Investigating Biological Mechanisms of Radiation Resistance in Advanced Stage Cervical Cancer

Fiona Ruiz
Washington University in St. Louis

Follow this and additional works at: https://openscholarship.wustl.edu/art_sci_etds

Part of the Cell Biology Commons, Molecular Biology Commons, and the Oncology Commons

Recommended Citation
https://openscholarship.wustl.edu/art_sci_etds/2016

This Dissertation is brought to you for free and open access by the Arts & Sciences at Washington University Open Scholarship. It has been accepted for inclusion in Arts & Sciences Electronic Theses and Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
Investigating Biological Mechanisms of Radiation Resistance in Advanced Stage Cervical Cancer

by

Fiona Ruiz

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
doctor of Philosophy

December 2019
St. Louis, Missouri
# Table of Contents

List of Figures ............................................................................................................................................v
List of Tables ................................................................................................................................................vii
Acknowledgments .........................................................................................................................................viii
Abstract ....................................................................................................................................................x

Chapter 1: Introduction .................................................................................................................................1

Chapter 2: Identification of genomic biomarkers for disease recurrence in chemoradiation treated
CESC ..........................................................................................................................................................3

   2.1 Introduction ........................................................................................................................................3

   2.2 Study design .......................................................................................................................................4

      2.2.1 Patient Cohort ..............................................................................................................................4

      2.2.2 Statistical analysis .........................................................................................................................5

      2.2.3 Targeted gene panel sequencing and mutation annotation .........................................................6

      2.2.4 Generation of stable shRNA mediated knockdown NSD1 cell lines .........................................8

      2.2.5 H3K36 methylation .....................................................................................................................8

      2.2.6 Clonogenic survival ....................................................................................................................9

      2.2.7 NSD1 knockdown cell line treatment response .......................................................................9

      2.2.8 RNAseq analysis .......................................................................................................................10

   2.3 Disease recurrence in WUSM patient cohort .....................................................................................10

   2.4 Using NGS to identify candidate gene biomarkers of disease recurrence after chemoradiation treatment ....................................................................................................................13

      2.4.1 Gene prioritization ......................................................................................................................13

      2.4.2 Candidate biomarker selection .................................................................................................16

   2.5 Role of NSD1 in CRT response .........................................................................................................16

      2.5.1 NSD1 biological functions .........................................................................................................16

      2.5.2 NSD1 role in disease ..................................................................................................................17

      2.5.3 Generation of NSD1 KD cell lines ...........................................................................................18

      2.5.4 Effect of NSD1 KD on cell sensitivity to single agent carboplatin and CRT treatment ..............21

      2.5.5 Differential transcriptional regulation induced in NSD1 KD cells .......................................24
Chapter 3: Role of HPV transcript expression in CESC treatment response ..........................36

3.1 Introduction ..................................................................................................................36

3.2 Study design ..................................................................................................................40

3.2.1 HPV genotyping .....................................................................................................40

3.2.2 Viral transcriptome and integration state ...............................................................40

3.2.3 Engineering E6 and E6* overexpressing cell lines ................................................40

3.2.4 p53 functionality in E6* and E6 overexpressing cell lines ........................................41

3.2.5 p21 functionality in E6* and E6 overexpressing cell lines ........................................41

3.2.6 Cell survival after CRT ..........................................................................................42

3.2.7 Cell response to RT treatment ...............................................................................42

3.3 HPV positive patients ..................................................................................................43

3.3.1 HPV genotypes detection and viral integration status ............................................43

3.3.2 Comparison of HPV 16 vs HPV other patient groups ............................................47

3.3.3 HPV 16 and relative HPV transcript expression correlate with poor patient survival outcomes ...........................................................................................................51

3.4 In vitro E6 and E6* overexpressing cell line characterization .......................................52

3.4.1 In vitro correlation of HPV transcript expression and RT sensitivity .......................53

3.4.2 E6 and E6* overexpression modulates p53 expression ...........................................54

3.4.3 E6* overexpression induces p21 mediated cellular senescence .................................56

3.4.4 In vitro Siha E6 and E6* cellular response to chemoradiation treatment .................58

3.4.5 E6* overexpression induces cellular senescence ....................................................63

3.5 Overall summary and proposed amendments to treatment guidelines ..........................63

Chapter 4: HPV negative cervical cancer ........................................................................65

4.1 HPV negativity is a poor prognostic marker for mortality in oropharyngeal and cervical cancer .............................................................................................................65

4.2 Study design .................................................................................................................69

4.2.1 Defining HPV negativity .........................................................................................69
4.2.2 Genomic sequencing.................................................................69
4.2.3 RNAseq analysis.................................................................70
4.3 Characterization of HPV negative cervical cancer tumors...........70
  4.3.1 Results of genomic analysis..............................................70
  4.3.2 Results of transcriptome analysis.....................................74
  4.3.3 Summary of HPV negative tumor profiling.......................77
4.4 Testing the efficacy of Palbociclib in HPV negative and HPV positive cancer cell lines..............................................................79
  4.4.1 Cell line selection............................................................79
  4.4.2 HPV negative cell line sensitivity to Palbociclib..................80
  4.4.3 Palbociclib induced G1 arrest and proliferation attenuation.....81
4.5 Summary of HPV negative CESC characterization.....................84
Chapter 5: Conclusions.................................................................85
References....................................................................................89
List of Figures

Figure 2.1: Progression-free and overall survival curves ......................................................12
Figure 2.2: Frequently mutated genes and their enrichment in recurrent patient cohort........14
Figure 2.3: Significantly mutated genes ..............................................................................15
Figure 2.4: NSD1 expression in ATCC cervical cancer cell lines ...........................................19
Figure 2.5: Generation of stable NSD1 knockdown cell line ................................................20
Figure 2.6: Effect of NSD1 knockdown on single agent carboplatin treatment sensitivity....22
Figure 2.7: Effect of NSD1 knockdown effect on CRT treatment sensitivity.........................23
Figure 2.8: Differential expression analysis of Caski shNSD1 vs shControl cells..............25
Figure 2.9: GSEA pathway analysis of DE genes .................................................................26
Figure 2.10: γH2AX p-S139 foci formation and clearance after 5μM carboplatin ..............28
Figure 2.11: Cell cycle analysis after 5μM carboplatin treatment ........................................29
Figure 2.12: γH2AX p-S139 foci formation and clearance after 2Gy radiation treatment......30
Figure 2.13: Cell cycle analysis after 0.5μM carboplatin and 4Gy radiation treatment.......31
Figure 3.1: HPV genotype significantly stratifies patient outcomes ..................................44
Figure 3.2: Viral state determined using DNA methodology ...............................................45
Figure 3.3: Viral state determined using RNAseq methodology ..........................................46
Figure 3.4: HPV E6, E6*I and E7 transcript expression in WUSM cohort .........................49
Figure 3.5: Patient outcomes stratified by relative HPV transcript expression .................50
Figure 3.6: Cervix cancer cell lines transcript expression and sensitivity to radiation treatment .................................................................................................................53
Figure 3.7: Generation of HPV E6* and E6 overexpressing cell lines and their effect on p53 ..................................................................................................................55
Figure 3.8: p21 stabilization and induction of cellular senescence

Figure 3.9: E6* and E6 overexpression effect on clonogenic potential

Figure 3.10: E6* and E6 overexpression do not alter G2/M cell cycle arrest following CRT treatment

Figure 3.11: Apoptosis is not the primary mechanism of cell death after CRT treatment

Figure 3.12: HPV E6 and E6* overexpression affects rates of γH2AX p-S139 foci clearance after RT treatment

Figure 4.1: Patient survival outcomes stratified by HPV status

Figure 4.2: SMGs upregulated in HPV negative tumors

Figure 4.3: SMGs downregulated in HPV negative tumors

Figure 4.4: Pathway analysis of HPV negative vs HPV positive cervical tumors in WUSM cohort

Figure 4.5: SMGs in HPV negative cervical cancer function in G1 to S phase cell cycle progression

Figure 4.6: Genotype and cyclin D1 expression across cervical cancer and head and neck squamous cell cancer cell lines

Figure 4.7: HNSCC cell line sensitivity to Palbociclib

Figure 4.8: G1 cell cycle arrest 24h after Palbociclib treatment

Figure 4.9: Proliferation of HNSCC cell lines after 0.25µM Palbociclib
List of Tables

Table 2.1: Targeted Gene Panel..........................................................7
Table 2.2: HPV Genotype Panel..........................................................7
Table 2.3: Patient baseline characteristics.............................................11
Table 3.1: Patient baseline characteristics..............................................48
Table 4.1: Frequency of mutations by HPV status in University of Alabama at Birmingham (UAB) patient cohort..........................................................71
Table 4.2: Co-occurrence of significantly mutated genes (SMGs) in HPV negative tumors..........................................................72
Table 4.3: Mutual exclusivity of significantly mutated genes (SMGs) in HPV negative tumors..........................................................72
Table 4.4: Significantly mutated genes (SMGs) enriched in HPV negative tumors............73
Acknowledgments

The work completed in this thesis dissertation would not have been possible without the support and guidance of my colleagues, mentors, friends and family. I’d like to begin by thanking my thesis mentor Julie Schwarz for your guidance in developing these thesis aims, your ability to always feedback to the larger clinical picture we were working towards and for allowing me the time to develop new skill sets, namely analysis of bioinformatic datasets, making this thesis work feasible. My lab mates and colleagues, especially, Rashmi Pillai, Naoshad Muhammad, Mike Zahner and Nishanth Gabriel; you have all helped immensely in the day to day grind of lab work and helped me to both develop my technical expertise as well as help develop my experimental planning. You have all made my time in lab enjoyable and I value all of hours spent together “in the trenches”. I’d like to thank all of my colleagues and collaborators in the Radiation Oncology department who have all been so supportive and helpful both in and out of the lab. I’d especially like to thank Jin Zhang, Stephanie Markovina and Perry Grigsby who have helped me in the development and execution of most of my thesis work, your advice has always been very valuable to me and I thank you all for your time in listening to and discussing these projects. Also, to my collaborator at the University of Alabama Birmingham, Akin Ojesina, you have always been so positive and such a joy to work with and be around, and I greatly value the mentorship you have provided me. Next I would like to thank my thesis committee who always came to our thesis updates ready and willing to advise me and steer these aims into the most productive directions, you have all been such great people to bounce ideas off of and a wealth of information for me to learn from and I truly value the time you graciously put
in to mentoring me through my PhD. I would also like to thank my MCB administrator Stacy Kiel who always made sure that paperwork was turned in on time and that my head was generally above water. Lastly to all my DBBS and MCB peers and the professors who are apart of these programs you have truly made my experience at WashU a fulfilling and enjoyable time filled with riveting science and great non-science memories as well.

To my friends and family, I would not have made it through this rigorous program without your love and support. You have always been such unfailing support systems in my life and whether you were near or far never determined how much that love and support was felt. I thank you all from the bottom of my heart and cannot wait to see what this next chapter of life holds for us.

Finally, to the patients and their families who were apart of the tumor banking study making this work possible, I thank you for your willingness to contribute to this research and to help future patients. Your strength and determination are remarkable, and this work will continue to contribute to additional work to help bring about better and more effective treatment strategies for better patient care.

Fiona Ruiz

Washington University in St. Louis

December 2019
Dedicated to my parents.
ABSTRACT OF THE DISSERTATION

Investigating Biological Mechanisms of Radiation Resistance in Advanced Stage Cervical Cancer

by

Fiona Ruiz

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, 2019

Professor Julie K Schwarz, Chair

The current standard of care treatment for locally advanced cervical cancer is curative intent pelvic radiation with concurrently administered platinum chemotherapy (CRT). This treatment strategy is effective for many patients, but 33-50% of patients treated with CRT develop disease recurrence. Metastatic and recurrent cervical cancer is an incurable condition, and many of the currently available treatments are associated with significant morbidity and mortality. Identifying these patients upfront is a challenge that clinicians face when developing treatment strategies. Previous studies used to catalog the genomic and transcriptomic landscape of cervical cancer lacked high quality corresponding clinical follow up data for patients, and patients were not treated uniformly, making the clinical interpretation of these alterations difficult. The goal of this thesis was to pair next-generation sequencing technology with well annotated prospectively collected clinical follow up data from patients uniformly treated with CRT to identify biomarkers present in pretreatment cervical tumor biopsies that correlate with...
disease recurrence and investigate the mechanism by which these biomarkers result in radiation treatment failure.

In this thesis I identified both human host and Human Papilloma Virus (HPV) related biomarkers that correlate with disease recurrence after standard of care CRT in a cohort of 88 advanced stage cervical cancer patients. Mutations in the human host genes \textit{FBXW7} and \textit{TP53} were associated with metastatic disease recurrence. All patients with \textit{NSD1} mutant tumors recurred exclusively within the pelvis, including two patients with co-occurring mutations of either \textit{FBXW7} or \textit{TP53}; therefore, this gene was selected for further \textit{in vitro} validation studies of radiation resistance. \textit{NSD1} is a histone methyltransferase (HMT) that has been previously identified as a poor prognostic indicator for both neuroblastoma and breast cancer patients. Engineered \textit{NSD1} knockdown cervical cancer cell lines exhibited decreased H3K36 methylation and transcriptional reprogramming of genes involved in cell extrinsic inflammatory pathways and cell intrinsic p53, apoptosis and estrogen response pathways. Loss of \textit{NSD1} was sufficient to induce resistance to single agent carboplatin and combination CRT treatment, validating it as a reliable biomarker for CRT treatment failure. In HPV positive cervical cancer cases, HPV genotype and high viral transcript expression were predictive of treatment failure after CRT. Downstream validation to determine whether high viral transcript expression is sufficient to induce radiation treatment resistance was achieved by overexpressing HPV 16 E6 and E6*I viral transcripts in the Siha cervical cancer cell line. Using this system, I demonstrated that increased E6*I expression led to the stabilization of p53 and p21 inducing cellular senescence, whereas E6 overexpression led to increased p53 degradation. Modulating either transcript resulted in reduced cell sensitivity to radiation treatment providing a biological basis for the observed clinical effect of these alterations.
Lastly, I collaborated with Dr. Akin Ojesina from the University of Alabama Birmingham to genomically characterize cervical cancer cases where HPV was undetectable using currently available technology. HPV negative cervical cancer accounts for 7-11% of all cervical cancer cases and these patients have worse survival outcomes compared to patients with HPV positive cervical cancer after standard of care CRT. We found that HPV negative tumors had an enrichment of mutations in genes that are involved in regulating entry into the cell cycle. One of these significantly mutated genes was \textit{CCND1}, which was also overexpressed in HPV negative tumors. Palbociclib is an FDA approved drug that targets the G1 cell cycle checkpoint through inhibition of CDK 4/6, which are the activating binding partners to cyclin D1. Using a panel of HPV negative and positive head and neck squamous cancer cell lines I confirmed that HPV negative cancer cell lines were uniquely sensitive to single agent Palbociclib treatment. These results suggest that the addition of Palbociclib may be a viable treatment strategy for this poor prognostic group.

