxide : biology and chemistry* **19**, 205-216, doi:10.1016/j.niox.2008.04.026 (2008).

51 Bredell, M. G. *et al.* Current relevance of hypoxia in head and neck cancer. *Oncotarget* **7**, 50781-50804, doi:10.18632/oncotarget.9549 (2016).

52 Swartz, J. E. *et al.* Poor prognosis in human papillomavirus-positive oropharyngeal squamous cell carcinomas that overexpress hypoxia inducible factor-1alpha. *Head & neck* **38**, 1338-1346, doi:10.1002/hed.24445 (2016).

53 Eustace, A. *et al.* A 26-gene hypoxia signature predicts benefit from hypoxia-modifying therapy in laryngeal cancer but not bladder cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 4879-4888, doi:10.1158/1078-0432.ccr-13-0542 (2013).

54 Buffa, F. M., Harris, A. L., West, C. M. & Miller, C. J. Large meta-analysis of multiple cancers reveals a common, compact and highly prognostic hypoxia metagene. *Br J Cancer* **102**, 428-435, doi:10.1038/sj.bjc.6605450 (2010).

55 Huang, X. *et al.* Hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation. *Mol Cell* **35**, 856-867, doi:10.1016/j.molcel.2009.09.006 (2009).

56 Chan, Y. C., Khanna, S., Roy, S. & Sen, C. K. miR-200b targets Ets-1 and is down-regulated by hypoxia to induce angiogenic response of endothelial cells. *J Biol Chem* **286**, 2047-2056, doi:10.1074/jbc.M110.158790 (2011).

57 Shivdasani, R. A. MicroRNAs: regulators of gene expression and cell differentiation. *Blood* **108**, 3646-3653, doi:10.1182/blood-2006-01-030015 (2006).

58 Sun, F. *et al.* Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. *FEBS letters* **582**, 1564-1568, doi:10.1016/j.febslet.2008.03.057 (2008).

59 Li, H., Rokavec, M., Jiang, L., Horst, D. & Hermeking, H. Antagonistic Effects of p53 and HIF1A on microRNA-34a Regulation of PPP1R11 and STAT3 and Hypoxia-induced Epithelial to Mesenchymal Transition in Colorectal Cancer Cells. *Gastroenterology* **153**, 505-520, doi:10.1053/j.gastro.2017.04.017 (2017).

60 Nelsen, C. J. *et al.* Short term cyclin D1 overexpression induces centrosome amplification, mitotic spindle abnormalities, and aneuploidy. *The Journal of biological chemistry* **280**, 768-776, doi:10.1074/jbc.M407105200 (2005).

61 del Rey, J. *et al.* Centrosome clustering and cyclin D1 gene amplification in double minutes are common events in chromosomal unstable bladder tumors. *BMC Cancer* **10**, 280, doi:10.1186/1471-2407-10-280 (2010).

62 McBride, M., Rida, P. C. G. & Aneja, R. Turning the headlights on novel cancer biomarkers: Inspection of mechanics underlying intratumor heterogeneity. *Molecular aspects of medicine* **45**, 3-13, doi:10.1016/j.mam.2015.05.001 (2015).

63 Gustafson, L. M. *et al.* Centrosome hyperamplification in head and neck squamous cell carcinoma: a potential phenotypic marker of tumor aggressiveness. *The Laryngoscope* **110**, 1798-1801, doi:10.1097/00005537-200011000-00004 (2000).

64 Syed, M. I. *et al.* Gamma tubulin: a promising indicator of recurrence in squamous cell carcinoma of the larynx. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* **140**, 498-504, doi:10.1016/j.otohns.2008.12.049 (2009).

65 Reiter, R. *et al.* Centrosome abnormalities in head and neck squamous cell carcinoma (HNSCC). *Acta oto-laryngologica* **129**, 205-213, doi:10.1080/00016480802165767 (2009).

66 Carroll, P. E. *et al.* Centrosome hyperamplification in human cancer: chromosome instability induced by p53 mutation and/or Mdm2 overexpression. *Oncogene* **18**, 1935-1944, doi:10.1038/sj.onc.1202515 (1999).

67 Reiter, R. *et al.* Aurora kinase A messenger RNA overexpression is correlated with tumor progression and shortened survival in head and neck squamous cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* **12**, 5136-5141, doi:10.1158/1078-0432.ccr-05-1650 (2006).

68 Bonilla-Velez, J., Mroz, E. A., Hammon, R. J. & Rocco, J. W. Impact of human papillomavirus on oropharyngeal cancer biology and response to therapy: implications for treatment. *Otolaryngologic clinics of North America* **46**, 521-543, doi:10.1016/j.otc.2013.04.009 (2013).

69 Bristow, R. G. & Hill, R. P. Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. *Nature reviews. Cancer* **8**, 180-192, doi:10.1038/nrc2344 (2008).

70 Rockwell, S., Dobrucki, I. T., Kim, E. Y., Marrison, S. T. & Vu, V. T. Hypoxia and radiation therapy: Past history, ongoing research, and future promise. *Curr Mol Med* **9**, 442-458 (2009).

71 Knuth, J. *et al.* Hypoxia-inducible factor-1 α activation in HPV-positive head and neck squamous cell carcinoma cell lines. *Oncotarget* **8**, 89681-89691, doi:10.18632/oncotarget.20813 (2017).

72 Jung, Y. S. *et al.* HPV-associated differential regulation of tumor metabolism in oropharyngeal head and neck cancer. *Oncotarget* **8**, 51530-51541, doi:10.18632/oncotarget.17887 (2017).

73 Brockton, N. *et al.* High stromal carbonic anhydrase IX expression is associated with decreased survival in P16-negative head-and-neck tumors. *International journal of radiation oncology, biology, physics* **80**, 249-257, doi:10.1016/j.ijrobp.2010.11.059 (2011).

74 Chang, T. C. *et al.* Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Molecular cell* **26**, 745-752, doi:10.1016/j.molcel.2007.05.010 (2007).

75 Tazawa, H., Tsuchiya, N., Izumiya, M. & Nakagama, H. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 15472-15477, doi:10.1073/pnas.0707351104 (2007).

76 Du, R. *et al.* Hypoxia-induced down-regulation of microRNA-34a promotes EMT by targeting the Notch signaling pathway in tubular epithelial cells. *PloS one* **7**, e30771, doi:10.1371/journal.pone.0030771 (2012).

77 Rietbergen, M. M. *et al.* Increasing prevalence rates of HPV attributable oropharyngeal squamous cell carcinomas in the Netherlands as assessed by a

validated test algorithm. *Int J Cancer* **132**, 1565-1571, doi:10.1002/ijc.27821 (2013).