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Novel Roles of Tobacco-associated Genes Underlying Disparate Survival in Appalachian Head and Neck Squamous Cell Carcinoma

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Novel Roles of Tobacco-associated Genes Underlying Disparate Survival in Appalachian Head and Neck Squamous Cell Carcinoma

Brenen William Papenberg

Dissertation submitted to the School of Medicine at West Virginia University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Cancer Cell Biology

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ABSTRACT

Novel Roles of Tobacco-associated Genes Underlying Disparate Survival in Appalachian Head and Neck Squamous Cell Carcinoma

Brenen William Papenberg

Head and neck squamous cell carcinoma (HNSCC) is an aggressive neoplasm primarily caused by tobacco consumption or human papillomavirus (HPV) infection. Incidence and mortality rates for HNSCC vary geographically. Appalachian residents consume tobacco products to a greater extent than national averages, a risk factor known to promote HPV-negative HNSCC through increased genomic instability. Male Appalachian patients display significantly worse relative survival than Appalachian females or non-Appalachian residents of either sex. Secondary analysis of available cancer registry outcome data from 2007-2013 indicates that white males with stage IV oral cavity/pharyngeal (OC/P) HNSCC are responsible for the decreased male survival observed within Appalachian regions. This disparity is seen in a subset of Appalachian states and is associated with lower relative survival in HPV-Associated subsites, suggesting a higher percentage of HPV-negative cancers occur in oral cavity regions typically associated with HPV infection. This increase is likely associated with higher smoking and smokeless tobacco usage in Appalachian states. An unbiased statistical analysis of gene copy number alterations (CNAs) from the Cancer Genome Atlas (TCGA)-HNSC national cohort indicates that smoking significantly correlates with amplification or deletions of genes found in several chromosomal cytobands. Of these, chromosome 11q13.3 and 11q13.4 (amplified) and chromosomes 9p21.3 and 9p24.1 (deleted) are the only altered segments that correlate with reduced overall survival in patients that smoked, independent of HPV status. 11q13 amplification is the most common CNA in HNSCC and enhances tumor progression and poor outcome due to protein overexpression. Transcriptome analysis of HNSCC with 11q13 amplification indicates that 13 out of 17 amplified genes are overexpressed, including the oncogenic cell gene CCND1 (cyclin D1). Recently, 3'UTRs of select mRNAs have been shown to function as positive regulators of cancer progression by sequestering tumor-suppressive microRNAs (miRNAs) as competing endogenous (ce)RNA. Thus, a novel mechanism of poor outcome in 11q13 amplified patients may be due to the 3'UTR regions of elevated 11q13 transcripts serving to titrate tumor-suppressive miRNAs, permitting increased oncogenic progression. The overall **hypothesis** of this dissertation is that HNSCC patients in Appalachia have lower survival due to a larger number of individuals harboring tumors with 11q13 amplification from tobacco-

induced damage, resulting in increased transcript expression that downregulates tumor-suppressive miRNA functions. Predictive algorithms and secondary TCGA data analysis indicate that the CCND1 3'UTR contains multiple miRNA response elements expected to bind known tumor-suppressive miRNAs. Patient outcome data indicates that CCND1-amplified patients with 3'UTR containing transcripts have poorer overall survival than patients with CCND1 overexpression lacking the 3'UTR. Stratification by CCND1 expression reveals several altered protein targets including CCNB1, TYMS, and SERPINE1, that share miRNA response elements with CCND1 mRNA. Identification of tumor-promoting effects from 11q13 transcripts provides alternative avenues for therapeutic intervention in this most aggressive form of HNSCC by identifying novel druggable transcription targets that can augment protein-based precision medicine strategies or by facilitating stratification of patients for clinical trials in patients with smoking-induced 11q13 amplification in Appalachia or other regions of high tobacco use.

Dedication:

I dedicate this dissertation to my partner and future wife Sarah. You are my light in the darkness and the wind in my sails. You have made me a better person than I could on my own and I cannot wait to spend the rest of my life with you. Thank you for your encouragement and confidence in my ability throughout my time as a graduate student.

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Glossary of Terms

9CFS - Common Fragile Site, Chromosome 9
ACS - American Cancer Society
AGO - Argonaute
AGO2 - Argonaute 2
AIAN - American Indian / Alaska Native (Race)
AJCC - American Joint Committee On Cancer
AKT - Protein Kinase B
ANO1 - Anoctamin 1, Calcium Activated Chloride Channel
ANOVA - Analysis of Variance
AP1 - Activator Protein 1
APA - Alternative Cleavage and Polyadenylation
API - Asian Or Pacific Islander (Race)
ARC - Appalachian Regional Commission
ARE - AU-Rich Element
ARE-BP - ARE-Binding Protein
AREG - Amphiregulin
Arp2/3 - Actin-Related Proteins 2 and 3
ATCC - American Type Culture Collection
ATM - Ataxia-Telangiectasia Mutated
B - Black (Race)
BA - Basal
BCL2L11 - Bcl-2-like Protein 11
BFB - Breakage-Fusion-Bridge
BID - BH3 Interacting Domain Death Agonist
BPDE - Benzo[2]Pyrene-7,8-Diol-9,10-Epoxyde
BRAF - B-Raf
BRFSS - Behavioral Risk Factor Surveillance System
C. elegans - Caenorhabditis elegans
CA9 - Carbonic Anhydrase 9

Glossary of Terms

CAK - CDK-Activating Kinase
CASP9 - Caspase 9
CCNB1 - Cyclin B1
CCND1 - Cyclin D1
CD8 - Cluster of Differentiation 8
CDC - Centers For Disease Control
CDH3 - Cadherin 3
CDK4 - Cyclin-Dependent Kinase 4
CDK6 - Cyclin-Dependent Kinase 6
CDKN2A - Cyclin-Dependent Kinase Inhibitor 2A
CDKN2B - Cyclin-Dependent Kinase Inhibitor 2B
CDSMD1 - CUB and Sushi Multiple Domains 1
ceRNA - Competing Endogenous RNA
CFS - Common Fragile Site
CI - Confidence Intervals
circRNA - Circular RNA
CL - Classical
CMG - CDC45-MCM-GINS
CNA - Copy Number Alteration
CPT1A - Carnitine Palmitoyltransferase 1A (Liver)
CRS - Cumulative Relative Survival
CTTN - Cortactin
DCP2 - Decapping Protein 2
DEFB108B - Defensin, Beta 108B
DGCR8 - Digeorge Syndrome Critical Region 8
DHCR7 - 7-Dehydrocholesterol Reductase
DISC - Death-Inducing Signaling Complex
DMEM - Dulbecco's Modified Eagle Media
DNA - Deoxyribonucleic Acid
DVL3 - Dishevelled Segment Polarity Protein 3
E1-7 - Early 1-7 (HPV)

E2F - E2 Transcription Factor	GISTIC - Genomic Identification Of Significant Targets In Cancer
E6AP - E6AP ubiquitin-protein ligase	GMAP - Galanin Message-Associated Protein
EGFR - Epidermal Growth Factor Receptor	GPR - G-protein Regulator 1
ENPP7P8 - Ectonucleotide Pyrophosphatase/Phosphodiesterase 7 Pseudogene 8	GRB2 - Growth Factor Receptor-Bound Protein 2
EPA - Environmental Protection Agency	gRNA - Guide RNA
ERAD - endoplasmic-reticulum-associated protein degradation response	GTP - Guanosine Triphosphate
ErbB - Erythroblastic Oncogene B	HER2 - Human Epidermal Growth Factor Receptor 2
ERBB2 - receptor tyrosine-protein kinase erbB-2	HIF1A - Hypoxia-Inducible Factor 1-alpha
ERK - Extracellular Signal-Related Kinase	HLA-DRA - Human Leukocyte Antigen DR Alpha Chain
EV - Empty Vector	HNSCC - Head And Neck Squamous Cell Carcinoma
FADD - Fas (Tnfrsf6)-Associated Via Death Domain	HOXD - Homeobox D
FAM86C1 - Family With Sequence Similarity 86, Member C1	HPV - Human Papillomavirus
FDA - Food And Drug Administration	HR - Hormone Receptor
FDR - False Discovery Rate	IARC - International Agency For Research On Cancer
FGF19 - Fibroblast Growth Factor 19	ICD-O - International Classification Of Diseases For Oncology
FGF3 - Fibroblast Growth Factor 3	ICOS - Inducible T-cell Costimulator
FGF4 - Fibroblast Growth Factor 4	IDeA - Institutional Development Awards
FGFR - Fibroblast Growth Factor Receptor	IGH - Immunoglobulin Heavy Chain Locus
FGFR1 - Fibroblast Growth Factor Receptor 1	IGHMBP2 - Immunoglobulin Mu Binding Protein 2
FGFR3 - Fibroblast Growth Factor Receptor 3	IHC - Immunohistochemistry
FRA11F - Fragile Site 11F	IMS - Inflamed / Mesenchymal
FRA11H - Fragile Site 11H	INBRE - IDeA Network of Biomedical Research Excellence
FRA9C - Fragile Site 9C	INK4 - Inhibitors Of CDK4
FS - Frameshift	ITGA2 - Integrin Subunit Alpha 2
G1 - Growth 1 (Cell Cycle)	KMT2D - Histone-Lysine N-Methyltransferase 2D
GAL - Galanin/GMAP Prepropeptide	KRT1 - Keratin, type II cytoskeletal 1
GATA3 - GATA Binding Protein 3	KRT9 - Keratin, Type I cytoskeletal 9
GDAC - Broad Genomic Data Analysis Center	L1-2 - Late 1-2 (HPV)
GEF - Guanine Nucleotide Exchange Factor	

LAG3 - Lymphocyte Activation Gene 3	mTOR - Mammalian Target Of Rapamycin
LAR - Leukocyte Common Antigen-Related	MYEOV - Myeloma Overexpressed
LC-MS - Liquid Chromatography Mass Spectrometry	NAACCR - North American Association For Central Cancer Registries
let-7 - lethal-7	NAD - Nicotinamide Adenine Dinucleotide
lin-14 - Abnormal Cell Lineage 14	NADSYN1 - NAD Synthetase 1
lin-4 - Abnormal Cell Lineage 4	NCBI - National Center for Biotechnology Information
lncRNA - Long Non-Coding RNA	NCCN - National Comprehensive Cancer Network
log2FC - log2 Fold Change	NCI - National Cancer Institute
LOOCV - Leave-One-Out Cross Validation	NF1 - Neurofibromin 1
LRP1B - Low-Density Lipoprotein Receptor-Related Protein 1B	NIH - National Institutes Of Health
MACC1 - Metastasis-associated in colon cancer protein 1	NKX2-1 - NK2 Homeobox 1
MAPK - Mitogen-Activated Protein Kinase	NNK - 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone
MAS - Mitochondrial Assembly	NNN - N-Nitrosornicotine
Mb - Megabases	NOTCH1 - Notch homolog 1
MDM2 - Murine Double Minute	NRG1 - Neuregulin 1
MEK - Mitogen-Activated Protein Kinase Kinase	NSD1 - Nuclear Receptor Binding SET Domain Protein 1
MIR3164 - MicroRNA 3164	OC/P - Oral Cavity And Pharyngeal
MIR3664 - MicroRNA 3664	ORAOV1 - Oral Cancer Overexpressed 1
MIR548K - MicroRNA 548K	ORF - Open Reading Frame
miRISC - MicroRNA-Induced Silencing Complex	PABC - Poly(A)-Binding Protein C
miRNA - MicroRNA 9	PAH - Polycyclic Aromatic Hydrocarbons
MM - miRNA-mutant Constructs	PCLO - Piccolo
MMP9 - Matrix Metalloproteinase 9	PCNA - Proliferating Cell Nuclear Antigen
MRE - MicroRNA Response Element	PCR - Polymerase Chain Reaction
MRGPRD - MAS-Related GPR, Member D	PD-L1 - Programmed Death Ligand 1
MRGPRF - MAS-Related GPR, Member F	PDX - Patient Derived Xenograft
mRNA - Messenger RNA	PI - Propidium Iodide
MRPL21 - Mitochondrial Ribosomal Protein L21	PI3K - Phosphoinositide 3-Kinase
MTL5 - Metallothionein-Like 5, Testis-Specific (Tesmin)	PIK3CA - Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic Subunit Alpha

POB - Pyridyloxo-Butyl	SOS1 - Son Of Sevenless Homolog 1
PPFIA1 - Liprin, Alpha 1	SOX2 - (Sex Determining Region Y)-box 2
PRAD1 - Parathyroid Adenomatosis	SSE - Structure-Specific Endonucleases
pRB - Retinoblastoma Protein	stRNA - Small Temporal RNA
PRC2 - Polycomb Repressive Complex 2	TCGA - The Cancer Genome Atlas
PTEN - Phosphatase And Tensin Homolog	TCGA-BLCA - TCGA Bladder Urothelial Carcinoma
PTH - Parathyroid Hormone	TCGA-BRCA - TCGA Breast Invasive Carcinoma
PXN - Paxillin	TCGA-ESCA - TCGA Esophageal Carcinoma
QGIS - Quantum Geographic Information System	TCGA-HNSC - TCGA Head-Neck Squamous Cell Carcinoma
Raf - Rapidly Accelerated Fibrosarcoma	TCGA-LUSC - Lung Squamous Cell Carcinoma
Ras - Rat Sarcoma	TME CoBRE - Tumor Microenvironment Center of Biomedical Research Excellence
RING - Really Interesting New Gene	TMEM16A - Transmembrane Member 16A
RNA - Ribonucleic Acid	TNFRSF6 - Tumor Necrosis Factor Receptor Superfamily Member 6
RNA5SP342 - 5S Ribosomal Pseudogene 342	TNRC6A - Trinucleotide Repeat-Containing Gene 6A Protein
RNase - RNA Sequencing	TP53 - Tumor Protein 53
RNF121 - Ring Finger Protein 121	TP63 - Tumor Protein 63
ROS - Reactive Oxygen Species	TPCN2 - Two Pore Segment Channel 2
RPKM - Reads Per Kilobase of Transcript, Per Million Reads	tRNA - Transfer RNA
RPPA - Reverse Phase Protein Lysate Microarray	TSB - Target Site Blocking
rRNA - Ribosomal RNA	TSNA - Tobacco-Specific Nitrosamine
RS - Relative Survival	TTF-1 - Thyroid Transcription Factor 1
RTK - Receptor Tyrosine Kinase	TYMS - Thymidylate Synthetase
S - Synthesis (Cell Cycle)	UCSC - University Of California Santa Cruz
SAES - Smoking-Associated Expression Signature	UNC93B6 - Unc-93 Homolog B6
SEER - Survival, Epidemiology, And End Reports	UTR - Untranslated Region
SERPINE1 - Plasminogen Activator Inhibitor 1	VEGF - Vascular Endothelial Growth Factor
SES - Socioeconomic Status	VEGFR - Vascular Endometrial Growth Factor Receptor
SH3 - SRC Homology 3	VIM - Vimentin
SHANK2 - Sh3 And Multiple Ankyrin Repeat Domains 2	W - White (Race)
Shh - Sonic Hedgehog	
snoRNA - Small Nucleolar RNA	

WHO - World Health Organization

Wnt - Wingless/Integrated

WV CTSI - West Virginia Clinical and Translational
Science Institute

Xist - X-inactivation specific transcript

XPO5 - Exportin 5

ZNF705E - Zinc Finger Protein 705E

Literature Review:

Head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) is an aggressive neoplasm that arises from squamous epithelial cells of the upper aerodigestive tract, encompassing the oral cavity, pharynx, and larynx(1). There are over 65,000 new HNSCC cases and 14,500 deaths estimated for 2020, with a five-year relative survival rate in the United States of 65% for all races and American Joint Committee on Cancer (AJCC) stages(2). Risk factors for the development of HNSCC include alcohol and tobacco use(3) and high-risk human papillomavirus (HPV) infection(4) (Figure 1).

Figure 1. Characteristics of head and neck squamous cell carcinoma.

Treatment of HNSCC depends on anatomical site (surgical accessibility) and stage of disease. For early stage patients (AJCC stage I or II), which represent 30-40% of new diagnoses(1), HNSCC is curable with surgical resection or radiotherapy alone(5). For patients with locally advanced disease (AJCC stage III or IV), which represent over 60% of new diagnoses(1), surgical resection followed by adjuvant radiotherapy or

chemoradiotherapy is used when surgical accessibility is feasible. Chemoradiotherapy alone is the curative standard of care when surgery is not feasible(5). Chemotherapeutic options for HNSCC include high dose cisplatin as the standard of care(5). To date, there are only two United States Food and Drug Administration (FDA) approved targeted therapies for HNSCC. The epidermal growth factor receptor (EGFR) inhibitor cetuximab was approved for use in 2006 to be used in certain circumstances, such as in cases with high EGFR where it was shown in clinical trials to increase 5-year survival when compared to radiotherapy alone(6), but platinum-based chemotherapy remains standard(5). However, if a patient tumor expresses the immune checkpoint protein programmed death ligand 1 (PD-L1), the patient is eligible for PD-L1 checkpoint inhibiting drugs such as pembrolizumab or

nivolumab(5). Pembrolizumab has been shown in clinical trials to increase 5-year survival of recurrent or metastatic patients when compared to radiotherapy alone as well as cetuximab and radiotherapy(7). The lack of additional therapeutics for HNSCC treatment underscores the persistent need for the continued development of new drugs against known important gene products, as well as continued identification and testing of novel targets for improved treatments in populations more refractory to conventional clinical approaches. Patient survival is largely dependent on stage of diagnosis as well as treatment regimens (Figure 2), highlighting the need for additional treatment options.

Widespread next-generation sequencing of HNSCC cohorts has identified driver mutations, common tumor-suppressor and oncogenes, and molecular subtypes(8–11), concluding that HPV-positive tumors harbor fewer mutations and copy-number alterations (CNAs) than HPV-negative tumors. This is in agreement with HPV-positive patients exhibiting better overall survival and stratifies HNSCC into two distinct prognostic

subtypes(12,13). HNSCC driver mutations identified in both HPV-positive and HPV-negative HNSCC are discussed throughout this review.

HPV-positive HNSCC incidence has been increasing and accounts for nearly 20% of all current HNSCC cases(14). HPV-positivity is significantly enriched in oropharyngeal tumors, where it is responsible for 60-80% of all HNSCC in this region(14–16). The remainder of HNSCC cases are HPV-negative and are described later in this review. High-risk HPV subtypes 16 and 18 are responsible for the majority of HPV-positive HNSCC, predominantly diagnosed in younger people after oral sex exposure(17) and is usually due to infection of the reticular epithelium lining the tonsillar crypts of the oropharynx(18–20).

HPV has a double-stranded, circular genome with genes important for early viral functions (E1-7) that mostly act by blocking host cell division and viral genome replication, as well as genes involved in late viral functions (L1-2) such as capsid development(21). Early viral proteins E6 and E7 are oncoproteins that target the tumor-suppressors retinoblastoma (pRb) and p53, respectively (Figure 3A). E6 disrupts normal cell growth inhibition by inhibiting p53, leading to degradation of p53 through ubiquitination by ubiquitin-protein ligase E3A (E6AP)(22). E7 targets pRb family members for degradation, which results in sequential activation of the transcription factor E2F, expression of S-phase genes and ultimately hyperproliferation. Degradation of pRb and release of E2F also leads to overexpression of the tumor-suppressor p16, which is used as a surrogate marker for HPV-positive

tumors as it is commonly overexpressed in HPV positivity tumors(23) (Figure 3B). As such, p16 immunohistochemistry (IHC) is currently used by the National Comprehensive Cancer Network (NCCN) as a diagnostic step to determine treatment of oropharyngeal cancers(5).

While treatment for HPV-positive tumors has largely remained the same as treatment of HPV-negative tumors, the prospect of treatment de-escalation has been proposed due to the increased chemo-radiosensitivity and curative rate of HPV-positive tumors(24–26). To this end, AJCC revised staging of HPV-positive tumors in AJCC-8, effective January 1, 2017, classifying most HPV-positive tumors as a lower stage(27). This is an important consideration for treatment guidance(5). However, recent studies have shown that some patients are undertreated using these guidelines, compromising patient safety(28). Prophylactic HPV vaccines have been approved for oropharyngeal cancer prevention, yet HPV-associated oropharyngeal incidence trends are not expected to reverse until after 2060(17). This expected delay is due to the vaccine being given to young individuals before sexual activity, the required time needed to achieve vaccine coverage in a population, and the time required to develop HPV-positive HNSCC(29).

HPV-negative HNSCC has a worse prognosis compared to HPV-positive HNSCC. This is mainly due to carcinogen-induced genomic instability that creates tumors with high mutational load, deleterious epigenetic alterations, large-scale chromosomal rearrangements and high intra-tumor heterogeneity (Figure 4). The aggregate effect of this genotoxic stress exerts a pleiotropic molecular impact on oncogenic processes that increases disease aggressiveness and lowers patient survival(8,9,30,31). The most common large-scale CNAs in HPV-negative HNSCC include amplifications of 7p11.2 (*EGFR*), 11q13.3 (*CCND1*), 8p24.21 (*MYC*), and 3p26.32 (*PIK3CA*) and homozygous deletion of 9p21.3 (*CDKN2A*). The most common somatic mutations in HPV-negative HNSCC include the tumor-suppressors *CDKN2A* and *TP53*. The most common CNA and somatic mutation in HPV-positive HNSCC is of *PIK3CA*(8).

To briefly describe these alterations, *EGFR* is part of the erythroblastic oncogene B (ErbB) family of receptor tyrosine kinases (RTKs) that responds to extracellular mitogens, driving downstream signaling via epidermal growth factor (EGF) binding, dimerization, autophosphorylation, and recruitment of adaptor proteins. EGFR has been shown to activate the Ras-Raf-MEK-ERK pathway described later in this review. The PI3K-AKT-mTOR

pathway, which consists of phosphoinositide 3-kinases (PI3Ks) such as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), protein kinase B (AKT), and mammalian target of rapamycin (mTOR) regulates downstream molecules that are mainly related to metabolism and cell cycle(32). Activation of the Ras-Raf-MEK-ERK pathway can also induce Wnt (wingless/integrated) and Shh (Sonic hedgehog) signaling, which in turn can activate the transcription factor Myc(33). Amplification of *EGFR*, amplification of *MYC*, or mutation to *PIK3CA* lead to aberrant downstream signaling of these pathways and altered expression of various genes(34).

Cyclin D1 (*CCND1*) is a cell cycle regulator that is regulated by cyclin-dependent kinase inhibitor 2A (*CDKN2A*). These proteins are discussed in-depth later in this review. Tumor protein 53 (*TP53*) is a tumor-suppressing transcription factor that regulates the cell cycle, apoptosis, and genomic stability(35).

Combining genetic characteristics and gene expression profiles of HPV-negative HNSCC stratifies the disease into three distinct subtypes: basal (BA), classical (CL), and inflamed/mesenchymal subtypes (IMS)(9) (Figure 5). Characteristics of the BA subtype include enriched hypoxia signaling (*HIF1A*, *CA9*, and *VEGF*), neuregulin signaling (*EGFR*, *AREG*, and *NRG1*), and overexpression of the epithelial marker *CDH3* and cytokeratins *KRT1* and *KRT9*. This subtype is descriptive of nearly 40% of all cases and has been shown to be preferentially responsive to EGFR-target therapies(36). Characteristics of the CL subtype include enrichment for polyamine degradation pathways, which is related to tobacco detoxification. CL is also divided by HPV status into CL-HPV and CL-nonHPV, which make up 8.0% and 18.2% of all cases, respectively. While these two groups have similarities, they are still distinct in certain pathways, including cell-cycle and cell-division regulation. The IMS

subtype features expression of genes related to T lymphocyte infiltration (*CD8*, *ICOS*, *LAG3*, and *HLA-DRA*), overexpression of mesenchymal markers (*VIM*), overexpression of matrix metalloproteinases (*MMP9*), and downregulation of the epithelial marker *CDH3* and cytokeratins *KRT1* and *KRT9*. IMS is also divided by HPV status into IMS-HPV and IMS-nonHPV, two distinct subtypes that share characteristics, but differ in cell-cycle and smoking-associated pathways. They are responsible for 12.9% and 20.9% of all cases, respectively(9). Patient outcome differs between all subtypes, with the HPV-related subtypes CL-HPV and IMS-HPV having

better overall survival than tobacco-related subtypes BA, CL-nonHPV, and IMS-nonHPV(9). Although many driving mutations in HNSCC have been extensively studied, most are related to alteration of protein expression. In Study 3, we evaluated the potential contributions to tumor progression through alteration of mRNA expression.

Cancer in Appalachia

Currently available data from the CDC show that cancer incidence and mortality rates have varied geographically (Figure 6) from 2013-2017 with higher rates present in the Appalachian region. The United States Appalachian region has a long history of diverse factors, such as low socioeconomic status (SES) and increased tobacco usage that contribute to the overall allostatic load responsible for increased cancer incidence and poorer patient outcomes(37). High cancer incidence in Appalachia has been recognized by the Appalachian Regional Commission (ARC) and the National Cancer Institute (NCI) as a health disparity since 1993, with high incidence rates remaining high in the region since that time(37–41).

**A. Incidence Rates (%) by State, All Cancers
2013-2017, All Races, Sexes, Ages**

**B. Death Rates (%) by State, All Cancers
2013-2017, All Races, Sexes, Ages**

Figure 6. Incidence (A) and Death (B) rates of U.S. by state.

The United States Appalachian region currently encompasses 205,000 square miles across 420 counties in 13 contiguous states surrounding the Appalachian Mountains(37). The legal definition of Appalachia has changed multiple times since the ARC was first established in 1965 through amendments to United States Code(42). The demographics of the region portray a population that is over 80% white and 42% rural, with the region reporting higher unemployment, lower income, and higher poverty rates than most of the rest of the United States(43).

Recently, cancer incidence in general has been shown to have disparate and increasing incidence in Appalachia compared to non-Appalachia, regardless of sex, race, region, or primary site(38). However, this incidence disparity has decreased over time for most primary sites, with the exception of HNSCC (oral cavity, pharynx, and larynx) and cancers of the lung, bronchus, and thyroid(38), most of which are tobacco-associated malignancies(38,39,44–47). Previous work evaluating oral cancer survival in Appalachia using 2004 Surveillance, Epidemiology, and End Results (SEER) data identified higher mortality in 10 Appalachian states including West Virginia, Alabama, Pennsylvania, Tennessee, Kentucky, Georgia, South Carolina, Mississippi,

BRFSS Current Smoker Prevalence by Year 1986-2010, 2011-2018

Figure 7. Smoking tobacco usage in United States, 1986-2010, 2011-2018.

North Carolina, and Ohio(48). Of the cancer risk factors endemic to Appalachia, age at diagnosis, travel distance to critical care centers, and a disproportionate presentation of patients at a late AJCC stage have been reported to have no impact on survival in Appalachian subpopulations(49,50). However, increased smoking and smokeless tobacco use for several decades within Appalachia have been linked to low SES and increased HNSCC incidence(51–53) and presumably contribute to HNSCC incidence and mortality disparities within Appalachia (Figure 7). A systematic evaluation within the Appalachian region to identify specific subregions or HNSCC sites responsible for driving disparate survival has not been conducted to date.

Tobacco and Cancer

There has been a presumed connection between tobacco usage and cancer development since Nazi Germany first investigated links between tobacco exposure and lung cancer in the late 1930s(54). The first clear evidence was published in 1954, which linked smoking habits to the mortality of British doctors(55). Evidence has mounted since then, and in 2004 the United States Surgeon General concluded that evidence is sufficient to infer a causal relationship between smoking tobacco usage and the development of tumors of the lung, larynx, oral cavity, pharynx, esophagus, pancreas, bladder, kidney, cervix, and stomach, as well as acute myeloid leukemia(56).

Incinerated tobacco products including cigarettes or cigars contain compounds that have been attributed to this relationship with cancer by previous work and the World Health Organization (WHO) International Agency for Research on Cancer (IARC). The most extensively investigated compounds include polycyclic aromatic hydrocarbons (PAHs), heavy metals, aldehydes, aromatic amines, and tobacco-specific nitrosamines (TSNAs). These compounds can be naturally produced by the tobacco plant or can be by-products from the combustion process, referred to as pyrolysis. Nicotine is a tobacco-generated alkaloid which the plant uses for defense against herbivorous insects(57) and is the addictive, stimulating substance within tobacco products due to its similarity to the neurotransmitter acetylcholine(58). Although nicotine is the addictive substance that drives tobacco consumption and uptake of tobacco-associated compounds, it has not been shown to be carcinogenic(56). PAHs are formed by the incomplete combustion of biological material. The United States Environmental Protection Agency (EPA) has identified 16 key PAHs, including benzo[a]pyrene, that cause or may cause various types of cancer(59,60), all of which are found in tobacco smoke(61). PAHs have been shown

to likely be involved in the carcinogenesis of lung, laryngeal, oral cavity, and cervical cancers(62). Heavy metals, including the human carcinogen cadmium(60,63), are absorbed from the soil into the tobacco leaves(64) and are released into tobacco smoke when incinerated(65). Heavy metals are likely to be involved in the carcinogenesis of lung cancer(62). Volatile carbonyl compounds, including acetaldehyde, formaldehyde, acetone, and 2-butanone, are formed from heating amino acids and sugars in the tobacco leaf(66) and are classified as possibly carcinogenic to humans by the IARC(60,67). Aldehydes are likely to be involved in the carcinogenesis of lung and nasal cancers(62). Aromatic amines, including 2-naphthylamine, are found in unburnt tobacco and are also formed as pyrolysis products(68) and are known human bladder carcinogens(60,69). TSNAs are another class of chemical compounds that have been implicated in cancer development and include *N*-Nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which have been shown to be carcinogenic(60,70). TSNAs do not occur naturally within the tobacco plant and are not only a product of tobacco combustion, but are products of aerobic bacteria during drying and curing of tobacco(71). TSNAs are likely to be involved in the carcinogenesis of lung, nasal, oral cavity, esophageal, liver, pancreatic, and cervical cancers(62).

Smokeless tobacco, which is a tobacco product intended to be consumed without combustion but instead by direct absorption through the oral mucosa in the form of chewing tobacco, snuff, or snus, has a shorter list of carcinogenic compounds due to the lack of combustion. Smokeless tobacco does contain PAHs (benzo[a]pyrene), heavy metals (cadmium), carbonylic compounds (formaldehyde), and TSNAs (NNK and NNN), among others(72). Although smokeless tobacco still contains similar carcinogenic compounds when compared to smoking tobacco, the levels of these compounds are lower in smokeless tobacco(73). Electronic cigarettes are an interesting area in the development of a safer form of tobacco consumption. Several studies have shown that electronic cigarettes deliver a lower concentration of TSNAs(74,75), but more research is required to determine how safe electronic cigarettes are in general or as a replacement for traditional smoking or smokeless tobacco(76).

The carcinogenic compounds within tobacco typically require metabolic activation, usually catalyzed by cytochrome P-450 enzymes, to convert them into a form that can covalently bind to DNA and form a DNA adduct (Figure 8). Differences in the efficiency of this metabolic activation or carcinogen detoxification may be able to explain cancer susceptibility between individuals(77). Two classes of tobacco-associated carcinogens that have been extensively studied concerning DNA adducts include PAHs and TSNAs. The molecular activation of benzo[a]pyrene (PAH) occurs through conversion to benzo[2]pyrene-7,8-diol-9,10-epoxides (BPDEs). This molecule forms adducts with deoxyguanosine to form BPDE-N2-deoxyguanosine(78). The molecular activation of NNN through α -hydroxylation creates the same intermediate as methyl hydroxylation of NNK, pyridyloxo-butyl (POB)-DNA adducts(79). There are several mechanisms that cells can use to remove tobacco-generated DNA adducts, such as alkyltransferases, nucleotide excision repair, mismatch repair, and double strand break repair(62,80). However, if the enzymes required for these repair mechanisms are unable to repair the DNA adduct, either through loss-of-function via mutation or being utilized elsewhere to repair widespread DNA damage, these DNA adducts can persist and be converted to genomic mutations through error-prone repair mechanisms or incorrect nucleotide insertion during translesion DNA synthesis(80) (Figure 9). Double stranded DNA breaks can also occur through oxidative damage to DNA linked to tobacco exposure(81). These double strand breaks can cause large chromosomal aberrations which can result in deletions and amplifications of genomic DNA(82). The tumor-suppressor p53 is one of the most important proteins for detecting and repairing DNA damage(83). Thus it is not surprising that mutations to *TP53* are the most common mutations in human malignancies(84), preventing the cell to properly address DNA adducts, resulting in the eventual accumulation of mutations and chromosomal aberrations(85,86).

Chromosomal Instability and Amplification of Chromosome 11q13

Chromosomal instability is a hallmark of cancer(87) that is defined as the rate of genetic aberrations seen within a cell or tumor and includes small mutations, insertions, and deletions. Instability also includes larger chromosomal rearrangements such as translocations and amplifications, and also includes generation of aneuploidy through gain or loss of entire chromosomes(88). Chromosomal instability is categorized into two main groups. The first is numerical chromosomal instability, which depicts the gain or loss of entire chromosomes. The second is structural chromosomal instability, which characterizes large chromosomal fragment changes that include chromosomal rearrangements and CNAs. CNAs include amplifications (more than two extra copies, +2), gene gains (two extra copies, +1), heterozygous deletions (missing one copy, -1), or homozygous deletions (missing both copies, -2)(88,89). Chromosomal instability has been linked to cellular transformation, tumor heterogeneity, metastasis, tumoral drug resistance, and worse patient outcomes(90–93).

CNAs that arise from chromosomal instability are important areas of research due to the multitude of lost tumor-suppressors and gained oncogenic drivers which shape tumor progression. CNAs can range in size from focal alterations that include relatively few genes and comprise less than 25% of the length of a chromosome arm or alterations smaller than 3 Mb, to larger arm-length alterations that can encompass the length of an entire chromosome arm(94,95). The shorter length of focal CNAs allows more straightforward determination of driving alterations, which remains difficult in large scale, arm-length alterations. Pan-cancer analyses have shown that many CNAs are specific to cancer type, but there are common deletions and amplifications found shared

amongst multiple cancers. The most common focal amplifications across multiple cancer types include *MYC*, *CCND1*, *ERBB2*, *CDK4*, *NKX2-1*, *MDM2*, *EGFR*, *FGFR1*, and *KRAS*- all of which are documented oncogenes(96,97). The most common focal deletions across multiple cancer types include those of *CDKN2A*, *PTEN*, and *NF1* which are all tumor-suppressors(96,97).

To briefly describe these alterations, receptor tyrosine-protein kinase erbB-2 (*ERBB2/HER2*), fibroblast growth factor receptor 1 (*FGFR1*), and *EGFR* are all RTKs that function in signaling transduction to activate the (*KRAS*)-Raf-MEK-ERK pathway(34) that is regulated by neurofibromin 1 (*NF1*)(98), Wnt/Shh signaling which activates *MYC*(33), and the PI3K-AKT-mTOR pathway that is regulated by phosphatase and tensin homolog (*PTEN*)(32). Thyroid transcription factor 1 (TTF-1) or NK2 homeobox 1 (*NKX2-1*) is a transcription factor important for lineage survival as well as enhancing EGFR-driven tumorigenesis(99). Murine double minute (*MDM2*) functions an oncogene by inhibiting p53-mediated transactivation(100,101). *CCND1* is a cyclin-dependent kinase 4 (*CDK4*)/*CDK6* regulator that is involved in cell cycle regulation and is regulated by *CDKN2A* that will be discussed in-depth later in this review.

Fragile sites within normal chromosomes are sites of genomic instability during DNA replication stress. There are two classes of fragile sites, including rare fragile sites that are expressed (experience breakage) in less than 5% of individuals and common fragile sites (CFSs) that are found in all individuals(102–107). Fragile sites are

usually located within large genes and have been linked to secondary structures of DNA and replication fork stalling due to replicative stress(107) (Figure 10). During replication, DNA strands are separated to expose the DNA template to DNA replication machinery, which allows for single stranded DNA to form secondary structures. If the replication fork is stalled due to replication stress, such as by tobacco-associated DNA-adducts as discussed in the previous section(108), more time is allowed for these secondary structures to form. This free, single strand DNA is then exposed to environmental damage and strand breaks may occur. Targeted cleavage of DNA by structure-specific endonucleases (SSEs) attempting to resolve the stalled fork can also lead to strand breaks(107). If stalled forks near fragile sites do not get resolved, DNA bridges may assist during nuclear division, which will lead to the mechanical breakage of DNA strands(107). Loss-of-heterozygosity or homozygous deletions can occur if multiple stalled forks are cleaved through deletion of the DNA between replication forks(107). If a double stranded DNA break remains unresolved during replication, it is possible for the broken strand to fuse to a sister chromatid and begin a breakage-fusion-bridge (BFB) cycle (Figure 11), which can lead to chromosomal amplification(109–111).