Altogether the results of this dissertation demonstrate the power of employing next-generation sequencing to a cohort of patients who were uniformly treated and had well annotated, prospectively collected clinical follow up data. Using this resource, I was able to identify novel biomarkers of disease recurrence after standard of care CRT and determine the proposed biological mechanism of CRT resistance in each case. I identified and validated that mutations in NSD1 and high HPV transcript expression can be used to identify patients prior to treatment who are likely to fail the standard of care. These patients may benefit from the addition of other chemotherapies or targeted agents into their initial treatment plan or alternatively radiation dose escalation could be employed. Additionally, in the case of cervical tumors in which HPV is undetected, these patients would likely benefit from Palbociclib treatment.
Chapter I: Introduction

Cervical cancer is the fourth leading cause of cancer incidence and mortality in women worldwide (1). In 2019 there are expected to be 13,170 new cervical cancer diagnosis and 4,250 cervical cancer deaths in the United States alone (2). Persistent HPV infection is causative for 91% of cervical cancer cases (3). Encoded within the HPV genome are two oncogenes E6 and E7 which target p53 and pRb for degradation respectively. Through E6 and E7, the virus immortalizes the host cell resulting in a hyperproliferative cell that facilitates viral replication. HPV infection itself, however, is not sufficient to induce cervical cancer. This hyperproliferation results in the development of non-cancerous cervical interepithelial neoplasms (CIN) 1 – 3 which can spontaneously regress. Over time, if HPV infection persists, the uncontrolled replication of the infected cell leads to the accumulation of mutations which can further deregulate and reprogram the cell leading to tumorigenesis.

Several efforts have been made to prevent the formation of cervical cancer, namely the HPV vaccine and routine pap smear screenings. Although the HPV vaccine is highly effective against the target HPV genotypes, and has the power to prevent HPV related cancers, it is not mandatory to receive it in the United States and this has resulted in sub-optimal vaccination rates which are inadequate to achieve herd immunity. Establishment of routine pap smears has been widely adopted, which has led to a decline in cervical cancer incidence due to early detection of cellular abnormalities that occur over the course of disease progression.

While cervical cancer screening has led to a decrease in disease incidence, the overall survival outcomes for patients that do develop cervical cancer have remained stagnant. Treatment strategies for cervical cancer rely on clinical tumor staging where stage 0 - stage IIA
tumors are treated primarily by surgical resection and stage IIB - stage IVA are treated with curative intent radiation treatment administered with concurrent platinum-based chemotherapy (4). However, 30-50% of patients will fail their chemoradiation treatment and therefore better diagnostic biomarkers are necessary to identify patients who are likely to fail the current standard of care treatment strategy.

Previous studies have demonstrated that HPV status, HPV genotype and viral DNA state are all prognostic indicators in cervical cancer patients. Cervical cancer patients with undetectable levels of HPV in their tumors have poor progression-free and overall survival outcomes compared to patients with HPV positive tumors. However, this HPV positive survival benefit is not binary and patients with HPV genotypes other than HPV 16 in their tumors have worse progression-free (PFS) and overall survival (OS) after treatment than patients with HPV 16 positive tumors (5,6). The viral DNA state has also been shown to confer differential survival outcomes in cervical cancer patients, where HPV integration into the host genome is associated with worse survival outcomes after treatment compared to patients with episomal viral DNA, although the biological mechanism to explain this clinical observation is poorly understood (7,8). These poor survival outcomes highlight the need for efficient biological biomarkers that can be used to identify patients likely to fail SOC therapy. In the remaining chapters I will detail how host mutations and HPV viral transcript expression affect chemoradiation treatment and what novel treatment strategies should be tested to target the underlying biology of these predictive biomarkers in order to better manage patient disease.
Chapter II: Identification of genomic biomarkers for disease recurrence in chemoradiation treated CESC

2.1 Introduction

In the United States 44% of newly diagnosed cervical cancer cases are localized to the cervix, 36% are regional with disease spread to regional lymph nodes and 15% present with metastatic disease. Cervical cancer survival outcomes are best for early disease stages with localized disease having a 91.8% 5-year survival. These survival outcomes decrease to 56.3% and 16.9% 5-year survival rates among patients with regional and distant disease respectively (2). The establishment of cervical cancer screening through routine pap smears has resulted in a decline in cervical cancer incidence in well developed countries, particularly amongst patients who have access to routine health screenings; however, the overall survival outcomes for patients that do develop cervical cancer have remained stagnant. Treatment planning relies on FIGO tumor staging and advanced stage cervical cancer (FIGO Stages IIB – IVA) is treated with curative intent chemoradiation (CRT) treatment, which consists of definitive pelvic irradiation plus the concurrent administration of cisplatin chemotherapy. For patients who present with advanced stage cervical cancer, 50% will fail CRT and develop disease recurrence, and these patients have dismal survival outcomes, as metastatic and recurrent cervical cancer is in most cases an incurable condition. These poor survival outcomes highlight the need for efficient
biomarkers that can be used to identify patients likely to fail standard of care (SOC) therapy prior to initiating treatment. Understanding the biological mechanism of these biomarkers can be used to develop novel treatment strategies that will better manage a patient’s disease.

Previous studies have aimed to catalog the mutational landscape of cervical cancer and have shown that although transformation by Human Papilloma Virus (HPV) is a driver in most cases (see Chapters 2 and 3), cervical cancer is characterized by a number of recurring mutations (9,10). However, patients in these studies were not uniformly treated, and follow-up data was not reliably collected for all patients. Although these studies provide extensive insight on the genomic alterations in this disease, they lack the ability to reliably correlate alterations with clinical patient outcomes. The objective of this study was to identify frequently altered genes using targeted gene exome sequencing and to assess whether these alterations, identified at the time of diagnosis, increased a patient’s risk of developing recurrent disease after completing standard of care CRT.

2.2 Study design

2.2.1 Patient cohort

The study population included 88 patients prospectively enrolled into a tumor-banking protocol (January 2008 through July 2011). This study was approved by the Institutional Review Board and all patients provided informed consent for sequencing (201105374). Tumor biopsies and blood were obtained prior to the initiation of therapy and stored at the Tissue Procurement Facility. Radiotherapy (RT) consisted of external beam irradiation and intracavitary brachytherapy per institutional guidelines. Tumor specimens from the 79 patients who received
and completed curative intent RT were used for downstream identification of candidate genomic CRT resistance biomarkers. Concurrent cisplatin or carboplatin chemotherapy was administered to 76 of these patients. Patients were followed at the following timepoints after the completion of CRT: 6 weeks, 3 months, 6 months, 12 months, 18 months, 2 years, 3 years and 5 years. Physical examination including a pelvic exam was performed at each visit. Patients received post treatment $^{18}$F-Flouro-deoxy-glucose positron emission tomography (FDG-PET) scans at 3 months post-therapy to assess treatment response; 54 patients had a complete metabolic response, 12 had partial metabolic response, 8 had progressive disease and 5 patients did not have a follow-up FDG-PET scan. Median follow-up time for patients alive at the time of last follow-up was 62 months (range: 25-92 months). At the time of last follow-up, 1 patient was alive with disease, 25 patients had died of cervical cancer, 7 patients died of intercurrent disease and the remaining 46 patients had no evidence of disease.

### 2.2.2 Statistical analysis

Tumor recurrence and overall survival (OS) were the primary endpoints of the study. Survival outcomes were measured from the completion of treatment. Recurrence was defined as new or progressive disease both locally within the pelvis and distantly outside the RT field. The Kaplan-Meier method and log rank test were used to determine differences in progression-free (PFS) and overall survival. R version 3.5.2 and the packages survminer and survival were used for the analysis. $P$ less than 0.05 was set as the threshold for significance for all study outcomes.
2.2.3 Targeted gene panel sequencing and mutation annotation

Tumor biopsies were sectioned and reviewed by a pathologist and only specimens with ≥ 60% neoplastic cellularity and < 20% necrosis were used for further analysis. DNA was extracted from fresh frozen tumor tissue using QIAamp DNA kit (Qiagen, USA) per manufacturer’s protocol. Illumina libraries were constructed with dual-index barcode sequences and enrichment was performed for target sequences (Table 2.1). Probes targeting 5 high-risk and 27 low-risk HPV genotypes were spiked in (Table 2.2). Sequence reads were aligned to GRCh37-lite_WUGSC_variant_2 (http://genome.wustl.edu/pub/reference/GRCh37-lite_WUGSC_variant_2/). Putative somatic point mutations were detected using tumor and normal Samtools r982,(11), Varscan 2.3.6 (12), Strelka 1.0.11 (13) and Somatic Sniper (14). Putative somatic indels were identified using GATK (gatk-somatic-indel 5336) (15), Pindel 0.5 (15) and Varscan 2.3.6. Variants were merged and filtered as previously described. In addition, putative variants were filtered to remove known germline dbSNPs (dbSNP 137) (16). Variants considered for downstream statistical analysis had a log-likelihood ratio ≥ 5, reference-allele-supporting reads ≥ 8 in the normal DNA, and variant-allele-supporting reads ≥ 3 in the tumor. Univariate cox regression was performed using R version 3.5.2 survival package, silent mutations were not considered in the analysis. Functional impact of variants were annotated using SIFT (17), SIFT Indel (18), Predict SNP2 (19), and Provean (20).
### Table 2.1: Targeted Gene Panel

<table>
<thead>
<tr>
<th>Gene 1</th>
<th>Gene 2</th>
<th>Gene 3</th>
<th>Gene 4</th>
<th>Gene 5</th>
<th>Gene 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACVR2A</td>
<td>CDKN2A</td>
<td>FAM194A</td>
<td>HTT</td>
<td>MYO15A</td>
<td>SF3B1</td>
</tr>
<tr>
<td>ADRB1</td>
<td>CEBPA</td>
<td>FBXW7</td>
<td>IDH2</td>
<td>NAV3</td>
<td>SIGIRR</td>
</tr>
<tr>
<td>AJUBA</td>
<td>CHGA</td>
<td>FGFR2</td>
<td>KCNMA1</td>
<td>NCOR1</td>
<td>SIN3A</td>
</tr>
<tr>
<td>AR</td>
<td>COL17A1</td>
<td>FGFR3</td>
<td>KDM5C</td>
<td>NCOR1P1</td>
<td>SMAD2</td>
</tr>
<tr>
<td>ARHGAP35</td>
<td>CTCF</td>
<td>FLT3</td>
<td>KMT2B</td>
<td>NDUFB2</td>
<td>SMAD4</td>
</tr>
<tr>
<td>ARID1A</td>
<td>CTNNB1</td>
<td>FLT4</td>
<td>KMT2C</td>
<td>NF1</td>
<td>SMC1A</td>
</tr>
<tr>
<td>ARID5B</td>
<td>DNMT3A</td>
<td>FOXA2</td>
<td>KMT2D</td>
<td>NFE2L2</td>
<td>SOX17</td>
</tr>
<tr>
<td>ASXL1</td>
<td>EGFR</td>
<td>FOXP2</td>
<td>LIFR</td>
<td>NFE2L3</td>
<td>SOX9</td>
</tr>
<tr>
<td>ATM</td>
<td>EGR3</td>
<td>HGF</td>
<td>LOC101930221</td>
<td>NOTCH1</td>
<td>SPOP</td>
</tr>
<tr>
<td>ATR</td>
<td>ELF3</td>
<td>HIST1H2BD</td>
<td>LRRK2</td>
<td>NSD1</td>
<td>STAG2</td>
</tr>
<tr>
<td>ATRX</td>
<td>ENSG00000200418</td>
<td>HIST1H2BF</td>
<td>MALAT1</td>
<td>PCBP1</td>
<td>STK11</td>
</tr>
<tr>
<td>ATXN1</td>
<td>ENSG00000253125</td>
<td>HIST1H2BN</td>
<td>MAP2K4</td>
<td>PIK3CA</td>
<td>TAF1</td>
</tr>
<tr>
<td>AXIN2</td>
<td>ENSG00000255498</td>
<td>HIST1H4F</td>
<td>MAP3K1</td>
<td>PIK3CG</td>
<td>TBP</td>
</tr>
<tr>
<td>B4GALT3</td>
<td>ENSG00000258430</td>
<td>HIST1H4K</td>
<td>MAP7</td>
<td>PIK3R1</td>
<td>TET2</td>
</tr>
<tr>
<td>BRCA1</td>
<td>ENSG00000267934</td>
<td>HIST4H4</td>
<td>MAPK1</td>
<td>POLQ</td>
<td>TGFB2</td>
</tr>
<tr>
<td>BRCA2</td>
<td>ENSG00000269941</td>
<td>HLA-A</td>
<td>MECOM</td>
<td>PPP2R1A</td>
<td>TLR4</td>
</tr>
<tr>
<td>BTN2A2</td>
<td>ENSG00000273000</td>
<td>HLA-B</td>
<td>MED1</td>
<td>PRX</td>
<td>TNRG6A</td>
</tr>
<tr>
<td>CASP8</td>
<td>EP300</td>
<td>HLA-C</td>
<td>MIR142</td>
<td>PTEN</td>
<td>TP53</td>
</tr>
<tr>
<td>CBFB</td>
<td>EPHA3</td>
<td>HLA-DRB1</td>
<td>MLLT3</td>
<td>RAD21</td>
<td>TSHZ2</td>
</tr>
<tr>
<td>CCDC97</td>
<td>EPPK1</td>
<td>HLA-F</td>
<td>MRGPRE</td>
<td>RB1</td>
<td>TSHZ3</td>
</tr>
<tr>
<td>CCND1</td>
<td>ERBB2</td>
<td>HLA-G</td>
<td>MT-ND6</td>
<td>RUNX1</td>
<td>U2AF1</td>
</tr>
<tr>
<td>CDK12</td>
<td>ERBB3</td>
<td>HLA-J</td>
<td>MTOR</td>
<td>RYR1</td>
<td>USP9X</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>ESPNP</td>
<td>HLA-L</td>
<td>MUC2</td>
<td>SETD2</td>
<td>WT1</td>
</tr>
<tr>
<td>CDKN1C</td>
<td>EZH2</td>
<td>HNRNPUL2</td>
<td>MUC4</td>
<td>SEZ6L2</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.2: HPV Genotype Panel

**HPV Genotype Probes**

#### High Risk HPV

- 16, 18, 31, 33, and 45

#### Low Risk HPV

- 6b, 11, 26, 30, 35, 39, 51, 52, 53, 54, 56, 57, 58, 59, 61, 66, 68a, 70, 72, 73, 82, 98, 99, 100, 104, 105, and 113
2.2.4 Generation of stable shRNA mediated knockdown NSD1 cell lines

NSD1 transcript and protein expression was quantified across a panel of 8 cervix cancer cell lines. Caski, C33A and Siha cells were selected for shRNA knockdown of NSD1. They were transfected with MISSION lentiviral transduction particles at a MOI of 2 (NSD1 TRCN0000238372 2.3x10^7 TU/ml or control SHC202V 4.6x10^7 TU/ml, Sigma-Aldrich, St. Louis, MO, USA) with polybrene per manufacturer’s protocol. Cells were incubated for 72hrs in fresh media before puromycin selection (0.4ug/ml). Establishment of stable NSD1 KD cell lines was confirmed by qRT-PCR and western blot analysis.