Figure 11. Diagrammatic representation of breakage-fusion-bridge mechanism.

When evaluating HNSCC for chromosomal aberrations, several alterations stand out and are common in an appreciable number of patients(8,112,113). CNAs within HNSCC vary by HPV status(8,112,114–117), with HPV-negative patients harboring a higher number of total CNAs, likely due to increased tobacco exposure(8,112,113).

The most common large-scale chromosomal amplification within HNSCC is the amplification of chromosome 11q13.3-q13.4. This amplification is found in nearly a third of all HPV-negative HNSCC patients and contains several genes of oncogenic interest, including the potential oncogenesis-promoting genes *CCND1*, *FADD*, and *CTTN*(8,113,118,119). The mechanism of 11q13 amplification has been linked to BFB(120) and appears to involve fragile sites *FRA11H*, and *FRA11F*(121,122). However, other mechanisms such as amplification involving segmental duplications, which do not require the loss of a telomere, have also been proposed(123).

Genes of note within the 11q13 amplicon (Figure 12) include the cell cycle regulator *CCND1* which will be discussed in depth later in this review. Briefly, cyclin D1 functions as the regulatory subunit of CDK4/6, whose activity is required for cell cycle transition from G1 to S(124). Overexpression of cyclin D1 protein leads to a constitutively active cell cycle and can lead to tumorigenesis(125). Oral cancer overexpressed 1 (*ORAOV1/LTO1*) has recently been shown to be involved in relief of reactive oxygen species (ROS)(126) and its overexpression has been shown to be a biomarker of progression(127). Liprin-α1 (*PPFIA1*) has been shown

to localize LAR phosphatase to cell focal adhesions and has been shown to be involved in cell invasion, cell spreading, and cancer cell signaling(128–130). Anoctamin-1 (*ANO1*)/Transmembrane member 16A (*TMEM16A*) is a calcium-activated voltage-gated chloride channel that has been shown to play a role in inhibiting apoptosis(131). Fas-associated death domain (*FADD*) is involved in apoptosis by mediating the formation of death-inducing signaling complexes (DISC) during programmed cell death(132). Although *FADD* has been associated with HNSCC metastasis, its tumor-promoting functions are poorly understood(133,134). Fibroblast growth factor 3 (*FGF3*) acts as a negative regulator of bone growth through fibroblast growth factor receptor 3 (*FGFR3*)(135). Amplification and overexpression of *FGF3* is associated with increased invasiveness in breast cancer(136). Fibroblast growth factor 4 (*FGF4*) functions in embryonic development` (137) through an undetermined fibroblast growth factor receptor and is involved in human stomach cancer(138). Fibroblast growth factor 19 (*FGF19*) functions as a hormone in the regulation of bile acid synthesis and signals through fibroblast growth factor receptor 4 (*FGFR4*)(139). *FGF19* amplification has been tied to autocrine signaling that promotes tumor growth(139). Cortactin (*CTTN*) is an actin-binding protein that regulates Arp2/3 and the branching of actin networks to facilitate cellular invasion, migration, and tumor metastasis(140–142). Myeloma overexpressed (*MYEOV*) appears to be involved in cell migration and is a prognostic marker in multiple myeloma and colon cancer(143,144). SH3 and multiple ankyrin repeat domains protein 2 (*SHANK2*) is a scaffold protein involved with actin dynamics at neuronal spines and synapses(145–147) and does not have an identified role in cancer.

Cyclin D1

CCND1, originally referred to as parathyroid adenomatosis (*PRAD1*), was first identified as a candidate oncogene in 1991 when it was found to be part of a genomic rearrangement with parathyroid hormone (PTH) in parathyroid adenomas, leading to its overexpression(148,149). Shortly after, it was implicated in genomic translocations in B-cell lymphoma where it was referred to as B-cell lymphoma 1 (*BCL1*)(150). *PRAD1* was then confirmed to be a novel cyclin. Cyclins were identified due to their cyclic accumulation and subsequent destruction that varied depending on stage of cell cycle through the fertilization of marine invertebrate eggs(151). At the time of this discovery, two types of human cyclins had been characterized: A-type, important for the early cell cycle, and B-type, which drove the cell into mitosis. *PRAD1* was similar in sequence to all cyclins, but could not fit into either group, suggesting it was part of a new cyclin family and was then renamed cyclin D1 (*CCND1*)(152,153). *CCND1* was then confirmed to be a nuclear protein that is required during cell cycle progression in G1(124).

The transcription regulation and cellular function of *CCND1* has been elucidated in great detail (Figure 13, left). The best-studied activators of cyclin D1 transcription are mitogenic growth factors, which act through mitogen-activated protein kinases (MAPKs)

in the canonical Ras-Raf-MEK-ERK pathway(154). Briefly, small proteins that can induce a cell to begin mitosis through extracellular signaling are referred to as mitogens. These mitogens interact with RTKs on the cell surface such as EGFR, fibroblast growth factor receptor (FGFR), and vascular endothelial growth factor receptor (VEGFR)(155). After receptor-ligand binding, RTKs signal through adapter proteins and guanine nucleotide

exchange factors (GEFs) such as growth factor receptor-bound protein 2 (GRB2) and son of sevenless homolog 1 (SOS1) which lead to the activation and release of the GTPase Ras (HRAS). HRAS will then begin a phosphorylation cascade through the Ras-Raf-MEK-ERK pathway which includes BRAF, mitogen-activated protein kinase kinase (MEK) and ultimately extracellular signal-related kinases (ERKs)(155). This pathway leads to the dimerization of Jun and Fos to produce the activator protein 1 (AP1) transcription factor and subsequent transcription of *CCND1*(154). Once transcribed, *CCND1* transcript can be regulated by both AU-rich elements (AREs) which stabilize the transcript and by microRNAs that can block translation or degrade the transcript(156). After translation, cyclin D1 protein functions as a regulatory partner with CDK4 or CDK6 (Figure 13, right). Cyclin D1 binds to CDK4/CDK6, allowing this complex to be activated by cdk-activating kinase (CAK)(157) where it enters the nucleus and then poly-phosphorylates pRB. This phosphorylation event causes a conformational change, allowing pRB to release pRB-bound E2F transcription factors which are responsible for the transcription of S phase genes(158,159). E2F target genes that are induced by this interaction include DNA polymerase alpha, thymidylate synthase, and ribonucleotide reductase, all of which are involved in DNA synthesis in S phase(160).

CCND1 has been established as a human oncogene via amplification or overexpression in breast cancer, lung cancer, melanoma, mantle cell lymphoma, and oral cancer(161). *CCND1* overexpression predominately occurs because *CCND1* is one of the most common CNAs in human cancer(96), but overexpression in mantle cell lymphoma is due to the translocation of *CCND1* that places it next to the immunoglobulin heavy chain locus (IGH)(162). The role of cyclin D1 in cancer is through increased CDK4/CDK6 activity, leading to increased pRB phosphorylation, hyperactivation of E2F-responsive genes and ultimately increased proliferation of tumor cells. However, there have been other non-canonical functions of cyclin D1 that have been suggested that can also contribute to its oncogenic ability. These functions fit into other hallmarks of cancer and include evading growth suppressors(163), activation of invasion and metastasis(164), enabling replicative immortality(165), inducing angiogenesis(166), and resisting cell death(167). The activity of CDK4/CDK6 is regulated by a family of tumor-suppressor proteins referred to as inhibitors of CDK4 (INK4). This family includes p16 INK4A, p15 INK4B, p18 INK4C, and p19 INK4D which inhibit CDK4/CDK6 by binding directly to the kinase(168,169). Therapeutic targeting of the proliferate functions of cyclin D1 have been an area of interest which has focused on using small molecule selective inhibitors of CDK4/CDK6. To date, there are three FDA approved CDK4/6 inhibitors:

palbociclib, abemaciclib, and ribociclib, all of which are approved for treatment of HR-positive and HER2-negative breast cancer(170,171). While clinical trials are still underway for other cancer types, HR-positive and HER2-negative breast cancer remains the only approved cancer for CDK4/6 inhibitor treatment(172).

Non-coding and Competing Endogenous RNA

A discrepancy between annotated RNA molecules and RNA molecules found within the human transcriptome has been noticed for some time(173–175). Recent studies have attempted to elucidate the function of some of these “junk” RNA molecules and have yielded new classes of RNA with new biological functions. To date there are many classes of RNA with varying functions including the canonical RNA involved in protein synthesis (messenger RNA, mRNA; ribosomal RNA, rRNA; transfer RNA, tRNA), RNA involved in DNA replication or post-transcriptional modification (small nucleolar RNA, snoRNA; guide RNA, gRNA) and RNA involved in regulatory functions (long non-coding RNA, lncRNA; microRNA, miRNA)(176), the focus of this review. Many of these RNA classes lack open reading frames (ORFs) and do not code for a protein product, which could solve the observed discrepancy.

One group of small regulatory RNA molecules are miRNAs, which are small RNAs that range from 20 to 30 nucleotides and do not have an ORF. The first miRNA was discovered in *Caenorhabditis elegans* in 1993 and was named small temporal RNA (stRNA) *lin-4*(177). It was then observed that *lin-4* had complementary sequences to 3' untranslated region (3'UTR) of the *C. elegans* gene *lin-14* and was important for its temporal regulation during development(178). It took some time for other miRNA to be observed in *C. elegans* or in other species. The next miRNA to be observed was *let-7* in *C. elegans*, which was again important for development. Excitingly, homologs for *let-7* were found in humans and other animals(179). Multiple laboratories quickly found over 100 different small RNAs that came from a stem-loop precursor and were also evolutionarily conserved. However, these did not seem to be temporally expressed and were hence renamed to miRNA(180–182). To date, there have been thousands of miRNAs described in humans alone(183,184).

Canonical biogenesis of miRNA begins with transcription by RNA polymerase II into pri-miRNAs(185) (Figure 14). These are then processed into pre-miRNAs by the microprocessor complex which includes RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and the ribonuclease III enzyme Drosha(186). These

pre-miRNAs are then exported into the cytoplasm by exportin 5 (XPO5) and are processed into a mature miRNA duplex by the RNase III endonuclease Dicer, which cleaves the hairpin loop(186,187). These free strands of miRNA can then be loaded into the Argonaute (AGO) family of proteins(188) to form the miRNA-induced silencing complex (miRISC)(189) and miRNA-induced gene regulation can occur.

The miRISC complex can regulate gene expression via multiple mechanisms. The majority of miRISC gene regulation occurs through complementary sequences between the miRISC-bound miRNA and sequences in other RNA molecules called miRNA response elements (MREs). The

degree of complementary interaction between the miRNA:MRE and the AGO in the miRISC determines the mechanism of regulation. If the interaction is fully complementary and AGO2 is in the miRISC, this will result in direct AGO2-dependent endonuclease activity and degradation of the RNA(190). However, most miRNA:MRE interactions in animals are not fully complementary, so target degradation occurs through another mechanism. This begins with recruitment of trinucleotide repeat-containing gene 6A protein (*TNRC6A/GW182*) and poly(A)-binding protein C (PABPC) which promotes deadenylation of the target transcript(191,192). Decapping of the transcript is then facilitated by decapping protein 2 (DCP2), which is then followed by target degradation by exonucleases(192,193). Not all miRISC interactions lead to immediate degradation of target RNAs through this mechanism. There is also evidence that miRISC can inhibit gene translation through blocking translation machinery initiating translation or inhibiting translation after it has initiated(190,194). It is also not necessary for

this regulatory mechanism to result in degradation of target RNA, as multiple studies have shown that these interactions can result in a decrease in target protein without changes in target mRNA levels(195–198). Due to this observation, it is possible for miRNA to be sequestered or “sponged” by target mRNAs, lncRNAs, and circRNAs with shared MREs to mitigate their regulatory role(199).

Regulatory RNAs that are longer than miRNAs include long non-coding RNAs (lncRNAs). lncRNAs are RNA molecules that are longer than 200 nucleotides and either lack an ORF or code for small peptides less than 100 aa(200). lncRNA were first described in 1990 with the discovery of the regulatory lncRNA *H19*(201) and the X-inactivation linked lncRNA *Xist*(202). lncRNA can exist in the genome as stand-alone RNAs that are transcribed and polyadenylated(203), or as antisense transcripts opposite of sense coding genes(204), or as pseudogenes that have lost their coding potential due to mutations(200), or as intronic sequences(205), or as other promoter-associated and enhancing RNAs(206).

There have been many described lncRNAs involved in a variety of functions including epigenetic and transcriptional decoys and regulators. The lncRNA HOTAIR functions as a recruiter for the polycomb repressive complex 2 (PRC2) and regulates chromatin state of genes, including *HOXD* which is involved in differentiation and limb development(207). lncRNAs can also function as molecular recruiters by recruiting epigenetic complexes during transcription. The DNA:RNA complementarity of lncRNAs allow them to target these complexes to specific genomic positions, something that is not possible with protein-driven recruitment(208). Some lncRNAs act as decoy RNA and compete for transcription factor binding(209) while others can directly inhibit RNA polymerase II(210), both resulting in a downregulation in transcription. On the other hand, lncRNAs can also function as coregulators to activate transcription, such as ncRNA_{CCND1} regulation during stress(154).

Aberrant expression levels of miRNAs and lncRNAs have been shown to be involved in cancer. In HNSCC, several oncogenic miRNA expression levels are altered, including *miR-21* and *miR-31*, which are well documented oncogenic miRNAs that are detectable in plasma(211–215) and indicate a poor prognosis. *miR-21* is the most frequently upregulated miRNA in HNSCC and is involved in drug resistance, invasion, cell survival, and metastasis(216). Well known targets of *miR-21* include the tumor suppressors *GRHL3* and *PTEN*(212,216–219). *miR-31* is also significantly upregulated in HNSCC and functions by targeting factor-inhibiting-factor (HIF),

subsequently activating HIF and its downstream targets, ultimately contributing to the development of HNSCC(220,221). Other aberrant miRNA expressions have been noted, especially in the case of gene amplification, but their cellular targets are still under investigation. Tumor suppressing miRNA can also experience expression changes in HNSCC, such as the *let-7* family which includes *let-7a-j* and *miR-98* that regulate cell renewal, cellular motility, and EMT regulation(222). The miR-99 family normally targets the mTOR signaling pathway to inhibit the phosphorylation of AKT. Downregulation of these miRNA, including through gene deletion, can lead to the initiation of HNSCC(223–225). Hundreds of lncRNAs have been shown to be differentially expressed in HNSCC(226) with the most extensively studied lncRNA being *MALAT1*, which is notably located on chromosome 11q13.1. High levels of *MALAT1* are associated with increased lymph node metastasis and poor patient prognosis(227,228).

The presence of MREs on cellular mRNA, lncRNA, and other RNA molecules leads to the possibility that there is another layer of complexity involved in RNA regulation due to these molecules all competing for the same pool of cellular miRNA. This concept was first discussed in 2011 and is referred to as the competing endogenous RNA (ceRNA) hypothesis(229). Briefly, the hypothesis states that since the levels of MREs and miRNAs are in balance, any change to this balance, be it either from an increase in the amount of MREs through target gene overexpression or a decrease in miRNA due to mutation, will also alter expression of other target RNAs that share the same MREs(229–232) (Figure 15). This is still a controversial hypothesis as there is both ample evidence, such as ceRNAs that alter expression of the tumor suppressor PTEN(233) or BRAF(234) and stoichiometric concerns that state that changes in miRNA target abundance is unlikely to cause effects on gene expression(235).

Despite these conflicting reports, ceRNAs have been implicated in several disease states including cardiovascular disease with roles in atrial fibrillation, myocardial infarction, and cardiac hypertrophy(236–238); neurodegenerative disorders with roles in Parkinson's and Alzheimer's disease(239,240); and cancer(241–245). It remains clear that additional studies are necessary to further bridge this gap of knowledge to either support or reject the ceRNA hypothesis.

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Study 1: Disparate Survival of Late-stage Male Oropharyngeal Cancer in Appalachia

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Abstract

The United States Appalachian region harbors a higher cancer burden than the rest of the nation, with disparate incidence of head and neck squamous cell carcinomas (HNSCC), including oral cavity and pharynx (OC/P) cancers. Whether elevated HNSCC incidence generates survival disparities within Appalachia is unknown. To address this, HNSCC survival data for 259,737 tumors from the North American Association for Central Cancer Registries (NAACCR) 2007-2013 cohort were evaluated, with age-adjusted relative survival (RS) calculated based on staging, race, sex, and Appalachian residence. Tobacco use, a primary HNSCC risk factor, was evaluated through the Behavioral Risk Factor Surveillance System (BRFSS) from Appalachian states. Decreased OC/P RS was found in stage IV Appalachian white males within a subset of states. The survival disparity was confined to human papillomavirus (HPV)-associated oropharyngeal cancers, specifically the oropharynx subsite. This correlated with significantly higher smoking and male smokeless tobacco use in most Appalachian disparity states. Lower survival of Appalachian males with advanced-stage HPV-associated oropharyngeal cancers suggests pervasive tobacco consumption likely generates more aggressive tumors at HPV-associated oropharynx subsites than national averages. Comprehensive tobacco and HPV status should therefore be evaluated prior to considering treatment de-intensification regimens for HPV-associated oropharyngeal cancers in populations with high tobacco consumption.

Introduction

HNSCC involves the epithelium of the oral cavity, pharynx and larynx. OC/P cancers are a major HNSCC subset, with nearly 11,000 deaths predicted in the US in 2020[1]. Risk factors include tobacco and alcohol use, and high-risk human papillomavirus (HPV) infection[2,3]. OC/P cancers are subdivided into HPV-associated oropharynx (HPV-associated) and non-HPV-associated oral cavity, hypopharynx, and nasopharynx (non-HPV-associated) cancers[4]. These designations are supported by studies indicating that non-HPV-associated cancers are primarily tobacco/alcohol induced, whereas HPV-associated cancers are predominantly caused by HPV infection[5]. Furthermore, HPV-negative or non-HPV-associated cancers consistently have poorer outcomes than HPV-positive or HPV-associated cancers, segregating these cancers as distinct diseases with differential clinical management[6,7]. While national incidence of non-HPV-associated cancers is decreasing due to tobacco cessation, HPV-associated cancers are increasing due to rising infection rates[8]. Regarding race, blacks with HNSCC present with more advanced disease, are older and have worse survival than whites, denoting a racial disparity[9–11]. Increased screening for HPV coupled with subsite analysis indicates that blacks with HNSCC have less HPV-positive cancer than whites, explaining differences in survival[12,13]. Consistent with this, HPV-associated cancers continue to increase in white males and in rural areas[4,14,15].

The Appalachian region encompasses 205,000 square miles across 420 counties in 13 contiguous states[16]. Forty-two percent of Appalachia is rural, with poverty rates in many counties 1.5 times above the national mean[16]. The region is over 80% white, with several states having higher percentages of oral and other HPV-associated cancers, and higher smoking and smokeless tobacco use[17–19]. Aggregate risk factors experienced by Appalachian residents contribute to a high allostatic load capable of generating cancer disparities in a region with low ethnic diversity[20]. Incidence disparities within Appalachia are well recognized[21] with rates higher for HPV- and tobacco-related malignancies[22–24]. Elevated risk factor exposure has been suggested as the prime reason for disparate incidence in Appalachian male HNSCC[24,25]. However, due to gaps in data availability and small patient numbers at the county level, details on how increased incidence impacts HNSCC survival in Appalachia have not been rigorously evaluated[25]. Here we have evaluated the RS of all HNSCC in the majority of Appalachian states from 2007-2013 and have identified an outcome disparity in white males with oropharynx cancers that corresponds with elevated smoking and smokeless tobacco use in the region.

Materials and Methods

HNSCC Cohort and Relative Survival Data

All methods were carried out in accordance with relevant guidelines and regulations. Survival data were generated by NAACCR and provided in Surveillance, Epidemiology, and End Results (SEER)*Stat approved under NAACCR IRB protocol 16-14, where all patient data was obtained following informed consent. Patients were diagnosed from 2007-2013 with tumors of International Classification of Diseases for Oncology (ICD-O)-3 histology types 8050-8084, 8120-8131 (squamous and transitional epithelium). Selecting all primary tumors matching selection criteria, the cohort includes tumors of the oral cavity (n=55,620/259,737, 21.4%) including ICD-O-3 site groups of tongue (C02.0-02.3, 02.9), floor of mouth (C04.0-04.9), and gum and other mouth (C03.0-03.9, C05.0, C05.8-05.9, C06.0-06.9); oropharynx (n=81,170/259,737, 31.3%) including ICD-O-3 site groups of tongue (C01.9, C02.4, C02.8), gum and other mouth (C05.1-05.2), tonsil (C09.0-09.9), oropharynx (C10.0-10.9), and “other oral cavity and pharynx” (C14.0-14.8); “other pharynx” (n=17,731/259,737, 6.8%) including ICD-O-3 site groups of nasopharynx (C11.0-11.9) and hypopharynx (C12.9-13.9); and larynx (n=60,296/259,737, 23.2%) including ICD-O-3 site group larynx (C32.0-32.9). Remaining tumors are additional primaries of miscellaneous ICD-O-3 site groups (n=44,916/259,737, 17.3%). The cohort includes variables denoting whether or not patients were Appalachian county residents at the time of diagnosis, and county economic status from the Appalachian Regional Commission (ARC)[26]. Appalachian states with survival data fit for use through the reporting period included New York, Pennsylvania, West Virginia, Kentucky, North Carolina, South Carolina, Georgia, and Alabama[27]. The majority of cases were white (n=221,939/259,737, 85.4%), stage IV (n=84,253/259,737, 32.4%), and male (n=193,647/259,737, 74.6%). Cases were evaluated using the Ederer II method and the “U.S. by race (W, B, AIAN, API) and Canada 1995-2012, Ages 0-99, State-county (modeled by varied state-county-ses)” life table. Included cases were microscopically confirmed for malignant behavior and followed at monthly intervals. Excluded cases were those that were diagnosed solely via death certificate or autopsy, and alive cases

with no survival time. Age-adjusted RS, defined as net survival measure representing cancer survival in the absence of other causes of death[28], was calculated using SEER*Stat for each patient stratification, with z-tests for significance testing between each group using an alpha level of 0.05. Cumulative (C)RS was used to compare groups with five years of available RS data and the maximum available follow-up RS was used to compare groups that failed to reach five years of follow-up.

Mapping

Maps were generated using 2014 cartographic boundary shapefiles from the United States Census Bureau[29] and Quantum Geographic Information System (QGIS) 3.2.2 software[30]. Appalachian state-Appalachian county refers to a county within Appalachia. Appalachian state-non-Appalachian county refers to a county outside of the Appalachian region, but within a state containing Appalachian counties. Non-Appalachian states lack Appalachian counties.

TCGA Cohort and Associated Clinical Data

Data from The Cancer Genome Atlas (TCGA) were obtained from the Broad Genomic Data Analysis Center (GDAC) Firehose 2016_01_28 TCGA-HNSC cohort consisting of 529 patients with tumors of the oral cavity (n=320/529, 60.5%), pharynx (n=92/529, 17.4%), and larynx (n=117/529, 22.1%). Clinical data and patient characteristics were retrieved from the level 4 TCGA clinical data file All_CDEs.txt and cBioPortal[31,32]. OC/P patients were stratified by HPV-associated or non-HPV-associated subsite defined by the Centers for Disease Control (CDC)[33] and by confirmed HPV status. All patients were evaluated for HPV in the All_CDEs.txt file, denoted by variable “hvp_status”. Kaplan-Meier P-values were calculated using Mantel-Cox log-rank test and were validated by an independent biostatistician.

Appalachian Tobacco Use Data and Analysis

Data for 2016 current smoker and smokeless tobacco user frequency, weighted frequency, prevalence, and confidence intervals for Appalachian and non-Appalachian counties were calculated and provided by BRFSS coordinators from Appalachian states. Current smoker status was calculated using CDC BRFSS Tobacco Use Question 1 and 2 from the 2016 questionnaire. Current smokeless status was calculated using CDC BRFSS Tobacco Use Question 3. Non-Appalachian state tobacco use data were acquired from CDC BRFSS[19].

Statistical significance was determined within states and between regions using G-tests for independence without Yates' correction using BRFSS frequency (N) values and an alpha value of 0.05. BRFSS recommends caution interpreting results with less than 50 respondents. Bonferroni correction was used where applicable to reduce type I error. Due to changes in sample composition and weighting methodology in 2011, data from years after 2011 cannot be directly compared to previous years[34].

Results

Identification of a Survival Disparity in White Appalachian Males with Stage IV Oral Cavity and Pharyngeal Cancer

The NAACCR cohort from 2007-2013 in this study contained 145,823 OC/P tumors and 57,805 laryngeal tumors (Table 1, Fig. 1a, Fig. S1). The eight NAACCR-reporting Appalachian states cover 67.6% of all Appalachian counties and 72.3% of the Appalachian population[35]. The OC/P and laryngeal cohorts reflect overall Appalachian demographics, with a higher percentage of white cases and lower percentage of ethnic minorities than non-Appalachia (Table 1). Except for lower CRS in Appalachian OC/P and laryngeal cancers, all other parameters were nearly identical to national averages in each disease (Table 1). The majority of OC/P and laryngeal cancer cases came from distressed or transitional Appalachian counties.

RS was measured among OC/P and laryngeal cancer patients by Appalachian or non-Appalachian residency at time of diagnosis (Fig. 1a, Fig S1). When stratified by American Joint Committee on Cancer (AJCC)-6 stages I to IV, stage IV patients had the highest patient numbers and lowest CRS for each disease (OC/P; n=63,396/116,595, CRS=45.5%; laryngeal; n=14,963/51,362; CRS=67.1%), and were selected for further evaluation (Fig. 1b and Fig. S1). OC/P and laryngeal patients were stratified by sex, race, and Appalachian residency (Fig. 1c, 1d; Fig S1). Survival analyses indicated that white Appalachian OC/P males (n=5,014/53,696; CRS=44.9%) displayed a significantly lower CRS compared to white non-Appalachian males (Fig. 1c; n=37,843/53,696; CRS=49.2%), $P=0.00001$. In OC/P, there was no significant difference in CRS between white Appalachian females (n=1,381/53,696; CRS=43.1%) and white non-Appalachian females (n=9,458/53,696; CRS=43.7%) (Fig. 1c) or between black Appalachian males (n=387/7,342; CRS=35.8%) and black non-Appalachian males (n=5,297/7,342; CRS=34.4%) (Fig. 1d). Black Appalachian females (n=116/7,342; three-year RS=46.1%) failed to reach five-year follow-up for CRS comparison, but there was no observed significance

between black non-Appalachian females at the latest available follow-up year ($n=1,540/7,342$; three-year $RS=34.8\%$) (Fig. 1d). No significant differences in CRS were observed for Appalachian laryngeal cancers between any sex or race (Fig. S1). White Appalachian males with stage IV OC/P were selected for further study due to their significantly different survival.

The Survival Disparity in White Appalachian Male Stage IV OC/P is Specific to Appalachian Counties within Select Appalachian States

To elucidate whether the observed stage IV white Appalachian male OC/P survival disparity is specific to Appalachian counties within Appalachian states, patients were stratified by Appalachian or non-Appalachian county and state residency. Appalachian states-Appalachian counties ($n=5,014/42,857$; $CRS=44.9\%$) displayed a significantly lower CRS compared to Appalachian states-non-Appalachian counties ($n=9,700/42,857$; $CRS=47.9\%$), $P=0.00017$ and non-Appalachian states ($n=28,143/42,857$; $CRS=49.6\%$), $P=0.00032$ (Fig. 2a and b). This indicates that the survival disparity is specifically due to cancer survival within Appalachia, not to contributions from adjacent non-Appalachian counties within Appalachian state borders. There was no significant difference in CRS between non-Appalachian states and Appalachian states-non-Appalachian counties (Fig. 2a).

To determine if the stage IV white Appalachian male OC/P survival disparity could be identified at the state level, patients within Appalachian counties were stratified by state residency. Significantly lower CRS was observed for Appalachian counties in Alabama ($n=626/31,423$; $CRS=39.7\%$), $P=0.0006$, Kentucky ($n=372/31,423$; $CRS=39.6\%$), $P=0.0101$, Pennsylvania ($n=1,695/31,423$; $CRS=45.3\%$), $P=0.0047$, and West Virginia ($n=587/31,423$; $CRS=35.3\%$), $P=0.007$ compared to non-Appalachian states ($n=28,143/31,423$; $CRS=49.6\%$) (Fig. 2c and d). All other analyzed Appalachian states did not have a significantly different CRS (Supplementary Figure S2).

The White Appalachian Male Stage IV OC/P Survival Disparity is Primarily Found in HPV-Associated Oropharyngeal Subsites

To determine if the stage IV white Appalachian male OC/P disparity is predominantly present within a specific OC/P subregion, cases were initially divided into non-HPV-associated or HPV-associated cancer subtypes for survival analysis. HPV-associated cancers frequently contain oral mucosa with HPV DNA, and are defined as HPV-associated oropharynx by the CDC[33]. Remaining subsites are not associated with HPV infection and

represent non-HPV-associated cancer (Fig. 3a). First, we evaluated the CDC definitions as predictors of HPV infection in HNSCC by using patients in the TCGA cohort, where all patients have known HPV status. Importantly, patients were evaluated for overall survival, since HPV is not a variable collected by NAACCR and thus could not be assessed in the Appalachian cohort. TCGA patients were additionally stratified as HPV-associated and non-HPV-associated using subsite information, then compared to outcomes based on confirmed HPV status. HPV-positive white male stage IV TCGA patients have an undefined median survival with five-year overall survival of 71%, whereas HPV-negative patients have median survival of 47.0 months and five-year overall survival of 38% (Fig. 3b). Similarly, HPV-associated cancer patients from the same cohort have median survival of 68.4 months and five-year overall survival of 61%, while non-HPV-associated patients have lower median survival of 30.1 months and five-year overall survival of 37%. While the survival trends regarding HPV status are in general agreement between the NAACCR and TCGA cohorts, the difference in five-year overall survival is greater when comparing HPV-positive (71%) and HPV-associated (61%) patients than between HPV-negative (38%) and non-HPV-associated (37%) patients. This suggests that the CDC non-HPV-associated subsite designation is a better predictor for HPV negativity than the HPV-associated subsite designation is for HPV positivity.

To elucidate the discordance between HPV-positive and HPV-associated patients in explaining the observed Appalachian survival differences, TCGA patients were separated into non-HPV-associated cancers and into the specific HPV-associated oropharynx subsites, with the HPV status determined for each grouping. Using this breakdown, non-HPV-associated cancers are mostly HPV-negative, whilst HPV-associated cancers contain a mixture of HPV-positive and HPV-negative cases (Fig. 3c). These findings potentially explain the discrepancy between HPV-positive and HPV-associated outcomes, and also point to confounding factors such as tobacco/alcohol use or socioeconomic status (SES) that may exist in patient cohorts stratified solely on the basis of HPV-association.

White male stage IV cancer cases in the NAACCR cohort were next stratified by HPV-association and Appalachian residency. Appalachian patients with HPV-associated cancers (n=3,449/33,281; CRS=53.1%) displayed a significantly lower CRS compared to HPV-associated patients in non-Appalachian states (n=20,399/33,281; CRS=57.7%), $P=0.0118$ (Fig. 3d). No significant difference was found in CRS between non-HPV-associated patients in Appalachian counties (n=1,577/33,281; CRS=29.5%) compared to non-HPV-

associated patients in non-Appalachian states (n=7,856/33,281; CRS=30.9%). These results suggest that the stage IV white Appalachian male OC/P survival disparity is driven by lower survival of HPV-associated oropharyngeal patients. Appalachian states with the overall stage IV white Appalachian male OC/P survival disparity identified in Figure 2c were evaluated separately and trended in a similar manner (Supplementary Figure 3).

White male stage IV patients in the NAACCR cohort were subsequently stratified by each HPV-associated oropharyngeal region to delineate the contributions of each subsite on Appalachian survival. A significant difference in survival was observed between patients with primary oropharynx tumors in Appalachia (n=325/2,807; CRS=24.4%) and non-Appalachian counties (n=649/2,807; CRS=36.9%), p=0.0399 as well as non-Appalachian states (n=1,833/2,807; CRS=37.5%), P=0.0394 (Fig. 3e). No significant differences were observed between patients with primary tonsil tumors in Appalachia (n=1,603/14,110; CRS=55.8%) and non-Appalachia states (n=9,240/14,110; CRS=63.3%) or counties (n=3,267/14,110; CRS=59.6%). Similarly, no significant differences were observed between patients with primary tongue tumors in Appalachia (n=1,579/14,345; CRS=54.8%) and non-Appalachian states (n=9,625/14,345; CRS=55.9%) or counties (n=3,141/14,345; CRS=55.1%) (Fig. 3f, 3g). Other oropharyngeal subsites lacked sufficient numbers for individual analysis but were combined with tonsil and (base of) tongue to generate an “other HPV-Associated” subset (Fig. 3a).

Regarding survival driven by oropharyngeal tumors, “other HPV-associated” and non-HPV-associated stratification reveals three distinct outcomes in non-Appalachia (Fig. 3h): “Other HPV-associated” (n=18,627/33,347; CRS=59.7%) has the best outcome, followed by oropharynx (n=1,833/33,347; CRS=37.5%), then non-HPV-associated (n=7,856/33,347; CRS=30.9%). However, in Appalachia, only two distinct outcomes are revealed: “Other HPV-associated” (n=3,129/33,347; CRS=56.7%) has the best outcome, whereas oropharynx (n=325/33,347; CRS=24.4%), has a statistically similar outcome to non-HPV-associated (n=1,577/33,347; CRS=29.5%) (Fig. 3h). These results suggest that the observed Appalachian survival disparity is largely driven by lower survival in the oropharynx subregion of HPV-associated stage IV male oropharyngeal patients.

High Appalachian Tobacco Use Correlates with Stage IV White Appalachian Male OC/P outcomes

The lower survival of stage IV white Appalachian male HPV-associated cancer compared to confirmed HPV-positive national cases implies that risk factors other than HPV are driving poorer survival in the Appalachian cohort (Fig. 3b). Since smoking and smokeless tobacco use are highest in Appalachia[19], and current smoker prevalence rates have remained higher in West Virginia (a state with all counties within Appalachia) than the nation for several decades[36] (Fig. 4a), we evaluated tobacco use prevalence rates in Appalachian states from state BRFSS registries stratified by Appalachian residency and sex using data from 2016 as a representative year.

In males, smoking prevalence rates were significantly higher in Appalachian counties compared to non-Appalachian counties, $P=4.441\text{e-}16$ and non-Appalachian states, $P<2.2\text{e-}16$. Specifically, male smoking prevalence rates were significantly higher in Appalachian counties compared to non-Appalachian counties in Kentucky ($P=2.80\text{e-}04$), New York ($P=2.55\text{e-}04$), Ohio ($P=0.02965$), and Virginia ($P=0.008904$) (Fig. 4b, top). Male smokeless tobacco prevalence rates were significantly higher in Appalachian counties compared to non-Appalachian counties, $P<2.2\text{e-}16$ and non-Appalachian states, $P<2.2\text{e-}16$. Specifically, male smokeless tobacco prevalence rates were significantly higher in Appalachian counties compared to non-Appalachian counties in Kentucky ($P<2.2\text{e-}16$), New York ($P=9.03\text{e-}7$), North Carolina ($P=5.44\text{e-}6$), Ohio ($P=1.87\text{e-}12$), South Carolina ($P=0.00419$), and Virginia ($P=6.63\text{e-}9$). Significant differences were also observed in Maryland and Pennsylvania, but these regions had fewer than 50 respondents.

West Virginia contains no non-Appalachian counties for comparison, but the smoking tobacco rate of 25.8% and the smokeless tobacco rate of 15.9% are similar to that of other states with significant differences in the region (Fig. 4b, top). Interestingly, except for Alabama, states with significantly higher male smokeless tobacco use include every state with a significant difference in CRS for stage IV white Appalachian male OC/P and HPV-associated cancers (Fig. 4d).

In females, average smoking prevalence rates were higher in Appalachian counties compared to non-Appalachian counties, $P<2.2\text{e-}16$, and non-Appalachian states, $P<2.2\text{e-}16$ (Fig. 4b, bottom) and were statistically different than that of Appalachian males (20.7% males vs. 19.2% females) (Fig. 4c). Smoking prevalence rates were significantly higher in Appalachian counties compared to non-Appalachian counties in Alabama ($P=1.5\text{e-}4$), Kentucky ($P=1.33\text{e-}9$), Maryland ($P=0.02284$), New York ($P=3.4\text{e-}9$), Ohio ($P=9.38\text{e-}5$),

Pennsylvania ($P=1.44e-4$), and Virginia ($P=0.0362$). Female smokeless tobacco prevalence rates were significantly higher in Appalachian counties compared to non-Appalachian counties, $P=8.76e-4$ and non-Appalachian states, $P=2.912e-05$, and were significantly different than that of Appalachian males (10.9% males vs. 1.2% females), $P<2.2e-16$ (Fig. 4c). Significant differences were observed in Kentucky and Maryland, although these regions had fewer than 50 respondents. These data indicate that significantly higher smoking and smokeless tobacco rates within Appalachia correlate with disparate survival of stage IV white Appalachian male OC/P patients in most Appalachian disparity states.

Discussion

With a majority of population and geographical coverage, our direct, non-exploratory analysis of HNSCC survival from available states representing all ARC-defined Appalachian subregions[16] identifies stage IV white Appalachian males with OC/P cancer as having lower cancer-related survival compared to non-Appalachian males. Based on available data, this stage- and sex-specific disparity is manifest within the Appalachian areas of four states. The disparity is restricted to the ICD-O-3 defined oropharynx (C10.0-10.9) within the CDC-defined HPV-associated oropharyngeal region. This disparity predominantly occurs in states with significantly higher male smoking and smokeless tobacco use, consistent with high risk-factor exposure in Appalachia known to contribute to increased HNSCC incidence[22,24,25].

Previous work evaluating Appalachian oral cancer survival using 2004 SEER data identified higher combined male and female survival in 10 states without consideration of stage or race[25]. When stratified by stage, race and sex, our multivariate analysis indicates that the only significant difference in CRS occurs in white Appalachian stage IV male OC/P patients diagnosed under AJCC-6[37]. RS values for stage IV white Appalachian and non-Appalachian male OC/P are higher than that of black males or black females, in agreement with a recognized national survival disparity for black OC/P cancers attributed to lower overall SES and cultural barriers[9,11]. In addition, female white stage IV OC/P patients have a lower CRS than male white stage IV OC/P regardless of Appalachian status, likely reflecting the lower rate of HPV-positive oropharyngeal disease in females[38,39]. Emerging national trends indicate that HPV-associated oropharyngeal cancers occur more frequently in younger, white male patients with limited tobacco use, and within rural areas[4,14,38]. These factors, coupled with blacks and white females having higher percentages of HPV-negative disease[12,13] are congruent with HPV-positive and/or HPV-associated white male oropharyngeal patients having higher survival at the national level.

OC/P patient populations consist of a mixture of HPV-negative and HPV-positive disease. White Appalachian males have a higher incidence in HPV-positive OC/P cancers, which would be expected to result in increased survival. However, stage IV-matched CRS for white Appalachian males is closer to the lower survival prevalence rates observed for white females than for non-Appalachian white males (Fig. 1c). Furthermore, male Appalachian stage IV HPV-associated oropharynx patients exhibit outcomes similar to non-HPV-associated male stage IV

disease within and outside of Appalachia. Of the recognized cancer risk factors endemic to Appalachia, age at diagnosis, travel distance to critical care centers and disproportionate presentation of patients at stage IV have been reported to have no impact on survival in Appalachian subpopulations[40,41] (Table 1). However, increased smoking and smokeless tobacco use within Appalachia have been linked to low SES and increased HNSCC incidence[42–44], and thus are presumably factors contributing to the decreased male stage IV oropharyngeal cancer survival.

Our findings also indicate that Appalachian smoking tobacco prevalence rates, represented by West Virginia, have been higher than national averages for several decades (Fig. 4a), and while Appalachian smoking tobacco use is significantly higher in males and females, smokeless tobacco use is primarily higher in males compared to national averages (Fig. 4b). Smokeless tobacco use has been specifically linked to increased cancer risk in OC/P and oropharyngeal subsites[45–48]. Elevated smoking and smokeless tobacco use in most Appalachian states with significantly different CRS corresponds with decreased CRS in white male stage IV OC/P cancers. This has the potential to result in the male patient population garnering a higher percentage of tobacco-induced HPV-negative disease at all oral subsites, including the oropharynx. While the lack of diagnostic HPV detection by p16 staining as a variable in the NAACCR cohort precludes direct analysis of viral status, segregation of white male stage IV OC/P patients by non-HPV-associated OC/P and HPV-associated oropharynx indicates that HPV-associated oropharyngeal cancer is responsible for the Appalachian male stage IV disparity within ICD-O-3 oropharynx codes. The predominant subsites under this delineation are C10.0 (vallecula), C10.1 (anterior surface of epiglottis), C10.2 (lateral wall of oropharynx), C10.3 (posterior wall of pharynx) and C10.4 (branchial cleft). These oropharyngeal regions exclude palatine and lingual tonsils, as well as most other HPV-associated oropharyngeal sites containing the reticular epithelium lining the tonsillar crypts that constitute the primary sites of oropharyngeal HPV infection and neoplasia[49–51]. Higher aggregate tobacco use by Appalachian males may therefore result in more frequent carcinogen-induced malignancy at oropharynx subsites, resulting in a greater percentage of HPV-negative disease in the oropharynx and corresponding worse CRS, similar to that observed in other tobacco-heavy oropharyngeal cohorts[3,52,53].

The Appalachian-specific OC/P and oropharynx-specific disparities found in this study were diagnosed under the AJCC-6 timeframe, having clinical and pathological staging guidelines independent of HPV status. Restaging

of HPV-positive oropharynx in 2018 under AJCC-8 due to the favorable prognosis of HPV-positive oropharyngeal cancers would shift most HPV-positive oropharynx in AJCC-6 to lower clinical stages. Such restaging would have the effect of potentially eliminating or downstaging the Appalachian male disparity described in this report. However, an additional ramification from this work is that male Appalachian or other populations with multifactorial oropharyngeal tobacco exposure may actually be under-staged using current AJCC-8 guidelines. This is an important consideration, since National Comprehensive Cancer Network (NCCN) guidelines recommend staging and treatment dependent on p16 status[52,54], where treatment de-escalation of HPV-associated oropharyngeal cancers continues to be evaluated[55–57]. Future efforts towards definitively determining the extent of HPV involvement in Appalachian OC/P and oropharynx through comprehensive p16 staining and PCR[58] will be required to better clarify the predominant factors underlying oropharynx-driven, stage-based disparities in past and future Appalachian cohorts.

Limitations and Implications

While the current study covers the majority of the Appalachian population and is the most comprehensive study of HNSCC survival of the region to date, lack of available qualified survival data from five Appalachian states prevented complete assessment of survival in the region. The association between heavy aggregate tobacco use and poor male stage IV oropharyngeal survival in states with significantly different CRS suggests that stage IV oropharyngeal patients from states with Appalachian regions containing similar tobacco use patterns may also harbor disparate outcomes. Our findings, in conjunction with the poor survival of HPV-positive oropharynx patients with high smoking histories[59,60], underscore the need for the comprehensive tobacco history of any HPV-positive oropharynx patient to be considered prior to treatment. In addition to smoking, the significantly higher smokeless tobacco use by the male Appalachian population further increases the risk of tobacco-induced cancer at HPV-associated oropharyngeal sites, leading to oropharynx tumors that are either HPV-negative, or are HPV-positive but exhibit aggressive HPV-negative tumor behavior.

Conclusions

These findings provide novel and in-depth insight into a specific demographic within a chronically underserved, rural population that is at higher clinical risk for poor OC/P outcome. Persistent high tobacco usage in Appalachia,

in spite of increased tobacco cessation efforts, reinforces the need for continued targeted risk awareness. This includes emerging forms of supposed safer nitrosamine-containing products, such as e-cigarettes, that contribute to the cumulative patient tobacco load[61]. Inclusion of patient use of these products as standard registry variables should be considered for improved monitoring of tobacco-related disparities in future populations. The described male oropharynx disparity may also be present in male patients from other regions with heavy smoking, smokeless tobacco[62] or betel-quinid[63] use, and should be monitored accordingly for similar poor survival and continued cessation intervention policies.

Competing interests

The author(s) declare no competing interests.

Author Contributions

B.W.P: Conceptualization, writing—original draft, data curation, formal analysis, and investigation. **J.L.A.:** Conceptualization and writing—review and editing. **S.M.M.:** Conceptualization, writing—review and editing. **E.T.I.:** Conceptualization and writing—review and editing. **P.A.M.:** writing—review and editing. **C.J.J.:** Conceptualization, writing—review and editing, supervision, methodology, software, and resources. **S.A.W.:** Funding acquisition, conceptualization, project administration, writing—original draft, review, editing and project supervision.

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Data Availability

The data that support the findings of this study are available upon application and IRB approval from NAACCR, thus these data are not in the public domain. Data are however available from the authors upon reasonable request and with prior approval by NAACCR.

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Figures and Tables

Figure 1. Identification of a Survival Disparity in White Appalachian Male Stage IV OC/P cancer. A. Flow diagram of procedures used to evaluate Appalachian-specific survival data. Boxes contain rationale and steps involved in the sequential stratification and cohort analysis for OC/P cancer. **B.** Survival analysis of Appalachian OC/P cancer stratified by clinical stage. CRS values are plotted for each year after diagnosis, with five-year (60 month) ratios evaluated across all AJCC-6 stages. **C** and **D.** Survival analysis of white (**C**) and black (**D**) stage IV OC/P cancer stratified by Appalachian residency and sex. CRS values are plotted as in (**B**). Patient N, five-year CRS with 95% CI and P-values between significant groups are shown at the top of each graph; n.s., not significant. Black patients were evaluated for significance at 36 months due to lack of complete female survival by 48 months and beyond.

Figure 2. State-level analysis of Appalachian OC/P survival. A. Disparate stage IV white Appalachian male OC/P survival is restricted to Appalachian counties. Plotted CRS values over time are shown for non-Appalachian states (black), non-Appalachian counties within Appalachian states (green) and Appalachian

counties within Appalachian states (red). Patient N, five-year CRS with 95% CI and P-values at 60 months are shown at the top of the graph; n.s., not significant. **B.** Mapping of the denoted geographic regions evaluated in **(A)**. **C.** Select Appalachian states with significantly different CRS values contain a survival disparity in OC/P cancer. Appalachian states with significant CRS values are shown plotted over time. Patient N, five-year CRS with 95% CI and P-values at 60 months for states with significant survival differences between Appalachian and non-Appalachian counties are shown at the top of the graph. **D.** Map of Appalachian region displaying states with significantly different stage IV white Appalachian male OC/P CRS. States with significant survival differences between Appalachian and non-Appalachian counties are in black, states with no available data are marked with a cross-hatched pattern.

Figure 3. The Stage IV White Appalachian Male OC/P survival disparity is predominant in HPV-associated oropharyngeal cancers. A. Schematic of the head and neck showing location of non-HPV-associated pharynx

and oral cavity (blue), and CDC-defined HPV-associated oropharynx (green) cancers. A subset of the CDC-defined HPV-associated oropharynx is denoted in orange. ICD-O-3 site groups and corresponding site codes for each cancer type are indicated (see Materials & Methods for detailed description of each site group). **B.** Overall survival between confirmed and HPV-associated OC/P subtypes. Kaplan-Meier analysis of overall survival of stage IV white males from the TCGA HNSC cohort stratified by HPV status (HPV-positive or HPV-negative) or by ICD-O-3-coded HPV association (HPV-associated and non-HPV-associated). P-values calculated using Mantel-Cox log-rank test, patient N, median and five-year overall survival for each group are noted. **C.** HPV status of OC/P subsites in stage IV white male TCGA patients. Percent of patients with confirmed HPV-negative (red) or HPV-positive (black) disease for each indicated subsite is shown. Denoted subsite groupings are indicated at the bottom as non-HPV-associated (blue), oropharynx (green), or other HPV-associated (orange). **D.** The stage IV white Appalachian male OC/P survival disparity is present in HPV-associated oropharyngeal cancer. CRS values are plotted over time for stage IV white Appalachian male OC/P patients stratified by Appalachian and non-Appalachian counties and HPV- or non-HPV-associated status. **E-G.** Stage IV white males stratified by HPV-associated oropharynx subsites. Oropharynx (**E**), tonsil (**F**), and tongue (**G**). CRS values are plotted over time with patients stratified by Appalachian counties (red), non-Appalachian counties (green) and non-Appalachian states (black). **H.** Stage IV white males stratified by non-HPV-associated, oropharynx, other HPV-associated subsites and Appalachian residency. For D-H, RS values are plotted over time with patients stratified by Patient N, five-year CRS with 95% CI and P-values at 60 months for each group are shown at the top of each respective graph; n.s., not significant.

b BRFSS Current Smoker and Smokeless Tobacco Use Prevalence by Region and Sex, 2016

c BRFSS Current Smoker and Smokeless Tobacco Use Prevalence by Region and Sex, 2016

Figure 4. High combined tobacco use in Stage IV white Appalachian male OC/P disparity states. A. Overall smoking prevalence rates in West Virginia (as a representation of Appalachia, gold) compared to the United States (grey). Years 2011-2018 cannot be directly compared to earlier years due to differences in weighting methodology across time. **B.** Elevated tobacco use by Appalachian males (*top*) and females (*bottom*). Data for 2016 smoking weighted prevalence estimates for males in Appalachian states stratified by county status (blue, Appalachian counties; gold, non-Appalachian counties; grey, non-Appalachian states), smoking (solid) or smokeless (cross-hatched) tobacco use with P-values calculated using G-test for independence without Yates' correction are shown at *top*. Error bars represent 95% CI. **C.** Combined Appalachia county smoking (solid) and smokeless (cross-hatched) weighted prevalence estimates with P-value calculated using G-test for independence without Yates' correction are shown above significant results. **D.** Mapping of Appalachian counties smokeless tobacco use. Appalachian counties with significantly higher (red) and non-significantly different (black) smokeless tobacco use are shown compared to non-Appalachian counties within each respective Appalachian state. West Virginia (hatched; non-applicable) lacks non-Appalachian counties for comparison.

2007 - 2013 NAACCR Cohort Characteristics†	Total OC/P and Laryngeal n = 214821 (100%)	Total OC/P n = 154525 (100%)	Appalachian OC/P n = 16366 (10.59%)	Non-Appalachian OC/P n = 138159 (89.41%)	Total Laryngeal n = 60296 (100%)	Appalachian Laryngeal n = 7614 (12.63%)	Non-Appalachian Laryngeal n = 52682 (87.37%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Age (years)	63.05 ± 12.01	62.35 ± 12.22	61.99 ± 12.05	62.39 ± 12.24	64.83 ± 11.26	63.73 ± 11.10	64.99 ± 11.28
	CRS ± SEM	CRS ± SEM	CRS ± SEM	CRS ± SEM	CRS ± SEM	CRS ± SEM	CRS ± SEM
Relative Survival (%)	56.9 ± 0.2	55.0 ± 0.2	52.7 ± 0.7	55.3 ± 0.2	60.1 ± 0.4	58.1 ± 1.1	60.4 ± 0.4
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Sex							
Male	160,108 (74.5)	111,900 (72.4)	11,779 (72)	100,121 (72.5)	48,208 (80)	6,021 (79.1)	42,187 (80.1)
Female	54,713 (25.5)	42,625 (27.6)	4,587 (28)	38,038 (27.5)	12,088 (20)	1,593 (20.9)	10,495 (19.9)
Race							
White	183,029 (85.2)	132,955 (86)	15,049 (92)	117,906 (85.3)	50,074 (83)	6,892 (90.5)	43,182 (82)
Black	23,385 (10.9)	14,769 (9.6)	1,123 (6.9)	13,646 (9.9)	8,616 (14.3)	661 (8.7)	7,955 (15.1)
Other	8,407 (3.9)	6,801 (4.4)	194 (1.2)	6,607 (4.8)	1,606 (2.7)	61 (0.8)	1,545 (2.9)
AJCC-6 Stage							
I	40,356 (18.8)	21,555 (13.9)	2,431 (14.9)	19,124 (13.8)	18,801 (31.2)	2,271 (29.8)	16,530 (31.4)
II	23,202 (10.8)	14,622 (9.5)	1,666 (10.2)	12,956 (9.4)	8,580 (14.2)	1,136 (14.9)	7,444 (14.1)
III	28,895 (13.5)	19,498 (12.6)	2,208 (13.5)	17,290 (12.5)	9,397 (15.6)	1,332 (17.5)	8,065 (15.3)
IV	79,924 (37.2)	64,794 (41.9)	7,105 (43.4)	57,689 (41.8)	15,130 (25.1)	1,968 (25.8)	13,162 (25)
Missing	40,938 (19.1)	33,023 (21.4)	2,868 (17.5)	30,155 (21.8)	7,915 (13.1)	850 (11.2)	7,065 (13.4)
Primary Site							
Oral Cavity	55,620 (25.9)	55,620 (36)	6,028 (36.8)	49,592 (35.9)	-	-	-
Oropharynx	81,174 (37.8)	81,174 (52.5)	8,551 (52.2)	72,623 (52.6)	-	-	-
Other Pharynx	17,731 (8.3)	17,731 (11.5)	1,787 (10.9)	15,944 (11.5)	-	-	-
Larynx	60,296 (28.1)	-	-	-	60,296 (100)	7,614 (100)	52,682 (100)
ARC Economy FY2017‡							
Distressed	1,842 (0.9)	1,112 (0.7)	1,112 (6.8)	-	730 (1.2)	730 (9.6)	-
At-Risk	3,491 (1.6)	2,303 (1.5)	2,303 (14.1)	-	1,188 (2)	1,188 (15.6)	-
Transitional	15,935 (7.4)	11,022 (7.1)	11,022 (67.3)	-	4,913 (8.1)	4,913 (64.5)	-
Competitive	2,402 (1.1)	1,701 (1.1)	1,701 (10.4)	-	701 (1.2)	701 (9.2)	-
Attainment	310 (0.1)	228 (0.1)	228 (1.4)	-	82 (0.1)	82 (1.1)	-
Not Applicable	190,841 (88.8)	138,159 (89.4)	-	138,159 (100)	52,682 (87.4)	-	52,682 (100)

† Includes data from Alabama, Alaska, Arizona, California, Colorado, Connecticut, Delaware, Florida, Georgia, Hawaii, Idaho, Illinois, Indiana, Iowa, Kentucky, Louisiana, Maine, Michigan, Mississippi, Montana, Nebraska, New Hampshire, New Jersey, New Mexico, New York, North Carolina, Pennsylvania, Rhode Island, Seattle, South Carolina, Utah, West Virginia, Wisconsin, and Wyoming.

‡ ARC County Economic Status designation for Appalachian counties[26] is categorized by Distressed (Worst 10% of U.S. Counties), At-Risk (Worst 10+ to 25% of U.S. Counties), Transitional (Worst 25% to Best 25% of U.S. Counties), Competitive (Best 10+ to 25% of U.S. Counties), and Attainment (Best 10% of U.S. Counties) based on National Index Value Rank which is indicated by Three-Year Average Unemployment Rate, Per Capita Market Income, and Poverty Rate.

SD: standard deviation; SEM: standard error of the mean

Table 1. Appalachian and Non-Appalachian OC/P and Laryngeal Patient Characteristics 2007-2013

Study 1 Supplemental Materials: Disparate Survival of Late-stage Male Oropharyngeal Cancer in Appalachia

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Supplementary Figure S1. Survival analysis of Appalachian Stage IV laryngeal cancer. A. Flow diagram of procedures used to evaluate Appalachian-specific laryngeal cancer survival data. Boxes contain sequential operational steps utilized in the stratification and cohort analysis, noting no difference in survival within the cohort. **B.** Survival analysis of Appalachian laryngeal cancer stratified by clinical stage. RS values are plotted for each year after diagnosis, with five-year (60 month) ratios evaluated across all AJCC-6 stages. **C** and **D.** Survival analysis of white (**C**) and black (**D**) stage IV laryngeal cancer stratified by Appalachian residency and sex. RS values are plotted as in (**B**). P-values between significant groups are shown at the top of each graph; n.s., not significant; NA, not available. Black patients were evaluated for significance at 48 months due to incomplete male survival at 60 months.

Supplementary Figure S2. Survival analysis of Appalachian states with non-significant OC/P survival. A. States lacking disparate RS of stage IV white Appalachian male OC/P. Plotted RS values over time are shown for non-Appalachian states (black), and Appalachian states with non-significant (n.s.) survival. Patient N, five-year CRS with 95% CI and P-values at 60 months for each significant Appalachian state are shown at the top of the graph. **B.** Map of Appalachian region displaying states with non-significant stage IV white Appalachian male OC/P survival. Colored states are non-significant, and significant states are in black; states with no available data are marked with a cross-hatched pattern.

Supplementary Figure S3. Survival analysis of Appalachian states with non-significant OC/P survival. A-D. Identification of states with disparate stage IV white Appalachian male HPV-associated oropharyngeal cancer survival. RS values were plotted for patients stratified as in Figure 3D for each OC/P disparity state. **A**, Alabama; **B**, Kentucky; **C**, Pennsylvania; **D**, West Virginia. Patient N, five-year CRS with 95% CI and P-values at 60 months for each Appalachian state are shown at the top of the graph; n.s., not significant.

Study 2: Copy Number Alterations Identify a Smoking-Associated Expression Signature Predictive of Poor Outcome in Head and Neck Squamous Cell Carcinoma

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Abstract

Cigarette smoking is a risk factor for the development of head and neck squamous cell carcinoma (HNSCC), partially due to tobacco-induced large-scale chromosomal copy-number alterations (CNAs). Identifying CNAs caused by smoking is essential in determining how gene expression from such regions impact tumor progression and patient outcome. We utilized The Cancer Genome Atlas (TCGA) whole genome sequencing data for HNSCC to directly identify amplified or deleted genes correlating with smoking pack-year based on linear modeling. Internal cross-validation identified 35 CNAs that significantly correlated with patient smoking, independent of human papillomavirus (HPV) status. The most abundant CNAs were chromosome 11q13.3-q14.4 amplification and 9p23.1/9p24.1 deletion. Evaluation of patient amplicons reveals four different patterns of 11q13 gene amplification in HNSCC resulting from breakage-fusion-bridge (BFB) events responsible for SAES amplification. Predictive modeling identified 16 genes from these regions that denote poorer overall and disease-free survival with increased pack-year use, constituting a smoking-associated expression signature (SAES). Patients with altered expression of signature genes have increased risk of death and enhanced cervical lymph node involvement. The identified SAES can be utilized as a novel predictor of increased disease aggressiveness and poor outcome in smoking-associated HNSCC.

Introduction

HNSCC is an aggressive neoplasm of the upper aerodigestive tract caused by exposure to tobacco, alcohol, and high-risk HPV infection(1). Over 53 000 new cases and 11 000 deaths are predicted to occur in the U.S. due to HNSCC in 2020(2) with five-year post-treatment survival near 50%(3). Cervical nodal metastasis is frequently found in HNSCC, and is an important prognostic indicator associated with late stage (American Joint Committee on Cancer (AJCC) stage III/IV) disease in aggressive smoking-associated HPV-negative cancers(4,5).

Widespread next-generation sequencing of HNSCC cohorts has identified driver mutations, common tumor suppressor/oncogenes, and molecular subtypes(6–9). Conclusions from this work indicate that HPV-positive tumors harbor fewer mutations and CNAs than HPV-negative cancer. This is congruent with HPV-positive patients exhibiting better overall survival, stratifying the disease into distinct prognostic subtypes(3,10). Over 50% of HPV-negative patients present with stage IV disease, where tobacco-generated tumors contain high genomic instability, increased lymphovascular invasion, enhanced therapeutic resistance, poor prognosis and decreased time of survival(6,7,11–13).

CNAs involved in HPV-negative HNSCC progression include gene gains (two extra copies), amplifications (more than two extra copies), loss of heterozygosity, and homozygous deletion of chromosomal segments(6,9,10,14). Widespread loss of chromosomal segments, along with concurrent frequency of mutations in tumor suppressor genes, supports the generalized concept that HNSCC is largely driven by genomic relief of tumor suppression(15). In addition, HPV-negative HNSCC is driven by overexpression of oncogenes in genomically amplified regions(16,17). Large-scale CNAs primarily arise through double-strand DNA breakage at fragile sites and subsequent telomeric loss(18). This initiates a BFB cycle through chromatid annealing, leading to uneven separation during mitosis and increased chromosome segment copy numbers(19).

While smoking-driven genomic damage is well known in HNSCC(10,20), the precise CNAs correlated with smoking have not been previously defined. Here, we utilized an unbiased, internally-validated statistical approach with the TCGA-HNSC cohort to identify chromosomal homozygous deletions and amplifications in HNSCC that correlated with patient smoking. Currently, this is the only publicly available HNSCC cohort that contains necessary molecular and clinical parameters for patients suitable for such evaluation. Within this cohort, amplification of genes within chromosome 11q13.3-q13.4 and homozygous deletions of genes within

chromosome 9p21.3/9p24.1 were the only CNAs that correlated significantly with cigarette pack-year usage in an appreciable percentage of patients. A gene subset with altered transcript expression from these regions constitute a smoking-associated expression signature (SAES) predictive of patient pack-year use, corresponding with elevated risk of death and enhanced regional lymph node metastasis. Alignment-based mapping of patient 11q13.3-q13.4 amplicons identifies underlying BFB scenarios responsible for amplified SAES genes. Collectively these results identify CNAs and corresponding genes in HNSCC that correlate positively with cigarette use, confirming known and identifying new oncogenic drivers that may serve as novel biomarkers and targets for improved clinical intervention in patients with documented smoking histories.

Materials and Methods

Samples and Clinical Data

Data were obtained from the 2016_01_28 TCGA-HNSC cohort, downloaded from Broad Genomic Data Analysis Center (GDAC) Firehose(21), consisting of 529 patients with tumors of the oral cavity (n=320/529, 60.5%), pharynx (n=92/529, 17.4%), and larynx (n=117/529, 22.1%). The majority of patients were male (n=386/529, 73%) and smoked (n=299/529, 56.5%), with mean 32.4 pack-years exposure. Patients were diagnosed from 1992-2013 with a mean age of 60.7 years. 20 patients were excluded that lacked copy-number or smoking history. Clinical data were retrieved from the level 4 TCGA data file All_CDEs.txt and cBioPortal(22). Alcohol use was calculated by multiplying Alcohol Frequency and Daily Drinks. Patients with no pack-year data were excluded from pack-year correlations. Statistical significance was determined using chi-square test and Bonferroni correction or one-way ANOVA and Tukey's post-hoc with an alpha of 0.05.

Determination of Smoking-Associated CNAs

Smoking-associated CNAs were determined by Genomic Identification of Significant Targets in Cancer (GISTIC)2(23) analysis of TCGA 2016_01_28 level 4 data for 24,776 genes(21). Briefly, raw GISTIC2 values within all_data_by_genex.txt were used for analysis. A linear regression model was used to assess the association between gene GISTIC2 value as a dependent variable and smoking in pack-years as an independent variable, adjusting for age, sex, and disease stage. For multiple testing, p-value sets were modeled using a beta-uniform mixture model(24), permitting selection of p-value cutoffs bracketing the false discovery rate (FDR). Genes that reached statistical significance (100 maximum) in each analysis, or the top 100 genes if significance was not reached, were analyzed further. Prediction modeling was validated using multiple methods. First, the dataset was randomly separated into training and validation sets, each containing 50% of patients. Second, the Leave-One-Out cross validation (LOOCV) method was used(25). For each gene in each method, a linear regression model was fit using pack-year value as the explanatory variable, and gene GISTIC2 value as the response using half of all samples (50%), or all but one sample (LOOCV). The fitted models were used to examine the accuracy of the remaining samples. Predictive capabilities were evaluated through concordance, sensitivity, and specificity after performed for each gene. Third, the "stability selection" method was used to assess the prediction for each gene, which combines the techniques of sub-sampling and randomized Lasso

algorithms(26). Validated genes were combined by cytogenetic sub-sub band (cytoband). Cytobands altered in fewer than 10% of patients and those dependent on HPV-status were excluded. Predicted CNA probabilities were graphed based on the fitted model, with data sorted by ascending pack-year. In the time-to-event analysis, survival functions by amplified genes (yes/no) were assessed by Kaplan-Meier and log-rank testing for overall and progression-free survival.

Evaluation of Common Mutations

Patient individual gene mutations and Total Mutations were obtained from TCGA-HNSC 2016_01_28 level 3 Mutation Packager Raw Calls and the cBioPortal “Mutated Genes” analysis(21,22). Data were stratified by smoking and CNA status. Smoking status was divided into “Never Smoker” (pack-year=0), “Ever Smoker” (pack-year>0), “Heavy Smoker” (pack-year>10), “Light Smoker” ($0 < \text{pack-year} \leq 10$) and “Low Risk” ($\text{pack-year} \leq 10$), similar to other reports(27).

Determination of the Smoking-associated Expression Signature

Expression levels of genes identified from the CNA analysis were determined using the TCGA-HNSC 2016_01_28 level 3 RNAseq gene expression dataset(21). Analysis was conducted using z-scores calculated from reads per kilobase of transcript, per million reads (RPKM) in normal adjacent tissues as baseline. Gene expression for overall survival was assessed by comparing the first quartile (lowest 25%) of expression to the fourth quartile (highest 25%) of expression levels. Genes where differential expression significantly affected overall survival were combined to create the SAES. A linear regression model was used to assess the association between the z-score as a dependent variable and smoking pack-year as an independent variable. LOOCV validation was performed as described. The predicted SAES gene expression probability is based on the fitted model, with patients sorted by ascending pack-year. For correlative analysis between lymph node (yes/no) and gene overexpression ($z\text{-score} > 2$) or underexpression ($z\text{-score} < -2$), odds ratios and the 95% confidence intervals (CI) for each gene was estimated from a fitted logistic model. Time-to-event survival functions were assessed using the Kaplan-Meier and log-rank testing for overall and progression-free survival (yes/no for overexpression). Cox models were used to assess the association between SAES genes and survival. Estimated relative risk of death was presented graphically based on the fitted model where the data were sorted by standardized SAES expression.

Amplicon Alignment and Segment Determination

Thresholded GISTIC2.0 CNA values were downloaded from the GDAC Firehose(21). Values were sorted and aligned in relation to the total length of the entire 11q13 chromosomal segment and flanking regions. Amplicon segments were derived from previous mapping studies(28–31), aided by natural breaks in the alignment data across cohort patients with 11q13 amplified genes. SAES gene positions were aligned using the University of California Santa Cruz (UCSC) Genome Browser with human genome assembly GRCh38/hg38(32).

Results

Cigarette Smoking Correlates with Specific Copy Number Alterations In HNSCC

To identify smoking-associated CNAs in HNSCC, CNAs containing amplified and deleted genes were extracted and independently evaluated for correlation with pack-year (Fig. 1A) (Supplementary Table S2). 1 775 amplified genes were identified that correlated significantly, mapping to 92 cytobands (Fig. 1A). To assess HPV-status as a potential confounding variable, identified genes were correlated with confirmed HPV-status. None of the 92 amplified cytobands exclusively correlated with HPV-positive tumors, whereas 17 cytobands showed selective correlation to HPV-negative tumors. The remaining two cytobands were amplified independent of HPV-status and further evaluated (Fig. 1B). As expected, smokers were more commonly HPV-negative than non-smokers (Supplementary Table S1).

Of the amplified genes that correlated with pack-year, genes located on chromosome 11q13.3 (n=20) and 11q13.4 (n=10) were from the only cytobands amplified independent of HPV status and in at least 10% of patients with pack-year data (Fig. 1C). This region contains the chromosomal fragile sites (CFSs) FRA11H and FRA11F and is flanked by two segmental duplications (SDs)(28–31,33–35). 11q13.3 amplification is significantly enriched in patients that smoked (85.6%) compared to non-smokers (65.1%) (Fig. 1D). Patients with any 11q13.3 gene amplification had a 36.3-month median overall survival and a 38.4% five-year overall survival rate, whereas non-amplified patients had a 65.7-month median overall survival and a 51.8% five-year overall survival rate (Fig. 1E). Disease-free survival for 11q13.3 amplified patients trended lower but failed to reach significance (Fig. 1F). Similarly, 11q13.4 amplified patients were significantly enriched for smokers (87.5%) compared to non-smokers (66.2%) (Fig. 1G). Patients with 11q13.4 gene amplification showed lower overall and progression-free survival, with a 26.8-month median overall survival, and 34.0% five-year overall survival rate. Non-amplified patients had a 65.7-month median overall survival and 50.4% five-year overall survival rate (Fig. 1H). Five-year disease-free survival for 11q13.4 amplified individuals was 38.4% compared with 56.5% in non-amplified cases (Fig. 1I). The majority of patients with 11q13.3 gene amplification also had 11q13.4 gene amplification (73.4%), consistent with the cytobands being contiguous. Due to lack of suitable external validation sets, multiple internal validation methods were used. Separating the dataset into training and validation sets for analysis demonstrated a high specificity (>95%) and concordance (>73.8%). LOOCV analysis on each significant gene also demonstrated

high specificity (>97.4%) and concordance (>74%). This unbiased approach independently confirms that amplification of genes within chromosome 11q13.3-q13.4 correspond with this region being the most frequent CNA in HNSCC(6,16), and that 11q13.3-q13.4 gene amplification correlates with cigarette use and poor HNSCC outcome as suggested in previous work(6,16).

In addition, our analysis identified five cytobands that correlated with homozygous deletion and pack-year (Fig. 2A). After evaluating by HPV-status as in Figure 1B, five genes from these cytobands were found to be deleted independent of HPV (Fig. 2B). One gene mapped to cytoband 9p24.1 and four genes mapped to cytoband 9p21.3 (Fig. 2C). This region contains an unnamed CFS (9CFS) and the CFS FRA9C, promoting chromosome breakage and gene deletion(36). Deletion of 9p21.3 was enriched for smokers (80.4%) compared to non-smokers (66.2%) (Fig. 2D). Patients with any 9p21.3 gene deletion had a 35.9-month median overall survival and a 38.5% five-year overall survival rate, whereas non-deleted patients had a 66.7-month median overall survival and a 51.2% five-year overall survival rate (Fig. 2E). Disease-free survival for 9p21.3 deleted patients failed to reach significance (Fig. 2F). Deletion of cytoband 9p24.1 was not enriched for smokers (Fig. 2G) and no difference in overall (Fig. 2H) or disease-free survival (Fig. 2I) was observed. Training and validation analysis demonstrated high specificity (>98%) and concordance (>69.5%).

The Majority of Smoking-associated Amplified Genes are Contained Within the Most Frequently Amplified 11q13 Segment in HNSCC.

The worse outcomes and higher clinical risk parameters associated with genes in 11q13.3.-11q13.4, combined with the variation in amplicon lengths led us to determine the underlying 11q13 amplification mechanisms. 87% of 11q13 amplified patients had distal 11q loss, suggesting BFB as the predominant amplification mechanism. Chromosomal alignment and clustering of all 11q13.3-q13.4-amplified patients from the TCGA-HNSC cohort identified three distinct alignment patterns of amplified regions, grouped as segments 1, 2 and 3 (Fig. 3A). All or part of the segment 2 region is present in every patient with 11q13.3-q13.4 amplification. Based on segment distribution and the FRA11H and FRA11F, four possible BFB scenarios account for all observed 11q13 amplification patterns in HNSCC (Fig. 3B). Scenario 1 occurs in 17% of patients, where the first break occurs at the telomeric end of FRA11H before Segment 3 to delete this region. This results in sister chromatid end fusion at segment 2. A subsequent second break at FRA11H between segment 1 and segment 2 results in inverted segment 1 loss, repeated through subsequent rounds of BFB to increase amplicon number (Fig. 3). Additional

scenarios (2-4) involving breakage at different FRA11H sites, FRA11F, or other combinations also occur at lower frequencies than scenario 1 (Fig. 3B). 13% of amplified patients have intact 11q telomeres, indicating that SD-mediated amplification occurs at lower frequency. Regardless, the resulting segment 2 region in all amplification events contains the 11q13.4-q13.4 chromosomal region that harbors the majority of identified smoking-associated genes (Fig. 1C).

Smoking-associated Copy-number Alteration Corresponds with Elevated Risk Factor Exposure

Patients with smoking-associated CNAs stratified by cytoband and smoking status were evaluated for clinical characteristics and additional HNSCC risk factors across the cohort (Supplementary Table S1). All patients with 11q13.3-q13.4 amplification (Total Amplified) had significantly higher mean pack-year usage than patients lacking amplification (Total Non-Amplified) (46.6 vs. 27.1). The majority of 11q13.3-q13.4 Amplified Smokers were male with stage IV disease, had positive lymph node involvement, higher alcohol consumption, higher pack-year and were HPV-negative. In addition, the majority of 9p21.3/9p24.1 Deleted Smokers were male with stage IV disease, had positive lymph node involvement, and were HPV-negative.