2.2.5 H3K36 methylation

NSD1 KD cell lines were evaluated for their target methylation of H3K36 mono-, di-, and tri-methylation. Cells were lysed with Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA), proteinase/phosphatase inhibitors and PMSF on ice for 30min and then sonicated using the Bioruptor UCD-200 (Diagenode) on high 5min. NuPAGE LDS Sample Buffer (4x) (Invitrogen) and SDS buffer (2x) were added, samples were boiled at 95°C for 10min and gel electrophoresed on 4-20% gradient gels (Mini-Protean TGX, Bio-Rad, Hercules, CA, USA), transferred to PVDF blot, and blocked with 5% TBST milk. Primary antibodies incubated at 4°C overnight; 1:1000 anti-NSD1 (N312/10) (UC Davis/NIH NeuroMab Facility, Davis, CA, USA), 1:1000 anti-histone H3 (di methyl K36) ab9049, 1:1000 anti-histone H3 (mono methyl K36) ab9048 (Abcam, Cambridge, UK), 1:1000 Tri-methyl-histone H3 (Lys36) (D5A7), 1:1000 Histone H3 (D1H2). Secondary and conjugated antibody incubated 1h at room temperature; 1:5000 Actin-HRP (sc-47778), 1:4000 anti-rabbit (sc-2357) and 1:3000 anti-mouse (sc-516102) (Santa Cruz Biotechnology, Dallas, TX, USA), were used with ECL chemiluminescent reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Blots were visualized and quantified using
the Bio-Rad ChemiDoc MP imaging system and Image Lab software (Bio-Rad, Hercules, CA, USA).

### 2.2.6 Clonogenic survival

Cells were plated at a density of $1 \times 10^6$ cells per well in six-well plates. The next day cells were treated with either vehicle (0.01% DMSO), 0.5uM or 5uM carboplatin alone; or vehicle and 0.5uM carboplatin 1h prior to single-fraction radiation treatment (2 and 4Gy). Cells were incubated for 48h then seeded at a density of 500 cells per well and incubated for an additional 10-12 days, until control wells formed visible colonies (> 50cells). Plates were fixed and stained with 2% crystal violet, 70% ethanol for 20min, rinsed in PBS and Milli-Q water and air-dried overnight at room temperature. Plating efficiency was calculated as the (number of colonies formed in control wells)/(500 cells seeded); and the surviving fraction was calculated as the (number of colonies formed after treatment)/(500 cells seeded x plating efficiency). All clonogenic assays were independently replicated three times.

### 2.2.7 NSD1 knockdown cell line treatment response

Cellular response to 5uM carboplatin and 0.5uM carboplatin plus radiation treatment was evaluated by cell cycle analysis and gamma H2AX foci staining. For cell cycle analysis, cells were treated, harvested 24 and 48h after treatment, and stained with propidium iodide. Flow cytometry analysis was performed on MacsQuant Analyzer and quantified using FlowJo. For each sample 5,000 events were collected. For γH2AX foci staining, cells were treated, harvested and fixed with 4% paraformaldehyde at 30m, 6h and 24h post-carboplatin treatment or 10m and 24h post-2Gy RT. Primary antibody 1:300 anti-phospho-Histone H2A.X (Ser139)(20E3)
incubated at 4C overnight. Secondary antibody 1:500 anti-rabbit Alexa Fluor 488 incubated 2h at room temperature. Slides were mounted using ProLong Gold Antifade Reagent with DAPI.

2.2.8 RNAseq analysis

RNA was extracted from Caski shControl and shNSD1 cell lines using the RNeasy Mini Kit (74104) (Qiagen, USA) per manufactures instructions. Samples with 0.5 – 1µg of RNA and RIN > 7 were used for library preparation and run using the NovaSeq S1 flow cell. Differential expression analysis was performed with raw gene read counts using the R package DESeq2 (21). The raw counts were log2 scaled using DESeq2 rlog function and principal component analysis was run using the DESeq2 plotPCA function. Differentially expressed genes with log2 fold changes of ≥ 1 or ≤ -1 and p-value < 0.01 were used for downstream GSEA pathway analysis and were visualized using the R package pheatmap.

2.3 Disease recurrence in WUSM patient cohort

Patient characteristics at the time of diagnosis for the 79 patients who completed their fully prescribed CRT treatment are shown in (Table 2.3). There were 48 patients who did not develop any disease recurrence within the study time and 31 who did develop disease recurrence; with 12 pelvic failures, 14 distant failures and 4 combination pelvic and distant failures (Figure 2.1A). Patients that experienced disease recurrence of any kind had significantly worse survival outcomes compared to patients with no evidence of disease recurrence (p< 0.0001) (Figure 2.1B). At the time of diagnosis there was no significant differences in age, race, stage, metastasis or histology between patients that eventually recurred versus those who did not. Patients that
developed disease recurrence did have higher incidence of lymph node involvement at the time of diagnosis (p = 0.009).

Table 2.3: Patient baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Curative Intent Treatment completed (n = 79)</th>
<th>No Recurrence (n = 48)</th>
<th>Recurrence (n = 31)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>54</td>
<td>54</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>25-85</td>
<td>25-81</td>
<td>39-85</td>
<td>ns</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>ns</td>
</tr>
<tr>
<td>Caucasian</td>
<td>58</td>
<td>36</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ib1</td>
<td>13</td>
<td>11</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ib2</td>
<td>11</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Iia</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Iib</td>
<td>28</td>
<td>15</td>
<td>13</td>
<td>ns</td>
</tr>
<tr>
<td>IIIa</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>IIIb</td>
<td>22</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>IVa</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>IVb</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PET lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>42</td>
<td>28</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Aortic</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Pelvic</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.009389</td>
</tr>
<tr>
<td>SCV</td>
<td>28</td>
<td>19</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>75</td>
<td>47</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Groin</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>Liver</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>Small cell</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td>67</td>
<td>41</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1: Progression-free and Overall Survival Curves
A) Kaplan-Meier curve for progression-free survival for all comers with completed curative intent RT (n = 79). B) Kaplan-Meier curves for cause-specific survival; 31 patients developed disease recurrence of any kind and 48 patients had no disease recurrence during the follow-up period. Patients that developed disease recurrence of any kind had significantly worse overall survival outcomes (p < 0.0001).
2.4 Using NGS to identify candidate gene biomarkers of disease recurrence after chemoradiation treatment

2.4.1 Gene prioritization

DNA extracted from the 79 tumor-blood paired samples were sequenced using a targeted gene panel of 211 known pan cancer associated genes (Table 2.1). Somatic variants were filtered for non-silent mutations with at least 8 reference-allele-supporting reads in the normal DNA, and at least 3 variant-allele-supporting reads in the tumor. Genes with these high confidence somatic mutations and a mutational frequency of at least 5% in the cohort were further analyzed for enrichment in patients who developed disease recurrence using fisher’s exact test, and their impact on patient risk of developing recurrence assessed by univariate cox regression analysis.

Sixty-eight patient tumors had at least one high confidence mutation called, and genes with a mutational frequency > 5% are shown in (Figure 2.2). As expected we identified recurrent alterations in PIK3CA (19.1%), PTEN (13.2%), HLA-B (13.2%), HLA-A (10.3%), FBXW7 (8.8%), MAPK1 (8.8%), ARID1A (7.4%), EP300 (7.4%), TP53 (5.9%), ERBB2 (5.9%), and CASP8 (5.9%) which have all been previously implicated in cervical cancer (9,10). Additionally, we found frequent mutations in the histone methyltransferases KMT2D (19.1%), KMT2C (16.2%), NSD1 (5.9%), and KMT2B (5.9%).

Mutations in FBXW7 (p = 0.018), and NSD1 (p = 0.013) were strongly associated with patients that developed disease recurrence of any kind (Figure 2.2, side panel). Univariate COX regression was used to identify mutated genes that impacted patient risk of CRT treatment failure and disease recurrence was the primary endpoint. The risk of disease recurrence was 9.75, 5.87
and 3.5 times higher in patients with *NSD1*, *TP53* or *FBXW7* mutant tumors respectively, compared to patients with wild type (WT) sequences (Figure 2.3A). There were 6 patients with *FBXW7* mutant tumors, 4 with *TP53* mutant tumors and 4 with *NSD1* mutant tumors. All patients with coding mutations in *NSD1* experienced in-RT-field failure and developed locally recurrent disease. Distant disease recurrence developed in 83.3% and 75% of patients with *FBXW7* and *TP53* mutant tumors respectively. Two patients had co-occurring mutations; *FBXW7/NSD1* and *TP53/NSD1*, and both experienced local disease recurrence (Figure 2.3B).

**Figure 2.2: Frequently mutated genes and their enrichment in recurrent patient cohort**

All panels are aligned with vertical tracks representing the 68 patients with high confidence somatic mutations. The data is sorted in order by disease recurrence (top panel) and mutational frequency cut off at 5%, silent mutations have been excluded (bottom panel). Top panel:
Clinical tracks for disease recurrence (no recurrence = blue, recurrence = grey), tumor HPV status (HPV positive = black, HPV negative = white), and Histology (Adenocarcinoma = light brown, Adenosquamous = yellow, Small Cell = pink, Squamous = dark brown); Side panel:
Frequency of alteration per gene (altered in no recurrence group = blue, altered in recurrence group = grey), fisher exact test was used to test association of mutation with disease recurrence (* p < 0.05). Plot made using the R package GenVisR waterfall function.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sample</th>
<th>Mutation</th>
<th>Predicted Effect</th>
<th>Tumor VAF</th>
<th>Recurrence Site</th>
<th>Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSD1</td>
<td>700514_754077</td>
<td>p.R2297T</td>
<td>Deleterious</td>
<td>15.28</td>
<td>Cervix/PALN</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>700514_712739</td>
<td>p.M1531fs</td>
<td>Deleterious</td>
<td>22.97</td>
<td>Cervix/PLN</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>700514_704012</td>
<td>p.S1008Y</td>
<td>Neutral</td>
<td>76.6</td>
<td>Cervix</td>
<td>NED</td>
</tr>
<tr>
<td></td>
<td>700514_736378</td>
<td>p.Q1058*</td>
<td>Deleterious</td>
<td>17.24</td>
<td>Cervix</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>700514_754077</td>
<td>p.S601F</td>
<td>Deleterious</td>
<td>24.32</td>
<td>Cervix/PALN</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>700514_728836</td>
<td>p.Q624*</td>
<td>Deleterious</td>
<td>16.95</td>
<td>Brain</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>700514_731985</td>
<td>p.R479*</td>
<td>Deleterious</td>
<td>26.67</td>
<td>Lung/MSLN</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>700514_707630</td>
<td>p.R479G</td>
<td>Deleterious</td>
<td>23.33</td>
<td>PALN/SCV</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>700514_707913</td>
<td>p.R512W</td>
<td>Deleterious</td>
<td>49.23</td>
<td>Bone/Liver</td>
<td>AWD</td>
</tr>
<tr>
<td></td>
<td>700514_755438</td>
<td>p.R543G</td>
<td>Deleterious</td>
<td>30.56</td>
<td>None</td>
<td>NED</td>
</tr>
<tr>
<td></td>
<td>700514_704806</td>
<td>p.C176F</td>
<td>Deleterious</td>
<td>61.54</td>
<td>MSLN/Liver</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>700514_711380</td>
<td>p.L194R</td>
<td>Deleterious</td>
<td>27.54</td>
<td>None</td>
<td>DICD</td>
</tr>
<tr>
<td></td>
<td>700514_740838</td>
<td>p.S6*</td>
<td>Neutral</td>
<td>78.05</td>
<td>Lung</td>
<td>NED</td>
</tr>
<tr>
<td></td>
<td>700514_736378</td>
<td>p.Q331*</td>
<td>Deleterious</td>
<td>19.44</td>
<td>Cervix</td>
<td>DOD</td>
</tr>
</tbody>
</table>

**Figure 2.3: Significantly mutated genes**

A) Univariate cox regression performed using R package survival, shown are all significant genes with a mutation frequency of 5% or higher. B) Table shows list of patients with mutations in one of the SMG’s. Superscript denotes patients with a mutation in more than one SMG. (DOD = dead of disease, DICD = died of intercurrent disease, AWD = alive with disease, NED = no evidence of disease).
2.4.2 Candidate Biomarker Selection

NSD1 and FBXW7 both passed the selection criteria for candidate biomarkers, namely they each had a mutational frequency > 5% in the cohort, were enriched in patients who developed disease recurrence after CRT treatment, and significantly increased patient risk of developing disease recurrence after CRT treatment. Patient’s with FBXW7 mutant tumors primarily developed metastatic disease recurrence after CRT treatment, indicating that alteration of FBXW7 may be a biomarker of disease metastasis in cervical cancer. Conversely, all patients with NSD1 mutant tumors recurred within the field of radiation, indicating that NSD1 alterations are a potential biomarker of CRT treatment resistance. Therefore, NSD1 was selected for in vitro biomarker validation to test whether its loss is sufficient to induce tumor cell resistance to CRT.

2.5 Role of NSD1 in CRT response

2.5.1 NSD1 biological functions

Nuclear receptor binding SET domain containing protein (NSD1), is a member of the NSD histone methyltransferase family and has functional roles as both a regulator of nuclear receptors and as a methyltransferase. NSD1 has two nuclear receptor interacting domains (NIDs) that allow it to have a bifunctional role as either an activator or repressor of its target nuclear receptors (22,23). This interaction can either be ligand independent for retinoic acid receptor (RAR) and thyroid hormone receptor (TR) or ligand dependent for RAR, TR, retinoid X receptor (RXR) and estrogen receptor (ER). As a methyltransferase NSD1 mono- and di-methylate’s histone targets H3K36 and H4K20 and non-histone targets NFkB p65 at K218 and K221 (24). H3K36 state is an important determinate in coordinating which DNA damage response will be
used to repair. H3K36me2 enhances double stranded break repair by non-homologous end joining (NHEJ) (25). Additionally, NSD1 dependent methylation of H3K36 coordinates the recruitment and elongation of RNAPol II to target genes (26). Several studies have identified regulation between NSD1 and NFkB p65. One study demonstrated that NSD1 dependent methylation of the NFkB p65 subunit enhanced NFkB activity, and NSD1 knockdown (KD) dramatically reduced NFkB activity (24). Another group found that activation of p65 corresponded with decreased H3K36me3 and inhibition of p65 increased NSD1 expression and restored H3K36me3 (27).