Differences in the Mutational Profile of Commonly Altered HNSCC Driver Genes in Smoking-associated Copy-number Altered HNSCC

Tobacco-associated HNSCC demonstrates characteristic mutational signatures that result in oncogene activation or loss of tumor suppression(6,37). Since 11q13.3-q13.4 amplification and 9p21.3 deletion occurs in a greater percentage of cigarette-smoking patients, we examined the mutational occurrence of the most frequently altered HNSCC genes in patients that smoked and contained these CNAs (Table 1). The top ten most commonly mutated cancer-related HNSCC genes were evaluated(6). When stratified by smoking status, the mutation rates of *LRP1B* (19.6% smokers; 8.2% non-smokers), *NSD1* (14.2% smokers; 4.1% non-smokers) and *CASP8* (8.3% smokers; 16.4% non-smokers) differed significantly. Smoking was also associated with 11q13.3, 11q13.4 and 9p21.3 gene CNA. Interestingly, the mutation rate difference of *TP53*, the most commonly mutated gene in HNSCC(6,9), did not differ significantly by smoking status (71.1% smokers; 63.9% non-smokers) when defining smokers as patients with an above zero pack-year or with a reported smoking history (Table 1, Supplementary Table S3). When patients were stratified by 11q13.3-q13.4 amplification, the mutation rates of *CASP8* (2.2% amplified; 13.1% non-amplified), *KMT2D* (0.7% amplified; 15% non-amplified), *NOTCH1* (16.2%

amplified; 17.4% non-amplified), *NSD1* (6.7% amplified; 13.7% non-amplified), *PCLO* (19.1% amplified; 14.2% non-amplified), and *TP53* (83.8% amplified; 64.1% non-amplified) were significantly different. 11q13.3-q13.4 amplification was also significantly associated with 9p21.3 deletion. When patients were stratified by 9p21.3/9p24.1 deletion, the mutation rates of *NOTCH1* (23% deleted; 13.5% non-deleted) and *TP53* (76.4% deleted; 65.1% non-deleted) were significantly different. 9p21.3/9p24.1 deletion was also significantly associated with 11q13.3-q13.4 amplification. *CDKN2A* mutation rate was also significantly different (8.9% deleted; 29.2% non-deleted); however, *CDKN2A* is located in the deleted 9p21.3 region. All other evaluated gene mutation rates were not significantly different across the cohort, regardless of smoking or CNA status.

Identification of the Smoking-associated Expression Signature in HNSCC Patients with Smoking-associated CNAs

To determine the expression of actionable drivers in smoking-associated CNAs, mRNA expression was analyzed for each cross-validated gene. Inclusion criteria were restricted to patients with full mRNA sequencing data and RPKM values for each gene, known copy-number status and smoking history. 260 patients contained suitable RNAseq data for analysis (Figure 4A); nine non-protein coding genes in the 11q13.3-q13.4 region (*DEFB108B*, *MIR3164*, *MIR548K*, *ENPP7P8*, *ZNF705E*, *UNC93B6*, *FAM86C1*, *MIR3664*, and *RNA5SP342*) did not have reported RPKM values and could not be analyzed. The remaining genes were assessed for expression differences between patients with CNAs and euploid patients. Patients with CNAs had significantly higher percentages of overexpression or underexpression of most genes (Figure 4B). Genes from this group with expression differences that significantly decreased overall survival were combined to generate a HNSCC CNA-based SAES (Fig. 4C). Ten genes did not alter overall survival based on expression and were summarily excluded (Supplementary Table S4, Supplementary Figure S1). Patients with overexpression or underexpression of one SAES gene demonstrated lower overall survival (Fig. 4D), where patients with overexpression or underexpression of at least three SAES genes also had significantly worse progression-free survival (Fig. 4E). The impact of SAES gene expression on survival was additive, with decreased survival observed in patients with increasing numbers of SAES genes (Fig. 4D and E, Supplementary Figure S2). Multivariate analysis with age, sex, AJCC stage, HPV status, extracapsular spread, perineural invasion, and lymphovascular invasion confirmed overexpression of *ANO1*, *CCND1*, *CTTN*, *FADD*, *MRPL21*, and *TPCN2* as independent predictors of overall survival in the SAES (Supplementary Table S5).

Increased Cigarette Smoking Predicts Smoking-associated CNAs and the Smoking Expression Signature

We evaluated patient pack-year data with smoking-associated gene CNA to determine if it could be used to determine CNA risk. Each gene was fit into a linear regression model, with pack-year as the explanatory variable and gene amplification for the response of one testing sample. This was used to determine the probability of CNA for each gene (Supplementary Table S6). Significant genes were combined to create average curves for log odds changes with smoking pack-year and the log odds of gene CNA in non-smokers across all genes, using parameter averages for linear regression models for each gene (Fig. 5A). A parallel analysis was utilized based on the gene RPKM values in the SAES with smoking pack-year to generate regression curves for overexpressed or underexpressed genes mapping to SAES regions (Fig. 5B). Only genes mapping to 11q13.3-q13.4 were significant for both analyses (Supplementary Table S7). Mean gene amplification and overexpression levels were predicted to increase with increasing pack-year value, correlating increased smoking levels with higher risk of 11q13.3-q13.4 amplification and SAES transcript overexpression in HNSCC.

Smoking-associated Expression Signature Genes Predict Increased Risk of Death and Cervical Lymph Node Metastasis

Since regional cervical lymph node metastasis in HNSCC is the primary clinical parameter for poor clinical outcome(38,39), we determined the relative risk of death and lymph node involvement in HNSCC patients with the SAES. All patients in the TCGA-HNSC cohort were evaluated regardless of smoking status to compensate for the presence of SAES in patients using other tobacco forms that can potentially result in tobacco-associated CNA(40). For each SAES gene, a positive correlation was observed between risk of death and standardized expression (Fig. 5C, Supplementary Figure S3). Overexpression of an eight-gene SAES subset predicted significantly an increased odds of lymph node metastasis, (Fig. 5D), identifying specific SAES genes that may drive poorer outcomes through increased metastatic risk.

Discussion

Genes mapping to the amplified 11q13.3-q13.4 cytobands and the deleted 9p21.3 and 9p24.1 cytobands were the only CNAs that correlated with pack-year in a significant number of HNSCC patients in this cohort. Patients with smoking-associated CNAs have worse overall survival, smoked more, were predominantly male, and HPV-negative. Correlation analysis of transcript expression of smoking-associated CNA genes identified an expression subset associated with poor outcome and nodal involvement. Increased CNA risk and transcript over- or underexpression are associated with elevated patient smoking levels, identifying a novel gene group that can be used for improved screening and target investigation for treatment of late stage HNSCC patients with smoking histories.

Correlation of clinical parameters with molecular markers from comprehensive genomic analyses has emerged as a powerful tool for prognostic analysis in cancer medicine(41,42). Integrated analysis of TCGA and other non-public HNSCC molecular cohorts have identified genomic and mutational patterns that have been instrumental in determining prognostic aspects of HPV involvement, molecular subtyping and mutational status associated with different HNSCC medical aspects(6,7,43–45). Many CNAs identified in these studies have been previously noted in HNSCC and proposedly due to tobacco-induced genomic instability(6,16,37). In addition, our analysis identified other smoking-associated CNAs that reached significance in an HPV-dependent manner (Figures 1B and 2B). Of these, genes mapping to amplified 3q23-q29 (*SOX2/TP63/PIK3CA*), deleted 2q22.1 (*LRP1B*) and 8p23.2 (*CSMD1*) have been previously noted to be amplified or deleted in HNSCC(46–48), supporting the utilized correlation methodology.

Although recent sequencing work supports near-mutual exclusion between tobacco-induced, HPV-negative tumors with tobacco-associated CNAs and HPV-positive tumors lacking tobacco-associated CNAs(6,37,49–51), other studies report smoking-associated CNAs present in HPV-positive patients, although at lower levels than HPV-negative cancers(52). HPV-positive patients with smoking histories have progressively worse outcomes corresponding with elevated pack year(53). Such patients would have an increased likelihood of smoking-associated CNAs and thus manifestation of the SAES in HPV-positive tumors, supporting the observed lack of HPV-positivity with smoking and smoking-associated CNAs revealed in this study.

The tobacco-associated CNAs have been well documented to negatively impact patient prognosis and have been an area of intense study for several decades(54–60). Tobacco-associated CNAs can occur early in malignant development and throughout dysplastic progression to invasive carcinoma(54). Initial TCGA analysis indicated that smoking-associated CNAs corresponded with *TP53* mutation and worse outcome in HPV-negative patients(6). Consistent with this, we found higher *TP53* mutation and smoking-associated CNA frequency, but note that *TP53* mutational status is independent of patient smoking history using a univariate approach (Supplementary Table S1). While this contrasts with previous work linking *TP53* mutation with cigarette smoking in HNSCC(61), it is consistent with the TCGA-HNSC Clinical vs. Mutation Level 4 D1V10 analysis, showing no relationship between *TP53* status and pack-year(6,21). This may be due to varying cutoffs of pack-year usage (Supplementary Table S3). Regardless, there is clear evidence that smoking leads to *TP53* mutation and increases DNA damage through continued cigarette use(62,63). Mutated *TP53* unable to properly surveil and/or repair double-strand DNA breaks will lead to increased instability during replicative stress(28,64). Distal 11q loss and deletion of the *ATM* DNA damage repair gene is frequent in 11q13 amplified HNSCC(16,18,65). Telomeric 11q loss is also associated with 11q22 co-amplification in 11q13-amplified HNSCC, with cooperativity between genes proposed as a selective pressure for co-amplification(6). We note that co-amplification of these regions in the TCGA cohort occurs at low frequency (3.6%) and that 11q22 gene amplification does not correlate with smoking. 11q13/11q22 co-amplification may therefore be driven by other factors that promote DNA breakage near the chromosome 11q telomere.

11q13 amplicon sizes vary from 0.65Mb to over 5Mb(34). Amplicon alignment allows for identification of conserved amplification patterns, providing insight into smoking-induced genomic damage. Segment alignment with FRA11H and FRA11F indicate that 11q13.3-q13.4 SAES genes cluster within segment 2. This is similar to previous 11q13 amplicon gene alignments in HNSCC, breast and other cancers(16,66,67). Harmonizing with these designations, the previously established core 1 alignment corresponds with segment 1, core 2 with segment 2, and cores 3 and 4 with segment 3(67). Over half of 11q13-amplified HNSCC patients exhibit the scenario 1 event involving successive breakage at FRA11F and FRA11H (Fig. 4B). Increased fragile site breakage involving FRA11F and FRA11H in BFB formation has been previously proposed as a tobacco-mediated mechanism utilized in 11q13 amplification(19,31,68), collectively suggesting that progressive breakage at these sites is preferentially favored during the scenario 1 BFB cycle. Irrespective of the amplification scenario,

segment 2 amplification levels and subsequent SAES gene expression is predicted to increase with elevated smoking rates, consistent with tobacco-induced fragile site involvement in generating SAES gene amplification. Simultaneous 11q13 amplicon gene overexpression implies cooperativity between gene products impacting multiple cancer hallmarks to maintain and enhance the neoplastic phenotype. Previous work on 11q13 amplicon genes has proposed that the cell cycle regulator *CCND1*, actin cytoskeletal protein *CTTN* and apoptosis scaffold *FADD* function as the primary 11q13 amplicon drivers in HNSCC, since amplification and overexpression of each of these genes accelerates patient mortality(54,56). These genes are included in the SAES and were validated as independent predictors of overall survival, underscoring their importance in promoting the poor outcomes observed in 11q13-amplified HNSCC cohorts(50,69). Additional amplified SAES genes with known roles in HNSCC include the *ORAOV1/LTO1*(70), *PPFIA1*(71), *ANO1*(72) and *FGF19*(73). Functions of the remaining SAES genes in HNSCC are largely unknown, warranting future investigation to determine their respective contributions in 11q13-amplified HNSCC.

CCND1 and *CTTN* amplification and overexpression corresponds with increased lymph node involvement in numerous cohorts, suggesting their use as biomarkers of aggressive disease(74,75). These genes predict increased metastatic lymph node risk (Fig 5D). Importantly, *CCND1* had the highest lymph node metastatic odds ratio in our study, congruent with overexpression utilized as a biomarker for increased metastatic risk(76). Apart from *CTTN*, the remaining SAES with significantly higher lymph node risk remain to be evaluated as independent markers of lymph node involvement and/or investigated as novel targets in aggressive disease. The importance of understanding how 11q13 amplification and resultant gene overexpression contributes to poor outcome in smoking-induced HNSCC is further underscored by increased resistance to chemotherapy and epidermal growth factor receptor targeting in 11q13-amplified HNSCC(77–79). This highlights a persistent need for earlier and precise diagnosis for patients with SAES CNA and expression status to provide improved clinical management of tobacco-positive, HPV-negative HNSCC.

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Competing Interests

The author(s) declare no competing interests.

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Figure Legends

Figure 1. 11q13.3 and 11q13.4 amplification in HNSCC is correlated with genomic instability resultant from smoking. **A.** Schematic depicting experimental determination of smoking correlated CNAs from the TCGA-HNSC dataset containing 509 patients with CNA and pack-year data. **B.** Segregation of smoking correlated CNAs based on HPV-status. CNAs independent of HPV are denoted in the center of the diagram. **C.** Diagrammatic representation of chromosome 11 with location of significantly smoking-associated amplified 11q13.3-q13.4 genes. Fragile sites FRA(11H), FRA(11F), and SDs are shown at their respective locations. Loci position and size are drawn to scale based on coordinates from the UCSC Genome Browser. **D.** Waterfall plot showing patient pack-year distribution stratified by 11q13.3 cytoband amplification status. **E-F.** Overall (**E**) and disease-free (**F**) survival analysis of patients with 11q13.3 amplification. **G.** Waterfall plot showing patient pack-

year distribution stratified by 11q13.4 amplification status. **H-I.** Overall (**H**) and disease-free (**I**) survival analysis of patients with amplification of 11q13.4.

Figure 2. 9p21.3 and 9p24.1 deletion in HNSCC is correlated with patient smoking status. A. Schematic depicting experimental determination of smoking correlated deletions from the TCGA-HNSC dataset. **B.** Smoking correlated homozygous deletions grouped by HPV-status. CNAs independent of HPV are in the center of the diagram. **C.** Diagrammatic representation of chromosome 9 with location of significantly smoking-associated deleted 9p21.3 and 9p24.1 genes. Fragile site FRA(9C) and the unnamed CRS (9CRS) are shown. Loci position and size are drawn to scale based on coordinates from UCSC Genome Browser. **D.** Waterfall plot showing patient pack-year distribution stratified by 9p21.3 cytoband deletion status. **E-F.** Overall (**E**) and disease-free (**F**) survival analysis of patients with 9p21.3 amplification. **G.** Waterfall plot showing patient pack-year distribution

stratified by 9p24.1 deletion status. **H-I.** Overall (**H**) and disease-free (**I**) Kaplan-Meier survival analysis of patients with amplification of 9p24.1.

Figure 3. 11q13 Amplification Patterns Identify a Distinct Chromosomal Segment Containing Smoking Overexpression Signature Genes in HNSCC. A. Heat map displaying all 11q13 amplified HNSCC patients aligned by amplicon frequency and size. Patient genomic status was categorized by amplification (>2 extra

copies; dark red), gene gain (2 extra copies; light red), heterozygous deletion (light blue), and homozygous deletion (dark blue). Fragile sites 11H (FRA11H; orange) and 11F (FRA11F; purple) and segmental duplications (SD; magenta) with respective genomic location and approximate coordinates are displayed at the top and on the left. Corresponding chromosome 11q cytoband positions are located on the right. Patients are grouped by decreasing amplicon length, with conserved breakage coordinates indicated by segment position. Segment 1 (red); segment 2 (blue); segment 3 (green) are noted with their respective estimated chromosomal ranges. **B.** Frequency of 11q13 amplification scenarios in HNSCC. BFB cycles illustrate each possible amplification event based on chromosomal alignment and frequency of occurrence in the TCGA-HNSC cohort. Fragile site, SD, and amplicon segment locations are color-coded as in (A).

Figure 4. Smoking-associated gene expression identifies a transcriptional signature that negatively impacts HNSCC outcome. **A.** Schematic diagram showing experimental determination of the SAES from the TCGA-HNSC dataset. 260 patients with full mRNA sequencing and pack-year data were evaluated as indicated. **B.** Percentage of patients overexpressed and amplified (red), underexpressed and deleted (blue) compared to

patients that are over- or underexpressed that lack a CNA (black) for each SAES gene. **C.** Genes with overall survival differences between the first and fourth quartile of transcript expression. The list constitutes the SAES in HNSCC. **D-E.** Overall (**D**) and disease-free (**E**) survival analysis of all patients harboring genes from the SAES. Black lines in each graph represent patients with no positive SAES genes, red line represents patients with one positive SAES gene. Light gray lines represent the additive range of patients with multiple positive SAES genes.

Figure 5. Predictive modeling and odds analysis of smoking-associated 11q13 genes in HNSCC survival and lymph node metastasis. **A-B.** Probability analysis of 11q13.3-q13.4 gene (*MTL5*, *MRPL21*, *TPCN2*, *CCND1*, *ORAOV1*, *FGF19*, *ANO1*, *FADD*, *PPFIA1*, *CTTN*, *SHANK2*, *DHCR7*, and *RNF121*) amplification (**A**) and 11q13.3-q13.4 SAES gene (*CCND1*, *CTTN*, *FADD*, *FGF19*, *MRPL21*, *MTL5*, *ORAOV1*, *PPFIA1*, *SHANK2*, and *DHCR7*) overexpression (**B**) in patients with increasing pack-year use. 95% confidence interval (95% CI) shown in shaded regions. **C.** Relative risk of death for overexpressed 11q13.3-q13.4 SAES genes (positive slope) and underexpression of 9p21.3 and 9p24.1 SAES genes (negative slope). Left vertical dashed line represents a z-score of -2 (underexpressed). Right vertical dashed line represents a z-score of 2 (overexpressed). Horizontal dashed line represents a relative risk of 1. **D.** Odds ratio analysis of SAES genes significant for lymph node metastasis due to 11q13.3-q13.4 overexpression. Total patients with lymph node

metastasis (LN) and total patients with overexpression (OX) are shown for each gene with the corresponding odds ratio (OR) and 95% CI.

Chr11 Amplification Genomic Alterations	Total n = 509 (100%)	Smokers n = 387 (100%)	Non-Smokers n = 122 (100%)	Total Amplified n = 136 (100%)	Amplified Smokers n = 120 (100%)	Amplified Non-Smokers n = 16 (100%)	Total Non-Amplified n = 373 (100%)	Non-Amplified Smokers n = 267 (100%)	Non-Amplified Non-Smokers n = 106 (100%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Total SNA	149.9 ± 190.2	154.9 ± 141.8	134.4 ± 294.8	144.6 ± 99.58	150.4 ± 102.9	101.8 ± 55.87	151.9 ± 214.2	156.9 ± 156.5	139.5 ± 316
Total CNA (Amp.)	333.8 ± 436.6	359.7 ± 459.3	251.5 ± 344.4	528.7 ± 475.3 *	541.3 ± 495.5 ‡	434.1 ± 272.1	262.7 ± 399.3 *	278.1 ± 417.9 ‡	224 ± 346.8
Total CNA (Del.)	97.27 ± 242	89.74 ± 202.6	121.1 ± 338	104.3 ± 218.2	97.22 ± 199.4	157 ± 331.1	94.72 ± 250.4	86.39 ± 204.3	115.7 ± 340.3
SNA	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
CASP8	52 (10.2)	32 (8.3) *	20 (16.4) *	3 (2.2) *	2 (1.7) ‡	1 (6.3)	49 (13.1) *	30 (11.2) ‡	19 (17.9)
CDKN2A	110 (21.6)	78 (20.2)	32 (26.2)	29 (21.3)	23 (19.2)	6 (37.5)	81 (21.7)	55 (20.6)	26 (24.5)
FAT1	112 (22)	89 (23)	23 (18.9)	22 (16.2)	21 (17.5)	1 (6.3)	90 (24.1)	68 (25.5)	22 (20.8)
KMT2D	57 (11.2)	41 (10.6)	16 (13.1)	1 (0.7) *	0 (0) ‡	1 (6.3) †	56 (15) *	41 (15.4) ‡	15 (14.2)
LRP1B	86 (16.9)	76 (19.6) *	10 (8.2) *	25 (18.4)	25 (20.8) †	0 (0) †	61 (16.4)	51 (19.1) †	10 (9.4) †
NOTCH1	87 (17.1)	70 (18.1)	17 (13.9)	22 (16.2) *	19 (15.8)	3 (18.8)	65 (17.4) *	51 (19.1)	14 (13.2)
NSD1	60 (11.8)	55 (14.2) *	5 (4.1) *	9 (6.6) *	9 (7.5) ‡	0 (0)	51 (13.7) *	46 (17.2) ‡	5 (4.7) †
PCLO	79 (15.5)	66 (17.1)	13 (10.7)	26 (19.1) *	23 (19.2)	3 (18.8)	53 (14.2) *	43 (16.1)	10 (9.4)
PIK3CA	88 (17.3)	71 (18.3)	17 (13.9)	20 (14.7)	17 (14.2)	3 (18.8)	68 (18.2)	54 (20.2)	14 (13.2)
TP53	353 (69.4)	275 (71.1)	78 (63.9)	114 (83.8) *	102 (85) ‡	12 (75)	239 (64.1) *	173 (64.8) ‡	66 (62.3)
CNA	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
11q13.3 (Amp.)	135 (26.5)	119 (30.7) *	16 (13.1) *	135 (99.3) *	119 (99.2) ‡	16 (100) ‡	0 (0) *	0 (0) ‡	0 (0) ‡
11q13.4 (Amp.)	103 (20.2)	93 (24) *	10 (8.2) *	103 (75.7) *	93 (77.5) ‡	10 (62.5) ‡	0 (0) *	0 (0) ‡	0 (0) ‡
11q13.3 and 11q13.4 (Amp.)	102 (20)	92 (23.8) *	10 (8.2) *	102 (75) *	92 (76.7) ‡	10 (62.5) ‡	0 (0) *	0 (0) ‡	0 (0)
9q21.3 (Del.)	164 (32.2)	139 (35.9) *	25 (20.5) *	65 (47.8) *	60 (50) ‡	5 (31.3)	99 (26.5) *	79 (29.6) ‡	20 (18.9) ‡
9p24.1 (Del.)	55 (10.8)	40 (10.3)	15 (12.3)	16 (11.8)	15 (12.5)	1 (6.3)	39 (10.5)	25 (9.4)	14 (13.2)
9q21.3 and 9q24.1 (Del.)	28 (5.5)	22 (5.7)	6 (4.9)	10 (7.4)	10 (8.3)	0 (0)	18 (4.8)	12 (4.5)	6 (5.7)
Chr9 Deletion Genomic Alterations	Total n = 509 (100%)	Smokers n = 387 (100%)	Non-Smokers n = 122 (100%)	Total Deleted n = 191 (100%)	Deleted Smokers n = 157 (100%)	Deleted Non-Smokers n = 34 (100%)	Total Non-Deleted n = 318 (100%)	Non-Deleted Smokers n = 230 (100%)	Non-Deleted Non-Smokers n = 88 (100%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Total SNA	149.9 ± 190.2	154.9 ± 141.8	134.4 ± 294.8	149.8 ± 137.4	155 ± 146.3	125.3 ± 81.85	150 ± 216.2	154.7 ± 138.9	137.9 ± 343.6
Total CNA (Amp.)	333.8 ± 436.6	359.7 ± 459.3	251.5 ± 344.4	388.4 ± 508.3	406.7 ± 519.4	304.2 ± 451	300.9 ± 384.4	327.6 ± 411.3	231.2 ± 293.9
Total CNA (Del.)	97.27 ± 242	89.74 ± 202.6	121.1 ± 338	120.8 ± 202.9	110.5 ± 193.3	168.7 ± 240	83.1 ± 262	75.6 ± 207.9	102.7 ± 368.6
SNA	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
CASP8	52 (10.2)	32 (8.3) *	20 (16.4) *	22 (11.5)	12 (7.6) †	10 (29.4) ‡	30 (9.4)	20 (8.7)	10 (11.4) ‡
CDKN2A	110 (21.6)	78 (20.2)	32 (26.2)	17 (8.9) *	12 (7.6) ‡	5 (14.7)	93 (29.2) *	66 (28.7) ‡	27 (30.7)
FAT1	112 (22)	89 (23)	23 (18.9)	46 (24.1)	32 (20.4) †	14 (41.2) ‡	66 (20.8)	57 (24.8) †	9 (10.2) ‡
KMT2D	81 (15.9)	65 (16.8)	16 (13.1)	33 (17.3)	29 (18.5)	4 (11.8)	48 (15.1)	36 (15.7)	12 (13.6)
LRP1B	86 (16.9)	76 (19.6) *	10 (8.2) *	31 (16.2)	28 (17.8)	3 (8.8)	55 (17.3)	48 (20.9) †	7 (8) †
NOTCH1	87 (17.1)	70 (18.1)	17 (13.9)	44 (23) *	37 (23.6) ‡	7 (20.6)	43 (13.5) *	33 (14.3) ‡	10 (11.4)
NSD1	60 (11.8)	55 (14.2) *	5 (4.1) *	22 (11.5)	21 (13.4)	1 (2.9)	38 (11.9)	34 (14.8) †	4 (4.5) †
PCLO	79 (15.5)	66 (17.1)	13 (10.7)	28 (14.7)	25 (15.9)	3 (8.8)	51 (16)	41 (17.8)	10 (11.4)
PIK3CA	88 (17.3)	71 (18.3)	17 (13.9)	25 (13.1)	19 (12.1) ‡	6 (17.6)	63 (19.8)	52 (22.6) †	11 (12.5) †
TP53	353 (69.4)	275 (71.1)	78 (63.9)	146 (76.4) *	127 (80.9) ‡	19 (55.9) †	207 (65.1) *	148 (64.3) ‡	59 (67)
CNA	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
11q13.3 (Amp.)	135 (26.5)	119 (30.7) *	16 (13.1) *	70 (36.6) *	64 (40.8) ‡	6 (17.6) †	65 (20.4) *	55 (23.9) ‡	10 (11.4)
11q13.4 (Amp.)	103 (20.2)	93 (24) *	10 (8.2) *	52 (27.2) *	48 (30.6) ‡	4 (11.8) †	51 (16) *	45 (19.6) ‡	6 (6.8)
11q13.3 and 11q13.4 (Amp.)	102 (20)	92 (23.8) *	10 (8.2) *	51 (26.7) *	47 (29.9) ‡	4 (11.8) †	51 (16) *	45 (19.6) ‡	6 (6.8)
9q21.3 (Del.)	164 (32.2)	139 (35.9) *	25 (20.5) *	164 (85.9) *	139 (88.5) ‡	25 (73.5) ‡	0 (0) *	0 (0) ‡	0 (0) ‡
9p24.1 (Del.)	55 (10.8)	40 (10.3)	15 (12.3)	55 (28.8) *	40 (25.5) ‡	15 (44.1) ‡	0 (0) *	0 (0) ‡	0 (0) ‡
9q21.3 and 9q24.1 (Del.)	28 (5.5)	22 (5.7)	6 (4.9)	28 (14.7) *	22 (14) ‡	6 (17.6) ‡	0 (0) *	0 (0) ‡	0 (0) ‡
* p<0.05 Total Amplified/Deleted vs Total Non-Amplified/Deleted or Total Smoker vs Total Non-Smoker by Pearson chi-squared test (bottom)									
† p<0.05 Amplified/Deleted Smoker vs Amplified/Deleted Non-Smoker or Non-Amplified/Deleted Smoker vs Non-Amplified/Deleted Non-Smoker by one-way ANOVA (top) or Pearson chi-squared test (bottom)									
‡ p<0.05 Amplified/Deleted Smoker vs Non-Amplified/Deleted Smoker or Amplified/Deleted Non-Smoker vs Non-Amplified/Deleted Non-Smoker by one-way ANOVA (top) or Pearson chi-squared test (bottom)									
SD: Standard Deviation									
Smokers: Pack-year > 0 or Patient Smoking History; Non-Smokers: Pack-year = 0 or no reported Patient Smoking History									

Table 1. Mutational Frequency of Common HNSCC-associated Genes in Smoking and non-Smoking Patients with 11q13.3 and 11q13.4 Gene Amplification

Study 2 Supplementary Materials: Copy Number Alterations

Identify a Smoking-Associated Expression Signature Predictive of Poor Outcome in Head and Neck Squamous Cell Carcinoma

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Chr11 Amplification Patient Characteristics	Total n = 509 (100%) Mean ± SD	Total Smokers n = 364 (100%) Mean ± SD	Total Non-Smokers n = 145 (100%) Mean ± SD	Total Amplified n = 136 (100%) Mean ± SD	Amplified Smokers n = 120 (100%) Mean ± SD	Amplified Non-Smokers n = 16 (100%) Mean ± SD	Total Non-Amplified n = 373 (100%) Mean ± SD	Non-Amplified Smokers n = 244 (100%) Mean ± SD	Non-Amplified Non-Smokers n = 129 (100%) Mean ± SD
Age (years)	60.7 ± 12.2	61.2 ± 11.0	59.1 ± 15.1	59.8 ± 12.4	60.1 ± 12.3	57.9 ± 13.4	61 ± 12.2	61.7 ± 10.4	59.3 ± 15.4
Smoking Usage									
Pack-years	32.4 ± 36.3	45.9 ± 35.3 *	0 ± 0 *	46.6 ± 41.7 *	54.4 ± 40.1 †	0 ± 0 †	27.1 ± 32.6 *	41.7 ± 32.2 †	0 ± 0 †
Alcohol Usage									
Weekly Drinks	20.3 ± 29.9	24.4 ± 31.7 *	5.0 ± 13.8 *	26.6 ± 30.8	28.5 ± 31.0 †	0 ± 0 †	17.7 ± 29.2	22.3 ± 31.9 †	5.5 ± 14.4 †
Survival (months)	30.3 ± 28.5	30.6 ± 29.8	29.1 ± 23.9	29.3 ± 27.3	29.7 ± 28.1	26.5 ± 20.5	30.6 ± 28.9	31.1 ± 30.5	29.5 ± 24.5
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Sex									
Male	375 (73.7)	290 (79.7) *	85 (58.6) *	106 (77.9)	97 (80.8) †	9 (56.3) †	269 (72.1)	193 (79.1) †	76 (58.9) †
Female	134 (26.3)	74 (20.3) *	60 (41.4) *	30 (22.1)	23 (19.2) †	7 (43.8) †	104 (27.9)	51 (20.9) †	53 (41.1) †
AJCC Stage									
I	26 (5.1)	16 (4.4)	10 (6.9)	6 (4.4)	6 (5)	0 (0)	20 (5.4)	10 (4.1)	10 (7.8)
II	83 (16.3)	57 (15.7)	26 (17.9)	17 (12.5)	15 (12.5)	2 (12.5)	66 (17.7)	42 (17.2)	24 (18.6)
III	95 (18.7)	67 (18.4)	28 (19.3)	16 (11.8)	15 (12.5)	1 (6.3)	79 (21.2)	52 (21.3)	27 (20.9)
IV	305 (59.9)	224 (61.5)	81 (55.9)	97 (71.3) *	84 (70)	13 (81.3)	208 (55.8) *	140 (57.4)	68 (52.7)
N Status									
N Positive	255 (50.1)	189 (51.9)	66 (45.5)	82 (60.3) *	73 (60.8) †	9 (56.3)	173 (46.4) *	116 (47.5) †	57 (44.2)
N Negative	182 (35.8)	127 (34.9)	55 (37.9)	37 (27.2)	30 (25)	7 (43.8)	145 (38.9)	97 (39.8)	48 (37.2)
Missing	72 (14.1)	48 (13.2)	24 (16.6)	17 (12.5)	17 (14.2)	0 (0)	55 (14.7)	31 (12.7)	24 (18.6)
HPV Status									
Positive	98 (19.3)	59 (16.2) *	39 (26.9) *	17 (12.5) *	15 (12.5)	2 (12.5)	81 (21.7) *	44 (18) †	37 (28.7) †
Negative	409 (80.4)	304 (83.5) *	105 (72.4) *	119 (87.5) *	105 (87.5)	14 (87.5)	290 (77.7) *	199 (81.6) †	91 (70.5) †
Missing	2 (0.4)	1 (0.3)	1 (0.7)	0 (0)	0 (0)	0 (0)	2 (0.5)	1 (0.4)	1 (0.8)
Chr9 Deletion Patient Characteristics	Total n = 509 (100%) Mean ± SD	Total Smokers n = 360 (100%) Mean ± SD	Total Non-Smokers n = 149 (100%) Mean ± SD	Total Deleted n = 191 (100%) Mean ± SD	Deleted Smokers n = 149 (100%) Mean ± SD	Deleted Non-Smokers n = 42 (100%) Mean ± SD	Total Deleted n = 318 (100%) Mean ± SD	Non-Deleted Smokers n = 211 (100%) Mean ± SD	Non-Deleted Non-Smokers n = 107 (100%) Mean ± SD
Age (years)	60.7 ± 12.2	61.2 ± 11.0	59.1 ± 15.1	61.5 ± 11.8	60.7 ± 11.3	65.5 ± 13.6 †	60.2 ± 12.3	61.5 ± 10.9 †	56.6 ± 15.0 †
Smoking Usage									
Pack-years	32.4 ± 36.3	45.9 ± 35.3 *	0 ± 0 *	34.5 ± 31.4	44.3 ± 28.8 †	0 ± 0 †	31.2 ± 38.9	47.0 ± 39.2 †	0 ± 0 †
Alcohol Usage									
Weekly Drinks	20.3 ± 29.9	24.4 ± 31.7 *	5.0 ± 13.8 *	21.2 ± 24.0	23.6 ± 24.3	1.1 ± 2.45	19.8 ± 32.7	24.9 ± 35.9 †	5.8 ± 15.1 †
Survival (months)	30.3 ± 28.5	30.6 ± 29.8	29.2 ± 23.9	29.4 ± 29.3	29.5 ± 29.5	28.9 ± 28.9	30.8 ± 28.0	31.4 ± 30.0	29.1 ± 21.9
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Sex									
Male	375 (73.7)	288 (80) *	87 (58.4) *	131 (68.6)	114 (76.5) †	17 (40.5) †	244 (76.7)	174 (82.5) †	70 (65.4) †
Female	134 (26.3)	72 (20) *	62 (41.6) *	60 (31.4)	35 (23.5) †	25 (59.5) †	74 (23.3)	37 (17.5) †	37 (34.6) †
AJCC Stage									
I	26 (5.1)	15 (4.2)	11 (7.4)	9 (4.7)	6 (4)	3 (7.1)	17 (5.3)	9 (4.3)	8 (7.5)
II	83 (16.3)	56 (15.6)	27 (18.1)	30 (15.7)	23 (15.4)	7 (16.7)	53 (16.7)	33 (15.6)	20 (18.7)
III	95 (18.7)	67 (18.6)	28 (18.8)	31 (16.2)	22 (14.8)	9 (21.4)	64 (20.1)	45 (21.3)	19 (17.8)
IV	305 (59.9)	222 (61.7)	83 (55.7)	121 (63.4)	98 (65.8)	23 (54.8)	184 (57.9)	124 (58.8)	60 (56.1)
N Status									
N Positive	255 (50.1)	188 (52.2)	67 (45)	97 (50.8)	79 (53)	18 (42.9)	158 (49.7)	109 (51.7)	49 (45.8)
N Negative	182 (35.8)	124 (34.4)	58 (38.9)	69 (36.1)	54 (36.2)	15 (35.7)	113 (35.5)	70 (33.2)	43 (40.2)
Missing	72 (14.1)	48 (13.3)	24 (16.1)	25 (13.1)	16 (10.7)	9 (21.4)	47 (14.8)	32 (15.2)	15 (14)
HPV Status									
Positive	98 (19.3)	59 (16.4) *	39 (26.2) *	12 (6.3) *	11 (7.4) †	1 (2.4) †	86 (27)	48 (22.7) †	38 (35.5) †
Negative	409 (80.4)	300 (83.3) *	109 (73.2) *	179 (93.7) *	138 (92.6) †	41 (97.6) †	230 (72.3)	162 (76.8) †	68 (63.6) †
Missing	2 (0.4)	1 (0.3)	1 (0.7)	0 (0)	0 (0)	0 (0)	2 (0.6)	1 (0.5)	1 (0.9)

* p<0.05 Total Amplified/Deleted vs. Total Non-Amplified/Deleted or Total Smoker vs Total Non-Smoker by Pearson chi-squared test (bottom)
† p<0.05 Amplified/Deleted Smoker vs Amplified/Deleted Non-Smoker or Non-Amplified/Deleted Smoker vs Non-Amplified/Deleted Non-Smoker by one-way ANOVA (top) or Pearson chi-squared test (bottom)
‡ p<0.05 Amplified/Deleted Smoker vs Non-Amplified/Deleted Smoker or Amplified/Deleted Non-Smoker vs Non-Amplified/Deleted Non-Smoker by one-way ANOVA (top) or Pearson chi-squared test (bottom)
SD: Standard Deviation
Smokers: Pack-year > 0 or Patient Smoking History; Non-Smokers: Pack-year = 0 or no reported Patient Smoking History

Supplementary Table S1. Clinical Characteristics of HNSCC Patients by Smoking, 11q13.3 and 11q13.4 Cytoband Amplification Status

Supplementary Table S2. Gene GISTIC2 Value and Pack-Year Linear Regression Analysis. Related

to Figures 1 and 2. Available on WVU Library EDT Website.

Mutations by Smoking Status	Total n = 420 (100%)	Never Smoker n = 122 (100%)	Ever Smoker n = 298 (100%)	Heavy Smoker n = 270 (100%)	Low-Risk n = 150 (100%)	Light Smoker n = 28 (100%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Total SNA	130.2 ± 271.9	134.4 ± 294.8	153.6 ± 146	157.7 ± 148.9	130.2 ± 271.9	109.1 ± 101.2
Total CNA (Amp.)	381.6 ± 486.4	251.5 ± 344.4 †	381.6 ± 486.4	397.8 ± 496.9 †‡	239.8 ± 331.9 ‡	171.9 ± 242.5
Total CNA (Del.)	96.1 ± 251.9	121.1 ± 338	85.68 ± 205.6	89.44 ± 213.3	108 ± 309.6	48.7 ± 96.21
SNA	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
CASP8	42 (10)	20 (16.4) *	22 (7.4) *	16 (5.9) ‡	26 (17.3) ‡	6 (21.4)
CDKN2A	89 (21.2)	32 (26.2) †	57 (19.1)	49 (18.1) †‡	40 (26.7) ‡	8 (28.6)
FAT1	85 (20.2)	23 (18.9) †	62 (20.8)	57 (21.1) †	28 (18.7)	5 (17.9)
KMT2D	65 (15.5)	16 (13.1) †	49 (16.4)	46 (17) †	19 (12.7)	3 (10.7)
LRP1B	70 (16.7)	10 (8.2) *†	60 (20.1) *	54 (20) †‡	16 (10.7) ‡	6 (21.4)
NOTCH1	70 (16.7)	17 (13.9) †	53 (17.8)	44 (16.3) †	26 (17.3)	9 (32.1)
NSD1	49 (11.7)	5 (4.1) *†	44 (14.8) *	42 (15.6) †‡	7 (4.7) ‡	2 (7.1)
PCLO	61 (14.5)	13 (10.7) †	48 (16.1)	45 (16.7) †	16 (10.7)	3 (10.7)
PIK3CA	80 (19)	17 (13.9) †	63 (21.1)	56 (20.7) †	24 (16)	7 (25)
TP53	286 (68.1)	78 (63.9) †	208 (69.8)	193 (71.5) †‡	93 (62) ‡	15 (53.6)
* p<0.05 Never Smoker vs Ever Smoker						
† p<0.05 Heavy Smoker vs Never Smoker						
‡ p<0.05 Low Risk vs Heavy Smoker						
SD: Standard Deviation						
Never Smoker: Pack-year = 0; Ever Smoker: Pack-year > 0; Heavy Smoker: Pack-year > 10; Low-Risk: Pack-year <= 10; Light Smoker: Pack-year <= 10 and > 0						

Supplementary Table S3. Mutational Frequency of common HNSCC-associated Genes by

Smoking Exposure. Related to Table 1.

11q13.3 Gene Expression	Total	Total Smokers	Total Non-Smokers	Amplified	Amplified Smokers	Amplified Non-Smokers	Non-Amplified	Non-Amplified Smokers	Non-Amplified Non-Smokers
	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)
Overexpressed									
ANO1	74 / 196 (37.8)	57 / 148 (38.5)	17 / 48 (35.4)	41 / 53 (77.4) *	37 / 48 (77.1) ‡	4 / 5 (80) ‡	33 / 143 (23.1) *	20 / 100 (20)	13 / 43 (30.2) ‡
CCND1	28 / 196 (14.3)	25 / 148 (16.9)	3 / 48 (6.3)	24 / 51 (47.1) *	22 / 46 (47.8) ‡	2 / 5 (40) ‡	4 / 145 (2.8) *	3 / 102 (2.9)	1 / 43 (2.3) ‡
CPT1A	21 / 196 (10.7)	19 / 148 (12.8)	2 / 48 (4.2)	11 / 24 (45.8) *	11 / 22 (50) ‡	0 / 2 (0)	10 / 172 (5.8) *	8 / 126 (6.3)	2 / 46 (4.3)
CTTN	57 / 196 (29.1)	48 / 148 (32.4)	9 / 48 (18.8)	50 / 53 (94.3) *	45 / 48 (93.8) ‡	5 / 5 (100) ‡	7 / 143 (4.9) *	3 / 100 (3)	4 / 43 (9.3) ‡
FADD	152 / 196 (77.6)	117 / 148 (79.1)	35 / 48 (72.9)	53 / 53 (100) *	48 / 48 (100) ‡	5 / 5 (100)	99 / 143 (69.2) *	69 / 100 (69)	30 / 43 (69.8)
FGF19	95 / 196 (48.5)	78 / 148 (52.7) *	17 / 48 (35.4) *	37 / 51 (72.5) *	37 / 46 (80.4) ‡	0 / 5 (0)	58 / 145 (40) *	41 / 102 (40.2)	17 / 43 (39.5)
FGF3	13 / 196 (6.6)	13 / 148 (8.8) *	0 / 48 (0) *	9 / 51 (17.6) *	9 / 46 (19.6) ‡	0 / 5 (0)	4 / 145 (2.8) *	4 / 102 (3.9)	0 / 43 (0)
FGF4	16 / 196 (8.2)	11 / 148 (7.4)	5 / 48 (10.4)	7 / 51 (13.7)	7 / 46 (15.2) ‡	0 / 5 (0)	9 / 145 (6.2)	4 / 102 (3.9)	5 / 43 (11.6)
IGHMBP2	62 / 196 (31.6)	49 / 148 (33.1)	13 / 48 (27.1)	26 / 30 (86.7) *	24 / 28 (85.7) ‡	2 / 2 (100) ‡	36 / 166 (21.7) *	25 / 120 (20.8)	11 / 46 (23.9) ‡
MRGPRD	38 / 196 (19.4)	30 / 148 (20.3)	8 / 48 (16.7)	16 / 34 (47.1) *	15 / 32 (46.9) ‡	1 / 2 (50)	22 / 162 (13.6) *	15 / 116 (12.9)	7 / 46 (15.2)
MRGPRF	9 / 196 (4.6)	7 / 148 (4.7)	2 / 48 (4.2)	2 / 34 (5.9)	2 / 32 (6.3)	0 / 2 (0)	7 / 162 (4.3)	5 / 116 (4.3)	2 / 46 (4.3)
MRPL21	47 / 196 (24)	40 / 148 (27)	7 / 48 (14.6)	24 / 28 (85.7) *	23 / 26 (88.5) ‡	1 / 2 (50)	23 / 168 (13.7) *	17 / 122 (13.9)	6 / 46 (13)
MTL5	60 / 196 (30.6)	52 / 148 (35.1) *	8 / 48 (16.7) *	15 / 20 (75) *	14 / 18 (77.8) ‡	1 / 2 (50)	45 / 176 (25.6) *	38 / 130 (29.2)	7 / 46 (15.2)
MYEOV	5 / 196 (2.6)	5 / 148 (3.4)	0 / 48 (0)	4 / 46 (8.7) *	4 / 43 (9.3) ‡	0 / 3 (0)	1 / 150 (0.7) *	1 / 105 (1)	0 / 45 (0)
ORAOV1	106 / 196 (54.1)	84 / 148 (56.8)	22 / 48 (45.8)	51 / 51 (100) *	46 / 46 (100) ‡	5 / 5 (100) ‡	55 / 145 (37.9) *	38 / 102 (37.3)	17 / 43 (39.5) ‡
PPFIA1	106 / 196 (54.1)	85 / 148 (57.4)	21 / 48 (43.8)	54 / 54 (100) *	49 / 49 (100) ‡	5 / 5 (100) ‡	52 / 142 (36.6) *	36 / 99 (36.4)	16 / 43 (37.2) ‡
SHANK2	16 / 196 (8.2)	15 / 148 (10.1)	1 / 48 (2.1)	13 / 52 (25) *	13 / 47 (27.7) ‡	0 / 5 (0)	3 / 144 (2.1) *	2 / 101 (2)	1 / 43 (2.3)
TPCN2	103 / 196 (52.6)	79 / 148 (53.4)	24 / 48 (50)	33 / 35 (94.3) *	31 / 33 (93.9) ‡	2 / 2 (100)	70 / 161 (43.5) *	48 / 115 (41.7)	22 / 46 (47.8)
11q13.4 Gene Expression	Total	Total Smokers	Total Non-Smokers	Amplified	Amplified Smokers	Amplified Non-Smokers	Non-Amplified	Non-Amplified Smokers	Non-Amplified Non-Smokers
	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)	OX / Amp (%)
Overexpressed									
DHCR7	60 / 196 (30.6)	52 / 148 (35.1) *	8 / 48 (16.7) *	33 / 35 (94.3) *	31 / 100 (100) ‡	2 / 50	27 / 161 (16.8) *	21 / 17.95	6 / 13.64
NADSYN1	29 / 196 (14.8)	25 / 148 (16.9)	4 / 48 (8.3)	26 / 35 (74.3)	23 / 74.19	3 / 75 ‡	3 / 161 (1.9) *	2 / 1.71	1 / 2.27 ‡
RNF121	60 / 196 (30.6)	39 / 148 (26.4) *	21 / 48 (43.8) *	23 / 27 (85.2) *	21 / 87.5 ‡	2 / 66.67	37 / 169 (21.9) *	18 / 14.52 ‡	19 / 42.22 ‡
9p21.3 Gene Expression	Total	Total Smokers	Total Non-Smokers	Deleted	Deleted Smokers	Deleted Non-Smokers	Non-Deleted	Non-Deleted Smokers	Non-Deleted Non-Smokers
	UX / Del (%)	UX / Del (%)	UX / Del (%)	UX / Del (%)	UX / Del (%)	UX / Del (%)	UX / Del (%)	OX / Amp (%)	OX / Amp (%)
Underexpressed									
C9ORF53	0 / 196 (0)	0 / 148 (0)	0 / 48 (0)	0 / 49 (0)	0 / 0 ‡	0 / 0	0 / 147 (0) *	0 / 0	0 / 0
CDKN2A	12 / 196 (6.1)	12 / 148 (8.1) *	0 / 48 (0) *	11 / 50 (22) *	11 / 25 ‡	0 / 0	1 / 146 (0.7)	1 / 0.96	0 / 0
CDKN2B	41 / 196 (20.9)	35 / 148 (23.6)	6 / 48 (12.5)	35 / 45 (77.8) *	30 / 76.92 ‡	5 / 83.33 ‡	6 / 151 (4) *	5 / 4.59	1 / 2.38 ‡
MTAP	28 / 196 (14.3)	20 / 148 (13.5)	8 / 48 (16.7)	21 / 28 (75) *	16 / 69.57 ‡	5 / 100 ‡	7 / 168 (4.2) *	4 / 3.2	3 / 6.98 ‡
9p24.1 Gene Expression	Total	Total Smokers	Total Non-Smokers	Deleted	Deleted Smokers	Deleted Non-Smokers	Non-Deleted	Non-Deleted Smokers	Non-Deleted Non-Smokers
	UX / Del (%)	UX / Del (%)	UX / Del (%)	UX / Del (%)	UX / Del (%)	UX / Del (%)	UX / Del (%)	OX / Amp (%)	OX / Amp (%)
Underexpressed									
PTPRD	0 / 196 (0)	0 / 148 (0)	0 / 48 (0)	0 / 16 (0)	0 / 0	0 / 0	0 / 180 (0)	0 / 0	0 / 0
* p<0.05 Total Amplified/Deleted vs Total Non-Amplified/Deleted or Total Smoker vs Total Non-Smoker by Pearson chi-squared test (bottom)									
† p<0.05 Amplified/Deleted Smoker vs Amplified/Deleted Non-Smoker or Non-Amplified/Deleted Smoker vs Non-Amplified/Deleted Non-Smoker by Pearson chi-squared									
‡ p<0.05 Amplified/Deleted Smoker vs Non-Amplified/Deleted Smoker or Amplified/Deleted Non-Smoker vs Non-Amplified/Deleted Non-Smoker by Pearson chi-squared									
OX: Overexpressed; UX: Underexpressed; Amp: Amplified; Del: Deleted									
Smokers: Pack-year > 0 or Patient Smoking History; Non-Smokers: Pack-year = 0 or no reported Patient Smoking History									

Supplementary Table S4. Smoking-associated Gene Overexpression of HNSCC Patients by Smoking, 11q13.3 and 11q13.4 Cytoband Amplification Status. Related to Figure 4.

Supplementary Figure S1. Determination of smoking-associated expression signature through effect of differential gene expression on overall survival. Overall Survival analysis of the differential expression levels for each identified gene comparing the first quartile (lowest 25%) to the fourth quartile (highest 25%). Genes with a significant effect on overall survival were combined into the smoking-associated expression signature.

SAES Gene Expression Effect on Overall Survival

SAES Gene Expression Effect on Disease-Free Survival

Supplementary Figure S2. Additive effect of SAES gene over- or underexpression on overall survival. Kaplan-Meier survival analysis comparing the effect of additional over- or underexpression of a SAES genes on overall (top) and disease-free (bottom) survival. Median survival and p-value for each signature are color-key on the right.

	Gene Copy-Number Alteration					Gene Expression				
	Cox Hazard Ratio	2.5% CI	97.5% CI	P-value		Cox Hazard Ratio	2.5% CI	97.5% CI	P-value	
ANO1	1.050	0.940	1.174	0.386		1.298	1.088	1.549	0.004	
C9orf53	0.688	0.493	0.959	0.027		0.971	0.933	1.010	0.142	
CCND1	1.045	0.928	1.177	0.463		1.122	1.003	1.255	0.043	
CDKN2A	0.705	0.507	0.981	0.038		0.977	0.895	1.066	0.605	
CDKN2B	0.836	0.601	1.162	0.286		0.917	0.797	1.054	0.222	
CPT1A	0.975	0.824	1.154	0.770		1.043	0.937	1.160	0.443	
CTTN	1.043	0.934	1.164	0.455		1.109	1.018	1.207	0.017	
DHCR7	1.125	0.973	1.300	0.112		1.117	0.998	1.250	0.054	
FADD	1.048	0.938	1.171	0.404		1.100	1.025	1.181	0.008	
FGF19	1.060	0.942	1.192	0.332		1.001	0.999	1.004	0.290	
FGF3	1.074	0.956	1.207	0.227		0.996	0.980	1.013	0.653	
FGF4	1.071	0.952	1.204	0.254		0.996	0.980	1.014	0.679	
IGHMBP2	1.019	0.871	1.191	0.818		1.040	0.982	1.102	0.178	
MRGPRD	1.058	0.912	1.227	0.459		0.994	0.940	1.051	0.823	
MRGPRF	1.040	0.897	1.205	0.604		0.848	0.657	1.093	0.203	
MRPL21	1.004	0.856	1.177	0.963		1.063	1.016	1.112	0.009	
MTAP	0.978	0.719	1.329	0.885		0.936	0.848	1.034	0.193	
MTL5	0.983	0.816	1.184	0.855		1.046	0.960	1.139	0.304	
MYEOV	1.081	0.957	1.221	0.211		1.148	0.919	1.433	0.223	
NADSYN1	1.125	0.973	1.300	0.112		1.037	0.881	1.220	0.664	
ORAOV1	1.048	0.931	1.180	0.438		1.030	0.980	1.083	0.245	
PPFIA1	1.045	0.937	1.167	0.428		1.053	0.995	1.113	0.073	
PTPRD	1.121	0.921	1.364	0.254		0.964	0.890	1.044	0.364	
RNF121	1.238	1.012	1.515	0.038		1.079	0.951	1.223	0.238	
SHANK2	1.028	0.919	1.150	0.625		1.199	0.964	1.491	0.102	
TPCN2	1.040	0.903	1.197	0.586		1.111	1.026	1.203	0.010	
DEFB108B	1.129	0.928	1.373	0.224						
ENPP7P8	1.090	0.910	1.306	0.350						
FAM86C1	1.121	0.919	1.368	0.259						
MIR3164	1.040	0.903	1.197	0.586						
MIR3664	1.105	0.977	1.249	0.114						
MIR548K	1.046	0.937	1.169	0.423						
RNA5SP342	1.129	0.928	1.373	0.224						
UNC93B6	1.177	1.008	1.374	0.039						
ZNF705E	1.129	0.928	1.373	0.224						

Supplementary Table S5. Multivariate Analysis Predicting Overall Survival using Age, Sex, AJCC Stage, HPV-Status, Extracapsular Spread, Perineural Invasion, and Lymphovascular Invasion

Relative Risk of Death Associated with SAES Gene Expression

Supplementary Figure S3. Relative risk of death of each smoking-associated expression signature gene. Pearson correlations between standardized gene expression (z-score) and risk of death (hazard ratio) for each individual SAES gene in Fig. 5C

	odds ratio	2.5% CI	97.5% CI	P-value
MTL5	1.013	1.005	1.020	8.05E-04
CPT1A	1.013	1.006	1.020	6.07E-04
MRPL21	1.013	1.006	1.021	2.69E-04
IGHMBP2	1.013	1.006	1.021	2.75E-04
MRGPRD	1.016	1.009	1.024	2.57E-05
MRGPRF	1.017	1.010	1.025	7.16E-06
TPCN2	1.017	1.009	1.024	1.24E-05
MIR3164	1.017	1.009	1.024	1.24E-05
MYEOV	1.017	1.010	1.024	4.16E-06
CCND1	1.016	1.010	1.024	3.92E-06
ORAOV1	1.016	1.010	1.024	3.92E-06
FGF19	1.016	1.010	1.024	3.92E-06
FGF4	1.016	1.010	1.024	3.92E-06
FGF3	1.016	1.009	1.023	6.15E-06
ANO1	1.017	1.011	1.025	1.37E-06
FADD	1.017	1.010	1.024	2.20E-06
PPFIA1	1.017	1.010	1.024	2.42E-06
MIR548K	1.017	1.010	1.024	2.42E-06
CTTN	1.016	1.010	1.024	3.36E-06
SHANK2	1.016	1.009	1.023	6.12E-06
MIR3664	1.015	1.008	1.022	3.13E-05
DHCR7	1.016	1.009	1.024	2.46E-05
NADSYN1	1.016	1.009	1.024	2.46E-05
UNC93B6	1.017	1.010	1.025	1.47E-05
ENPP7P8	1.016	1.009	1.025	4.12E-05
FAM86C1	1.016	1.009	1.025	7.30E-05
ZNF705E	1.015	1.008	1.023	1.43E-04
DEFB108B	1.015	1.008	1.024	1.25E-04
RNA5SP342	1.015	1.008	1.024	1.25E-04
RNF121	1.015	1.008	1.024	1.51E-04
MTAP	1.004	0.998	1.010	0.177
C9orf53	1.003	0.997	1.009	0.294
CDKN2A	1.003	0.997	1.008	0.362
CDKN2B	1.003	0.997	1.009	0.266
PTPRD	1.001	0.992	1.008	0.822

Supplementary Table S6. Predictive Probability Linear Regression of Gene CNA by GISTIC2 Value and Pack-Year. Related to Figure 5.

	Estimate	Standard Error	z.value	P.value
ANO1	0.006343591	0.004242823	1.495134	0.135
CCND1	0.012633468	0.005410455	2.33501	0.020
CPT1A	0.013633059	0.005939486	2.295327	0.022
CTTN	0.017000566	0.004766874	3.566397	0.000
DHCR7	0.021053314	0.004994869	4.214988	0.000
FADD	0.01377474	0.005896312	2.336162	0.019
FGF19	0.011723851	0.004431076	2.645825	0.008
FGF3	0.021877574	0.007099945	3.081372	0.002
FGF4	-0.000668514	0.007599246	-0.087971	0.930
IGHMBP2	0.006795303	0.004361989	1.557845	0.119
MRGPRD	0.00612869	0.004965211	1.234326	0.217
MRGPRF	0.002650902	0.009437755	0.280883	0.779
MRPL21	0.010992611	0.004682154	2.347768	0.019
MTL5	0.015144763	0.004639637	3.264213	0.001
MYEOV	0.018813873	0.010098192	1.863093	0.062
NADSYN1	0.021029108	0.005666638	3.711038	0.000
ORAOV1	0.009501594	0.004406822	2.15611	0.031
PPFIA1	0.015513346	0.004739885	3.272937	0.001
RNF121	-0.002647988	0.004567647	-0.579727	0.562
SHANK2	0.014984733	0.00651749	2.299157	0.021
TPCN2	0.006648249	0.004262356	1.559759	0.119
C9orf53	<i>* all values are 0 in C9orf53</i>			
CDKN2A	1.00E-02	7.51E-03	1.33E+00	0.183
CDKN2B	5.53E-03	4.86E-03	1.14E+00	0.255
MTAPP	3.41E-03	5.66E-03	6.03E-01	0.547
PTPRD	<i>* all values are 0 in PTPRD</i>			

Supplementary Table S7. Predictive Probability Linear Regression of Gene Over- or Underexpression by Z-score and Pack-Year. Related to Figure 5.

Study 3: The CCND1 3'-UTR as a Potential Driver of Poor Outcome in Chromosome 11q13-amplified Head and Neck Squamous Cell Carcinoma

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Running title: CCND1 3'-UTR in HNSCC progression

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Keywords: CCND1, competing endogenous RNA, 3'-UTR, microRNA, head and neck cancer

Abstract

Background: Three prime untranslated regions (3'-UTRs) of messenger (m)RNAs can serve as competing endogenous (ce)RNAs through the binding and titration of regulatory micro (mi)RNAs. Inactivation of tumor-suppressive miRNAs by UTRs with shared miRNA response elements (MREs) promotes cancer progression through permissive expression of miRNA-targeted oncogenic transcripts. Chromosome 11q13 amplification drives the progression of head and neck squamous cell carcinoma (HNSCC) and other cancer types through oncogenic protein overexpression. However, the collective ability of 3'-UTR regions in 11q13 overexpressed transcripts to function as oncogenic ceRNAs is unclear.

Methods: We utilized TargetScan to predict MREs for the 3'-UTR of 11q13 genes and The Cancer Genome Atlas (TCGA) to analyze and stratify gene, exon, and protein expression values. Western blotting was used to determine protein expression. mRNA in-situ hybridization (mRNA-ISH) was used to quantify mRNA expression and localization in HNSCC cell lines.

Results: Six transcripts from the most commonly amplified 11q13 genes in HNSCC are overexpressed and maintain the 3'-UTR. Of these, the *CCND1* 3'-UTR is predicted to bind 31 tumor suppressive miRNAs, thus has oncogenic ceRNA characteristics. Overexpression of the 3'-UTR encoded from *CCND1* exon 5 denotes worse clinical outcome. HNSCC cells lines with 11q13 amplification overexpress the *CCND1* 3'-UTR in the cytoplasm, where it can function as a ceRNA. Correlation analysis of global protein expression in patients with *CCND1* 3'-UTR overexpression identified significant protein overexpression from genes with 3'-UTR MREs homologous with *CCND1*. Four proteins (BID, RB1, PXN, MACC1) were positively correlated in human papillomavirus (HPV)-positive, and eight proteins (SERPINE1, TYMS, DVL3, ITGA2, BCL2L11, CCNB1, GATA3, and E2F1) were positively correlated in HPV-negative HNSCC. Similar correlation analysis of four other 11q13-amplified cancer types identified SERPINE1, GATA3 and ITGA2 as common upregulated proteins in tumors with *CCND1* 3'-UTR overexpression, suggesting conservation of the potential *CCND1* 3'-UTR ceRNA mechanism.

Conclusions: An integrated approach across multiple patient cohorts in different cancers identified several candidate genes potentially regulated by high *CCND1* 3'-UTR overexpression and ceRNA activity, which may be utilized for additional therapeutic strategies to improve the outcomes of patients with *CCND1* amplification and protein overexpression.

Background

HNSCC is an aggressive neoplasm of the upper aerodigestive tract caused by tobacco, alcohol, and high-risk HPV infection(1–3). HPV-negative patients have a worse outcome than HPV-positive patients, stratifying the disease into distinct prognostic subtypes(4–6). TCGA and others have identified large-scale genomic copy number alterations (CNAs) in HNSCC(7,8). Of these, amplification of the 11q13 chromosome region occurs in 25-50% of cases and is the most frequent CNA in HPV-negative HNSCC(9,10). Patients with 11q13 amplification have more metastatic disease, shorter time to recurrence and die sooner than HPV-negative patients lacking amplification(11,12). The overexpressed G1/S cell cycle promoting protein cyclin D1, encoded from the *CCND1* gene, is accepted as the main oncogenic driver in 11q13 amplified cancer through increased cyclin-dependent kinase (CDK)4/6 activation and inhibition of the tumor suppressing retinoblastoma protein (pRB)(12–15). *CCND1* amplification and protein overexpression are correlated with decreased patient survival and increased progression in HNSCC(16–19).

Current understanding of 11q13 amplification in mediating HNSCC progression is largely based on the study of protein products from amplified genes(20,21). However, the role of the noncoding regions of overexpressed mRNA transcripts from any 11q13 amplicon gene (e.g., 5' and 3'-UTR regions) has not been evaluated. Non-coding UTR regions are known to contribute to cancer progression(22,23). Such transcripts can function as ceRNAs that operate via several mechanisms(24–27). One mechanism is titration of miRNAs through competitive binding of miRNAs shared with different transcripts containing homologous MREs(26,28,29). Binding of miRNAs to transcripts results in transcript degradation, or blocks translation without substantial degradation(30,31). Both mechanisms reduce protein expression from shared MRE-containing transcripts. Many studies in cancer have shown oncogenic ceRNA effects by long non-coding (lnc) pseudogenes and non-shortened 3'-UTR regions(16,29,32–36). Oncogenic ceRNAs thus sequester tumor-suppressive miRNAs at MREs shared with oncogenic target transcripts. This reduces the available levels of tumor-suppressive miRNAs, allowing transcripts normally degraded/blocked to persist and translate proteins(37). The ultimate result of such ceRNA activity is relief of tumor suppression that enhances neoplastic progression(29,38).

Global ceRNA networks exist in HNSCC(39,40), where dysregulation of miRNA networks causes neoplastic gene expression(41–44). Such studies infer that the non-coding regions in 11q13 amplified transcripts may contribute to the aggressive advancement of HNSCC as ceRNAs. 11q13 genes can be amplified up to 80-fold

in HNSCC, increasing transcript levels 16-48 times above baseline(9,13,17,45). As such, highly overexpressed transcripts from the 11q13 amplicon may serve as ceRNAs in driving HNSCC. In this report we have evaluated the predicted MREs in the 3'-UTR regions of the most commonly overexpressed protein-coding genes from the 11q13 amplicon. The CCND1 3'-UTR region was the only overexpressed transcript containing a higher number of tumor suppressive MREs. Patients with CCND1 3'-UTR overexpression have worse overall survival. The CCND1 3'-UTR localizes to the cytoplasm in 11q13 amplified HNSCC cells, allowing it to serve as a ceRNA. Correlation analysis of protein expression from transcripts with MREs shared with the CCND1 3'-UTR identified four proteins with a positive correlation in HPV-positive HNSCC (BID, RB1, PXN, and MACC1) and eight positively correlated in HPV-negative HNSCC (SERPINE1, TYMS, DVL3, ITGA2, BCL2L11, CCNB1, GATA3, and E2F1) in patients with CCND1 3'-UTR overexpression. Overexpression of several of these proteins is also positively correlated with CCND1 3'-UTR overexpression in other cancers with 11q13 amplification. This informatics-based approach provides new candidate protein and miRNA targets for further mechanistic interrogation of the underlying causes of poor clinical outcomes in 11q13 amplified cancers.

Methods

Clinical and Survival Data

Clinical and survival data were obtained from the 2016_01_28 TCGA head-neck squamous cell carcinoma (TCGA-HNSC) cohort, downloaded from Broad Genomic Data Analysis Center (GDAC) Firehose(46). Data were retrieved from the level 4 TCGA data file All_CDEs.txt and cBioPortal(47). TCGA-HNSC consists of a total of 529 patients. However, only 263 patients had RNAseq data and were used for determining survival analyses. Patients were also evaluated for HPV in the All_CDEs.txt file, denoted by variable “hvp_status”, which was used to stratify the cohort into confirmed HPV-positive and HPV-negative subsets. Kaplan-Meier P-values were calculated using Mantel-Cox log-rank test with an alpha of 0.05.

Determination of MiRNA Response Elements and Genetic Characteristics

We used the University of California Santa Cruz (UCSC) Genome browser(48) and National Center for Biotechnology Information (NCBI) AceView(49) to determine lengths of open reading frames (ORFs), 3'-UTRs, exons, and introns. Predicted MREs for each gene were downloaded from TargetScan using the Conserved site context++ scores file(50). Genes from the 11q13 amplicon were extracted and individual miRNAs were categorized as tumor-suppressive, oncogenic, or indeterminate using OncomiRDB(51). Additional miRNAs were identified by manual evaluation of the literature.

Copy Number and Expression Analysis of Genes, Exons, and Proteins

CNAs were determined by Genomic Identification of Significant Targets in Cancer (GISTIC)2(52) analysis of TCGA 2016_01_28 level 4 data for 24,776 genes(46). Briefly, raw GISTIC2 values within all_data_by_genex.txt were used to categorize patients by amplification (more than two extra copies), gene gain (two extra copies), homozygous deletion, or heterozygous deletion. Expression levels for genes were obtained from TCGA-HNSC (N=263), TCGA bladder urothelial carcinoma (TCGA-BLCA) (N=56), TCGA breast invasive carcinoma (TCGA-BRCA) (N=791), TCGA esophageal carcinoma (TCGA-ESCA) (N=185), and TCGA lung squamous cell carcinoma (TCGA-LUSC) (N=223) 2016_01_28 level 3 RNAseq gene expression datasets(46). Expression levels for exons were obtained from TCGA-HNSC 2016_01_28 level 3 RNAseq exon expression dataset(46). Gene and exon analyses were conducted using log2 fold change (log2FC) normalization calculated from reads per kilobase of transcript, per million reads (RPKM) in normal adjacent tissues as baseline. High and low

expression of each gene or exon was assessed by comparing the first quartile (lowest 25%) of expression to the fourth quartile (highest 25%). Changes in global mRNA expression were determined using the log2FC ratio between high and low CCND1 expression levels. Protein expression levels were obtained from TCGA-HNSC (N=179), TCGA-BLCA (N=50), TCGA-BRCA (N=630), TCGA-ESCA (N=126), and TCGA-LUSC (N=149) 2016_01_28 level 3 RPPA AnnotateWithGene dataset(46). Protein expression analysis was conducted using the provided normalized values. Statistical significance was determined using Pearson correlation or one-way analysis of variance (ANOVA) and Tukey's post-hoc with an alpha of 0.05.

Western Blotting and Antibodies

Western blotting was conducted as described(53) and visualized with autoradiography film (E3012, Denville Scientific) or captured by an Amersham Imager 600 (GE Healthcare Bio-Sciences). Antibodies used were anti-cyclin D1 (sc-718, 1:1000; Santa Cruz Biotechnology) and anti- β -actin (#8457, 1:1000; Cell Signaling Technology).

Cell Lines and Cell Culture

Cell lines used were UMSCC19 and MSK921(54), MDA1586(55), UPCI:SCC040 and UPCI:SCC136(56) and HaCaT(57). UMSCC47(58) was purchased from the American Type Culture Collection (ATCC). All lines were propagated in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

RNA In-Situ Hybridization

RNA in-situ hybridization was performed as previously described(59). Briefly, custom Stellaris® FISH Probes were designed against CCND1 3'UTR by utilizing the Stellaris® RNA FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner (Version 2017). The CCND1 3'UTR were hybridized with Cy5. Stellaris RNA FISH Probe set labeled with (Biosearch Technologies, Inc.), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocolscells. Cells were grown on coverglass, permeabilized, and subsequently hybridized with a buffer containing custom fluorescent probes. Cells were then DAPI stained to visualize nuclei and mounted onto slides for microscopic analysis using a Nikon A1R confocal microscope.

Corrected total cell fluorescence was then calculated for each cell line. Statistical significance was determined one-way ANOVA and Tukey's post-hoc with an alpha of 0.05.

Determination of CCND1-targeting miRNAs

To determine genes that share MREs for *CCND1*-targeting miRNAs to compare global and per-gene expression changes, the TargetScan Predicted Targets (default predictions) file was downloaded(50). *CCND1* was extracted and miRNAs predicted to bind the 3'-UTR were used to find other genes with shared miRNA. Genes that are targeted by any shared miRNA are referred to as "targets". Genes that are not targeted by any of the predicted *CCND1*-targeting miRNA are "controls."

Survival Analyses

Survival was assessed using overall survival months and vital status supplied from TCGA-HNSC, TCGA-BLCA, TCGA-BRCA, TCGA-ESCA, and TCGA-LUSC clinical datasets. Data were stratified by applicable parameters and graphed with the Kaplan-Meier method. Statistical significance was determined using Mantel-Cox log-rank test with an alpha of 0.05.

Results

The 11q13 amplification is associated with overexpressed transcripts that harbor tumor-suppressing miRNA response elements

Based on previously-defined subdivisions in breast cancer(20), the 11q13 amplicon was analyzed for amplification frequency found within TCGA-HNSC patients. The highest frequency of amplification was seen in core 2, with all genes being amplified in more than 20% of patients (Figure 1A). The protein-coding genes within this region were evaluated for 3'-UTR length and expression of the exon containing the 3'-UTR within TCGA-HNSC patients. Several amplified genes contain long 3'-UTRs and displayed 3'-UTR overexpression. These include *CCND1*, *ORAOV1*, *ANO1*, *FADD*, *PPFIA1*, and *CTTN* in greater than 10% of patients (Figure 1B). To determine if these long 3'-UTRs harbored MREs for tumor-suppressing miRNAs, TargetScan Conserved site context++ scores were used to predict MREs and miRNA were classified as tumor-suppressive, oncogenic, or undetermined. Four 11q13 genes had over ten predicted tumor-suppressive MREs: *CCND1* (N=50), *ANO1* (N=11), *PPFIA1* (N=13), and *SHANK2* (N=47) (Figure 1C). *CCND1* was selected for further evaluation due to the unusually high amount of predicted tumor suppressive MREs in the 3'-UTR, its high tumor-suppressive miR:oncomiR ratio (Supplementary Figure S1), and because the 3'-UTR is overexpressed in HNSCC. Several well-known tumor-suppressive miRNAs are predicted to bind to the *CCND1* 3'-UTR, including *miR-1-3p*, *miR-16-5p*, *miR-17-5p*, and *let-7-5p* (Figure 1D). The potential ability to bind and sequester a wide variety of tumor-suppressing miRNAs could contribute to the poor survival seen in patients with genomic *CCND1* amplification (Figure 1E).

CCND1 3'-UTR is overexpressed in 11q13 the cytosol of amplified cells and is associated with poor patient survival

The *CCND1* transcript is encoded from five exons, with exon five containing the last 165nts of the ORF and the 3,192nts 3'-UTR (Figure 2A). After normalizing exon RPKM expression values, all five exons are expressed in their entirety in HNSCC patients. After stratifying patients by amplification (two or more extra copies), gene gain (two extra copies), euploid (no extra copies), heterozygous deletion (loss of one copy), or homozygous deletion (loss of both copies), *CCND1* expression is highest in amplified patients, where amplification and gene gain events exhibit elevated expression compared to euploid patients (Figure 2B). When comparing overall survival

for HNSCC patients with high exon 5 expression (highest quarter) to patients with low exon 5 expression (lowest quarter), patients with high exon 5 expression have decreased median (23.3 vs 90.6 months) and 5-year (32.7% vs 55.7%) overall survival (Figure 2C).

Since nuclear export of the *CCND1* transcript is required for oncogenic transformation(60), the ability of the 3'-UTR to function as a ceRNA likely occurs in the cytoplasm. To determine the localization of the 3'-UTR in 11q13-amplified HNSCC, we evaluated the expression level and subcellular localization of the *CCND1* transcript in HNSCC cell lines. As expected, cyclin D1 protein overexpression was observed in HNSCC cell lines containing 11q13 amplification compared to lines lacking amplification (Figure 3A). To measure *CCND1* 3'-UTR levels in these lines, we performed mRNA-ISH using probes homologous to sequences in the 3'-UTR. 11q13 amplified HNSCC lines contained 2-4-fold higher *CCND1* 3'-UTR levels compared to non-amplified cell lines (Figure 3B). Confocal microscopic evaluation of mRNA-ISH lines determined that the *CCND1* 3'-UTR is largely enriched in the cytosol of 11q13 amplified cells (Figure 3C), supporting a role for a cytoplasmic ceRNA function, as well as confirming that the 3'-UTR is not truncated when overexpressed in HNSCC.