2.5.2 NSD1 role in disease

Germline mutations in NSD1 is causative for Soto’s overgrowth syndrome (28). NSD1 alterations have recently been implicated in head and neck squamous cell cancer (HNSCC). Within the HNSCC TCGA cohort a subset of patients was characterized by their tumor’s hypomethylation of H3K36, where there was an enrichment of H3K36M and NSD1 mutations (29). Hypomethylation caused by NSD1 mutations was found to downregulate transcript expression of inflammatory cytokines resulting in less immune infiltration and described as immune cold tumors (30). In HPV negative HNSCC NSD1 alterations have been associated with favorable progression-free (PFS) and overall survival (OS) outcomes (31–33). Conversely, HNSCC patients with HPV positive and NSD1 mutant tumors had worse survival outcomes compared with HPV positive NSD1 WT sequence tumors (33). The difference between HPV negative and HPV positive NSD1 mutant tumors was found to involve regulation of ERCC5, which functions as a nuclease in nucleotide excision repair (NER). In HPV negative NSD1 mutant HNSCC tumors ERCC5 was downregulated and NSD1 KD in HPV negative HNSCC cell lines resulted in ERCC5 downregulation and sensitization to cisplatin (33). The HPV positive
NSD1 mutant HNSCC tumors did not have any significant difference in ERCC5 transcript expression. In our WUSM cohort all the NSD1 mutations were in HPV positive tumors and they corresponded with poor PFS and OS. Results from our cohort and the HNSCC study identifies a difference in NSD1 biology between HPV positive and HPV negative tumors. In HPV negative tumors functional NSD1 corresponds with worse survival outcomes and mutations confer better survival, whereas in HPV positive tumors WT NSD1 is favorable and mutations result in worse survival outcomes. The reasons behind these differences remains unclear. Hypermethylation and transcriptional repression of NSD1 is also a poor prognostic indicator in neuroblastoma, glioma and breast cancer patient cohorts (27,34). We therefore wanted to test the functional consequences of NSD1 knockdown in a panel of human cervical cancer cell lines which included both HPV positive and HPV negative cell lines.

2.5.3 Generation of NSD1 KD cell lines

NSD1 protein and transcript expression was assessed in a panel of 8 cervical cancer cell lines (Figure 2.4). The cervical cancer cell lines evaluated all have WT NSD1 sequences (35).

The HPV positive Caski and Siha cell lines, and HPV negative C33A cell line were stably transfected with non-mammalian targeting control and NSD1 targeting shRNA’s and KD was confirmed by western blot (Figure 2.5A). Caski and C33A both had high endogenous NSD1 expression and shNSD1 KD achieved an average fold decrease of 4.97 ± 2.44 and 3.87 ± 0.428 respectively. Siha had low NSD1 expression and Siha shNSD1 had an average fold decrease of 1.77 ± 0.428 (Figure 2.5B). shNSD1 cell lines had lower H3K36 mono-, di- and trimethylation compared to shControl cell lines (Figure 2.5C-D).
Figure 2.4: NSD1 expression in ATCC cervical cancer cell lines
NSD1 expression A) qRT-PCR normalized transcript expression and B) western blot protein expression
Figure 2.5: Generation of stable NSD1 knockdown cell line

A-B) Confirmation of NSD1 KD in Caski, C33A and Siha cell lines by western blot and band quantification. C-D) Western blot of H3K36 histone targets and band quantification (normalized to H3).
2.5.4 Effect of NSD1 KD on cell sensitivity to single agent carboplatin and CRT treatment

Control and shNSD1 cell lines were treated with increasing doses of carboplatin alone or with 0.5μM carboplatin 1 hour prior to single-fraction radiation and clonogenic potential was assessed by the gold standard colony formation assay. NSD1 KD cell lines had higher surviving fraction with increasing dose of carboplatin compared to shControl cells (Figure 2.6). The shNSD1 cell lines also had higher surviving fraction with increasing RT dose compared to shControl cell lines (Figure 2.7).
Figure 2.6: Effect of NSD1 knockdown on single agent carboplatin treatment sensitivity

A) Representative wells from colony formation assay of cells treated with increasing dose of carboplatin. Surviving fractions for B-D) Caski, C33A and Siha shControl and shNSD1 cell lines (solid line = shControl, dashed line = shNSD1).
Figure 2.7: Effect of NSD1 knockdown effect on CRT treatment sensitivity
A) Representative wells from colony formation assay of cells treated with 0.5μM carboplatin and increasing dose of radiation treatment. Surviving fractions for B-D) Caski, C33A and Siha shControl and shNSD1 cell lines (solid line = shControl, dashed line = shNSD1).
2.5.5 Differential transcriptional regulation induced in NSD1 KD cells

NSD1 has functional domains characteristic of both transcriptional activators and repressors and has been shown to coordinate RNApol II transcription (26). For this reason, we wanted to test the impact of NSD1 knockdown on gene transcription. RNAseq analysis was performed in Caski shControl and shNSD1 cell lines to ascertain genes under NSD1 transcriptional regulation. Differential expression analysis was performed using DESeq2 and genes with a log2 fold change > 1 or < -1 and an adjusted p-value < 0.05 were analyzed for pathway enrichment using GSEA. Caski shNSD1 cells had 3551 genes upregulated and 2717 downregulated compared to Caski shControl cells, suggesting that NSD1 preferentially acts as a transcriptional repressor of its target genes (Figure 2.8).

GSEA pathway analysis was performed to identify enrichment of DE genes in hallmark gene pathways, significant pathways were filtered for FWER p-value < 0.05 and at least 35% of the pathway enriched with DE genes. NSD1 KD resulted in the upregulation of several cell extrinsic pathways; Inflammatory response, TNFA signaling via NFkB, IFN alpha and IFN gamma response and cell intrinsic pathways; Estrogen response late, P53 pathway and Apoptosis (Figure 2.9).
Figure 2.8: Differential expression analysis of Caski shNSD1 vs shControl cells
A) Principle component analysis showing separate clustering of control and NSD1 KD Caski samples. B) Volcano plot of log2 fold change vs -log10 p-value for differentially expressed genes in RNAseq library (yellow = DE > 1, purple = DE < -1). C) Heatmap of 6,268 differentially expressed genes with log2 fold change > 1 or < -1 and p-value < 0.05 generated using pheatmap in R.
**Figure 2.9: GSEA pathway analysis of DE genes**
A-B) Cell extrinsic and intrinsic hallmark pathways upregulated in Caski shNSD1 Caski cells. C) Table for all significant hallmark pathways differentially regulated in Caski shNSD1 cells with at least 35% DE genes contributing to pathway enrichment.
2.5.6 NSD1 KD effect on cellular response to single agent carboplatin and CRT treatment

Following 5uM carboplatin treatment γH2AX foci continued to accumulate up to 24h after treatment and the amount of γH2AX positive nuclei did not differ between shControl and shNSD1 cell line (Figure 2.10). Despite acquiring a similar amount of DNA damage shNSD1 cells were less sensitive to high carboplatin treatment. Cell cycle analysis showed that both treated shControl and shNSD1 cells accumulated in early S-phase 24hrs after high carboplatin treatment. In Caski and Siha shControl cells this S-phase accumulation persisted at 48hrs post-carboplatin treatment, whereas the shNSD1 cells had progressed into G2/M. C33A cells, which are HPV negative and p53 mutant, also had transient S-phase accumulation 24h after treatment which had progressed into G2/M at 48h (Figure 2.11).

After radiation treatment (RT) initial γH2AX accumulation did not differ between shControl and shNSD1 cell lines. At 24h post-RT treatment C33A and Siha cells had significant clearance of γH2AX foci irrespective of NSD1 status. Caski cells retained high amounts of γH2AX foci 24h after CRT treatment and NSD1 loss did not affect foci clearance (Figure 2.12). Cell cycle analysis showed that all cells treated with CRT accumulated in G2/M phase 24h post treatment. At 48h post treatment cell accumulation shifted from G2/M to Sub-G1 in both shControl and shNSD1 cells (Figure 2.13). This increase in Sub-G1 cells may be indicative of apoptotic cells, however subsequent validation is necessary to discern whether these cells died by apoptotic or necrotic mechanisms.
**Figure 2.10: γH2AX p-S139 foci formation and clearance after 5µM carboplatin**

A) Representative images of γH2AX foci (green) and nuclei stained by DAPI (blue) immunofluorescence of shControl and shNSD1 cell lines. B-D) Quantification of H2AX foci positive cells (>3 foci/nuclei)
**Figure 2.11: Cell cycle analysis after 5μM carboplatin treatment**

A,C,E) Representative histograms for cell cycle distribution 24h and 48h after 5μM carboplatin treatment. B,D,F) Quantification of total cells in each cell cycle phase.
**Figure 2.12: γH2AX p-S139 foci formation and clearance after 2Gy radiation treatment**

A) Representative images of γH2AX foci (green) and nuclei stained by DAPI (blue) immunofluorescence of shControl and shNSD1 cell lines. B-D) Quantification of H2AX foci positive cells (>3 foci/nuclei)
Figure 2.13: Cell cycle analysis after 0.5µM carboplatin and 4Gy radiation treatment
A,C,E) Representative histograms for cell cycle distribution 24h and 48h after 5µM carboplatin treatment. B,D,F) Quantification of total cells in each cell cycle phase.
2.5.7 Loss of NSD1 is sufficient to induce resistance to CRT treatment

Loss of NSD1 through shRNA mediated knockdown was sufficient to induce cellular resistance to carboplatin treatment. RNAseq analysis identified both cell intrinsic and extrinsic pathways that were upregulated with loss of NSD1, indicating that NSD1 is a negative transcriptional regulator of these pathways. NSD1 has already been previously described to inhibit ligand bound estrogen receptor (22). Our results further demonstrate that NSD1 is a negative regulator of ER as NSD1 KD leads to upregulation of estrogen response pathway. ER has been previously reported to regulate p53 via NFkB (36). Both p53 and NFkB signaling pathways were upregulated in NSD1 KD cells, most likely due to restored ER signaling. The upregulation of inflammatory pathways and p53 mediated apoptosis pathways we see in vitro may contribute to CRT resistance seen in NSD1 mutant patients. The upregulation of p53 and apoptotic pathways in the Caski NSD1 cell KD line may contribute to the resistance to carboplatin and CRT treatment. Enhanced inflammation in the tumor microenvironment has also been well established to induce resistance to RT, and inflammatory pathways were also upregulated in NSD1 KD cells. However, the effect of this upregulations remains to be validated in these cervix cancer NSD1 KD models.

Following carboplatin treatment both NSD1 expressing and KD cells accumulated DNA damage quantified by γH2AX foci formation. In response to carboplatin treatment NSD1 proficient cells engaged a sustained S phase cell cycle arrest, whereas NSD1 KD cells had an initial transient S phase accumulation 24h after carboplatin which transitioned into G2/M cell cycle accumulation by 48h post treatment. Altogether these results exhibit a model whereby NSD1 is required for sustained S phase arrest following carboplatin and loss of this arrest may induce the shNSD1 cells resistance to carboplatin treatment.
NSD1 KD decreased sensitivity to combination carboplatin radiation treatment. Both NSD1 expressing and KD cells had comparable induction of DNA damage following CRT treatment and NSD1 status did not have an effect on γH2AX clearance. Following CRT treatment both NSD1 proficient and KD cell lines had an initial G2/M cell cycle arrest that was maximal at 24h post treatment and decreased at 48h post treatment, leading to accumulation in Sub-G1 phase.

**2.5.8 Overall summary and future directions**

NSD1 and FBXW7 mutations were enriched in patients that developed disease recurrence and significantly increased the patient’s risk of disease recurrence. Patients with FBXW7 mutations recurred primarily outside of the cervix and this alteration may be a biomarker for disease metastasis. In the context of this dissertation I did not validate FBXW7 as a biomarker, however future studies should focus on characterizing FBXW7 loss in cervical cancer cell lines. To test whether FBXW7 mutations induce tumor metastasis generation of FBXW7 KD cell lines could be used to test their ability to migrate by *in vitro* scratch assays.

Patients with NSD1 mutations recurred exclusively within the pelvis indicating that alteration of NSD1 may be a biomarker for resistance to CRT treatment in cervical cancer patients. NSD1 KD was evaluated *in vitro* and loss of NSD1 was sufficient to induce cell resistance to carboplatin treatment and decreased cell sensitivity to CRT treatment. NSD1 status did not impact cell DNA damage accumulation or clearance, as quantified by γH2AX foci. Subsequent characterization of cell response to carbo and CRT demonstrated that NSD1 was necessary for persistent S phase cell cycle arrest following high carboplatin treatment. When NSD1 is lost cells are able to escape S phase accumulation and move into G2/M, where they can
use homologous recombination to correct DNA damage, which may be why shNSD1 cells resist carboplatin and CRT treatment. Although shControl and shNSD1 cell lines acquired similar levels of DNA damage, the NSD1 deficient cell lines had better survival after treatment suggesting that they are able to overcome the damage and continue to proliferate.

RNAseq analysis identified upregulation of inflammatory response and estrogen receptor pathways in NSD1 deficient Caski cells. To confirm whether the differential expression of these genes is directly caused by NSD1 mediated H3K36 methylation we are collaborating with Dr. Jackie Payton to perform Chip-seq on our Caski shControl and shNSD1 cells. Using probes for H3K36me2 we will combine the Chip-seq identified genes with our RNAseq transcriptome data to identify the genes that are transcriptionally regulated by NSD1 dependent H3K36 methylation. With this combined dataset we will be able to perform pathway analysis to confirm which pathways NSD1 activates or inhibits. Some of the pathways of interest that we would like to confirm are the hallmark inflammatory pathways and estrogen receptor response that was identified in our differential expression analysis. NSD1 prognosis has proven to be tissue of origin dependent and one such cause may be the estrogen sensitivity of the cell. Cervix is an estrogen sensitive tissue, like breast and brain, which both have poor prognosis when NSD1 is lost, however NSD1 mutations in the larynx, an estrogen insensitive tissue, leads to favorable patient outcomes. Identifying the relationship between NSD1 status and estrogen receptor signaling could be essential to understanding the tissue specific differences between NSD1 mutations effect on the cell. The altered estrogen signaling may also be causative for the upregulation in inflammatory response seen in Caski shNSD1 cells. HPV negative NSD1 mutant HNSCC patient tumors have been analyzed and shown to have an immune cold signature, this is opposite to our *in vitro* data which identified upregulation of inflammatory signals. Again, this
difference in response could be due to differences in estrogen sensitivity of the cell. Additionally, in HNSCC patients’ differences in prognosis were found between HPV negative and HPV positive NSD1 mutant tumors, where HPV positive NSD1 mutant tumors conferred poor outcomes for patients similar to our cervix cohort. Estrogen signaling has been shown to regulate HPV gene transcriptions and therefore these HPV status differences in NSD1 biological effect could be due to differences in regulation of the estrogen response.
Chapter III: Role of HPV transcript expression in CESC treatment response

3.1 Introduction:

Current clinical guidelines for newly diagnosed cervical cancer patients do not require testing for HPV status. Treatment strategies for cervical cancer rely on clinical tumor staging where stage 0 - stage IIA tumors are treated primarily by surgical resection and stage IIB - stage IVA are treated with curative intent radiation treatment, which is administered with concurrent platinum-based chemotherapy (4). However, as mentioned in Chapter II 50% of patients fail their chemoradiation treatment. HPV status has been shown to correlate with patient outcomes and therefore it could be used as a diagnostic biomarker to identify patients who are likely to fail the current standard of care treatment strategy. These patients could benefit from alternative chemotherapeutic or immunotherapy treatment strategies that may be more effective at treating their cervical cancer.

Persistent HPV infection is causative for 90% of cervical cancer cases (3). HPV is a DNA virus that infects cells of the basal epithelium. Viral replication is dependent on host cell mechanisms of replication and splicing in order to produce progeny viruses. Host cells will only replicate the viral genome during S phase of the cell cycle when DNA synthesis of the host genome is underway. Therefore, the virus must induce a state of constitutive entry into the cell cycle so it can continue to reproduce. Encoded within the HPV genome are two oncogenes E6 and E7 which target p53 and pRb for degradation respectively. Rb regulates entry into the cell cycle and its inhibition is necessary to progress from G1 into S phase. HPV achieves this cell cycle entry through E7 mediated degradation of Rb, inducing a state of unregulated cell cycle.
entry and replicating both host and viral DNA. Host cells tightly regulate and monitor the DNA replication process to ensure that deleterious mutations do not arise during the process. When HPV DNA is present it forms complex viral and human DNA structures that the host cell recognizes as foreign and induces an antiviral cell response. To overcome this detection strategy HPV E6 targets p53 for protein degradation, leading to the cells inability to integrate DNA damage signals with cell fate determinants resulting in cell survival. Altogether, through E6 and E7, the virus immortalizes the host cell resulting in a hyperproliferative cell that facilitates viral replication. HPV infection itself, however, is not sufficient to induce cervical cancer. Instead over time, if HPV infection persists, the uncontrolled replication of the infected cell leads to the accumulation of mutations which can further deregulate and reprogram the cell leading to tumorigenesis.

Only 10% of HPV viral infections result in persistent HPV gene expression, as the majority of HPV infections are cleared by the host immune system. Host related factors that influence the ability to clear HPV infection are poorly understood, although it is known that HPV related proteins promote tumor immune evasion. Persistent expression of HPV related genes is associated with integration of HPV viral DNA into the host genome, although in some cases viral DNA is maintained in tumor cells as an episome. Factors regulating HPV DNA integration into the host genome are poorly understood. Host genome sites where HPV integration frequently occurs have been reported throughout the genome and are found within fragile sequence sites and areas of open chromatin (37). HPV integration into the host genome can result in transcription of viral-host fusion proteins and influence transcriptional activity of neighboring host genes via a pseudo-promoter effect (10). Studies are ongoing to determine whether
consistent patterns of expression of viral-host fusion proteins or viral pseudo-promoter effects are associated with tumor progression and response to treatment.

It is well established that HPV drives tumorigenesis in cervical cancer; however, much less is known about how various states of persistent viral infection and expression of viral genes affect the tumor response to cancer therapy, including radiation and chemotherapy. Previous studies have demonstrated that HPV status, HPV genotype and viral DNA state are all prognostic indicators in cervical cancer patients. Cervical cancer patients whose tumors have detectable HPV DNA have better survival outcomes compared to patients where HPV is not detected using currently available technologies (38). Additional studies have found that the HPV positive survival benefit is variable with non-HPV 16 genotypes conferring worse progression-free (PFS) and overall survival (OS) after treatment than patients with HPV 16 positive tumors (6). The viral DNA state has also been shown to confer differential survival outcomes in cervical cancer patients, where HPV integration into the host genome is associated with worse survival outcomes after treatment compared to patients with episomal viral DNA, although the biological mechanism to explain this clinical observation is poorly understood (39,7,8).

These HPV survival differences are not exclusive to cervical cancer. Approximately 20% of oropharyngeal squamous cell cancer (OPSCCs) are HPV related, and patients with HPV related OPSCC have favorable outcomes after chemoradiation (40). P16 protein expression, determined by immunohistochemistry (IHC), is used clinically as a surrogate biomarker of HPV positivity, although it should be noted that not all p16 IHC positive head and neck cancers have detectable HPV DNA and/or RNA (41). p16 is a negative regulator of the cell cycle and inhibits the inhibitory phosphorylation of Rb. Previous studies have shown that p16 expression is influenced by Rb expression. In HPV infected cells E7 degrades Rb and this has been correlated
with an overexpression of p16. However, p16 overexpression can also occur in non-HPV infected cells making this surrogate biomarker imperfect in determining between HPV infected lesions and those with p16 de novo mutations. Despite this, studies using p16 IHC as a surrogate for HPV status, have demonstrated that patients with p16 IHC positive tumors had better survival compared to p16 IHC negative head and neck cancers (41). Due to this, p16 IHC status is tested in all newly diagnosed OPSCCs, has been included in the new staging system for OPSCC and patients with p16 positive OPSCCs are considered for treatment de-escalation in the context of clinical trials. More recently, HPV genotype differences are being appreciated in HPV positive HNSCC where HPV 18 genotype has been identified as an independent poor prognostic factor of overall survival (42). In addition, expression of alternative transcripts such as HPV E1-E4 has been identified as a candidate biomarker for treatment resistance in HPV positive head and neck cancer.

Altogether HPV is an important candidate prognostic biomarker for both CESC and oropharyngeal HNSCC, specifically the HPV 16 genotype is a good prognostic marker. However, the understanding of why other HPV genotypes confer worse prognosis is not well understood. One potential reason for these genotype differences may be that some HPV genotypes are more transcriptionally active compared to the HPV 16 genotype. It is also currently unknown to what extent and by what mechanism expression of HPV alternative transcripts influence response to chemoradiation. The purpose of this study was to investigate transcriptomic variations between HPV 16 and other HPV genotypes in cervical cancer patients and elucidate how the relative HPV transcript expression, including the expression of alternatively spliced forms of HPV E6, affects cellular response to chemotherapy and radiation treatment.
3.2 Study design:

3.2.1 HPV genotyping

DNA isolated from 88 pre-treatment tumors were sequenced as described in section 2.2.3. Probes for 32 unique HPV genotypes were used and HPV positivity was defined as any sample with HPV reads detected for one of the 32 genotypes. The primers for HPV 16 and 18 genotypes completely tiled the HPV genome, whereas all other HPV genotypes had partial gene tiling.

3.2.2 Viral transcriptome and integration state

RNA was extracted and 68 patients had at least 0.5ug of RNA isolated with RIN > 7, which was sufficient for library preparation and RNAseq was performed using the Illumina HiSeq 3000. Raw sequencing reads were aligned to reference human and HPV viral genomes in GRCh38.d1.vd1 using STAR 2.7.0f with chimSegmentMin set to 18. STAR generated chimeric junction files were parsed for reads that were chimeric between human and HPV references and the corresponding alignment information and nucleotide sequences were extracted. The human-HPV chimeric reads were verified using BLAT and BLAST to confirm presence of HPV integration at one or more locations. Additionally, viral read counts for sequences aligned to HPV E6, E6*I and E7 were normalized by reads per kilobase of transcript per million mapped reads (RPKM).

3.2.3 Engineering E6 and E6* overexpressing cell lines

ATCC cell lines Caski, Siha and SW756 were used to evaluate endogenous expression of HPV E6, E6*I and E7 transcripts and determine cell sensitivity to radiation treatment by alamar blue.
Siha had the lowest transcript expression for all HPV transcripts evaluated and was selected to engineer cell lines for the overexpression of HPV 16 E6*I and E6. The mammalian expression vector pCMV-6 was used as the expression vector for exogenous HPV viral transcript expression. IDT mini genes for E6 and E6* orfs were restriction enzyme digested with 10U/ul sgfI and 10U/ul mluI, ligated into pCMV-6 vectors using T4 DNA ligase, and transformed into 5-alpha competent E.coli (New England Biosciences, (NEB)) overnight and then selected using 25ug/ml kanamycin LB agar plates. Midiprep of plasmids was performed using ZymoPure II Plasmid Midiprep Kit (ZymoPure) according to manufacturer’s protocol. Purified vectors were transfected into Siha cells using Lipofectamine 3000. Transformed cells were G418 selected and resulting cell pools were validated using qRT-PCR for vector E6* and E6.

3.2.4 p53 functionality in E6* and E6 overexpressing cell lines

Western blot analysis was performed to assess p53 protein expression across the Siha cell lines. Conjugated antibody 1:5000 p53-hrp and 1:3000 actin-hrp were incubated 1h at room temperature. RNA isolated was trizol extracted and cDNA was synthesized using High Capacity RNA-to-cDNA kit per manufacturer’s protocol. Taqman probes for the p53 target genes; CDKN1A, NOXA, PUMA, BAX, GADD45A, and MDM2 were used to assess p53 transcription factor function.

3.2.5 p21 functionality in E6* and E6 overexpressing cell lines

P21 (CDKN1A) protein expression was assessed by western blot, primary antibody incubation overnight at 4C 1:1000 anti-p21 (Cell Signaling Technology) and secondary antibody incubation 1:4000 anti-rabbit at room temperature. For proliferation assays, cells were plated and counted 3-, 7-, 9- and 12-days post-plating. For SA-β-galactosidase (SA-β-gal) staining, cells were plated
and grown in culture for 7-days, harvested and stained per manufacturers protocol. SA-β-gal positive cells were quantified as number of cells per image frame, in non-overlapping regions. For cell cycle analysis, cells were harvested and stained with propidium iodide and flow cytometry was performed using the MacsQuant analyzer and FlowJo software.

### 3.2.6 Cell survival after CRT

Cells were plated and the next day treated with vehicle (0.01% DMSO) or 0.5µM carboplatin 1h prior to radiation treatment (2 and 4Gy). For cell viability, alamar blue was used 5 days post-treatment and viability was normalized to untreated control samples. For colony formation assay, cells were incubated for 2 days after treatment and then re-plated at 500 cells/well. Cells grew for 10-12 days, until untreated control wells reached colonies of > 50 cells. Plating efficiency (PE) was calculated as (# of colonies in control) / (500 cells seeded) and surviving fraction (SF) was calculated as (# of colonies in treated wells) / PE*(500 cells seeded). Cell lines were normalized to their own untreated control wells.

### 3.2.7 Cell response to RT treatment

Cell cycle and apoptosis were assayed using flow cytometry. For the following assays cells were plated and then next day treated with 0.5µM carboplatin plus 4Gy radiation, cells were harvested at 24 and 48hrs following treatment. For cell cycle analysis, cells were washed and then stained with propidium iodide. For apoptosis, cells were washed then stained with Annexin-V and propidium iodide. Flow cytometry analysis was performed on MacQuant Analyzer and quantified on FlowJo. For each sample 5,000 events were collected. For γH2AX foci staining cells were treated, harvested and fixed with 4% paraformaldehyde at 10m, 2h, 6h, 12h and 24h post-2Gy RT. Primary antibody 1:300 anti-phospho-Histone H2A.X (Ser139) (20E3) incubated
at 4C overnight. Secondary antibody 1:500 anti-rabbit Alexa Fluor 488 incubated 2h at room temperature. Slides were mounted using ProLong Gold Antifade Reagent with DAPI.

### 3.3 HPV positive patients

#### 3.3.1 HPV genotypes detection and viral integration status

In our 88-patient cohort, 87.5% of patients had detectable HPV DNA for at least one of the genotypes that were probed for. HPV 16 and 18 genotypes were most common with 48 HPV-16 positive tumors and 9 HPV-18 positive. Eighteen patients had non-16 or -18 HPV genotypes detected; 4 HPV-33, 3 HPV-45, 3 HPV-59, 1 HPV-31, 1 HPV-56, 1 HPV-58, 1-HPV 68a and 1 HPV-82. Two patients had over 10 HPV genotypes detected in their tumors, for these samples one had HPV-16 present and the other had HPV-18 present. Patients with non-HPV 16 genotypes detected, or many HPV genotypes detected were grouped together as HPV other. As expected, patients with HPV 16 positive tumors had better PFS (p = 0.051) and OS (p = 0.002) compared to HPV other patients and patients with no detectable HPV (Figure 3.1).
Viral integration was assessed using both DNA and RNA based methodologies. DNA based viral integration analysis was limited to HPV 16 and HPV 18 positive patient samples as only these genotypes had complete DNA probe tiling of their viral genome. There were 39 samples with episomal HPV DNA and 20 with integrated HPV DNA, 3 of which also had episomal HPV DNA detected, and 1 was undetermined. HPV integration of HPV 16 and 18 patients using DNA methodology did not significantly stratify patients PFS or OS (Figure 3.2). Additionally, there was no difference in viral state between HPV 16 and HPV 18 samples (Fig 3.2C).

For the 58 HPV positive patients with RNAseq data available viral integration was assessed, this method allowed the analysis to extend to all the HPV genotypes present in our cohort. Integrated HPV was detected in 44 samples (26 HPV-16, 8 HPV-18 and 10 HPV other)

Figure 3.1: HPV genotype significantly stratifies patient outcomes
A) Progression-free and B) overall survival curves stratified by HPV genotype.
and episomal HPV was detected in 14 samples (11 HPV-16 and 3 HPV other). Patients with integrated HPV using RNAseq approach had less favorable PFS ($p = 0.067$) and OS ($p = 0.057$) compared to patients with episomal HPV (Figure 3.3). Viral state was not significantly different between HPV 16 and HPV other samples, however the RNA method detected less non-integrated, episomal, samples compared to the DNA method (Fig 3.3C).

![Graph A](image)

![Graph B](image)

**Figure 3.2: Viral state determined using DNA methodology**
A) Progression-free and B) overall survival curves stratified by HPV integration for HPV 16 and HPV 18 positive patients only. C) Episomal and integrated samples by HPV genotype
Figure 3.3: Viral state determined using RNAseq methodology
A) Progression-free and B) overall survival curves stratified by HPV integration for HPV 16 and non-HPV 16 positive tumors (n = 58). C) Episomal and integrated samples by HPV genotype

<table>
<thead>
<tr>
<th>Strata</th>
<th>HPV 16</th>
<th>HPV other</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Episomal</td>
<td>11</td>
<td>3</td>
<td>0.2203</td>
</tr>
<tr>
<td>Integrated</td>
<td>26</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>
3.3.2 Comparison of HPV 16 vs HPV other patient groups

There were no significant differences in tumor staging, lymph node involvement or metastasis at the time of diagnosis between HPV 16 and HPV other genotype patient groups. HPV 16 and HPV other patient groups had no difference in radiation treatment intent or treatment completion. Despite there being no difference in clinical characteristics or treatment strategies non-HPV 16 patients experienced more disease recurrence (p = 0.014) and increased mortality (p = 0.025) after radiation treatment compared to HPV 16 positive patients (Table 3.1).

RNA from 68 patient biopsies was isolated and RNAseq was performed. Viral expression of E6, E6*I and E7 were quantified and compared between HPV 16 (n = 36) and HPV other (n = 17) patient groups. The HPV E6* and E7 transcripts were more highly expressed then HPV E6 in both HPV 16 and HPV other groups. HPV other tumors had higher HPV E6 (p = 0.0085) and E6* (p = 0.025) transcript expression compared to HPV 16 tumors. There was no difference in HPV E7 transcript expression between the HPV groups. (Figure 3.4).