Target RNAs sharing *CCND1* MREs are not globally altered by *CCND1* 3'-UTR overexpression

To determine if *CCND1* 3'-UTR overexpression exerts a global ceRNA effect on mRNA expression, we utilized TargetScan predicted targets as well as TCGA mRNA gene expression data to separate gene expression into genes targeted by the same miRNA predicted to target *CCND1* from miRNAs that are not predicted to bind the *CCND1* 3'-UTR (Figure 4A). 7,396 genes share MREs with *CCND1* ("targets"), while 6,230 do not ("controls"). When comparing gene expression normalized to log2FC, no differences were observed in the expression levels between these global gene groups in either HNSCC as a whole, or stratified by HPV-status (Figure 4B).

Target transcript degradation and downregulation is not the only mechanism employed by ceRNA. Target proteins can be downregulated through inhibiting translation, sequestering transcripts, or direct degradation of the transcript through exonuclease activity(31,61). These alternative translation inhibitory mechanisms may not lead to accelerated degradation of target transcripts and would not be discernible in a global context (Figure 4C). Therefore, it is possible that target proteins can be downregulated due to *CCND1* amplification and overexpression through one of these non-degradative mechanisms, and/or that the *CCND1* 3'UTR effect is only manifest against a small number of targets that would not be discernible by global evaluation (Figure 4D).

CCND1 3'-UTR overexpression correlates with a subset of overexpressed proteins protein expression in HNSCC and other 11q13-amplified cancers

To evaluate alternative readouts of potential *CCND1* 3'-UTR ceRNA activity, TCGA RPPA data and correlated protein expression was correlated with increasing *CCND1* 3'-UTR expression (Figure 5A). Although the TCGA reverse phase protein lysate microarray (RPPA) dataset consists of only 160 different proteins, differences in protein expression were identified that correlated with *CCND1* 3'-UTR expression. In HPV-positive patients, eight proteins are negatively correlated significantly with increasing *CCND1* 3'-UTR expression. Of these, all but the transcripts of *CDKN2A*, *C12ORF5*, and *CTNNA1* shared MREs with *CCND1* (Figure 5D). We also observed four proteins (*BID*, *RB1*, *PXN*, and *MACC1*) that positively correlated significantly with increasing *CCND1* 3'-UTR expression and the transcripts of which share MREs with *CCND1* (Figure 5B). In HPV-negative patients, nine proteins negatively correlated significantly with increasing *CCND1* 3'-UTR expression. Transcripts of *PRKCA*, *MACC1*, *AKT1/2/3*, and *BAK1* share MREs with *CCND1* (Figure 5C). In addition, 12 proteins positively correlate significantly with increasing *CCND1* 3'-UTR expression (Figure 5C). Of these, the transcripts of *SERPINE1*, *TYMS*, *DVL3*, *ITGA2*, *BCL2L11*, *CCNB1*, *GATA3*, and *E2F1* share MREs with the *CCND1* 3'-UTR (Figure 5D). Evaluating mRNA expression for these target proteins revealed altered transcript expression when stratified by *CCND1* 3'-UTR expression levels. Expression levels of *E2F1*, *SERPINE1*, and *TYMS* were significantly different when stratified by *CCND1* 3'-UTR expression, suggesting that *CCND1* expression is connected to target protein expression through control of translation or transcript degradation. The expression levels of the remaining transcripts were not significantly different when stratified by *CCND1* 3'-UTR expression levels, but may be regulated by a ceRNA mechanism that does not utilize target transcript degradation.

In addition to HNSCC, several other cancer types have high levels of 11q13 amplification, including bladder(62), breast(63), esophageal squamous(64) and lung squamous cell carcinoma(65). To determine if the potential *CCND1* 3'-UTR ceRNA mechanism utilized in HNSCC is also present in these cancers, a parallel analysis was performed on TCGA-BLCA, TCGA-BRCA, TCGA-ESCA, and TCGA-LUSC. In TCGA-BLCA, while there were several significantly positively correlated proteins that also shared *CCND1* MREs, no potential targets were shared with TCGA-HNSC. *IGFBP2* and *PARP1* are significantly negatively correlated and shared between TCGA-BLCA and TCGA-HNSC, but the transcripts do not share *CCND1* MREs (Figure 6A). In TCGA-BRCA,

PARP1, CCNB1, ANXA1, DVL3, EIF4EBP1, PRKCA, and MYC were significantly negatively correlated similarly to TCGA-HNSC, but only the transcripts of CCNB1, DVL3, and PRKCA share *CCND1* MREs. Interestingly, shared positive correlations include ITGA2 and GATA3, which do share *CCND1* MREs in the transcripts and are also seen in TCGA-HNSC (Figure 6B). In TCGA-ESCA, LCK was significantly negatively correlated and was shared with TCGA-HNSC. Significantly positively correlated genes that were shared with TCGA-HNSC include both SERPINE1 and ITGA2, which transcripts share *CCND1* MREs (Figure 6C). In TCGA-LUSC, ITGA2 was the only significantly positively correlated protein, with the transcript sharing *CCND1* MREs present in TCGA-HNSC (Figure 6D). Other shared proteins were discordantly regulated across cancer types (Figure 6E).

When evaluating mRNA expression levels of proteins that are shared between *CCND1* and TCGA-HNSC, the expression levels of *ITGA2* and *GATA3* in TCGA-BRCA and *ITGA2* and *SERPINE1* in TCGA-ESCA were all significantly different when stratified by *CCND1* 3'-UTR expression, suggesting that *CCND1* 3'-UTR expression levels has a role in regulating expression of these targets. In contrast, *ITGA2* in TCGA-LUSC was not significantly different, suggesting a different regulatory mechanism of transcript and/or protein expression in this cancer type.

Discussion

In this study, we propose a ceRNA network involving a novel function of *CCND1* 3'-UTR that may compete for tumor-suppressing miRNA, leading to dysregulation of target transcripts and alteration of the expression of target proteins with tumor-promoting functions. We identified several proteins that had altered expression when stratified by high and low expression of the 3'-UTR exon of *CCND1*, including *TYMS*, *SERPINE1*, *CCNB1*, *DVL3*, *ITGA2*, *GATA3*, and *E2F1*. The 3'-UTR of *CCND1* has been confirmed to bind and regulated by multiple miRNA, including miRs-15, 16, 17, and 20. In addition to miRNA, the transcript is also regulated post-transcriptionally by AU-rich element (ARE)-binding proteins (BPs) at seven putative sites that help regulate and stabilize the transcript(66). There is precedent for ceRNA networks in HNSCC as several ceRNA networks have already been described in HNSCC, but these do not include *CCND1*(39,67,68). The concept of amplification leading to the overexpression of transcripts that can serve as ceRNA has also been previously described. These include the lncRNA *PVT1* that is amplified in gastrointestinal cancer(69), the frequently amplified oncogene *CMYC*(70), and *MYEOV* in lung cancer, which arises from the same amplification event as *CCND1*(71). The *CCND1* 3'-UTR also has a function in oncogenic transformation of cells by acting as scaffolding for eIF4E and subsequent assembly of ribonucleotides responsible for the modulation of cellular growth(60). The binding of any miRNA, ARE-BP, and other 3'-UTR-mediated functions, including any potential ceRNA-mediated function, requires the 3'-UTR to not undergo alternative cleavage and polyadenylation (APA) that is typically found in cancer cells(72). Recent studies have shown that this does not seem to occur for the *CCND1* 3'-UTR(73). These reports and our results allow for the potential for ceRNA-mediated function.

This study has identified several proteins that have altered expression when stratified by high and low expression of *CCND1* 3'-UTR, including *TYMS*, *SERPINE1*, *CCNB1*, *DVL3*, *ITGA2*, *GATA3*, and *E2F1*. Most of these proteins have described roles in tumor progression. Thymidylate synthase (encoded by *TYMS*) has a role in DNA synthesis through the generation of thymidine deoxynucleotides, which is an important need of quickly dividing cells found in tumors(74,75). It has become a recent area of interest for targeting due to this role(76). Endothelial plasminogen activator inhibitor (encoded by *SERPINE1*) functions as the main inhibitor of tissue plasminogen activator and urokinase(77). However, a paradoxical role in cancer has been observed with overexpression of *SERPINE1* being linked to tumor cell proliferation, migration, and invasion(77–80) and has

been interesting as of late for targeting with small molecule inhibitors(81). G2/mitotic-specific cyclin B1 (encoded by *CCNB1*) is a cell cycle regulatory protein that complexes with CDK1 to regulate early cell cycle events(82) such as condensation of chromosomes(83). *CCNB1* is similar to *CCND1* in that it regulates cell cycle and aberrant expression can lead to increased cell cycle and increased tumor progression(84–88). Segment polarity protein disheveled homolog DVL-3 (encoded by *DVL3*) functions in the regulation of cell proliferation(89) and overexpression of *DVL3* has been linked to increased cellular proliferation and migration(90,91). Integrin alpha-2 (*ITGA2*) makes up part of the $\alpha 2\beta 1$ integrin duplex and is involved in cell adhesion and cell-surface-mediated signaling and has been implicated in tumor progression(92–96). GATA binding protein 3 (encoded by *GATA3*) is a transcription factor that binds GATA DNA sequences and functions in the development of tissues(97) and T helper cells(98), secretion of interleukins, and also induces maturation of breast epithelial cells(99). Its role in cancer is controversial, as it has been shown to have tumor-promoting(100) and tumor-suppressing roles(101), likely due to different transcriptional targets. E2F1 (encoded by *E2F1*) is a transcription factor involved in the CDK-pRB pathway that is responsible for regulating the transcription of S phase genes(102), including *TYMS*(103). Due to its function, overexpression of E2F1 has been implicated as a tumor-promoter in numerous cancer types(104–106) as well as a tumor-suppressor(107) depending on its transcriptional targets.

Importantly, all identified target genes share confirmed MREs with *CCND1*. Perhaps the most well-known is its ability to bind hsa-miR-16(108) which is shared with *ITGA2*(109). *CCND1* has also been shown to bind hsa-miR-19(110), shared with *BCL2L11*(111). Both *BCL2L11* and *E2F1* share hsa-miR-17(111,112), hsa-miR-20(111,112), hsa-miR-106(113,114), and hsa-miR-302(115,116) with *CCND1*(117,118). *SERPINE1* shares hsa-miR-34(119) and hsa-miR-449(120) binding with *CCND1*(121,122). *TYMS* shares hsa-miR-203 binding(123) with *CCND1*(124). The miRNA hsa-miR-372 is shared between *ITGA2*(125), *E2F1*(126), and *CCND1*(127). Lastly, *DVL3* shares binding sites for hsa-let-7(128) with *CCND1*(121). Other microRNA, namely hsa-miR-142, that have been confirmed to bind *CCND1*(129) have not yet been confirmed to bind *CCNB1* or *GATA3* are only predicted by TargetScan at this time.

This study also has implications in other cancer types with high 11q13 amplification rates. In esophageal squamous carcinoma, the expression levels of *ITGA2* and *SERPINE1* were altered when stratified by *CCND1* 3'-UTR expression. *SERPINE1* has been demonstrated as a prognostic marker in esophageal carcinoma with

overexpression being significantly higher in cancer patients and significantly correlates with smoking and patient age(130). Overexpression of SERPINE1 protein in esophageal squamous carcinoma has been linked to the promotion of invasion and migration through the activation of AKT and ERK signaling pathways(80). In lung cancer, ITGA2 is overexpressed in patients and is associated with clinical development of cancer(131), which is supportive of the findings in this study. In breast cancer, the expression levels of GATA3 and ITGA2 were significantly different when stratified by high and low *CCND1* 3'-UTR expression. Paradoxically, these proteins are reported to have both tumor-suppressive and tumor-promoting roles. Silencing of *ITGA2* has been shown to promote cell migration(125), yet overexpression of ITGA2 can also lead to promoted cell proliferation(94). Loss of GATA3 is associated with invasive growth and poor prognosis(132), yet a stabilizing mutation found in GATA3 can lead to a growth advantage in tumors(133,134)). Overexpression of GATA3 through a ceRNA-mediated mechanism may mimic this stabilization effect. Stratification by *CCND1* 3'-UTR may elucidate the paradoxical roles of GATA3 and ITGA2 in breast cancer. Overall, this study underscores the importance of determining the expression of *CCND1* mRNA, which may lead to better patient stratification for treatment or clinical trials.

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Figures and Tables

Figure 1. The 11q13 amplicon contains genes with long 3'-UTRs that have tumor-suppressing MREs. A. The 11q13 amplified region displaying amplification frequency (grey) with defined cores of amplification (orange, red, green, blue). **B.** Genes within the core 2 region of the 11q13 amplicon with ORF length, 3'-UTR length, amplification frequency, and 3'-UTR exon overexpression frequency. **C.** Predicted tumor-suppressing MREs of genes within the 11q13 amplicon. **D.** Selection of tumor-suppressive miRNAs predicted to bind *CCND1* 3'-UTR. **E.** Overall survival Kaplan-Meier graph of *CCND1* euploid patients (black) and *CCND1* amplified patients (red). Survival analysis was performed using Mantel-Cox log rank test with an alpha of 0.05.

Figure 2. The 3'-UTR of CCND1 is expressed in patients and is associated with poor survival. A. Diagrammatic representation of *CCND1* mRNA with UTRs (black), ORFs (yellow) and introns (magenta) locations and lengths. **B.** Exon-specific RNA expression values normalized to euploid patients (z-score). **C.** Overall survival Kaplan-Meier graph of low *CCND1* exon 5 expression (black) and high *CCND1* exon 5 expression (red). Survival analysis was performed using Mantel-Cox log rank test with an alpha of 0.05.

Figure 3. Cyclin D1 protein and mRNA is overexpressed in amplified cell lines and mRNA localizes to cytosol. A. Western blot of amplified and non-amplified HNSCC cell lines for cyclin D1 and beta-actin. **B.** Corrected total cell fluorescence (CTCF) of amplified and non-amplified cell lines for *CCND1* 3'-UTR mRNA-ISH. #: $p < 0.05$ compared to HaCat; †: $p < 0.05$ compared to MDA-1586; ‡: $p < 0.05$ compared to UMSCC48. Statistical significance was determined using ANOVA and Tukey's post-hoc with an alpha of 0.05. **C.** Representative images of MDA-1586 (non-amplified) and UPCI:SCC040 (amplified) cell line *CCND1* 3'-UTR mRNA ISH.

Figure 4. Global mRNA expression is not altered by CCND1 3'-UTR overexpression. A. Project outline describing determination of CCND1 MRE sharing target mRNAs and determination of global mRNA expression. **B.** Global mRNA expression changes in High CCND1 3'-UTR expression:low CCND1 3'-UTR expression ratio (log2FC) between CCND1 MRE target genes (blue) and control genes (yellow) in TCGA-HNSC overall, HPV-negative, and HPV-positive cohorts. **C.** Mechanisms of ceRNA-mediated regulation of cellular transcripts include degradative (left) and sequestration mechanisms. **D.** Proposed mechanism of CCND1 3'-UTR-induced ceRNA-related regulation.

Figure 5. Target protein regulation related to *CCND1* 3'-UTR expression in TCGA-HNSC. **A.** Project outline describing determination of *CCND1* MRE sharing target mRNAs **B-C.** Pearson correlation between target protein RPPA value and *CCND1* 3'UTR log2FC in TCGA-HNSC HPV-positive (**B**) and HPV-negative (**C**) patients. Blue boxes represent significantly negatively correlated proteins and red boxes represent significantly positively correlated proteins. Proteins are labeled as non-significantly correlated (grey), significantly correlated (black), or significantly correlated and also sharing *CCND1* MREs (red). **D.** Target protein transcript MREs shared with *CCND1* in HPV-negative HNSCC. **E.** Target protein mRNA expression values (log2FC) stratified by high (blue) and low (yellow) *CCND1* 3'-UTR expression in HPV-positive (left) and HPV-negative (right) patients. Statistical significance was determined using Pearson correlation or one-way ANOVA and Tukey's post-hoc with an alpha of 0.05.

Figure 6. Target protein regulation related to *CCND1* 3'-UTR expression in other 11q13-amplified cancers.

A-D. Pearson correlation between target protein RPPA value and *CCND1* 3'UTR log2FC in TCGA-BLCA (**A**), TCGA-BRCA (**B**), TCGA-ESCA (**C**), and TCGA-LUSC (**D**) patients. Blue boxes represent significantly negatively

correlated proteins and red boxes represent significantly positively correlated proteins. Proteins are labeled as non-significantly correlated (grey), significantly correlated (black), significantly correlated and also sharing *CCND1* MREs (red), significantly correlated and shared with TCGA-HNSC (green), or significantly correlated and shared with TCGA-HNSC and *CCND1* (blue). **E.** Target protein transcript MREs shared with TCGA-HNSC. Alterations found in TCGA-HNSC are labeled in green and alterations in both TCGA-HNSC and *CCND1* are labeled in blue. **F.** Target protein mRNA expression values (log2FC) stratified by high (blue) and low (yellow) *CCND1* 3'-UTR expression in TCGA-BRCA, TCGA-ESCA, and TCGA-LUSC patients. Statistical significance was determined using Pearson correlation or one-way ANOVA and Tukey's post-hoc with an alpha of 0.05.

Study 3 Supplementary Materials: Copy Number Alterations

Identify a Smoking-Associated Expression Signature Predictive of Poor Outcome in Head and Neck Squamous Cell Carcinoma

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Supplementary Figure S1. Predicted MREs of genes within the 11q13 amplicon. Predicted tumor-suppressing MREs (blue) and oncomiRs (red) were evaluated for each gene within the 11q13 amplicon. Calculated tumor-suppressor:oncomiR ratio is displayed below each gene.

Addendum to Study 3: The CCND1 3'-UTR as a Potential Driver of Poor Outcome in Chromosome 11q13-amplified Head and Neck Squamous Cell Carcinoma

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Running title: CCND1 3'-UTR in HNSCC progression

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Keywords: CCND1, competing endogenous RNA, 3'-UTR, microRNA, head and neck cancer

Background

In Study 3, we have identified potential roles of *CCND1* 3'-UTR in the progression of HNSCC. However, patients that overexpress *CCND1* Exon 5 also overexpress *CCND1* Exon 1 (Figure 1, Study 3 Figure 2). In this addendum to Study 3, we aim to separate the effects of *CCND1* ORF expression and *CCND1* 3'-UTR expression on patient survival as well as establish cellular mechanisms of *CCND1* 3'-UTR ceRNA-mediated effects on observed differences in patient survival utilizing preliminary *in vitro* cell biology studies.

Methods

Cell Lines and Cell Culture

Cell lines used were MDA1586(1) and UPCI:SCC040(2). All lines were propagated in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cell lines expressing engineered *CCND1* constructs were selected using 10 µg/ml blasticidin (#A1113903, Gibco). Cell lines stably infected with pLKO.1-puro *CCND1* shRNA or scramble control shRNA were generated by 20 µg/ml puromycin (#P9620, Sigma-Aldrich) selection following standard methods.

Development of *CCND1* Constructs

A plasmid containing an HA-tag at the 3' end of the *CCND1* ORF (*CCND1*-HA) was purchased from Addgene (#9050). The ORF-HA sequences were PCR amplified to produce a 1,158 bp *Bam*HI-*Sal*I fragment that was then subsequently ligated into pLenti CMV Blast empty (w263-1) (#17486, Addgene) that was cut using the same restriction enzymes to produce the *CCND1*-ORF (ORF) construct. A 1,158 bp *Bam*HI-*Xho*I fragment was made in parallel for ligation to a 3'-UTR fragment and then into pLenti CMV Blast empty. A *CCND1* plasmid containing the 3'-UTR(3,4) provided by Kathy Borden (University of Montreal) was PCR amplified to produce a 3,212 bp *Xho*I-*Sal*I fragment that was ligated to the 3' end of the 1,158 bp *Bam*HI-*Xho*I ORF fragment to create the *CCND1*-FL (FL) construct. The ORF was made refractory to two *CCND1* shRNAs (TRCN0000295873 and TRCN0000295874) using a site-directed mutagenesis kit (#200523, Agilent), following manufacturer's protocol to produce the final ORF and FL constructs. A frameshift mutation was generated by insertion of a guanine base immediately after the ATG start site in the +4 position to create the *CCND1*-FS (FS) construct. The pLenti CMV Blast empty served as the EV construct.

Western Blotting and Antibodies

Western blotting was conducted as described(5) and visualized with autoradiography film (E3012, Denville Scientific) or captured by an Amersham Imager 600 (GE Healthcare Bio-Sciences). Antibodies used were anti-cyclin D1 (sc-718, 1:1000; Santa Cruz Biotechnology), anti-HA-Tag (#3724, 1:1000; Cell Signaling Technology), anti-GFP (Clontech Labs Living Colors® JL-8), and anti-β-actin (#8457, 1:1000; Cell Signaling Technology).

Quantitative RT-PCR

Total cellular RNA was extracted with Trizol Reagent (#15596026, Ambion) following manufacturer's instructions. Eluted RNA was then treated with Turbo DNA-free kit (#AM1907, Invitrogen) to remove DNA. Concentrations of RNA were evaluated using a Nanodrop 2000 Spectrophotometer. 1 µg of total RNA was converted to cDNA using the iScript cDNA synthesis kit (#1708890, Bio-Rad). RT-qPCR was performed using the SsoAdvanced Universal SYBR Green Supermix (#1725271, Bio-Rad) and primers specific to the 3'-UTR of *CCND1*. Relative expression was calculated using the $\Delta\Delta CT$ method using *GAPDH* as a control endogenous gene.

RNA In-Situ Hybridization

RNA in-situ hybridization was performed as previously described(6). Briefly, custom Stellaris® FISH Probes were designed against *CCND1* 3'UTR by utilizing the Stellaris® RNA FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner (Version 2017). The *CCND1* 3'UTR were conjugated to Cy5 for fluorescent visualization. Cells were grown on coverglass, permeabilized and hybridized with a buffer containing the labeled *CCND1* probes following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocolscells. Cells were counterstained with DAPI to visualize nuclei and mounted onto slides for microscopic analysis using a Nikon A1R confocal microscope. Corrected total cell fluorescence was calculated for each cell line. Statistical significance was determined one-way ANOVA and Tukey's post-hoc with an alpha of 0.05.

Cell Cycle Analysis

MDA-1586 cells were grown to confluence and maintained for five days in order to induce contact-inhibition-mediated cell synchronization. (<https://www.future-science.com/doi/pdf/10.2144/01306rv01>). UPCI:SCC040 cells were synchronized using a double thymidine block as previously described (<https://pubmed.ncbi.nlm.nih.gov/5165443/>). Cells were then fixed and stained with propidium iodide (PI) as previously described(7,8) before flow cytometry analysis. Technical triplicates of both MDA-1586 and UPCI:SCC040, as well as biological triplicates of UPCI:SCC040 were assessed by flow cytometry. Flow cytometry experiments were performed in the West Virginia University Flow Cytometry & Single Cell Core

Facility, which is supported by the National Institute of Health grant U51 GM104942 (WV CTSI) and the Institutional Development Awards (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health grant numbers P30GM121322 (TME CoBRE) and P20GM103434 (INBRE). Data were analyzed using FCS Express 6 (De Novo Software).

Results

Exogenous overexpression of *CCND1* constructs in HNSCC cell lines

To delineate effects of the *CCND1* ORF and *CCND1* 3'-UTR on cancer progression, multiple *CCND1* constructs were generated (Figure 2A). An empty vector (EV) control plasmid of pLenti Blast was used as a backbone for the cloning of *CCND1* sequences and also serves as a control for downstream studies. A construct expressing green fluorescent protein (GFP) was generated by cloning *GFP* into EV to generate a control cell line to serve as a control in downstream experimentation. The *CCND1*-HA ORF was mutated using site-directed mutagenesis to form a *CCND1*-HA ORF refractory against two different *CCND1*-targeting shRNAs designed to knockdown of endogenous *CCND1* protein expression. This *CCND1*-HA ORF was then cloned into EV to form the ORF construct in order to evaluate effects of *CCND1*-HA ORF alone. The full-length *CCND1* 3'-UTR was subsequently cloned into ORF to generate the full-length *CCND1*-HA sequence (FL). This construct was subsequently mutated at the +4 position by to introduce a frameshift that ablates expression of exogenous cyclin D1 protein (FS) to evaluate overexpression of *CCND1* 3'-UTR alone without loss of mRNA secondary structure that may be required for ceRNA effects.

These constructs were introduced into the *CCND1*-diploid HNSCC cell line MDA-1586 using lentiviral infection to evaluate gain-of-function effects via *CCND1* overexpression. Successful transduction and construction expression were evaluated by western blotting, RT-qPCR, and mRNA-FISH depending on produced protein and/or mRNA products. Western blot visualization displayed successful stable expression of *CCND1* constructs with the presence of the HA epitope as a band of increased molecular weight reacting with to and anti-cyclin D1 in the MDA-1586-ORF and MDA-1586-FL cell lines (Figure 2B). Since overexpression of 3'-UTR cannot be assessed by western blot due to lack of protein production, RT-qPCR was utilized to visualize expression of *CCND1* 3'-UTR in MDA-1586 cell lines. Both lines showed increased relative expression of *CCND1* 3'-UTR in MDA-1586-FL and MDA-1586-FS cell lines (Figure 2C). To further assess the expression of *CCND1* constructs in MDA-1586 cells, mRNA-FISH was utilized which also allowed assessment of sub-cellular location of *CCND1* mRNA. Microscopic analysis indicated overexpression of *CCND1* mRNA constructs in the cytosol of MDA-1586 cell lines (Figure 2D).

To account for potential oncogene-addiction introduced through 11q13 amplification and subsequent enhanced endogenous *CCND1* overexpression, the engineered *CCND1* constructs were introduced into the 11q13-amplified HNSCC cell line UPCI:SCC040. Visualization of protein expression by western blotting indicated successful overexpression of the ORF and FL *CCND1* constructs (Figure 2E). To test the ability of these constructs to resist shRNA knockdown, UPCI:SCC040 cells expressing ORF, FL and ATG? were stably infected with an anti-*CCND1* shRNA. Western blot visualization revealed successful knockdown of endogenous *CCND1* without impacting recombinant *CCND1* overexpression (Figure 2F).

Exogenous *CCND1* 3'-UTR overexpression does not appear to alter HNSCC cell cycle progression

Due to the role of cyclin D1 in the progression of cell cycle, we sought to investigate the effects of overexpression of the *CCND1* 3'-UTR on cell cycle to assess 3'-UTR-mediated effects. Cell cycle was assessed using PI staining of synchronized HNSCC cell lines. MDA-1586 gain-of-function analysis displayed a modest increase of cells in the G2-M phase, suggesting increased proliferation of cells overexpressing the FL or FS constructs (Figure 3A). However, MDA-1586 cells were synchronized using a non-conventional method and the experimental results are only one run of analysis at only one time point (24 hours). Further analysis was conducted using the UPCISCC:040 cell, due to the aforementioned potential for oncogene addiction in the 11q13-amplified background that maybe necessary to visualize any changes in *CCND1*-amplified cells. Double thymidine block synchronization followed by biological triplicate (each repeat containing technical triplicates) revealed no differences in cell cycle at 6, 12, or 24 hours in transfected UPCISCC:040 cells (Figure 3B). This suggests that *CCND1* 3'-UTR overexpression does not impact HNSCC progression by modulating increase in cell proliferation or changes in cell cycle kinetics.

Future Directions

In Study 3, we identified differentially expressed proteins that were correlated with *CCND1* 3'-UTR expression changes. In this Addendum, we show that *CCND1* ceRNA-mediated effects on tumor progression do not act through increased cell cycle progression. However, the target proteins identified in Study 3 may function through other tumor-enhancing mechanisms, including increased proliferation, invasion, migration, apoptosis resistance, and DNA synthesis. Other assays such as BrdU incorporation or further optimized PI staining assays may be able to better evaluate potential changes on the cell cycle. These possibilities, coupled with the survival differences in patients with high *CCND1* 3'-UTR expression, underscore the need for further studies. The different *CCND1* expression constructs provide useful tools for the evaluation of any ceRNA-mediated effects in HNSCC cell lines in future work.

While TCGA offers a useful tool for evaluating global mRNA expression changes, evaluation using *CCND1* expression vectors is still vital for understanding *CCND1* ceRNA-mediated effects on mRNA. For determining transcriptional changes specific to the *CCND1* 3'-UTR, isolated RNA from UPCISCC:040 parental and EV, FL, ORF and FS overexpressing cells will be DNase and RiboZero treated before RNAseq in 2x150 format at 50M reads. Data will be evaluated for differential gene expression(9). Highly upregulated transcripts shared by FL and FS overexpressing cells compared with control and ORF lines will be considered novel candidates for *CCND1* ceRNA activity. 3'-UTR regions from candidate targets will be evaluated by DIANA Tools, TargetScan and the literature for shared microRNA response elements (MREs) with the *CCND1* 3'-UTR. As TCGA results suggest, evaluation of target proteins may be more useful than purely transcriptomic analysis. To evaluate changes in protein expression, cells will be lysed with the EasyPrep Mini MS prep kit (Fisher) and subjected to label-free quadrupole Orbitrap liquid chromatography tandem mass spectroscopy (LC-MS/MS)(10) with the assistance of the WVU BioNano Research Facility. SIEVE, Compound and Proteome Discoverer will be used for peptide identification, protein assembly and quantification(11). Relative protein values will be compared for FL and FS overexpressing cells to control and ORF cells, with overexpressed proteins in FL and FS cells evaluated for MREs shared with the *CCND1* 3'-UTR. We anticipate multiple transcripts and proteins will be upregulated in the FS and FL cell lines compared to controls, including those identified in patient samples (Study

3 Fig. 5D). Upregulated proteins in FL cells may be due to cyclin D1 protein overexpression, rather than ceRNA by the 3'-UTR. These cases will be compared with the FS results.

To test the ability of the *CCND1* 3'UTR as a ceRNA for putative target expression, the 3'-UTR regions for candidate and discovered ceRNA targets will be subcloned into a luciferase reporter vector and assayed(12). Constructs will be stably transfected into HNSCC *CCND1* KD cells with overexpression of EV, ORF, FL and FS transcripts (Figure 2A). Target-driven luciferase activity will be measured. To determine if target overexpression is dependent on *CCND1* ceRNA activity, the established seed region of MREs for miRNAs in each respective target 3'-UTR (Fig. 6D) will be mutated. Knockdown cells expressing miRNA-mutant constructs (MM) will be generated and the activity of the target 3'-UTR luciferase reporter compared to the *CCND1* ORF, FL and FS control cells. These results will be validated using target site blocking (TSB) oligonucleotides (Qiagen miRCURY) against the 3'-UTR MREs for each target. TSBs will be transfected into control ORF, FL and FS cells, and target protein and mRNA expression will be measured. To control for specificity, TSBs will be transfected into MM cells and assayed for target expression. We predict overexpression of *CCND1* FL and FS to increase candidate ceRNA target expression compared to ORF constructs due to 3'-UTR titration of shared miRNAs. TSB transfection is expected to block *CCND1* and target transcript MREs, producing an inhibitory effect.

Proliferation will be evaluated using CyQuant NF (Fisher) and Ki67 immunolabeling of fixed cells. Anchorage independent growth will be assayed for colony formation in soft agar as described(13). Apoptosis will be evaluated using annexin V staining and quantified by flow cytometry. HNSCC invasiveness will be evaluated with each cell line using an organotypic assay as described(14,15). Degree and extent of invasion will be quantified using a calculated index based on histologic imaging of H&E stained sections using Metamorph software as previously detailed(16). MM cell lines with known ceRNA effects for candidate targets will be evaluated in accordance with appropriate assays, with target expression monitored by western blotting and RT-qPCR. FL and FS overexpression are anticipated to effect one or more hallmarks to a greater extent than ORF and EV. The FS construct may not enhance all of these processes in some cases due to lack of coincident cyclin D1 protein expression, but will provide additional evidence that the 3'-UTR contributes to one or more aspects of HNSCC progression.

11q13 amplification and *CCND1* overexpression occurs in other tobacco-associated malignancies(17–20). Correlation analyses identify several shared overexpressed candidate *CCND1* 3'-UTR ceRNA targets (e.g., *SERPINE1* and *ITGA2*) (Study 3 Figure 6), further underscoring the significance of the proposed work. The outcome of these future directions addressing regulation of the miRNA network by *CCND1* transcript overexpression will provide unique insights into the consequence of 11q13 amplification and associated poor outcomes in HNSCC.

Figure 1. Patient overall survival stratified by *CCND1* exon expression. Survival analysis of patients with high *CCND1* exon 1 (blue) or exon 5 (red) overexpression (N=65) or low exon 1 (green) exon 5 (black) expression (N=65) using the Broad HNSC Level 3 exon expression dataset. Z-scores were normalized to diploid values. Survival analysis was performed using Mantel-Cox log rank test with an alpha of 0.05.

Figure 2. Development of *CCND1* constructs and expression in HNSCC cell lines. **A.** *CCND1* expression constructs subcloned into pLenti-Blast CMV (Addgene). EV; empty vector control. GFP; GFP control. ORF; cyclin D1 open reading frame. FL; complete *CCND1* transcript with ORF and 3'UTR. FS; point mutant frame-shifted at position +4 in the ORF. **B.** Protein expression of *CCND1* constructs. Western blotting of lysates from disomic MDA-1586 HNSCC cells transduced with the constructs in (A) and blotted as indicated. **C.** Ectopic expression of *CCND1* 3'-UTR regions. RNA from the indicated cells was amplified by RT-qPCR using primers targeting the *CCND1* 3'-UTR. Bars represent mean with SD error bars. Statistical significance was determined using ANOVA and Tukey's post-hoc with an alpha of 0.05. ****: p<0.0001; **: p<0.01. **D.** Representative images of RNA-FISH for non-amplified MDA-1586 and amplified UPCI:SCC40 HNSCC lines. *CCND1* transcripts display abundant cytoplasmic localization to serve as ceRNA. Bar = 20 μm. **E.** Protein expression of *CCND1* constructs. Western

blotting of lysates from amplified UPCI:SCC040 HNSCC cells transduced with the constructs in (A) and blotted as indicated. **F.** *CCND1* knockdown and re-overexpression of HA-tagged ORF and FL constructs in 11q13 amplified UPCI:SCC040. EV; empty vector sh Sramble control.

Figure 3. Cell cycle analysis of *CCND1* constructs in HNSCC cell lines. A. MDA-1586 and B. UPCISCC:040 *CCND1* overexpressing lines were synchronized, PI labeled and sorted by FACS. Analysis was conducted using FCS Express 5. Statistical significance was determined using ANOVA and Tukey's post-hoc with an alpha of 0.05.

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General Discussion:

This dissertation describes studies that illuminate the relationship between Appalachian HNSCC survival, tobacco-induced DNA damage, and novel functions of the tumor progression driver cyclin D1. The first Study details how increased tobacco exposure in the Appalachian region can contribute to decreased survival of AJCC stage IV HNSCC patients with primary tumors within the HPV-associated oropharynx. The second Study establishes large-scale chromosomal damage that is correlated with increasing patient pack-year value and identifies several candidate genes of unknown or unappreciated function in HNSCC for future studies. The third Study outlines a potential ceRNA-driven mechanism of cyclin D1, a well-known tumor progression driver in HNSCC while the Addendum to Study 3 explores this potential mechanism *in vitro*. Together, these studies expand on the observed HNSCC disparity within Appalachia, correlate tobacco-use to 11q13 amplification and subsequent overexpression of a subset of genes, and link an 11q13 gene to novel functions (Figure 1). Results from this dissertation could impact patient treatment regimens and clinical trial stratification by solidifying tobacco-use and 11q13 gene, including *CCND1*, expression levels as important diagnostic criteria.

Figure 1. Dissertation overview detailing three studies.

Although oral cavity and pharyngeal cancer incidence and mortality disparities in Appalachia have been documented in the past(1,2), the work outlined in Study 1 further describes these disparities by separating patients by AJCC stage, sex, race, and primary site. Thus, this approach utilizes a comprehensive methodology that includes cancer-specific survival data from the majority of Appalachian counties and the non-Appalachian counties within the same states. The results from this work are definitive descriptions of oral cavity and pharyngeal survival in the Appalachian region, pinpointing the observed disparity to AJCC-6 stage IV white males with tumors in the HPV-associated oropharynx. This suggests that these patients have a higher prevalence of HPV-negative cancer, which has been shown to have poorer survival(3–6), within classically defined HPV-positive subsites. This difference is potentially driven by an increased use of smoking and smokeless tobacco amongst males in the region. The implication of these findings is that because tobacco remains the primary cause of HNSCC(7) and Appalachia harbors the highest tobacco-use rates in the country(8), accurate tobacco histories of patients is an important predictor of clinical outcome, regardless of the anatomical location of the tumor. This work also suggests that the recent discussion of treatment de-intensification of patients with HPV-associated oropharyngeal cancers(9,10) should be revisited, and that tobacco-exposure needs to be included in this discussion as a treatment qualifier. Importantly, p16 immunohistochemistry (IHC) is one of the criteria used when determining treatment for HNSCC patients(11). This diagnostic indicator is well supported(12,13), yet not a perfect indicator of HPV positivity due to other chromosomal alterations that can occur to skew these results to lead to false positives and false negatives(14). *CDKN2A* is often deleted in HNSCC(3) which would lead to a false negative result even if the patient is HPV-positive. Conversely, *CCND1* would elicit the same loss of p16 negative-feedback loop by releasing more E2F transcription factor, which would also lead to an accumulation of p16 and could lead to be a false positive. These scenarios are detailed in Figure 2.