The relative expression of HPV viral transcript expression was tested as a prognostic indicator. Transcript expression for HPV E6, E6* and E7 was grouped as high (RPKM > upper quartile) and low (RPKM < upper quartile). High HPV E6* expression was a poor prognostic indicator for progression-free (p = 0.047) and overall survival (p = 0.033) (Figure 3.5 A,D). High E7 expression also had worse PFS (p= 0.11) and OS (p = 0.18) (Figure 3.5, B,E). Relative expression of HPV E6 alone was insufficient to stratify patient survival outcomes (Figure 3.5, C,F).
Table 3.1: Patient baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>HPV 16 (n = 48)</th>
<th>HPV other (n = 29)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ib1</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Ib2</td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IIa</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IIb</td>
<td>15</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>IIIa</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IIIb</td>
<td>12</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>IVa</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>IVb</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lymph Nodes at Diagnosis</td>
<td>24</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Aortic</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Pelvic</td>
<td>19</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>SCV</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Radiation Treatment Intent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curative</td>
<td>45</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Palliative</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Completed Treatment</td>
<td>46</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Recurrence</td>
<td>16</td>
<td>18</td>
<td>p = 0.015</td>
</tr>
<tr>
<td>Pelvic</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Distant</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>No data</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Overall Survival</td>
<td></td>
<td></td>
<td>p = 0.035</td>
</tr>
<tr>
<td>Alive</td>
<td>32</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Dead</td>
<td>16</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4: HPV E6, E6*I and E7 transcript expression in WUSM cohort
Comparison of HPV transcript expression between HPV 16 (pink) and non-HPV 16 positive (brown) patient tumors (* p < 0.05, ** p < 0.01)
Figure 3.5: Patient outcomes stratified by relative HPV transcript expression

Patients were grouped based upon relative HPV transcript expression, high > Q3 and low < Q3 for A,D) HPV E6*I, B,E) HPV E7 and C,F) HPV E6.
3.3.3 HPV 16 and relative HPV transcript expression correlate with poor patient survival outcomes

With respect to clinical practice cervical cancer tumors are not routinely tested for HPV status or HPV genotype. However, it has been appreciated in the literature that HPV positivity is a favorable prognostic biomarker for patient’s progression-free and overall survival. Additional studies have demonstrated that within the HPV positive group there is a spectrum of survival outcomes and patients with non-HPV 16 infected tumors have worse PFS and OS compared to patients with HPV 16 positive tumors. HPV viral state has also been described as a prognostic indicator for patient survival, where patients with HPV integrated tumors have worse PFS and OS.

In our prospectively collected cohort of patients uniformly treated with standard of care chemoradiation, I confirmed that patients with HPV DNA positive tumors had better survival outcomes after chemoradiation than HPV DNA undetected tumors. Furthermore, patients with HPV genotypes other than HPV 16 in their cervical tumors had worse survival outcomes after chemoradiation than HPV 16 positive tumors. In our cohort viral integration was quantified by both DNA and RNA methodology. Viral integration using DNA sequencing in HPV 16 and 18 positive tumors did not significantly stratify patient survival outcomes, however HPV integration defined by RNAseq did stratify patient PFS and OS. Univariate cox regression determined that infection of non-HPV 16 genotypes (HR= 2, p = 0.01548) but not RNA based viral integration (HR = 2, p = 0.1139) had an increased risk of developing disease recurrence. Altogether our cohort study confirmed that HPV status is an important prognostic indicator, however it is not a binary indicator and HPV genotype can be used as a prognostic biomarker of patient survival outcomes.
With regards to confirming previous studies I also wanted to determine what contributed to the HPV genotype differences. I ruled out clinical diagnostic differences as HPV 16 and non-HPV 16 patient groups had no significant difference in tumor stage, lymph node involvement at diagnosis, metastasis at diagnosis, treatment intent or treatment completion. However, patients in the HPV 16 group had less disease recurrence and better overall survival. In our cohort we found that non-HPV 16 positive tumors had significantly higher transcription of E6* and E6 compared to HPV 16 positive tumors. Transcript expression of HPV E6* significantly stratified patient’s progression-free and overall survival. I hypothesized that the difference in HPV transcript expression of E6 and E6* might be causative for the survival differences seen between the different HPV genotypes. In the following section I tested this using existing cervical cancer cell lines and engineering HPV 16 E6 and E6* overexpressing cell lines.

3.4 In vitro E6 and E6* overexpressing cell line characterization

HPV oncogenes E6 and E7 immortalize cells by degrading p53 and Rb respectively, initiating a state of uninhibited cell cycle entry and unregulated DNA damage response. HPV relies on host cellular machinery to transcribe and splice its various viral genes. In the normal viral lifecycle of high-risk HPV genotypes splicing of E6 into E6*I occurs in order to transcribe E7 (43). However, it has been demonstrated that HPV E6*I is a functional protein and acts as an antagonist of E6 function by blocking its association with target proteins such as p53 (44).
3.4.1 In vitro correlation of HPV transcript expression and RT sensitivity

RNA was isolated from Caski, Siha and SW756 cervical cancer cell lines and qRT-PCR was preformed to quantify the relative expression for HPV E6, E6*I and E7. Caski (HPV 16) had higher transcript expression of E6, E6*I and E7 compared to Siha (HPV 16) and SW756 (HPV 18) (Figure 3.6A). Cellular RT sensitivity was determined by Alamar Blue cell viability 5 days after ionizing radiation treatment. The high transcript expressing Caski cell line was the least sensitive to increasing doses of radiation compared to low HPV transcript expressing Siha (4gy: \( p = 0.032 \)) and SW756 (4gy: \( p = 0.017 \)) (Figure 3.6B). Altogether these results confirm that high viral transcript expression is correlated with resistance to radiation treatment, supporting what we saw in our patient cohort.

Figure 3.6: Cervix cancer cell lines transcript expression and sensitivity to radiation treatment
A) Caski, Siha and SW756 relative HPV transcript expression by qRT-PCR. B) Cell sensitivity to increasing doses of radiation treatment assessed by alamar blue (blue = Caski, orange = Siha, grey = SW756)
3.4.2 E6 and E6* overexpression modulates p53 expression

The low expressing Siha cell line was selected to test the independent effect of increasing transcript expression of E6 and E6*I on cell sensitivity to CRT treatment. HPV 16 E6 and E6*I protein coding sequences were ligated into pCMV-6 mammalian expression vectors and transfected into Siha cells using lipofectamine 3000. The vector transcript sequences of E6 and E6*I have been codon optimized for human cell expression and differ from native HPV 16 transcript sequences but result in the same amino acid sequences. Primers specific to the vector HPV 16 E6 and E6*I sequences were used to confirm vector specific transcript expression by qRT-PCR (Figure 3.7, A-B).

The primary target of HPV E6 mediated degradation is p53. Engineered cell lines were assessed for their ability to degrade or stabilize p53 protein, which was quantified by western blot. Siha E6 overexpressing cells had lower protein expression than WT Siha (p = 0.0107). E6* is an antagonist of E6 mediated degradation and Siha E6* overexpressing cells had higher p53 protein expression compared to WT Siha (p = 0.0107) (Figure 3.7, C-D). To test whether p53’s function as a transcription factor was affected by viral transcript expression, I performed qRT-PCR for 6 p53 target genes. The Siha E6* overexpressing cell line had higher CDKN1A (p = 0.021) and NOXA (p = 0.0089) transcript expression, and the Siha E6 overexpressing cell line had lower PUMA (p = 0.045) transcript expression compared to the WT Siha cell line. BAX, GADD45A and MDM2 transcript expression was not affected by either E6 or E6* overexpression (Figure 3.7, E-K).
Figure 3.7: Generation of HPV E6* and E6 overexpressing cell lines and their effect on p53
A,B) HPV vector specific expression of HPV 16 E6* and E6 across the cell lines. C,D) p53 protein expression western blot and band quantification (n=3). E-K) p53 target gene expression, E-F) upregulated in the Siha E6* cell line
3.4.3 E6* overexpression induces p21 mediated cellular senescence

*CDKN1A* encodes the p21 protein which is a direct target of p53 regulation. Siha E6* cells had higher p21 protein expression compared to WT Siha (Figure 3.8). The functionality of p21 was assessed in the three Siha cell lines; namely cellular proliferation, cell cycle phase and cellular senescence. The p53 and p21 low Siha E6 cell line had the fastest rate of proliferation followed by WT Siha and Siha E6* grew the slowest (Figure 3.8B).

When p53 is stabilized it activates p21 which in turn inhibits Cdk2, resulting in G1 cell cycle arrest. Although Siha E6* cells upregulated p21 protein there was no increased accumulation of G1 arrested cells compared to WT Siha cells (Fig 3.8 C-D). Previous studies have demonstrated that expression of HPV E7 bypasses p21 mediated cell cycle arrest resulting in abrogation of this G1 checkpoint (45). HPV E7 remains intact and expressed across Siha WT, Siha E6* and Siha E6 cell lines, therefore although p21 is being upregulated with E6* overexpression, no G1 cell accumulation occurs due to HPV E7 mediated ablation of the expected p21 induced G1 cell cycle arrest.

High p53 and p21 expression can also induce cellular senescence (46–48). Cells were plated and grown for 8 days and then stained for SA-β-galactosidase expression and senescent (SA-β-gal positive) cells were quantified. Siha E6* cells had more SA-β-gal positive cells compared to WT Siha (p = 0.029) and Siha E6 cell lines (p = 0.057) (Figure 3.8 E-F). There was no difference in SA-β-gal positivity between Siha E6 and WT Siha cells (p = 0.2).
**Figure 3.8: p21 stabilization and induction of cellular senescence**

A) p21 protein expression by western blot. B) Proliferation assay (black = Siha, brown = Siha E6, blue = Siha E6*) C-D) Cell cycle phase histogram and quantitation (n=3). E-F) Representative images of SA-β-Galactosidase staining (arrow = SA-β-Gal positive) and quantification of SA-β-Gal positive cells (* p< 0.05)
3.4.4 *In vitro* Siha E6 and E6* cellular response to chemoradiation treatment

Cell sensitivity to CRT treatment was assessed using the gold standard colony formation assay. In this assay cells are plated and the next day they are treated with 0.5µM Carboplatin 1hr prior to radiation treatment. Cells are allowed to recover for two days and then are re-plated at a low cell density and grown until untreated control cells form colonies of at least 50 cells. Both E6* and E6 overexpression decreased cell sensitivity to increasing doses of radiation treatment (Figure 3.9)

![Image of colony formation assay wells](image)

**Figure 3.9: E6* and E6 overexpression effect on clonogenic potential**
A) Representative image of colony formation assay wells. B) Normalized surviving fraction of Siha (black) and Siha E6* (blue) and C) Siha (black) and Siha E6 (brown).
Cell cycle analysis by flow cytometry was performed 24 and 48hrs after CRT treatment. Overexpression of E6* and E6 did not alter the cells ability to engage G2/M cell cycle arrest following treatment (Figure 3.10). Therefore, HPV E6 and E6* overexpression did not abrogate the cells ability to engage this cell cycle checkpoint. Apoptosis was also quantified 24 and 48hrs after CRT treatment using Annexin/PI staining and flow cytometry to identify cells undergoing apoptosis. Etoposide treatment was used as a positive control for apoptosis induction and all three cell lines had engaged their apoptotic pathways following treatment. However following CRT treatment cells were PI positive and Annexin negative, indicating that these dead cells did not undergo apoptosis following CRT treatment (Figure 3.11).

DNA damage induction and repair were quantified by immunofluorescence imaging of γH2AX p-Ser139 foci staining. Cells were treated with 2Gy radiation and then fixed at 10min, 2, 6, 12 and 24 hours after treatment to visualize the amount of DNA damage induced in each cell line and their ability to repair it. Siha E6* cells had a trend of higher γH2AX foci positive cells at baseline, and all cell lines had maximal γH2AX positive cells between 10m-6h. After 24h Siha E6 cells had cleared the γH2AX foci’s back to untreated levels, whereas Siha and Siha E6* retained γH2AX foci positive cells (p = 0.017) (Figure 3.12).
Figure 3.10: E6* and E6 overexpression do not alter G2/M cell cycle arrest following CRT treatment
Cell cycle analysis by flow 24 and 48hrs after 0.5µM carboplatin and 4Gy radiation treatment. (dark pink = 24h, light pink = 48h). A-B) Representative flow and quantification of Siha, C-D) Siha E6* and E-F) Siha E6.
**Figure 3.11: Apoptosis is not the primary mechanism of cell death after CRT treatment**

Apoptosis analysis by flow 24 and 48hrs after 0.5µM carboplatin and 4Gy radiation treatment. (dark pink = 24h, light pink = 48h). A) Representative flow plots of Annexin V and Propidium Iodide staining in Siha, Siha E6* and Siha E6 using etoposide as a positive control for apoptosis, B-D) Siha, Siha E6* and Siha E6 quantified staining.
Figure 3.12: HPV E6 and E6* overexpression effect rates of γH2AX p-S139 foci clearance after RT treatment

A) Representative images of γH2AX foci (green) and nuclei stained by DAPI (blue) immunofluorescence of Siha, Siha E6* and Siha E6 cell lines. B) Quantification of γH2AX foci positive cells (>3 foci/nuclei) at 10m, 2h, 6h, 12h, and 24h post-2Gy radiation treatment.
3.4.5 E6* overexpression induces cellular senescence

Taken together the data indicates that overexpression of E6* induces cellular senescence via stabilization of p53 and downstream activation of p21. Siha E6* cells had slower rates of proliferation and higher incidence of cellular senescence compared to Siha WT cells. Conversely, overexpression of E6 further decreased p53 protein stabilization and p21 was not activated. E6 overexpression increased the rate of proliferation and these cells did not undergo significant levels of cellular senescence. Future work should determine whether this senescence is seen in vivo by growing these overexpressing cell lines in mice and assess whether E6* induces cell senescence.

Cellular senescence is a state in which the cell no longer proliferates but is still viable. These cells alter the tumor microenvironment because of their senescence associated secretory profile (SASP) and can reprogram cells of the microenvironment to a tumor favorable phenotype (49). Since senescent cells are non-proliferative they are resistant to radiation treatment. However, they can be targeted using senolytic chemotherapies (50). The efficacy of these treatments should be tested in Siha E6* overexpressing cell lines to determine whether targeting the senescent cells sensitize this cell line to RT treatment.

3.5 Overall summary and proposed amendments to treatment guidelines

Current clinical guidelines do not require HPV testing of newly diagnosed cervical cancer. Instead patient treatment strategies are dictated by tumor FIGO stage at the time of diagnosis. However, 50% of advanced stage cervical cancer patients treated with chemoradiation
fail the standard-of-care treatment strategy. Based upon the results found in this chapter I propose that new guidelines should be adopted requiring HPV testing of newly diagnosed cervical cancer cases. In this study we demonstrated that non-HPV 16 positivity was a poor predictive biomarker of disease response to the standard of care chemoradiation treatment. Furthermore, patient tumors with non-HPV 16 genotypes had higher viral transcript expression of HPV E6 and E6*I. High HPV E6*I expression significantly increased patient risk of developing disease recurrence and was a predictive biomarker of poor disease response to CRT treatment.