Since p16 IHC is currently paramount in determining the course of patient treatment, this could lead to disastrous results, even if this impacts a low number of patients. A better solution would be to perform CCND1, CDKN2A, and HPV PCR alongside p16 staining(15,16). Unfortunately, with the advent of AJCC 8th edition putting more emphasis on HPV status by downstaging patients if they are HPV-positive by p16 staining, we are beginning to see evidence of potential undertreatment and worse outcomes of patients due to the under-staging and less aggressive treatment proposed in the AJCC 8th edition(9). Overall, these studies, coupled with the results of Study 1, underscore the need for continued pursuit to fully understand molecular, behavioral, and geographical differences in the staging and treatment of HNSCC patients regardless of etiology. Future studies could track the effectiveness of smoking cessation campaigns in Appalachia, as well as effects of prophylactic HPV vaccination on HNSCC stage IV white male survival and how this effects patient survival over time. If the disparity does not normalize to national outcomes, it is imperative that continued government funding to the ARC, as well as continued education and smoking cessation campaigns will continue to be necessary for reducing HNSCC risk and improving survival in the Appalachian region. Such an approach can also be extrapolated to areas of disproportional smoking tobacco, smokeless tobacco(17), or betel-quid use(18).

HNSCC is largely conceptualized by the field as being driven by genomic relief of tumor suppression(19). However, HNSCC also harbors many copy-number gains and amplifications, the most common of which is the 11q13 amplicon. Chromosome 11q13 amplification is found in nearly one third of all patients and is associated with lower survival(3,20). Although it is believed 11q13 amplification and other CNAs in HNSCC are associated with DNA damage arising from carcinogenic insult due to cigarette smoking and associated DNA damage(7,21), the precise CNAs correlated with smoking have not been directly defined. The work outlined in Study 2 seeks to close this knowledge gap by correlating the copy number of 24,776 genes with the pack-year data of 509 patients from the well-annotated TCGA cohort to generate a smoking-associated copy number and expression landscape in HNSCC. This work resulted in identifying a smoking-associated expression signature (SAES) that mapped to four distinct chromosomal cytobands in known CNAs, including deletions of genes within 9p21.3 and 9p24.1 with the vast majority being amplifications of genes within 11q13.3 and 11q13.4. All underexpressed and overexpressed genes within the SAES reduced patient overall survival and were correlated with an increased relative risk of death. Interestingly, there is a near-linear positive correlation between probability of amplification of 11q13.3-q13.4 and smoking pack year as well as between overexpression of 11q13.3-q13.4 SAES genes. The correlation of these CNAs coupled with this predictive aspect with increased smoking tobacco usage underscores the importance of collecting complete tobacco use histories during patient consultation and diagnosis. This history is simple to collect from the patient and may be useful to both researchers and clinicians to predict probability of smoking-associated DNA damage and expression SAES genes which could better guide early treatment. In addition to the known drivers of HNSCC within these CNAs, such as *CCND1* and *CTTN*(22,23), we have also identified candidate drivers of HNSCC where overexpression reduces patient survival. A subset of these candidate drivers also correlates with lymph node metastasis, which is an important prognostic indicator of patient outcome(24,25). These candidate genes include the mitochondrial ribosomal protein L21 (*MRPL21*), LAR protein tyrosine phosphatase-interacting protein alpha-1 (*PPFIA1*), fibroblast growth factor 19 (*FGF19*), two pore segment channel 2 (*TPCN2*), and E3 ubiquitin ligase RING finger protein 121 (*RNF121*). *MRPL21* encodes a large mitochondria ribosomal subunit, where proper stoichiometry is critical for mitoribosome assembly(26). There is no defined role for *MRPL21* in tumor progression, but its cellular function suggests a role in regulating tumor cell metabolism. *PPFIA1* encodes a LAR phosphatase-interacting protein that localizes to and regulates the disassembly of focal adhesions(27). Liprin-alpha1 has a roles in regulating

cell motility, invasion, and transmembrane signaling(28,29). *FGF19* encodes a fibroblast growth factor that functions as a hormone in the regulation of bile acid synthesis, signaling through the fibroblast growth factor receptor 4 (*FGFR4*)(30) that is linked to tumor-promoting autocrine signaling(30). *TPCN2* encodes a cation-selective ion channel that releases calcium ions from lysosomal stores but does not have a defined role as a tumor promoter(31). *RNF121* encodes an E3 polytopic membrane protein with five transmembrane regions thought to be part of the endoplasmic-reticulum-associated protein degradation response (ERAD) that downregulates several proteins in invertebrates(32). *RNF121* does not have a well-defined role in tumor progression as it has been shown to be both a tumor promoter(33) and tumor suppressor(34), with a defined role in NF- κ B activation(35). Future studies that expand our understanding of how 11q13 amplification and resultant gene overexpression of these poorly defined candidate genes contributes to poor patient outcome in smoking-associated HNSCC is further underscored by the increased resistance to chemotherapy and EGFR targeting in 11q13-amplified HNSCC(36–38). This highlights a persistent need for earlier and precise diagnosis for patients with SAES CNA and expression status to provide improved clinical management of tobacco-positive, HPV-negative HNSCC.

The 11q13 amplicon contains several genes that have been linked to increased tumor progression, including *CCND1*(19), *CTTN*(39), *FADD*(40), and *ORAOV1*(41). These genes drive tumor progression through gene product protein-protein interactions. However, other functions of the mRNA transcript itself, independent of protein function, could drive tumor progression. In Study 3, we explored the potential competing endogenous effects of the RNA of these genes on altering expression of other cellular RNAs and ultimately altering cellular expression of proteins involved in either tumor-suppressive or tumor-promoting roles. We observed that the overexpression of the *CCND1* 3'-UTR is associated with reduced patient survival and also contains 31 MREs for tumor-suppressive microRNAs. When correlating expression of *CCND1* 3'-UTR from RNAseq analysis and protein expression from reverse phase protein lysate microarray RPPA analysis, we identified several proteins of interest with increased differential expression encoded by transcripts containing MREs shared with the *CCND1* 3'-UTR. These proteins include TYMS, SERPINE1, CCNB1, DVL3, GATA3, E2F1, and ITGA2. All of these proteins have been linked to tumor progression, although their roles in HNSCC are only partially known(42,43,52–61,44,62,63,45–51). The added effect of these overexpressed tumor promoters creates an additional layer of complexity to the oncogenic role of overexpressed cyclin D1. Elucidating the full impact of

CCND1 mRNA overexpression, through 3'-UTR-mediated ceRNA sequestration or through cyclin D1 protein overexpression is imperative in understanding how to best stratify patients for treatment with CDK4/6 inhibitors that only impact *CCND1* protein function. The amplification of 11q13 may not be enough in and of itself to justify stratification for CDK4/6 inhibitors. As shown in the PALOMA-1 clinical trial that stratified patients by *CCND1* amplification and the results did not support that stratification by *CCND1* amplification improves patient outcome(64,65). Alternatively, there is evidence that stratification by *CCND1* transcript expression is a poor predictor of response rates of other chemotherapy drugs(66). The results in this Study coupled with these stratification results suggest that *CCND1* 3'-UTR expression levels may be an important way to predict patient outcome from chemotherapeutic intervention. In the Addendum to Study 3, we created *CCND1* expression vectors that overexpress different segments of the *CCND1* transcript using *in vitro* assays to examine for the presence of a ceRNA-mediated effect on tumor progression. These constructs contained the ORF alone, full length *CCND1*, and a frameshift *CCND1* mutant that does not produce cyclin D1 protein. Exogenous expression was confirmed using various *in vitro* assays including western blotting, RT-qPCR, and mRNA-FISH in both the non-11q13-amplified HNSCC cell line MDA-1586 and the 11q13-amplified HNSCC cell line UPCI:SCC040. Effect on cell cycle progression was also evaluated, with mixed results. For this reason, additional laboratory investigation is required to confirm that the suggested ceRNA effect is supported. Future experiments that examine effects of these proteins on tumor progression, such as transcriptome RNAseq or proteome mass spectrometry using the developed *CCND1* constructs will be imperative for complete understanding of the dynamics at play. If the ceRNA effect is supported, there is potential to uncover new actionable targets that are deregulated due to the ceRNA effect. Additionally, confirming shared microRNA that interact with these target proteins will be an important first step to direct targeted anti-microRNA therapy approaches to mediate *CCND1* 3'-UTR-driven tumor progression.

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Cortactin Phosphorylation by Casein Kinase 2 Regulates Actin-Related Protein 2/3 Complex Activity, Invadopodia Function, and Tumor Cell Invasion

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Abstract

Malregulation of the actin cytoskeleton enhances tumor cell motility and invasion. The actin-binding protein cortactin facilitates branched actin network formation through activation of the actin-related protein (Arp) 2/3 complex. Increased cortactin expression due to gene amplification is observed in head and neck squamous cell carcinoma (HNSCC) and other cancers, corresponding with elevated tumor progression and poor patient outcome. Arp2/3 complex activation is responsible for driving increased migration and extracellular matrix (ECM) degradation by governing invadopodia formation and activity. Although cortactin-mediated activation of Arp2/3 complex and invadopodia regulation has been well established, signaling pathways responsible for governing cortactin binding to Arp2/3 are unknown and potentially present a new avenue for anti-invasive therapeutic targeting. Here we identify casein kinase (CK) 2 α phosphorylation of cortactin as a negative regulator of Arp2/3 binding. CK2 α directly phosphorylates cortactin at a conserved threonine (T24) adjacent to the canonical Arp2/3 binding motif. Phosphorylation of cortactin T24 by CK2 α impairs the ability of cortactin to bind Arp2/3 and activate actin nucleation. Decreased invadopodia activity is observed in HNSCC cells with expression of CK2 α phosphorylation-null cortactin mutants, shRNA-mediated CK2 α knock-down, and with the CK2 α inhibitor Silmitasertib. Silmitasertib inhibits HNSCC collective invasion in tumor spheroids and orthotopic tongue tumors in mice. Collectively these data suggest that CK2 α -mediated cortactin phosphorylation at T24 is critical in regulating cortactin binding to Arp2/3 complex and pro-invasive activity, identifying a potential targetable mechanism for impairing HNSCC invasion.

Implications: This study identifies a new signaling pathway that contributes to enhancing cancer cell invasion.

Visual Overview: <http://mcr.aacrjournals.org/content/molcanres/17/4/987/F1.large.jpg>.

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Introduction

Cell invasion from the primary tumor is responsible for initiating the metastatic cascade and increasing cancer lethality (1, 2). Invasion is initiated in part through the action of invadopodia, actin-based membrane protrusions produced by tumor cells that mediate dissemination by degrading restrictive extracellular matrix (ECM) proteins through enzymatic matrix metalloproteinase (MMP) activity (3). Invadopodia contain a central filamentous (F-) actin core surrounded by an integrin-based adhesion ring complex that anchors the structure to allow focal matrix degradation and tumor cell protrusion through the basement membrane (4). Cortactin and actin-related protein (Arp) 2/3 complex are essential protein components involved in invadopodia precursor core formation required for subsequent MMP recruitment and membrane protrusion (5, 6). Cortactin overexpression is common in several cancer types including head and neck squamous cell carcinoma (HNSCC), resulting in enhanced motility, invasion, and invadopodia activity (7, 8). Cortactin binding to Arp2/3 complex activates Arp2/3 actin nucleation activity, enhancing cellular actin polymerization to form branched F-actin networks (6, 8–10). Cortactin also directly binds F-actin and bundles newly-formed filaments, providing an overall stabilizing effect on the Arp2/3-F-actin network required for invadopodia formation (5, 6, 11). Previous work has shown that a DDW motif within the cortactin N-terminal acidic (NTA) domain is central in mediating Arp2/3 activation and branched actin network formation (7, 12–14). This region is similar to the Arp2/3 binding motif found in the acidic region of the Verprolin, Central, Acidic (VCA) domain of the Wiskott–Aldrich Syndrome protein (WASP)-family of Arp2/3 nucleation promotion factors (NPF). Although the cortactin DDW motif is well established as the region responsible for Arp2/3 binding, posttranslational or other modifications of amino acids in the NTA region that regulate binding have not been reported. Tyrosine and serine phosphorylation of cortactin residues in the carboxyl-terminal region are essential for invadopodia formation, cellular invasion, and tumor metastasis through multiple mechanisms ultimately involving activation of WASP NPFs (6, 7, 15–18). In addition, comprehensive phosphorylation site mapping by mass spectroscopy has identified NTA phosphorylation sites in close proximity to the DDW motif (19). This raises the possibility that phosphorylation of one or more of these residues may serve to govern Arp2/3 binding and invadopodia function in invasive cancer.

Casein kinase (CK) 2 is a ubiquitously expressed, constitutively active serine/threonine kinase consisting of two catalytic subunits (α or α') and two β regulatory subunits (20). Increased CK2 expression correlates with cell-cycle progression, apoptosis resistance, and tumor cell motility in various cancers (20). Overexpressed CK2 enhances HNSCC tumor cell motility (20). CK2 phosphorylates the cortactin homologue HS1 at an unidentified site(s) in the NTA region (21), as well as residues near the DDW region in the NPFs neuronal (N)-WASP and WAS protein family member 2 (WASF2; WAVE2, WASP family Verprolin-homologous protein 2; refs. 22–25). Here we show that CK2 phosphorylation of threonine (T) 24 in the cortactin NTA impairs binding to and activation of Arp2/3 complex. Cortactin T24 and CK2 are required for efficient invadopodia formation and ECM degradation activity in HNSCC cell lines. Treatment of established and primary HNSCC cells with the selective CK2 inhibitor Silmitasertib

impairs invadopodia function and regional HNSCC invasion. These results identify a new mechanism of invadopodia regulation that can be targeted to impair HNSCC invasion.

Materials and Methods

Cell culture, lentiviral infection and transfection, siRNA

HNSCC cell lines OSC19 and UMSCC1 were acquired and maintained as described (26). MDA1586 cells were obtained in March 2014 from Barbara Frederick (University of Colorado, Denver, CO). All HNSCC lines were authenticated by STR profiling at the University of Arizona Genetics Core in June 2017. PCR-based mycoplasma testing (13100-01; Southern Biotech) was conducted on OSC19 and UMSCC1 lines in March 2015 and were free of contamination. The MDA1586 line was not tested for mycoplasma. HEK293T/17 cells were obtained in April 2013 from Robert Wysolmerski (West Virginia University, Morgantown, WV). NIH3T3 cells were obtained in June 2017 from Ivan Martinez (West Virginia University, Morgantown, WV) and maintained for ≤ 10 passages. These lines were not authenticated or tested for mycoplasma. Cells were propagated in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin for < 6 months. OSC19 and UMSCC1 cells stably infected with pLKO.1-puro cortactin shRNA or CK2 α shRNA were generated by clonal puromycin selection following standard methods (CSNK2A1: TRCN0000380839, TRCN0000027627; CTTN: TRCN0000040275).

UMSCC1 cells stably infected with pLU-Luc2 expressing luciferase were generated following standard methods. Murine cortactin rescue OSC19 and UMSCC1 cells containing cortactin shRNA stably infected with pLenti CMV Hygro cortactin constructs were generated by subsequent clonal hygromycin selection. Complete cortactin knockdown in OSC19 and UMSCC1 cells was achieved by transfection of cortactin-targeting siRNA (ON-TARGETplus SMARTpool L-010508-00-0020; Dharmacon) using a Nucleofector I (Amaxa Biosystems).

Western blotting, antibodies, and immunoprecipitation

Western blotting was conducted as described (27) and visualized with autoradiography film (E3012; Denville Scientific) or captured by an Amersham Imager 600 (GE Healthcare BioSciences). Antibodies used were: anti-cortactin clone 4F11 (1 μ g/mL; ref. 26), anti-pS473 AKT (#4060, 1:1,000; Cell Signaling Technology), anti-panAKT (#2920, 1:1,000; Cell Signaling Technology), anti- β -actin (#8457, 1:1,000; Cell Signaling Technology), anti-CK2 α (#2656, 1:500; Cell Signaling Technology), anti-DYKDDDDK (FLAG) clone 2EL-1B11 (MAB3118, 1:500; Millipore), and anti-Arp3 (#07-272, 1:500; EMD Millipore). Immunoprecipitation was conducted from cells lysed in 50 mmol/L Tris Buffer pH 8.0 with 10 mmol/L EDTA and 1% NP-40 (28). Clarified lysates (1 mg) were incubated with 50 μ L of FLAG M2 affinity resin (A2220; Sigma-Aldrich) for 2 hours at 4°C. Immune complexes were collected by centrifugation, washed twice with Tris buffer, separated by SDS-PAGE, and Western blotted with antibodies as described above.

Gelatin degradation assay, invadopodia characterization, and microscopy

Cells were plated on Oregon Green 488-conjugated gelatin (G13186; Invitrogen) coated coverslips (29). In cases of inhibitor treatment, cells were allowed to attach for 1 hour, then incubated

for 12 or 24 hours with Silmitasertib (S2248; Selleckchem) as indicated. Cells were rinsed in PBS, fixed with 10% buffered formalin (SF100-4; Fisher Scientific), and labeled as described (29). Antibodies used were 4F11 (1:500) or anti-FLAG (1:500). Primary antibodies were visualized using Alexa Fluor 647 conjugated goat anti-mouse secondary antibody (A21235, 1:500; Invitrogen). F-actin was visualized with rhodamine-conjugated phalloidin (R415, 1:1,000; Invitrogen). Coverslips were mounted using ProLong Gold antifade with DAPI (P36935; Invitrogen). Images for quantifying gelatin degradation and knockdown/rescue expression were acquired with a Zeiss Axio Imager Z2 epifluorescent microscope equipped with an AxioCam MRm CCD camera and AxioVision software using LD Plan-Neofluar 40X/0.6 Corr and Plan-Apochromat 63X/1.4 oil objectives (Carl Zeiss Microscopy). Acquisition parameters were held constant within comparison groups. Confocal images were acquired using a Zeiss Axio Imager Z1 LSM510 confocal microscope with EC Plan-Neofluar 40X/1.30 and Plan-Apochromat 63X/1.4 oil objectives and Zen2009 software (Carl Zeiss Microscopy). All representative images were level adjusted to enhance contrast and brightness as needed and resized using Photoshop CC 2018 (Adobe Systems). Gelatin images were corrected for uneven illumination via bandpass filtering using ImageJ software (NIH). Degradation and invadopodia formation was quantified as described previously (29), with $n \geq 70$ lentiviral infected or ≥ 100 inhibitor-treated cells evaluated for each condition. FLAG-stained control images were thresholded against nonspecific staining using ImageJ software. Cells above threshold values were considered positive for rescue construct expression and used for quantitation. For therapeutic treatments and RNAi stable cell lines, degradation and cell areas were determined by Image J (NIH) on an individual cell basis. Data represent the mean values normalized to control degradation area per cell area from at least three independent experiments. Invadopodia precursors were determined by colocalization of actin and cortactin at sites lacking gelatin degradation. Active invadopodia were determined by colocalization of actin, cortactin, and gelatin degradation. Data represent the mean from at least three independent experiments. Phase contrast images were acquired using a Zeiss Axiovert 200M microscope equipped with an AxioCamMR CCD camera using a Plan-Neofluar 10 $\times$ /0.30 objective and AxioVision software (Carl Zeiss Microscopy).

CK2 α kinase assay

In vitro kinase assays were performed as described (30). Briefly, 0.25, 0.5, or 1 μ g of purified GST-wild-type (WT) or T24A cortactin NTA fusion proteins were incubated with 8 ng CK2 α (#14-445; Millipore) and 10 μ Ci 32 P γ -ATP (#NEG002A500UC; PerkinElmer) at 30°C for 10 minutes. Reactions were terminated with hot SDS sample loading buffer. Proteins were visualized by autoradiography. Purified N-WASP GST-VCA (0.5 μ g) and GST (1 μ g) were used as respective positive and negative controls.

In vitro cortactin phosphorylation binding assay

Purified WT or T24A cortactin proteins (2.5 μ g) were bound to 4F11-conjugated protein G magnetic beads (#10003D; Life Technologies). Immune complexes were incubated in the presence or absence of activated CK2 α (75 ng; #V4482; Promega) and ATP (500 nmol, #BP413-25; Fisher Scientific) at 30°C for 15 minutes. Reactions were washed twice with 10 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 0.5 mmol/L EDTA. Complexes were washed

once with 10 mmol/L Tris pH 7.4, 10 mmol/L EDTA, and incubated with 50 ng Arp2/3 complex (#RP01-A; Cytoskeleton) at 4°C for 30 minutes. Following incubation, binding complexes were washed once with 10 mmol/L Tris Buffer pH 7.4 with 25 mmol/L NaCl, 10 mmol/L EDTA, 1% NP-40, then boiled and Western blotted with antibodies against cortactin and Arp3.

Actin polymerization assay

Actin polymerization experiments were conducted as described previously (31). Reactions contained 2 μ mol/L actin (10% pyrene-labeled), 75 nmol/L Arp2/3 complex, 100 nmol/L cortactin, or 50 nmol/L GST-VCA (#VCG03; Cytoskeleton), and/or varying amounts of CK2 α (#14-445; Millipore) as indicated. For reactions with CK2 α , GST-VCA, or cortactin mutants were preincubated with CK2 α and 500 nmol ATP for 15 minutes at room temperature prior to addition to the actin polymerization reaction.

PDX-derived cell lines

Patient-derived xenograft (PDX) tumors and cell lines were established as described (32). WVUSCC-AR2 and WVUSCC-AR5 were derived from surgical specimens of alveolar ridge HNSCC in compliance with West Virginia University Institutional Review Board approved protocol #1310105737A033. PDXs were developed in compliance with West Virginia University Institutional Animal Care and Use Committee approved protocol #15-0302.6 by placing approximately 1 mm tumor fragments into subcutaneous pockets in the flanks of anesthetized 8- to 10-week-old NOD/SCID- γ (NSG) mice. Tumor fragments were overlayed with Matrigel (354234; Corning) and incisions were closed using wound clips. Mice were weighed and monitored for tumor growth on a weekly basis. PDX tumors were passed into new NSG mice and/or used to generate cell lines once tumors reached ~ 1 cm in greatest dimension.

For cell line derivation, PDX tumors were minced and digested in DMEM supplemented with 20% FBS and 1 mg/mL collagenase IV (17104019; Gibco). Digested tissues were plated onto NIH3T3 fibroblasts senesced with 4 μ g/mL mitomycin C (BP2531; Fisher Scientific) and cultured in DMEM:F12 1:1 supplemented with 10% FBS, 400 ng/mL hydrocortisone (H0888; Sigma), 50 μ g/mL gentamycin (15750060; Gibco), 5 μ mol/L ROCK inhibitor (S1049; Selleckchem), 0.5 ng/mL recombinant human EGF (PHG0311; Gibco), and 10 ng/mL cholera toxin (C8062; Sigma). Both WVUSCC-AR2 and -AR5 were derived in August 2017 and maintained for ≤ 10 passages. Derived lines were verified using cytokeratin 14 staining (ab15462; Abcam). Neither STR profiling nor mycoplasma detection was performed on these cell lines. Prior to utilization in gelatin degradation or spheroid invasion assays, PDX-derived cell lines were plated directly onto cell culture dishes for one to two passages to remove the fibroblast population. Gelatin degradation and spheroid invasion assays were performed in DMEM supplemented with 10% FBS.

In vitro tumor spheroid invasion

3D spheroid invasion assays were performed as previously described (26). A total of 1×10^4 (OSC19) or 2.5×10^4 (UMSCC1 and WVUSCC-AR5) cells were plated into individual wells coated with 1.5% noble agar for 24 hours (UMSCC1) or 48 hours (OSC19 and WVUSCC-AR5) to form spheroids. For each line, spheroids were collected, resuspended in 500 μ L of 2 mg/mL rat tail collagen I (354236; Corning), and plated into individual wells

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of a 24-well plate precoated with 400 μ L solidified 2 mg/mL collagen I. Plates were incubated for 1 hour at 37°C, then overlaid with 1 mL DMEM supplemented with 10% FBS and 1% penicillin–streptomycin containing DMSO or 10 μ M Silmitasertib. Spheroid invasion was visualized at the indicated time points by phase contrast microscopy using a Zeiss Axiovert 200M microscope equipped with an AxioCamMR CCD camera using a Plan-Neofluar 5X/0.15 objective and Axiovision software (Carl Zeiss Microscopy). Maximal radial distances for invaded cells were calculated using Axiovision software, with invasive distance determined as the difference between the initial and final maximum radius for each invaded spheroid.

Orthotopic tongue tumors and invasion analysis

Tongue tumor establishment was adapted from previous work (33). A total of 2.5×10^4 luciferase expressing UMSCC1 cells were injected into the tongues of 8- to 10-week-old NSG mice (purchased from the West Virginia University Transgenic Animal Core Facility). Mice were maintained using transgenic dough diet (S3472; Bioserve) and weighed every 2 to 3 days. Tumor growth was monitored by bioluminescent imaging using 150 mg/kg D-luciferin (122796; Caliper Life Sciences) injected intraperitoneally, followed by *in vivo* whole-body bioluminescence imaging using an IVIS Lumina-II system and Living Image 4.0 software (PerkinElmer). Tumors were allowed to establish for 1 week, then mice were divided equally into two groups based on approximate tumor size. Mice were given 50 mg/kg Silmitasertib in DMSO or DMSO alone by oral gavage twice daily for 3 weeks. Mice were subsequently euthanized, tongues excised, processed, and stained for histologic analysis.

To quantify invasion parameters, whole tongue histologic images were cropped to encompass the tumor invasive front and analyzed using ImageJ. Images were processed with the colour deconvolution 1.5 plugin using H&E or H&E2 presets. Resultant colour_1 images were 25% contrast enhanced before conversion into binary images. ROIs were selected for particles above 15,000 pixel units and manually verified by overlay onto the original H&E image to remove artifacts. Invasive protrusions were defined as projections at the leading edge of the tumor surrounded by stroma on three sides and identified on the binary image using the polygon selection tool. Invasive distance was determined as the difference between the farthest edge of the protrusion and the protrusion base.

Statistical analysis

Differences in mean values between groups were evaluated using Student or Welch *t* test. Significance was determined at $P < 0.05$ utilizing GraphPad Prism 7 software. Error bars represent $\pm$ SEM.

Results

Cortactin threonine 24 is required for Arp2/3 complex binding and activation

Phosphorylation of serine (S) 11, T13, and T24 in the murine cortactin NTA domain has been reported (19). The proximity of these residues to the canonical Arp2/3 binding motif consisting of amino acids 20–22 (DDW) have the potential to regulate Arp2/3 binding (Fig. 1A). To determine if these residues influence cortactin binding to Arp2/3, FLAG-tagged murine cortactin constructs were generated that contained serine to alanine (S11A) and

threonine to alanine (T24A) phosphorylation-null mutations. T13 was not evaluated because it is not conserved in human cortactin. Co-immunoprecipitation studies indicate that S11A cortactin bound endogenous Arp2/3 at levels similar to WT cortactin, whereas T24A cortactin failed to effectively bind Arp2/3 despite retaining the DDW binding motif (Fig. 1B). Threonine to aspartic acid (T24D) phosphomimetic cortactin bound Arp2/3 at reduced levels compared with WT (Fig. 1B). These data demonstrate that both the DDW motif and T24 are required for optimal Arp2/3 complex binding. Furthermore, reduced Arp2/3 binding resultant from the addition of negative charge at amino acid 24 (T24D) suggests that phosphorylation may play a negative-regulatory role.

To assess the impact of T24 on Arp2/3 actin nucleation, recombinant human WT, Δ DDW, and T24A cortactin proteins were expressed in bacteria and purified (Fig. 1C). When evaluated in pyrene-labeled actin assembly assays, WT cortactin displayed slower polymerization kinetics compared with the N-WASp VCA domain, whereas the Δ DDW mutant failed to activate Arp2/3 as previously reported (Fig. 1D; refs. 16, 34, 35). T24A cortactin demonstrated intermediate activity, with reduced nucleation levels compared with WT cortactin and increased nucleation compared with Δ DDW (Fig. 1D). Taken together these data identify T24 in the cortactin NTA as a critical residue required for optimal cortactin-mediated Arp2/3 binding and activation regardless of phosphorylation status.

Cortactin T24 is required for invadopodia-mediated ECM degradation

Cortactin is essential for initiating invadopodia formation, maturation, and ECM degradation in part due to NTA-mediated Arp2/3 binding (36, 37). To determine the role of cortactin T24 in invadopodia function, a panel of cortactin knockdown-rescue cell lines stably expressing FLAG-cortactin mutant constructs were produced in invasive UMSCC1 (Fig. 2) and OSC19 (Supplementary Fig. S1) HNSCC cell lines. Both lines spontaneously produce invadopodia and degrade ECM (26, 38). Although individual cortactin siRNA (siCTTN) and shRNA (shCTTN) treatment resulted in decreased cortactin expression and matrix degradation in each case (Fig. 2D; Supplementary Figs. S1B and S2), sequential exposure to cortactin siRNA in stable shRNA cells resulted in efficient and reliable cortactin knockdown (KD; Fig. 2; Supplementary Figs. S1 and S2). Cortactin KD cells were used for subsequent experimentation to minimize the possibility of residual endogenous cortactin masking the effects of re-expressed FLAG-cortactin mutants. FLAG-WT cortactin expression in KD cells partially restored the amount of active invadopodia formation in UMSCC1 cells (Fig. 2A–C) and fully restored ECM degradation in UMSCC1 (Fig. 2A and B) and OSC19 (Supplementary Fig. S1A–S1C) cell lines. FLAG- Δ DDW enhanced invadopodia precursor formation but failed to rescue active invadopodia and ECM degradation (Fig. 2A–C; Supplementary Fig. S1A–S1C). Similarly, both FLAG-T24A and FLAG-T24D cortactin restored invadopodia precursor formation while failing to induce invadopodia maturation above KD levels, with active invadopodia and ECM degradation levels for both mutants similar to that of FLAG- Δ DDW cortactin (Fig. 2A–C; Supplementary Fig. S1A, S1C, and S1D). These results suggest that Arp2/3 binding and activation facilitated by cortactin T24 is required for effective cortactin-mediated invadopodia formation and ECM degradation in HNSCC cells.

Figure 1.

Cortactin T24 is required for binding and activation of Arp2/3 complex.

A, Diagram representing cortactin functional domains. NTA, N-terminal acidic domain; R1-R6, repeats regions with F-actin binding site indicated; Helix, alpha helical domain; PRR, proline rich region; SH3, Src homology 3 domain. NTA domain with position of S11 and T24 in context of the DDW region is shown. **B**, Immunoprecipitation analysis of Arp2/3 binding to cortactin NTA mutants. HEK293T/17 cells transfected with FLAG-empty vector (EV), FLAG-wild-type cortactin (WT) or the indicated FLAG-cortactin mutants. Immune complexes were Western blotted with antibodies against cortactin (top) and Arp3 (bottom). 1:10 diluted total cell lysates were Western blotted as indicated. **C**, Coomassie blue staining of the indicated purified recombinant human cortactin proteins. **D**, Effect of cortactin T24A on Arp2/3 complex activation. Fluorometric evaluation of actin polymerization over time with the indicated cortactin mutants incubated with Arp2/3 complex and pyrene-labeled actin. Polymerization curves: WT cortactin (blue), T24A cortactin (dark green), and Δ DDW cortactin (purple). N-WASP VCA domain (black) was used as a positive control; negative controls include Arp2/3 complex plus actin (pale green) and actin alone (red). Polymerization curves are representative from three independent experiments.

CK2 phosphorylation of cortactin T24 regulates interaction with Arp2/3 complex

The importance of T24 in Arp2/3 activation and invadopodia function, along with prior identification of T24 as a cortactin phosphorylation site, led us to identify the kinase(s) responsible for phosphorylating T24. Computational analysis of the sequences flanking T24 was performed by seven independent predictive algorithms, six of which suggested that CK2 α had the highest probability of phosphorylating cortactin T24 (Supplementary Table S1). To test this, kinase assays were conducted using GST-tagged cortactin WT and T24A NTA fusion proteins

with purified active CK2 α . The N-WASP VCA domain was used as a positive control, because previous studies have shown this region to be a CK2 α substrate (23, 25). Increasing amounts of GST-WT-NTA were efficiently phosphorylated by CK2 α , whereas no phosphorylation was evident in GST-T24A-NTA (Fig. 3A). These data indicate that cortactin T24 can serve as a CK2 α substrate, and that T24 is the only residue targeted by CK2 α in the NTA region.

To determine if CK2 α phosphorylation of cortactin T24 effects binding to Arp2/3 complex, recombinant human WT or T24A cortactin proteins were pre-incubated with or without

Figure 2.

T24 is required for cortactin-mediated invadopodia formation and ECM degradation in HNSCC cells. **A**, UMSCC1 cells with stable shRNA scramble control (Ctl) or anti-cortactin shRNA combined with siRNA knockdown (KD) were transduced with murine FLAG-WT, -ΔDDW, -T24A, and -T24D cortactin lentiviruses. Cells were plated on Oregon Green (OG)-488 gelatin coated coverslips for 12 hours, fixed, and labeled with anti-FLAG and rhodamine phalloidin (Actin). Gelatin panels are pseudo-colored white; degradation is evident as black areas indicating loss of fluorescence. Scale bar represents 20 μ m. **B**, Quantification of gelatin matrix degradation for control (Ctl), cortactin KD, and FLAG-cortactin expressing UMSCC1 cells. **C**, Quantification of invadopodia precursors (left) and active invadopodia (right) numbers from the lines assayed in **B**. Data represents the mean $\pm$ SEM of $n \geq 100$ cells for each line analyzed from at least three independent experiments. All gelatin degradation conditions were normalized to Ctl UMSCC1 cells. n.s., not significant; *, $P < 0.05$, Welch's or Student t test vs. Ctl (**B**, **C**); †, $P < 0.05$, Student t test vs. wild type cortactin rescue (WT; **C**). **D**, Total cell lysates from (**A**) evaluated for endogenous and FLAG-cortactin expression by immunoblotting with antibodies against cortactin (top) and β -actin (bottom).

CK2 α , then mixed with purified Arp2/3. Phosphorylation of WT cortactin by CK2 α reduced binding of Arp2/3 complex to background levels (beads alone) whereas no impact on T24A was observed (Fig. 3B). To ascertain the impact of CK2 α phosphorylation on cortactin-mediated Arp2/3 activation, actin assembly assays were conducted with CK2 α -phosphorylated cortactin and N-WASP VCA domain. As previously determined, CK2 α phosphorylation of N-WASP VCA results in a modest reduction of Arp2/3 NPF activity (black vs. grey, Fig. 3C; ref. 25). Similarly, WT cortactin incubated with increasing amounts of CK2 α prior to inclusion in polymerization assays resulted in a dose-dependent suppression of actin assembly, suggesting that CK2 α phosphorylation impairs the ability of cortactin to activate Arp2/3 complex (Fig. 3C). Although Arp2/3 can be activated by direct phosphorylation from multiple kinases (39–41), CK2 α had no direct effect on Arp2/3 activation (orange vs. pale green, Fig. 3C), suggesting that the inhibitory effect on Arp2/3 activity is due to phosphorylated cortactin in these assays. To determine if the CK2 α -targeted T24 residue is responsible for the observed inhibitory effect on Arp2/3 activity, polymerization assays were conducted with WT and T24A cortactin proteins following incubation with

CK2 α . Although CK2 α inhibited the ability of WT cortactin to activate Arp2/3 complex (blue vs. red, Fig. 3D), preincubation of CK2 α with T24A cortactin exhibited no additional inhibitory effect on Arp2/3 nucleation activity (green vs. purple, Fig. 3D). Collectively these data indicate that cortactin phosphorylation at T24 by CK2 α reduces the ability of cortactin to bind and activate Arp2/3 complex-mediated branched actin network formation.

CK2 α is required for optimal HNSCC invadopodia function

To determine if CK2 α impacts invadopodia function, CK2 α expression was stably knocked down in OSC19 and UMSCC1 cells using anti-CK2 α shRNAs targeting two different regions in the CK2 α transcript. Both shRNAs reduced CK2 α expression to nondetectable levels in each cell line (Fig. 4A). Neither line expressed the alternative CK2 α' isoform in control or CK2 α knockdown cells (data not shown). CK2 α knockdown in OSC19 cells reduced the level of active invadopodia by 71% to 88% and ECM degradation by 63% to 73%, whereas knockdown in UMSCC1 cells reduced active invadopodia formation by 68% to 92% and ECM degradation by 36% to 60% (Fig. 4B–D). Although these data indicate that CK2 α is a key mediator of invadopodia

Figure 3.

CK2 α phosphorylation of cortactin T24 inhibits Arp2/3 complex binding and activation. **A**, Cortactin T24 is a CK2 α phosphorylation site. Autoradiogram of active CK2 α incubated with the increasing amounts (0, 0.25, 0.5, and 1 μ g) of GST-WT-NTA or GST-T24A-NTA cortactin fusion proteins. GST (1 μ g) and the GST-VCA domain of N-WASP (0.25 μ g) were used as respective negative and positive phosphorylation controls. Positions of autophosphorylated CK2 α , GST-VCA, and cortactin NTA proteins are indicated on the left; autoradiogram is representative of three independent experiments. **B**, CK2 α phosphorylation at cortactin T24 ablates binding to Arp2/3 complex. Purified recombinant human WT and T24A cortactin proteins (2.5 μ g) were bound with an anti-cortactin antibody to protein G beads. Immune complexes were preincubated with or without 75 ng active CK2 α , washed and incubated with 50 ng purified Arp2/3 complex. Co-immunoprecipitated complexes were Western blotted for cortactin (top) and Arp3 (bottom). 4F11-bound protein G beads were used as a negative control for nonspecific binding (Beads). Arp2/3 complex (5 ng) was used as positive control for Arp3 immunoblotting. Blot is representative of two independent experiments. **C**, Cortactin phosphorylation by CK2 α inhibits cortactin-mediated Arp2/3 actin polymerization. WT human cortactin or GST-VCA proteins were preincubated with the indicated amounts of active CK2 α and evaluated for effects on Arp2/3 activity. Polymerization curves are representative from three independent experiments. **D**, Phosphorylation of T24 is responsible for the inhibitory effect of CK2 α on cortactin-mediated Arp2/3 activation. Human WT and T24A cortactin proteins were preincubated with or without 30 ng active CK2 α and evaluated for effects on Arp2/3-mediated actin assembly. Polymerization curves are representative from three independent experiments.

maturation and function, neither invadopodia formation nor matrix degradation was entirely abolished. This would indicate that alternative signaling pathways impinging on cortactin and other invadopodia proteins remain active in the absence of CK2 α expression.

The CK2 α inhibitor Silmitasertib suppresses invadopodia function in HNSCC cells

Silmitasertib (CX-4945) is an orally bioavailable small molecule ATP-competitive inhibitor that targets CK2 α kinase activity and is currently undergoing clinical trials in multiple cancer types

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Figure 4.

CK2α is required for optimal HNSCC invadopodia function. **A**, Evaluation of CK2α expression in stable scramble control (Ctl) and CK2α shRNA HNSCC cells. Cells were lysed and Western blotted with antibodies against CK2α (top) and β-actin (bottom). **B**, Representative confocal images of OSC19 and UMSCC1 cells with Ctl shRNA and with each anti-CK2α shRNA (shCK2α #1 and #2). Cells were plated on OG-488 gelatin coverslips for 12 hours then labeled with an anti-cortactin antibody and rhodamine phalloidin (Actin). Gelatin is pseudo-colored white. Scale bar represents 20 μm. **C**, CK2α knockdown decreases invadopodia-mediated ECM degradation. Quantification of matrix degradation area per cell area for Ctl and each anti-CK2α shRNA in the indicated cell lines. Degradation data were normalized to Ctl condition for each cell line. **D**, CK2α knockdown decreases invadopodia numbers. Amount of invadopodia precursors (left) and active invadopodia (right) per cell is shown for control and shCK2α OSC19 and UMSCC1 cells. Data in **C** and **D** represent the mean + S.E.M. of $n \geq 100$ cells analyzed from at least three independent experiments. n.s., not significant; *, $P < 0.05$, Welch t test vs. Ctl.

(NCT01199718, NCT02128282, NCT00891280; ref. 20). To determine the impact of Silmitasertib on HNSCC tumor cell-mediated invadopodia formation and ECM degradation, established HNSCC cell lines were treated with increasing Silmitasertib concentrations and evaluated for effects on invadopodia activity and ECM degradation. Dose-dependent decreases in ECM degradation were observed at concentrations above 0.5 $\mu\text{mol/L}$ in all evaluated HNSCC lines (Fig. 5A and B). The greatest impairment of gelatin degradation was seen at 10 $\mu\text{mol/L}$ (Fig. 5A and B), comparable to effective CK2-specific growth-inhibitory doses in several cancer cell lines (20, 42). At this concentration, active invadopodia formation was significantly diminished in OSC19 and UMSCC1 cells, whereas MDA1586 cells displayed nonsignificant decreases (Fig. 5C). To determine if the invadopodia inhibitory effect of Silmitasertib was directly due to altering CK2 α -mediated cortactin T24 phosphorylation, antibodies against phosphorylated cortactin T24 peptides (pT24) were designed and purified. Attempts by two different commercial vendors failed to generate a pT24-specific antibody (not shown). Therefore, we evaluated the phosphorylation status of S473 (pS473) in AKT following Silmitasertib treatment as a surrogate marker for drug efficacy, as this site is known to be phosphorylated by CK2 α (20). MDA1586 cells treated with 1 or 10 $\mu\text{mol/L}$ Silmitasertib had decreased pS473 AKT after 24 hours, the same timeframe used in ECM degradation assays (Supplementary Fig. S3). OSC19 and UMSCC1 cells had decreased pS473 AKT after treatment with 10 $\mu\text{mol/L}$ Silmitasertib for 12 hours, the time used for matrix degradation assays in these lines (Supplementary Fig. S3).

The fact that CK2 α phosphorylates multiple targets aside from cortactin (20) raises the possibility that the inhibitory effect of Silmitasertib may be due to impairing phosphorylation of additional proteins involved in invadopodia function. To determine the extent of cortactin-specific CK2 α phosphorylation in invadopodia-mediated ECM degradation, UMSCC1 cells expressing FLAG-WT, FLAG-T24A, and FLAG-T24D cortactin were treated with 1 $\mu\text{mol/L}$ Silmitasertib and evaluated for additional suppressive effects on matrix degradation (Fig. 5D and E). Silmitasertib diminished ECM degradation in cells expressing FLAG-WT cortactin by 51%, similar to the reduction observed in nontransfected cells (Fig. 5D vs. B). Neither FLAG-T24A nor FLAG-T24D expressing cells treated with Silmitasertib demonstrated reductions in ECM degradation levels from baseline vehicle-treated controls (Fig. 5D and E). Although these results do not entirely negate alternative CK2-dependent signaling pathways in invadopodia regulation, it does suggest that cortactin T24 is the primary CK2 α target in governing HNSCC invadopodia function.

To evaluate the anti-invadopodia effect of Silmitasertib in more translationally-relevant models, PDXs were derived from a well- and a moderately-differentiated HNSCC surgical sample. PDX tumors maintained original patient tumor architecture, displaying collective invasion and keratin pearls characteristic of differentiated HNSCC (Fig. 6A). Primary cell lines derived from these PDX tumors form invadopodia and spontaneously degrade gelatin within 24 hours (Fig. 6B). Both lines exhibit tight colony morphology under cell culture conditions consistent with HNSCC lines derived from epithelial HNSCC (Fig. 6B; refs. 32, 43). Treatment of WVUSCC-AR2 and WVUSCC-AR5 with Silmitasertib yielded similar results to those observed in established lines, with gelatin degradation impaired 38% to 62% in WVUSCC-AR2 cells and 56% to 66% in WVUSCC-AR5 cells at

and above 0.5 $\mu\text{mol/L}$ (Fig. 6C and D). Similarly, 10 $\mu\text{mol/L}$ Silmitasertib treatment suppressed active invadopodia by 76% in WVUSCC-AR2 and 91% in WVUSCC-AR5 (Fig. 6E). Collectively these data indicate that CK2 α kinase activity is essential for maximal ECM degradation ability in HNSCC.

Silmitasertib inhibits HNSCC invasion

To determine if Silmitasertib impacts HNSCC invasion, we initially utilized a 3D *in vitro* assay designed to model collective invasion typically observed in differentiated HNSCC. Tumor spheroids generated from OSC19, UMSCC1, and WVUSCC-AR5 PDX lines were embedded between layers of collagen I. WVUSCC-AR2 cells failed to form spheroids and could not be used in this assay. Spheroids were treated with 10 $\mu\text{mol/L}$ Silmitasertib or vehicle (DMSO) for 48 hours (Fig. 7A and B). Silmitasertib significantly reduced 3D collective invasion in all assayed lines (Fig. 7B). We next evaluated the ability of Silmitasertib to control invasion in the tongues of mice harboring orthotopic tumors. Luciferase-expressing UMSCC1 cells injected into the tongues of NSG mice formed detectable tumors within 1 week (Fig. 7C). Mice were divided into two groups containing similar tumor size as determined by *in vivo* bioluminescence, with one group treated with vehicle and one with Silmitasertib. Mice dosed twice daily for 3 weeks displayed a nonsignificant reduction in tumor growth compared with controls, similar to previous single agent xenograft studies (Fig. 7C; ref. 20). To negate potential bias due to unequal tumor size, four equivalent tumors from each group were selected for further assessment. In-depth evaluation of tumor margins from tongues excised after 4 weeks revealed alterations in invasive characteristics of Silmitasertib-treated mice (Fig. 7D–I). Tumors in drug-treated mice had a less ragged appearance at the invasive front, exhibiting shorter and smaller collective cell protrusions into the tongue stroma compared with control mice (Fig. 7D–G). In addition, tongues from Silmitasertib-treated mice had reduced perineural invasion of nerves adjacent to the invasive front (Fig. 7H and I). No difference was seen in the size or invasive distance of detached collective groups within the invasive front (Supplementary Fig. S4). Collectively these data support a role for CK2 α signaling in driving several pro-invasive behaviors associated with poor patient outcome in HNSCC (2, 44).

Discussion

Proteolysis of restrictive tissue barriers is essential to all steps in the metastatic cascade and increasing evidence indicates that the proteolytic activity of invadopodia is required for invasive breaching of ECM barriers (1). Signals that govern invadopodia are dynamic and highly regulated, requiring coordinated activity of several oncogenic pathways in parallel to achieve maximal efficiency (1). Active assembly and turnover of cellular F-actin networks is required for initiating invadopodia formation and subsequent maturation, involving recruitment and activation of membrane-bound and secreted MMPs to mediate ECM proteolysis (1). Breakdown of existing F-actin networks, in conjunction with Arp2/3 activation in invadopodia, is responsible for actin network turnover necessary for productive branched F-actin formation that drives membrane protrusion. Early recruitment of cortactin is necessary for invadopodia initiation, where phosphorylation of C-terminal tyrosines 421, 470, and 486, along with serines 405 and 418, occurs downstream of growth factor and integrin signaling (45). These phosphorylation events create

Figure 5.

Silmitasertib-mediated CK2 inhibition reduces invadopodia function in established HNSCC cell lines. **A**, Representative confocal images of MDA1586, OSC19, and UMSSC1 cells plated on OG-488 gelatin coverslips for 1 hour before treatment with vehicle (DMSO) or 10 μmol/L Silmitasertib for 24 hours (MDA1586) or 12 hours (OSC19 & UMSSC1; optimal ECM degradation times for each line). Cells were fixed and labeled with an anti-cortactin antibody and rhodamine phalloidin (Actin). Gelatin is pseudo-colored white. Scale bar represents 20 μm. **B**, CK2α inhibition decreases invadopodia-mediated ECM degradation. Quantification of gelatin matrix degradation area per cell area in HNSCC cell lines treated with the indicated Silmitasertib concentrations. **C**, CK2α inhibition decreases invadopodia numbers. Amount of invadopodia precursors (left) and active invadopodia (right) per cell is shown for Silmitasertib-treated HNSCC cell lines. Degradation data were normalized to DMSO-treated (0) cells for each cell line. Data in **B** and **C** represent the mean ± SEM of $n \geq 100$ cells analyzed from at least three independent experiments. n.s., not significant; *, $P < 0.05$, Welch or Student t test vs. DMSO for each cell line. **D**, Quantitation of gelatin matrix degradation area per cell area for UMSSC1 cells expressing FLAG-WT, -T24A, or -T24D cortactin treated with DMSO or 1 μmol/L Silmitasertib. Data represent the mean ± SEM of $n \geq 75$ cells analyzed from at least three independent experiments. n.s., not significant; *, $P < 0.05$, Welch or Student t test vs. DMSO for each condition. **E**, Representative confocal images of UMSSC1 cells expressing FLAG-WT, -T24A, or -T24D cortactin plated on OG-488 gelatin coverslips for 1 hour before treatment with vehicle (DMSO) or 1 μmol/L Silmitasertib for 12 hours. Cells were fixed and labeled with an anti-FLAG antibody and rhodamine phalloidin (Actin). Gelatin is pseudo-colored white. Scale bar represents 20 μm.

Figure 6.

Silmitasertib inhibits invadopodia function in HNSCC PDX cells. **A**, Establishment of HNSCC PDX tumors. Hematoxylin and eosin stained patient tumor tissue and PDXs. Patient tumors were from the alveolar ridge (AR). Scale bar represents 200 μm. **B**, Invadopodia formation in HNSCC PDX cells. Representative confocal images of WVUSCC-AR2 (AR2) and WVUSCC-AR5 (AR5) PDX cell lines. Cells were plated on OG-488 gelatin coverslips for 24 hours and labeled with an anti-cortactin antibody and rhodamine phalloidin (Actin). Gelatin is pseudo-colored white. Scale bar represents 20 μm. 10× representative phase contrast images (Phase) of each line are shown on the right. Scale bar represents 100 μm. **C**, Confocal images of WVUSCC-AR2 and WVUSCC-AR5 cells plated on OG-488 gelatin coverslips, allowed to attach for 1 hour, then treated with vehicle (DMSO) or 10 μmol/L Silmitasertib for 24 hours. Cells were labeled with an anti-cortactin antibody and rhodamine phalloidin (Actin). Gelatin is pseudo-colored white. Scale bar represents 20 μm. **D**, CK2α inhibition decreases invadopodia-mediated ECM degradation in PDX-derived cell lines. Quantification of matrix degradation area per cell area for WVUSCC-AR2 and WVUSCC-AR5 PDX cell lines treated with the indicated Silmitasertib concentrations. Degradation data were normalized to DMSO-treated cells for each cell line. **E**, CK2α inhibition decreases invadopodia numbers in PDX-derived cell lines. Determination of amount of invadopodia precursors (left) and active invadopodia (right) in WVUSCC-AR2 and WVUSCC-AR5 cells. Data represent the mean ± SEM, of $n \geq 100$ cells analyzed from at least three independent experiments. *, $P < 0.05$, Welch t test vs. DMSO for each cell line.

binding sites for scaffolding platforms that recruit N-WASP and WAVE2, ensuring that activation of Arp2/3 is maintained throughout the invadopodia cycle (6). Cortactin also recruits the F-actin severing protein cofilin to invadopodia, providing the necessary machinery for cyclic actin network breakdown and regrowth during invadopod extension (16). In addition to these important C-terminal functions, the ability of the NTA

region to directly bind Arp2/3 is also essential for invadopodia formation and ECM degradation, presumably through direct Arp2/3 activation and prolonged stabilization of Arp2/3-F-actin networks (10, 16).

Regarding the ability of the cortactin NTA to bind Arp2/3, this study reveals two distinct findings. First, that T24 is required for Arp2/3 binding, because mutation of this residue ablates (A)

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or reduces (D) the association of cortactin with Arp2/3 in co-immunoprecipitation assays. T24 is two residues C-terminal to the well-defined DDW Arp2/3 interaction motif that is conserved in other Arp2/3 NPFs (6). This places T24 in close proximity to contribute to Arp2/3 binding. Although the crystal structure of the cortactin NTA bound to Arp2/3 complex has not been reported, previous chemical crosslinking and three-dimensional reconstruction studies indicate that the NTA region primarily binds the Arp3 subunit in the complex, with the most N-terminal residues spanning the Arp2–Arp3 interface to contact the Arp2 subunit (Visual Overview; refs. 9, 34). Thus, T24 may contribute essential hydrogen bonding with recipient polar residues near the basic side chains on Arp3 involved in electrostatic binding to the acidic NTA residues. This concept is supported by the requirement of W22 in the DDW motif for Arp2/3 binding, along with the lack of an equivalent threonine in the VCA region of WASp proteins (16, 34, 35). Although T24A cortactin appears incapable of binding Arp2/3, it can activate Arp2/3 actin nucleation in conditions where Δ DDW cortactin remains inactive (Fig. 1D). This may be due to T24 having a lower affinity for Arp3 than the DDW region and/or the high cortactin molar levels required to stimulate NPF activity *in vitro*. Nonetheless, the requirement for cortactin T24 in invadopodia-mediated ECM degradation supports an essential biological role for this residue in a non-phosphorylated context.

Second, our study shows that T24 phosphorylation serves to negatively regulate cortactin binding to and activation of Arp2/3 complex (Fig. 3B–D). Addition of the phosphate group to T24 likely imparts a steric and electrostatic disruption, preventing the DDW and other interacting residues in the NTA from initiating and/or maintaining binding to Arp3. Steric interference may be the predominant mechanism, since T24D cortactin can bind Arp2/3 at reduced levels (Fig. 1B), indicating that negative charge alone is insufficient to completely prevent Arp2/3 association. Phosphorylation of T24 therefore serves to block the ability of cortactin to bind Arp2/3, similar to the regulation of the Arp2/3-F-actin regulatory proteins coronin 1B and cofilin (46, 47). Although we did not determine if phosphorylation of T24 serves to release cortactin from existing Arp2/3-F-actin branchpoints, phosphorylation of Arp2/3-bound cortactin T24 may be an

additional mechanism to dissociate cortactin from these regions. Such a mechanism could function in facilitating breakdown and recycling of invadopodia Arp2/3-F-actin networks.

The ability of CK2 α to phosphorylate cortactin T24 supports a wider role for this kinase in actin regulation. Previous work identified T24 as a putative but unconfirmed CK2 α phosphorylation site in the NTA region of myeloid-cell specific cortactin homolog HS1 (21). In addition, CK2 α phosphorylates two adjacent sites in the A domain of WASp family proteins (22–25). These studies report conflicting results on Arp2/3 activity, with phosphorylation of WASp enhancing and in N-WASp and WAVE2 inhibiting VCA-stimulated Arp2/3-mediated actin polymerization. Our results with CK2 α -phosphorylated N-WASp VCA domain also shows an inhibitory effect on Arp2/3 activation (Fig. 3C), suggesting that CK2 α effects may be differential to specific WASp family proteins.

Cortactin T24A and T24D expression in KD HNSCC lines impairs active invadopodia formation and subsequent ECM degradation (Fig. 2A–C; Supplementary Fig. S1A and S1C), supporting *in vitro* evidence that T24 phosphorylation prevents Arp2/3 binding and activation necessary for invadopodia activity. The importance of CK2 α -mediated cortactin T24 phosphorylation is further supported by the inability of Silmitasertib to further suppress ECM degradation in cortactin T24A or T24D expressing cells (Fig. 5D and E). CK2 α -specific knockdown or pharmacologic inhibition in cells would therefore be expected to reduce phosphorylation of cortactin and WASp family proteins, resulting in net increased Arp2/3 activity as observed from *in vitro* studies. The fact that CK2 α knockdown or kinase blockade also impairs invadopodia formation and function is paradoxical, but can be explained by cyclic CK2 α phosphorylation and dephosphorylation to regulate cortactin binding to and activation of Arp2/3 in invadopodia during maturation and elongation. Dephosphorylation of cortactin T24 and WASp NPFs by unknown phosphatases would enable these NPFs to promote Arp2/3 activation, actin polymerization, and invadopodia activity. Following network breakdown during actin turnover, CK2 α phosphorylation would return NPFs to the inactive state to await the next cycle of actin assembly. Although similar cyclic regulation of Src and cofilin has been shown to be essential for invadopodia function

Figure 7.

Silmitasertib impairs collective invasion in HNSCC. **A**, Representative phase contrast microscopy images of OSC19, UMSCC1, and WVUSCC-AR5 tumor cell spheroids embedded in collagen I (0 hour) then incubated in complete media containing 10 μ M Silmitasertib or vehicle (DMSO) for 48 hours. Red circles indicate the maximum radial distance at indicated time points. Scale bar represents 100 μ m. **B**, Quantitation of maximal collective invasive distance of each cell line at 48 hours with vehicle (0) or Silmitasertib (10 μ M/L). Data represent the mean $\pm$ SEM of $n \geq 10$ spheroids per cell line and condition analyzed from at least three independent experiments. *, $P < 0.05$, Welch or Student *t* test vs. DMSO. **C**, Effect of Silmitasertib on HNSCC orthotopic tumor growth. Bioluminescent monitoring of UMSCC1 cells orthotopically injected into tongues of NSG mice. Tumors were allowed to establish for 1 week prior to administration of Silmitasertib or equal volume vehicle (DMSO). Data represent mean $\pm$ SEM from two independent experiments. DMSO, $n = 9$ mice; Silmitasertib, $n = 7$ mice. n.s., not significant; Student *t* test vs. DMSO. **D**, Silmitasertib inhibits orthotopic HNSCC invasion. Representative hematoxylin and eosin stained invasive front of orthotopic UMSCC1 tongue tumors from mice receiving Silmitasertib or vehicle (DMSO) for 3 weeks. Black lines show tumor-encompassing RIO borders determined by software analysis. Black box in DMSO tumor indicates the invasive protrusion shown in **F**. White box in DMSO tumor denotes region containing perineural invasion (PNI) shown in **I**. Black arrow indicates direction of invasion toward tongue base. M, skeletal muscle; T, tumor. Scale bar represents 100 μ m. **E**, Invasive distance of tumor protrusions from mice treated with Silmitasertib or vehicle (DMSO). Data represent the mean $\pm$ SEM of two serial sections from $N = 4$ tumors; $n = 34$ protrusions per condition. *, $P < 0.05$, Student *t* test vs. DMSO. **F**, Representative image of an invasive protrusion from vehicle-treated mice shown in the black box in **D**. The protrusive region was traced in black to denote the tumor protrusion from surrounding muscle-containing stroma. M, skeletal muscle; T, tumor protrusion. Scale bar represents 100 μ m. **G**, Area of invasive protrusions from mice treated with Silmitasertib or vehicle (DMSO). Data represent the mean $\pm$ SEM of two serial sections from $N = 4$ tumors; $n = 34$ protrusions per condition. *, $P < 0.05$, Welch *t* test vs. DMSO. **H**, Percentage of lingual nerves displaying PNI in tumors from mice treated with Silmitasertib or vehicle (DMSO). Data represent the mean $\pm$ SEM of two serial sections from $N = 4$ tumors; $n \geq 60$ nerves per condition. *, $P < 0.05$, Student *t* test vs. DMSO. **I**, Representative image of PNI shown in the white box in the DMSO-treated mouse in **D**. N, nerve; T, tumor cells. Scale bar represents 50 μ m.

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and provides support for this model (38, 46), confirmation of T24 phosphorylation within invadopodia leading to altered actin dynamics along with identification of the T24 phosphatase are necessary to fully confirm this proposed mechanism.

Locoregional control of HNSCC dissemination is problematic, where perineural invasion and invasive metastatic spread to cervical lymph nodes accelerates patient decline (44, 48, 49). Cortactin and CK2 α expression are elevated in HNSCC and individually correspond with poorer patient outcomes (7, 20). CK2 knockdown and Silmitasertib treatment in HNSCC cells has antiproliferative and antimetastatic properties *in vitro* and in mice, providing support for the utility of Silmitasertib in HNSCC (20). The anti-invasive effect of Silmitasertib shown here is likely due in part to disruption of the invadopodia actin assembly through combined inhibition of multiple Arp2/3 NPFs. We note that T24 cortactin mutants, CK2 α knockdown, and Silmitasertib treatment in HNSCC cells do not completely abolish invadopodia formation, degradation activity or invasion. Activation of invadopodial Arp2/3 NPFs through alternative scaffolding or lipid-based signaling pathways that can bypass CK2 inhibition can account for this residual activity (1). However, our results indicate that CK2 α inhibition by a single agent does significantly impair collective HNSCC invasion. Given the current paucity of treatment options for invasive HNSCC, combining Silmitasertib with additional drugs known to impair invadopodia function by blocking additional invadopodia and motility pathways (8) has the potential to provide novel treatment options for controlling invasive spread of late-stage HNSCC harboring elevated cortactin and/or CK2 expression.

Disclosure of Potential Conflicts of Interest

S.A. Weed reports receiving a commercial research grant from Gilead Sciences. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

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Molecular Cancer Research

Cortactin Phosphorylation by Casein Kinase 2 Regulates Actin-Related Protein 2/3 Complex Activity, Invadopodia Function, and Tumor Cell Invasion

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When the Smoke Clears m⁶A from a Y Chromosome-Linked lncRNA, Men Get an Increased Risk of Cancer

A. Rouf Bandy¹, Brenen W. Papenberg², and Ludmila Prokunina-Olsson¹

Long noncoding RNAs (lncRNA) have been implicated in many diseases, including cancer. Although these disease-associated effects have been mostly attributed to the ability of lncRNAs to function as regulatory noncoding transcripts, there is growing evidence that lncRNAs may also encode functional micropeptides. In the current issue of *Cancer Research*, Wu and colleagues report a micropeptide encoded by a Y chromosome-

linked lncRNA that may explain the higher incidence of esophageal cancer in male smokers. Furthermore, this report provides broader insights related to the molecular epidemiology of male-dominant and smoking-driven cancers and may also help explain some cancer-related associations with mosaic Y chromosome loss.

See related article by Wu et al., p. 2790

Long noncoding RNAs (lncRNA) are generally defined as long (>200 nucleotides) transcripts that do not encode proteins. lncRNAs include some well-known regulatory transcripts that affect cancer initiation and progression, such as *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1), *HOTAIR* (HOX transcript antisense intergenic RNA), *UCA1* (urothelial carcinoma associated 1), *BCYRN1* (brain cytoplasmic RNA 1), and *HULC* (highly upregulated in liver cancer; ref. 1). Despite the “noncoding” designation, multiple short open reading frames (ORF) for putative micropeptides can usually be predicted within lncRNAs. An annotation of a 53-amino acid peptide, HOX-AS3, which inhibits tumorigenesis in the colon by metabolic reprogramming, provides one of a few examples demonstrating functional properties of micropeptides rather than their encoding lncRNAs (2). However, due to limited understanding of their functions and lack of high-throughput methods for their annotation, these putative lncRNA-encoded micropeptides are mostly overlooked.

In the current issue of *Cancer Research*, Wu and colleagues (3) present their discovery of a 21-amino acid micropeptide (designated as YY1BM) encoded by a Y chromosome-linked lncRNA, *LINC00278*. They demonstrated the role of YY1BM in tumorigenesis and cancer survival through its interaction with smoking exposure in male esophageal squamous cell carcinoma (ESCC). ESCC is one of the deadliest cancers in the world, with average 5-year survival rates remaining under 20%. Tobacco smoking is considered a major environmental risk factor for ESCC. Men are four times more likely to develop ESCC than women, which might be partially explained by the differences in smoking rates reported as 52.9% and 2.4% in Chinese men and women, respectively (4), with comparable ratios in other populations as well. Male-specific factors, both hormonal and genetic, such as those encoded by the Y chromosome, might be contributing to

this complex disease, in interaction with environmental exposures. This combination of risk factors is not specific to ESCC and might also be relevant to other male-dominant and smoking-driven cancers, such as of the head and neck, bladder, lung, and colon. However, molecular mechanisms that can explain this gender bias in several common cancers, especially in smokers, remain largely unclear. In their study, Wu and colleagues hypothesized that some of these answers might be literally encoded within the Y chromosome and chose to focus on lncRNAs.

The authors found that the expression of three Y chromosome-encoded lncRNAs was downregulated in esophageal tumors compared to adjacent normal tissues; of those, only downregulation of *LINC00278* was validated in an additional set of samples. Next, they experimentally demonstrated that one of the four ORFs predicted within this lncRNA encoded a 21-amino acid (2.12 kDa) micropeptide. Through rigorous *in vitro* and *in vivo* work, they demonstrated how downregulation of *LINC00278*, and therefore the reduced production of the corresponding micropeptide, could lead to ESCC in male smokers. Specifically, they showed that the micropeptide could bind the YY1 transcription factor, thereby blocking its interaction with the androgen receptor (AR). On the basis of these findings, the micropeptide was designated as the YY1-binding micropeptide (YY1BM). Low YY1BM expression resulted in reduced apoptosis in ESCC xenografts and tissues by decreasing the AR-regulated eEF2K expression, which controls protein synthesis. This effect was particularly noticeable under cellular stress conditions of severe nutrient deprivation, which might mimic the microenvironment of rapidly growing tumor cells. Thus, YY1BM might be inhibiting tumorigenesis by mediating cross-talk between pathways involving AR signaling, protein synthesis, and cell metabolism. These findings could also justify using AR-targeted therapies for men with ESCC and other cancers to be identified affected by decreased YY1BM expression.

Smoking is the major environmental risk factor for many cancer types, but the molecular mechanisms of smoking exposure are still only partially understood. It is also unclear whether smoking has different cancer-related effects in men and women, and if so, why? Wu and colleagues provide the most unexpected answer to this question. Dynamic regulation of transcriptional outcomes through the N⁶-methyladenosine (m⁶A) posttranscriptional RNA modification emerged as a mechanism of cellular adaptation to physiological and environmental conditions (5). This process is regulated by a combination of proteins with specific functions to add/write (methyltransferases), erase (demethylases), or read m⁶A on various transcripts. By

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exposing cells to cigarette smoke condensate, the authors identified a complex of METTL3/14 and WATP as a writer, ALKBH5 as an eraser and YTHDF1 as a reader, all affecting translation of *LINC00278* into YY1BM. Of these proteins critical for m⁶A function, only expression of ALKBH5 (an eraser) was increased by smoking exposure, through its promoter hypomethylation. Thus, it appears that smoking exposure facilitates m⁶A clearance from *LINC00278* by activating the expression of ALKBH5 (m⁶A eraser), resulting in decreased production of YY1BM, thereby contributing to tumorigenesis. Because YY1BM can be produced only in men, women with equal smoking exposure would not have the same cancer-related molecular consequences of smoking.

The authors also linked the reduced expression of *LINC00278* with decreased survival of men with ESCC, particularly in smokers. It was assumed that this association is related to the decreased production of YY1BM, given its antitumorigenic properties. However, this might not be entirely attributable to the expression or the functional effects of this lncRNA, considering several competing or overlapping mechanisms. First of all, *LINC00278* is adjacent to the *ZFY* gene, which is one of the genes affected by the extreme downregulation of chromosome Y gene expression (EDY), a phenomenon associated with age and smoking in men (6). Because *ZFY* was affected by EDY in multiple nontumor as well as tumor tissues, mRNA expression of *LINC00278* (not tested by that study) might also be affected by EDY in general and not specifically in ESCC. Focusing on lncRNAs, Wu and colleagues did not analyze any mRNA expression, including that of *ZFY*. Thus, it is difficult to disentangle whether *LINC00278* expression is the sole reason for the reported association with decreased survival, given that other genes in this region may be equally downregulated.

Downregulation of *LINC00278* might also be a molecular manifestation of the age- and smoking-dependent mosaic loss of Y chromosome (mLOY), which affects a fraction of somatic cells already in normal tissues and is further enhanced in tumors (7, 8). Although both mLOY and EDY could serve as informative biomarkers, molecular mechanisms underlying cancer-related consequences of these events are still unclear. Downregulation of *ZFY* has been suggested to have some cancer-related functional significance, but with limited experimental evidence. On the basis of the results presented by Wu and colleagues, downregulation or loss of *LINC00278* expression might offer at least a partial explanation of why mLOY and EDY are likely to be associated with cancer, especially in smokers. First, both these mechanisms would result in a decrease of *LINC00278* expression, and thus, of YY1BM. Second, smoking-mediated clearance of m⁶A from *LINC00278* would further prevent the translation of this micropeptide from *LINC00278*, which might already be decreased through mLOY or EDY. In other words, by affecting m⁶A-dependent translation of

YY1BM, smoking would likely increase cancer risk even without mLOY. However, the risk might be stronger in smokers with mLOY, due to downregulation of YY1BM by two different mechanisms.

Wu and colleagues also demonstrated that intratumoral treatment with recombinant YY1BM significantly reduced tumor growth and improved the ESCC survival in male xenograft mouse models. A recent study showed that another lncRNA-encoded micropeptide inhibited triple-negative breast cancer progression (9). Micropeptides are attractive as therapeutic agents due to their high specificity, potency, and low toxicity. Insulin is a well-studied example of a natural 51-amino acid minipeptide encoding an essential hormone, which is used as the main drug to treat type I diabetes. On the basis of cell membrane penetration and solubility, mini- and micropeptides might be more optimal than large proteins encoded by the bona fide protein-coding genes. Numerous micropeptides encoded by noncoding RNAs, including lncRNAs, might offer treasure troves of natural small-molecule-type peptides of possible biological significance. A recent genome-wide functional study has given a broad look at the translation of ORFs outside of canonical coding sequences (10). Among those, about 100 lncRNA-encoded micropeptides were found to regulate cell growth, highlighting their potential importance in cancer and other diseases. While these studies need extensive validation, we might expect some new cancer drugs to be found among naturally existing lncRNA-encoded micropeptides.

Of multiple findings from this study of significance across several fields, we will reiterate several main points: micropeptides encoded by short lncRNA-encoded ORFs might have major cancer-related functions; these functions may be modified by environmental risk factors such as smoking and contribute to gender bias; micropeptides could be the source of naturally existing small molecules and potential anticancer therapeutic agents. The molecular mechanism presented by Wu and colleagues offers a plausible explanation for the higher risk of ESCC in men than women, and smoking men, specifically. This study should inspire follow-up analyses in other smoking-driven cancers affecting both sexes, but with a disproportional male:female ratio.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

When the Smoke Clears m⁶A from a Y Chromosome–Linked lncRNA, Men Get an Increased Risk of Cancer

A. Rouf Banday, Brenen W. Papenberg and Ludmila Prokunina-Olsson

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July 14, 2020

Brenen W. Papenberg
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Dear Mr. Papenberg:

This letter is sent to provide approval for you to use NAACCR's logo in your dissertation that will be titled "Novel Roles of Tobacco-associated Genes Underlying Disparate Survival in Appalachian Head and Neck Squamous Cell Carcinoma" which contains the results of IRB #16-14. The use of the logo is approved for the example you provided in your 7-13-2020 email "Use of NAACCR Logo".

Please remember to cite the CiNA dataset you used. For example, the selected citation for his standard CiNA file used is: SEER*Stat Database: NAACCR Incidence Data - CiNA Analytic File, 1995-2014, for Expanded Races, Standard File, Weed - Appalachia Survival (which includes data from CDC's National Program of Cancer Registries (NPCR), CCCR's Provincial and Territorial Registries, and the NCI's Surveillance, Epidemiology and End Results (SEER) Registries), certified by the North American Association of Central Cancer Registries (NAACCR) as meeting high-quality incidence data standards for the specified time periods, submitted December 2016.)

Please contact me if you have any further questions.

Sincerely,

Charlie Blackburn
COO
NAACCR, Inc.

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Cancer cell biologist with seven years graduate research experience studying disparate populations through secondary analysis of cancer registry data and bioinformatic interrogation of The Cancer Genome Atlas. Have also identified novel oncogenic roles of amplified and overexpressed transcripts associated with tobacco-induced DNA damage in head and neck squamous cell carcinoma.

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Doctoral research projects under Scott Weed focused on identifying a novel health disparity for tobacco-correlated head and neck squamous cell carcinoma, determining tobacco-correlated gene amplifications, and identifying novel functions of cell cycle transcripts.

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Microbiology Department, Laboratory Assistant · May 2012 – May 2013

Undergraduate research project under Larry Halverson focused on biofilm formation of *Pseudomonas putida* and its ability to colonize maize rhizospheres.

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Dissertation Title: *Novel Roles of Tobacco-associated Genes Underlying Disparate Survival in Appalachian Head and Neck Squamous Cell Carcinoma*

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Scientific Presentations

Oral Presentations

Papenberg, BW, Ingles, J, Feng, J, Allen, J, Markwell, S, Hames, R, Interval, E, Montague, A, Patel, R, Coad, J, Wen, S, Weed, SA. Molecular characterization of the mortality disparity in tobacco-associated Appalachian HNSCC. **NAACCR Annual Conference**. Pittsburgh, PA. June 2018.

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Manuscripts

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Contributions

Dundar B, Markwell SM, Sharma NV, Olson CL, Mukherjee S, Brat DJ. Methods for in vitro modeling of glioma invasion: Choosing tools to meet the need. *Glia.* 2020;

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