In vitro characterization of high HPV E6* and E6 transcript expression revealed that increasing expression of either transcript was sufficient to induce CRT treatment resistance. Each transcript induced treatment resistance through differential regulation of p53 and downstream p21 activation. Increasing E6 expression inhibited p53 and p21 activity, resulting in enhanced DNA repair of RT induced lesions. High E6* expression inhibited E6 induced degradation of p53, resulting in p53 and p21 activation and induction of cellular senescence.

Based upon these results I propose that patient treatment planning should include HPV genotype and relative HPV E6 and E6* transcript expression. Current standard-of-care practice for patients presenting with tumor stage IIB – IVA is platinum-based chemotherapy and radiation treatment. Under the proposed guidelines patient’s treatment planning would be personalized based upon the tumor HPV status and HPV transcript expression. Patients with HPV 16 positive tumors and low transcript expression would continue to receive CRT treatment. For patients with high E6* expressing tumors, radiation treatment could be followed by adjuvant senolytics to target the RT resistant senescent cells. Patients with high E6 expression could benefit from pairing radiation treatment with DNA damage response inhibitors.
Chapter IV: HPV negative cervical cancer

4.1 HPV negativity is a poor prognostic marker for mortality in oropharyngeal and cervical cancer

In addition to cervical cancer, HPV is known to cause about 70% of vulvar and vaginal cancers, 90% of anal cancers, 60% of penile cancers and around 60-70% of oropharyngeal cancer cases. In a US population-based study it was estimated that 34,800 cancers per year were likely to be caused by HPV with 38.8% originating from the oropharynx and 31.3% from the uterine cervix (51). Previously published studies have demonstrated that for both oropharyngeal and cervical cancer HPV negativity is a poor prognostic indicator for overall survival. Based upon these findings the American Society of Clinical Oncology (ASCO) established a guideline on mandatory HPV screening in newly diagnosed oropharyngeal squamous cell carcinomas (52). This screening uses immunohistochemical (IHC) staining of primary tumor biopsies for p16, an established surrogate biomarker of HPV infection. Although IHC staining is a reliable test strategy that is easy to perform in the clinical laboratory setting, it has a high rate of calling false positive HPV cancers (53). Since HPV status is a strong prognostic marker of patient survival it is imperative to correctly identify patients with HPV negative cancer. More sensitive clinical HPV testing strategies employ DNA based PCR and RNA based RT-PCR to probe for panels of high-risk HPV genotypes. The DNA based PCR method informs the clinician on which HPV genotypes are present, however it does not inform as to whether the virus is transcriptionally active (54). Therefore, the current gold standard of clinical HPV testing is RT-PCR to detect transcriptionally active HPV. However, RNA based HPV detection is not routinely employed in
the clinical laboratory setting due to it being time consuming and technically difficult (54). In the research setting the current gold standard of HPV testing is RNAseq based detection of HPV viral transcripts. For HNSCC the TCGA definition of HPV negativity was less than 1,000 HPV transcript reads per tumor sample(55). The TCGA cervix cancer standard for defining HPV negativity was any tumors with less than 1 HPV transcript read per million (RPM) human transcript reads (10).

The American Cancer Society (ACS) has established guidelines for cervical cancer screening which recommends that women ages 21-29 receive cytology-based pap smears every 3 years and women ages 30-65 should receive pap smears and HPV testing every 5 years (56). The HPV testing routinely used is DNA based PCR testing for a panel of high-risk HPV genotypes. However, upon cervical cancer diagnosis there are currently no employed guidelines for HPV testing of primary cervix tumors. In clinical practice all cervical cancer cases are assumed to be HPV infected, however retrospective studies have repeatedly confirmed that 7-11% of cervical cancer patients have no detectable HPV in their tumors. As mentioned, previous studies have demonstrated that HPV negativity is a poor prognostic indicator for a cervical cancer patient’s progression-free and overall survival. Therefore, it would benefit patient care to identify those patients with HPV negative primary cervix cancers prior to treatment.

In our WUSM cohort we initially determined HPV status by DNA probes testing for 32 HPV genotypes using next-generation sequencing. This strategy was used to model the clinically practiced DNA based PCR assay that is commonly used for HPV detection. HPV negativity was defined as a tumor sample having no HPV DNA reads aligned to one of the 32 HPV genotypes probed for. Based upon this definition we identified 11 patients with HPV negative tumors in our cohort. Ten of these patients also had additional RNAseq data which we used to validate HPV
negativity using the gold standard RNA based method. RNAseq transcript reads were aligned to HPV reference genomes and the TCGA standard of < 1 HPV read per million human transcript reads was used to validate HPV negativity. All 10 of the DNA defined HPV negative tumor samples were also defined as HPV negative based upon the RNA method. The patients in our WUSM cohort that were categorized as having HPV negative tumors had significantly worse overall survival after curative intent CRT compared to patient’s with HPV positive tumors (p = 0.0053) (Figure 4.1).

To date there have been no comprehensive studies investigating the underlying biological differences between HPV negative and positive cervical cancer. Our own institutional study was underpowered to detect statistically significant differences in gene mutation enrichment between HPV negative and HPV positive cervical cancer tumors. To overcome this limitation, we collaborated with Dr. Akin Ojesina at the University of Alabama in Birmingham (UAB) to comprehensively analyze the largest cohort of HPV negative cervical cancer cases to date (HPV negative tumors n = 35, HPV positive tumors n = 430). The primary goal of this collaboration was to characterize the genomic landscape of HPV negative cervical cancer tumors and identify transcriptional differences between HPV negative and HPV positive tumors. Additionally, we wanted to identify clinically actionable targets that were uniquely modified in HPV negative cervical cancer tumors and validate whether HPV negative cell lines would be sensitive to the proposed experimental targeted therapy.
Figure 4.1: Patient survival outcomes stratified by HPV status

Kaplan-Meier survival analysis for progression-free (PFS) and overall survival (OS) outcomes in WUSM patients A) All comers and B) patients who completed curative intent radiation treatment stratified by HPV status.
4.2 Study design:

4.2.1 Defining HPV negativity

The UAB cohort defined HPV negative tumors as < 1 HPV read per million human transcript reads using RNAseq data (n=24). The WUSM cohort used DNA sequencing to define HPV status, previously described in 2.2.1. Any sample that had no detectable HPV reads for the 32 HPV genotypes probed for were defined as HPV negative (n=11) (Table 2.2). For the 11 WUSM HPV negative samples, 10 had corresponding RNAseq data. The RNAseq transcript reads were run against HPV genotypes HPV 16, 18, 31, 33, 45, 52, 56, 58 and 59 and HPV negativity was verified using the cut off of < 1 HPV read per million transcript reads. All 10 WUSM samples called as HPV negative by DNA probes were also negative using the RNA cut offs.

4.2.2 Genomic sequencing

Whole-exome sequencing was performed on 379 tumor-blood pairs from the UAB patient cohort. Genes were filtered for those that had a MutSig2CV p-value < 0.1 in either HPV negative or HPV positive tumors, and any gene that had 1 mutation in an HPV negative tumor and no mutations in HPV positive tumors. Fisher’s exact test was used to identify filtered genes significantly enriched for mutations (SMG) in HPV negative cervical cancer tumors. Filtered SMGs were also analyzed for both co-occurrence of mutations and mutual exclusivity of mutations with other SMGs. Targeted gene exome sequencing for 211 pan cancer associated genes (Table 2.1) was performed on 88 tumor-blood pairs from the WUSM cohort. Combined
analysis of UAB and WUSM cohort samples were restricted to patients with at least one mutation in one of the genes in the targeted WUSM gene panel. These genes were filtered using the previously described methodology and fisher’s exact test was again used to identify SMGs enriched in HPV negative cervical cancer tumor samples.

**4.2.3 RNAseq analysis**

RNA sequencing was performed on RNA extracted from 355 UAB and 68 WUSM cohort tumors. The UAB and WUSM cohorts were analyzed independently and used as test and validation datasets to assess differences in transcript expression between HPV negative and HPV positive cervical cancer tumors. Wilcoxon-test for significance was used to identify SMGs with differential transcript expression and p < 0.05. Additionally, differential expression (DE) analysis was performed using DESeq2 on the 10 HPV negative and 58 HPV positive tumors from the WUSM cohort. DE genes used for subsequent GSEA pathway analysis had a log2 fold change > 1 or < -1 and p-adj < 0.01. Pathway analysis was restricted to hallmark gene sets that had at least 10% of the pathway contributed from DE genes and an FDR < 0.01.

**4.3 Characterization of HPV negative cervical cancer tumors**

**4.3.1 Results of genomic analysis**

Collectively 1569 genes from the whole-exome sequencing of UAB cohort samples met at least one of the initial filtering criteria and fisher’s exact comparison was performed to identify genes more frequently mutated in HPV-negative tumors. Eight SMGs were identified and had false discovery rates (FDR) < 0.1 (Table 4.1).
In HPV negative tumors ZNF331 mutations co-occurred with CTCF (p = 0.007623) and ARID1A (p = 0.00659) mutations; and KIAA1012 mutations co-occurred with MC5R mutations (p = 0.000198) (Table 4.2). TP53 mutations found in HPV negative tumors were mutually exclusive with ARID1A (p = 0.027), ZNF33I (p = 0.0466), and NCAPH2 (p = 0.0466) mutations (Table 4.3). Combined analysis of the UAB and WUSM cohorts included 33 HPV-negative and 418 HPV-positive cervical tumors. TP53, ARID1A, PTEN, ARID5B, CTNNB1, CTCF and CCND1 were all identified as SMGs in HPV negative tumors (Table 4.4).

**Table 4.1:** Frequency of mutations by HPV status in University of Alabama at Birmingham (UAB) patient cohort

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative frequency of HPV negative tumors with mutation (%) (n = 24)</th>
<th>Relative frequency of HPV positive tumors with mutation (%) (n = 355)</th>
<th>FDR p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>50.0</td>
<td>3.1</td>
<td>2.65E-07</td>
</tr>
<tr>
<td>ARID1A</td>
<td>33.3</td>
<td>5.4</td>
<td>2.05E-02</td>
</tr>
<tr>
<td>RICTOR</td>
<td>20.8</td>
<td>0.6</td>
<td>1.01E-02</td>
</tr>
<tr>
<td>ARHGEF2</td>
<td>20.8</td>
<td>1.4</td>
<td>3.05E-02</td>
</tr>
<tr>
<td>ZNF331</td>
<td>16.7</td>
<td>0.3</td>
<td>3.14E-02</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>16.7</td>
<td>0.3</td>
<td>3.14E-02</td>
</tr>
<tr>
<td>KIAA1012</td>
<td>16.7</td>
<td>0.3</td>
<td>3.14E-02</td>
</tr>
<tr>
<td>MC5R</td>
<td>12.5</td>
<td>0</td>
<td>4.41E-02</td>
</tr>
</tbody>
</table>
### Table 4.2: Co-occurrence of significantly mutated genes (SMGs) in HPV negative tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Co-occurrence</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCF WT</td>
<td>ZNF331 WT 19</td>
<td>0.007623</td>
</tr>
<tr>
<td>CTCF Mutant</td>
<td>ZNF331 Mutant 1</td>
<td></td>
</tr>
<tr>
<td>ARID1A WT</td>
<td>ZNF331 WT 16</td>
<td>0.00659</td>
</tr>
<tr>
<td>ARID1A Mutant</td>
<td>ZNF331 Mutant 4</td>
<td></td>
</tr>
<tr>
<td>MC5R WT</td>
<td>KIAA1012 WT 20</td>
<td>0.000198</td>
</tr>
<tr>
<td>MC5R Mutant</td>
<td>KIAA1012 Mutant 0</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4.3: Mutual exclusivity of significantly mutated genes (SMGs) in HPV negative tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutually exclusive</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53 WT</td>
<td>ARID1A WT 5</td>
<td>0.027</td>
</tr>
<tr>
<td>TP53 Mutant</td>
<td>ARID1A Mutant 7</td>
<td></td>
</tr>
<tr>
<td>ZNF331 WT</td>
<td>TP53 WT 8</td>
<td>0.0466&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZNF331 Mutant</td>
<td>TP53 Mutant 4</td>
<td></td>
</tr>
<tr>
<td>NCAPH2 WT</td>
<td>TP53 WT 8</td>
<td>0.0466&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>NCAPH2 Mutant</td>
<td>TP53 Mutant 4</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> one-tailed test
<table>
<thead>
<tr>
<th>Cohort</th>
<th>Gene</th>
<th>Relative frequency of HPV negative tumors with mutation (%)</th>
<th>Relative frequency of HPV positive tumors with mutation (%)</th>
<th>FDR p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAB</td>
<td></td>
<td>(n = 22)</td>
<td>(n = 343)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TP53</td>
<td>54.55</td>
<td>4.08</td>
<td>9.29E-08</td>
</tr>
<tr>
<td></td>
<td>ARID1A</td>
<td>36.36</td>
<td>6.12</td>
<td>9.35E-03</td>
</tr>
<tr>
<td></td>
<td>PTEN</td>
<td>31.82</td>
<td>6.71</td>
<td>3.14E-02</td>
</tr>
<tr>
<td></td>
<td>ARID5B</td>
<td>18.18</td>
<td>0.58</td>
<td>9.78E-03</td>
</tr>
<tr>
<td></td>
<td>CTNNB1</td>
<td>18.18</td>
<td>0.87</td>
<td>1.64E-02</td>
</tr>
<tr>
<td></td>
<td>CTCF</td>
<td>18.18</td>
<td>1.17</td>
<td>2.52E-02</td>
</tr>
<tr>
<td>UAB + WU</td>
<td></td>
<td>(n = 33)</td>
<td>(n = 418)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TP53</td>
<td>45.45</td>
<td>3.83</td>
<td>8.82E-09</td>
</tr>
<tr>
<td></td>
<td>ARID1A</td>
<td>33.33</td>
<td>6.22</td>
<td>9.61E-04</td>
</tr>
<tr>
<td></td>
<td>PTEN</td>
<td>30.3</td>
<td>5.74</td>
<td>1.75E-03</td>
</tr>
<tr>
<td></td>
<td>ARID5B</td>
<td>21.21</td>
<td>0.72</td>
<td>6.43E-05</td>
</tr>
<tr>
<td></td>
<td>CTNNB1</td>
<td>15.15</td>
<td>0.96</td>
<td>6.69E-03</td>
</tr>
<tr>
<td></td>
<td>CTCF</td>
<td>15.15</td>
<td>1.44</td>
<td>1.84E-02</td>
</tr>
<tr>
<td></td>
<td>CCND1</td>
<td>9.09</td>
<td>0.24</td>
<td>4.11E-02</td>
</tr>
</tbody>
</table>

Table 4.4: Significantly mutated genes (SMGs) enriched in HPV negative tumors
4.3.2 Results of transcriptome analysis

The SMGs *ARHGEF2, CCND1* and *CTNNB1* all had higher transcript expression in HPV negative tumors from both the UAB and WUSM patient cohorts (Figure 4.2). *NCAPH2* had lower transcript expression in HPV negative tumors in both patient cohorts and *CTCF* and *RICTOR* had lower transcript expression in HPV negative tumors from the UAB cohort (Figure 4.3). Furthermore, genes found to be differentially expressed between HPV negative and HPV positive tumors in the WUSM cohort contributed to hallmark E2F targets (FDR < 0.001) and G2M checkpoint pathways (FDR = 0.002347) (Figure 4.4).

**Figure 4.2: SMGs upregulated in HPV negative tumors**
A) SMGs upregulated in HPV negative tumors from the UAB patient cohort and B) WUSM patient cohort
Figure 4.3: SMGs downregulated in HPV negative tumors
A) SMGs downregulated in HPV negative tumors from the UAB patient cohort and B) WUSM patient cohort
Figure 4.4: Pathway analysis of HPV negative vs HPV positive cervical tumors in WUSM cohort

Differentially expressed genes with log2 fold change > 1 or < -1 and p-value < 0.05 were used for GSEA pathway analysis. A) Significant pathways altered in HPV negative vs HPV positive patient tumors. B) Volcano plot of log2 fold change vs -log10 p-value for differentially expressed genes in RNAseq library (yellow = DE > 1, purple = DE < -1). C) Table of significantly altered pathways from GSEA analysis.
4.3.3 Summary of HPV negative tumor profiling

The seven SMGs identified in the UAB and WUSM combined genomic analysis have converging biological roles in coordinating G1 to S phase cell cycle progression (Figure 4.5). Additionally, HPV negative tumors had higher CCND1 transcript expression in both the UAB and WUSM cohorts. Entry into the cell cycle is tightly regulated at the restriction point by Rb, which binds the E2F transcription factor, blocking cell cycle progression. Cyclin D1 and CDK4/6 complexes inhibit Rb via hyperphosphorylation resulting in the release of E2F from Rb, allowing the transcription of E2F target genes that are necessary for progression into S-phase. Overexpression of cyclin D1 results in constitutive hyperphosphorylation of Rb and ultimately loss of G1 cell cycle arrest. In HPV positive cervical cancer, cells overcome G1 cell cycle arrest by expression of HPV E7 which targets Rb for proteasomal degradation. Although both HPV positive and HPV negative cervical cancers have deregulated cell cycle entry, the biological mechanism for which this is achieved differs. The constitutive inhibition of Rb via cyclin D1 overexpression identified in HPV negative tumors is clinically actionable and can be targeted using the FDA approved CDK 4/6 inhibitor Palbociclib.
Figure 4.5: SMGs in HPV negative cervical cancer function in G1 to S phase cell cycle progression

Converging functions of significantly mutated genes (SMGs) in G1 to S cell cycle progression and regulation at the restriction point (R-point). (Grey = human proteins, brown = HPV proteins, bold = SMGs, yellow = upregulated in HPV negative tumors and purple = downregulated in HPV negative tumors).
4.4 Testing the efficacy of Palbociclib in HPV negative and positive cancer cell lines

4.4.1 Cell line selection

Palbociclib restores G1 cell cycle arrest by inhibiting CDK4/6 thus blocking cyclin D1 from phosphorylating Rb, but wild-type (WT) Rb is necessary for Palbociclib to be effective. Both C33A and HT3, HPV negative cervix cancer cell lines, have mutated RB1 [Figure 4.6A] and neither cell line showed sensitivity to Palbociclib treatment (data not shown) [Cosmic CLP ref]. RB1 was not frequently mutated in cervical cancer patients from either UAB or WUSM cohorts, therefore although I could not evaluate Palbociclib efficacy in HPV negative CESC cell lines this should still be a viable treatment strategy for patients with HPV negative CESC. To evaluate the efficacy of Palbociclib treatments in HPV negative versus HPV positive cell lines I turned to head and neck squamous cell carcinoma (HNSCC) cell lines. The HPV negative Fadu and HPV 16 positive SCC-47 and SCC-154 HNSCC cell lines were used to evaluate efficacy of Palbociclib treatment in HPV positive and HPV negative cancer cell lines. All three HNSCC cell lines had WT RB1 genotypes, and the Fadu also had overexpression of cyclin D1 (Figure 4.6A).
### Figure 4.6: Genotype and cyclin D1 expression across cervical cancer and head and neck squamous cell cancer cell lines

A) HPV status, RB and TP53 genotypes across cell lines. B) Cyclin D1 protein expression by western blot analysis

### 4.4.2 HPV negative cell line sensitivity to Palbociclib

Cells were plated and then the next day were treated with increasing doses of single agent Palbociclib. Five days after treatment, cell viability was quantified using Alamar Blue and treated samples were normalized to untreated controls. HPV negative Fadu cells were sensitive to all doses of Palbociclib tested (0.25µM, p < 0.05; 0.5µM – 5µM, p < 0.001) (Figure 4.7A). Palbociclib had no effect on the HPV positive cell line’s viability. Combination Palbociclib and radiation treatment was also evaluated. Again, cells were plated and the next day treated with Palbociclib 1hr prior to radiation treatment and after five days cell viability was evaluated using Alamar Blue. The combination of Palbociclib with radiation treatment did not show significant sensitization in any of the cell lines tested, shown are representative results for 0.25µM Palbociclib plus radiation (Figure 4.7B).
4.4.3 Palbociclib induced G1 arrest and proliferation attenuation

The effect of Palbociclib on G1 cell cycle arrest was evaluated by treating cells with vehicle (0.01% DMSO) or 0.25µM Palbociclib and harvesting them 24 and 48hrs after treatment. Cells were stained with propidium iodide and analyzed by flow cytometry. G1 cell cycle arrest was maximal 24hrs after Palbociclib treatment with an average increase in G1 cells, across three independent replicates, of 13.6%, 2.2% and 2.0% for Fadu, SCC-47 and SCC-154 cells respectively (Figure 4.8). Attenuation in proliferation was evaluated by treating cells with either vehicle or 0.25µM Palbociclib and counting cells at 6-, 9- and 12-days post-treatment. A representative day 12 plate was crystal violet stained and is shown in Figure 4.9D. Fadu cells treated with Palbociclib had attenuated growth rates compared to vehicle treated controls (D9 p< 0.05, D12, p< 0.01) (Figure 4.9A). The HPV positive SCC-47 and SCC-154 cell lines showed no delays in growth rate after treatment with Palbociclib (Figure 4.9B-C).
Figure 4.8: G1 cell cycle arrest 24h after Palbociclib treatment

Representative histograms of A) HPV negative Fadu and B-C) HPV positive SCC-47 and SCC-154 for cell cycle analysis 24h after 0.25µM Palbociclib treatment (grey = vehicle, blue = 0.25µM Palbociclib). D) Average increase in G1 cell cycle arrest 24h after 0.25µM Palbociclib treatment (Avg ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Fadu</th>
<th>SCC-47</th>
<th>SCC-154</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.6 ± 2.0</td>
<td>2.2 ± 2.4</td>
<td>2.0 ± 1.4</td>
</tr>
</tbody>
</table>
**Figure 4.9: Proliferation HNSCC cell lines after 0.25µM Palbociclib**

Cells were counted at 6-, 9-, and 12-days post-Palbociclib treatment. A) Proliferation curves after Palbociclib treatment (solid = vehicle and dashed = 0.25µM Palbociclib). B) Representative crystal violet stained day 12 plate.
4.5 Summary of HPV negative CESC characterization

HPV status is a powerful prognostic biomarker of CESC patients’ progression-free and overall survival. In our institutional cohort we found that patients with HPV negative cervical tumors did not achieve disease control and had higher mortality rates than patients with HPV positive tumors. Our results highlight the need for routine HPV testing of primary cervix tumors to identify patients upfront that have HPV negative tumors. These patients are likely to fail their standard of care CRT treatment and would likely benefit from alternative treatment plans.

In our collaborative effort to genomically characterize HPV negative cervical tumors we identified seven SMGs with converging roles in cell cycle entry and progression, and CCND1 (cyclin D1) transcript was overexpressed in HPV negative tumors from both patient cohorts. The FDA approved CDK4/6 inhibitor, Palbociclib was evaluated for efficacy as a single agent therapeutic in HPV negative and HPV positive HNSCC cell lines. In response to single agent Palbociclib treatment the HPV negative Fadu cell line exhibited increased G1 cell cycle arrest 24hrs after treatment, prolonged proliferation attenuation up to 12 days after treatment and decreased cell viability compared to untreated controls. The HPV positive SCC-47 and SCC-154 cell lines showed no significant effects on G1 cell cycle arrest, proliferation rate or cell viability after Palbociclib treatment. Altogether these results indicate that HPV negative cell lines are uniquely responsive to single agent Palbociclib treatment and patients with HPV negative cervical cancer tumors could benefit from the addition of Palbociclib into their treatment plan.
Chapter V: Conclusion

Current treatment strategies for cervical cancer rely on FIGO tumor staging, with low stage tumors receiving surgery and advanced stage tumors being treated with curative intent radiation and concurrently administered platinum-based chemotherapy. However, 50% of advanced stage cervical cancer patients will fail their first line radiation therapy which results in poor morbidity and mortality. The goal of this dissertation was to identify biological biomarkers of disease recurrence after chemoradiation treatment using next-generation genomic sequencing and RNAseq profiling of pretreatment tumors in a well annotated and uniformly treated advanced stage cervix cancer cohort. I found that human host mutations, HPV viral variation, and distinct biology in HPV negative cervical cancer affected disease recurrence in patients.

Using a targeted exome gene panel, I found that alterations in FBXW7 and NSD1 correlated with patients that experienced disease recurrence and increased the risk of developing recurrent disease as calculated by univariate cox regression. For patients with FBXW7 mutations the disease recurrence was primarily distant, and NSD1 mutations resulted exclusively in local disease recurrence. NSD1 was selected for in vitro validation and its loss was sufficient to induce resistance to carboplatin single-agent treatment and combination carboplatin with radiation treatment. The NSD1 KD cells exhibited hypomethylation at H3K36 and differential expression analysis of Caski shNSD1 cells identified upregulation of estrogen response pathways, inflammatory profiles and p53 response pathways. This potential regulation between NSD1 and estrogen receptor may explain why NSD1 alterations confer tissue specific prognosis, with estrogen sensitive tissues having poor prognosis and estrogen insensitive tissues have good prognosis with NSD1 mutant tumors. Future experiments using Chip-seq to identify which
differentially expressed genes are regulated by NSD1 mediated H3K36me2 will be useful in mapping the transcriptional regulation of NSD1. Alterations in NSD1 is emerging as prognostic in many cancer types and further characterization in vitro and in vivo will be necessary to describe the mechanism by which NSD1 alterations affect treatment sensitivity. In our cervix cancer shNSD1 cell lines I found that loss of NSD1 did not affect γH2AX foci formation or clearance but it did alter cell cycle arrest following carboplatin treatment. In WT cell lines carboplatin induced an S-phase arrest that persisted up to 48hrs after treatment. The NSD1 KD cells initially had an increase in S-phase cells at 24h, but after 48hrs these cell lines were able to progress into G2/M, suggesting that NSD1 is required for the S-phase cell cycle arrest. Future experiments as to whether NSD1 mediates this directly or via transcriptional regulation will need to be assessed and additional in vivo models should be evaluated to determine how the alteration in inflammatory profile affects the tumor microenvironment and the effect that has on in vivo treatment response.

Previous studies have already shown that HPV status is prognostic in HPV related cancers, with HPV negative conferring worse progression-free (PFS) and overall survival (OS) outcomes compared to patients with HPV positive tumors. The HPV positive group however is heterogeneous in their outcomes and the goal of my dissertation was to identify which HPV positive patients would have worse survival outcomes. I found that patients with non-HPV 16 genotypes had worse PFS and OS and had higher transcript expression of HPV E6 and E6*I. The relative transcript expression of E6*I was sufficient to significantly stratify patient outcomes and along with HPV E6 it was evaluated in vitro. I quantified HPV transcript expression in existing cervix cancer cell lines and found that the high transcript expressing Caski cell line (HPV 16 positive) was most resistant to radiation treatment compared to the low expressing Siha (HPV
16) and SW756 (HPV 18) cell lines. To evaluate the individual effects each HPV transcript had on cell sensitivity to radiation treatment I engineered Siha cells to exogenously express either HPV 16 E6 or HPV 16 E6*. Overexpression of E6* led to protein stabilization of p53, activation of p21 which resulted in a less proliferative senescent state which was resistant to combination carboplatin and radiation treatment. Conversely overexpression of E6 further decreased p53 expression, had no p21 activation and the cells were hyperproliferative and able to repair their RT induced γH2AX foci faster than Siha WT cells. Altogether these results showed that although HPV E6 and E6* both result in CRT treatment resistance they work through different mechanisms. Therefore, the treatment strategies to target these alterations should be different. Patients with high HPV E6* transcript expressing tumors would benefit from the addition of adjuvant senolytics combined with radiation treatment, and patients with High E6 transcript expressing tumors may benefit from the addition of DNA repair inhibitors in combination with radiation treatment. Future work should be done to evaluate these mechanisms in vivo and determine whether these treatment strategies are sufficient to reverse the radiation resistant phenotype.

Lastly, in collaboration with Dr. Akin Ojesina at UAB I evaluated the genomic and transcriptomic landscape of HPV negative cervical cancer tumors. We found that mutations in ARID1A, ARID5B, CTCF, CCND1, CTNNB1, PTEN and TP53 were all significantly enriched (SMG) in HPV negative tumors and additionally CTNNB1 and CCND1 were significantly upregulated in HPV negative tumors. All of the SMGs have converging biological functions in G1 to S phase cell cycle progression and therefore treatment with the CDK 4/6 inhibitor Palbociclib was evaluated for efficacy in HPV negative versus HPV positive cell lines. Palbociclib blocks cyclin D1 mediated inhibition of Rb allowing the cell to restore the nature G1
checkpoint arrest, however in order to be efficacious the cell must be Rb competent. Our HPV negative cervix cancer cell lines were Rb mutant, so I used head and neck squamous cell lines to evaluate Palbociclib sensitivity. The HPV negative Fadu cell line had increased G1 arrest, decreased proliferation and decreased cell viability when treated with Palbociclib alone. Since Palbociclib is already an FDA approved drug I propose that a clinical trial for HPV negative cervix cancer patients should be opened evaluating the use of Palbociclib treatment either preceding or following radiation treatment to evaluate whether these patients will benefit from this treatment strategy.

Altogether the results of this dissertation have identified three biological biomarkers that predict patients who will have poor treatment response to chemoradiation treatment. Additional pre-clinical studies should be done to further evaluate the mechanism by which NSD1 mutations confer treatment resistance. HPV status should be evaluated for all newly diagnosed cervical cancer patients and HPV transcript expression of HPV E6 and E6*I should be quantified for HPV positive patients. In vivo models for the E6 and E6* overexpression should be used to confirm our in vitro results and test the efficacy of combine DDR inhibitors or senolytics with radiation treatment respectively. Finally, a clinical trial should be opened to test whether Palbociclib provides better disease management for HPV negative cervical cancer patients.
References


36. Qin C, Nguyen T, Stewart J, Samudio I, Burghardt R, Safe S. Estrogen up-regulation of p53 gene expression in MCF-7 breast cancer cells is mediated by calmodulin kinase IV-


