Investigation of MACROD2 as a Biomarker of Treatment Resistance in Human Papillomavirus-Related Head and Neck Cancer

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology and Laboratory Medicine
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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide. Infection by human papillomavirus (HPV) has caused a rise in HNSCC cases. Unfortunately, a cohort of HPV+ HNSCC patients exhibit treatment resistance. At present, no known treatment resistant biomarkers have been identified. We completed genomic characterization of a local HPV+ HNSCC cohort and observed MACROD2 deletions in the treatment failure dataset. Functional validation revealed siRNA and shRNA MACROD2 knockdown in HNSCC cells led to elevated cell viability and colony formation. Interestingly, shMACROD2 caused no change in cisplatin sensitivity, but a significant increase in radiation resistance in HPV+ HNSCC. Transcriptomic, proteomic, and immunofluorescence studies demonstrated that MACROD2 depleted HPV+ HNSCC cells displayed elevated hypoxia and altered DNA damage response signatures suggestive of possible mechanisms of radioresistance. Further work is needed to understand how to overcome these molecular mechanisms of radiation resistance in MACROD2 deficient HPV+ HNSCC.
Keywords

Head and Neck Cancer, Human Papillomavirus, Chemoradiation, Treatment Resistance, MACROD2, Hypoxia, γ-H2AX
Summary for Lay Audience

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide. Recently, infection by human papillomavirus (HPV) has caused a rapid rise in HNSCC cases. Although patients with HPV-associated HNSCC (HPV+ HNSCC) generally respond well to chemotherapy and radiation treatment, a cohort of patients exhibit treatment resistance resulting in cancer recurrence. At present, no known molecular drivers for treatment resistance in HPV+ HNSCC have been identified. We have completed a genomic characterization of HPV+ HNSCC tumours from patients from our treatment area. We observed that genetic changes in a gene called MACROD2 were more frequent in patients who failed treatment. Thus, we hypothesized that mimicking the genetic change in MACROD2 in cell line models would prove that this gene drives resistance to chemotherapy and radiation in HPV+ HNSCC.

To accomplish this, we used HPV+ HNSCC cells and inhibited the expression of the MACROD2 gene. Inhibiting MACROD2 led to elevated cell aggressiveness in the HPV+ HNSCC cell lines. Interestingly, MACROD2 inhibited cells were not resistant to chemotherapy, but had a significant increase in radiation resistance across several HPV+ HNSCC cell lines.

To understand how loss of MACROD2 gene expression leads to radiation resistance, we examined our cells at the transcript and protein level. Our results show MACROD2 deficient HPV+ HNSCC cell lines exhibit a distinct hypoxia signature, describing a low oxygen environment. Tumour hypoxia has been previously associated with radiation resistance. Also, MACROD2 inhibition was associated with an altered DNA damage response (DDR), also implicated in cancer aggressiveness and treatment resistance. Consequently, a suggested mechanism of radiation resistance in MACROD2 inhibited cells is an elevated hypoxia and altered DDR.

These findings provide a better understanding of the molecular basis of treatment resistance in HPV+ HNSCC. Our findings have the potential to stratify patients based on MACROD2 expression, so those harbouring MACROD2 mutations may seek alternatives to radiation therapy in the treatment of their HPV+ HNSCC.
Co-Authorship Statement

All chapters were written by Allie Dawson and edited by Dr. Anthony Nichols and Dr. John Barrett.

All experiments were performed by Allie Dawson. Bioinformatic analysis of RNASeq and RPPA data was performed by Amir Hossein Karimi.
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<th>Definition</th>
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<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia-telangiectasia mutated and Rad3-related</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Ca9</td>
<td>Carbonic anhydrase 9</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR-associated protein 9</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CI</td>
<td>Chromosome Instability</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DEL</td>
<td>Deletion</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-free survival</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
</tr>
<tr>
<td>DUP</td>
<td>Duplication</td>
</tr>
<tr>
<td>E Region</td>
<td>Early Region</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ESCC</td>
<td>Esophageal squamous cell carcinoma</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GEA</td>
<td>Gene enrichment analysis</td>
</tr>
<tr>
<td>Glut1</td>
<td>Glucose Transporter 1</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papillomavirus</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemical</td>
</tr>
<tr>
<td>INV</td>
<td>Inversion</td>
</tr>
<tr>
<td>L Region</td>
<td>Late Region</td>
</tr>
<tr>
<td>LCR</td>
<td>Long Control Region</td>
</tr>
<tr>
<td>LFC</td>
<td>Log2 fold change</td>
</tr>
<tr>
<td>MACROD2</td>
<td>Mono-ADP ribosylhydrolase 2</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
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<tr>
<td>NHEJ</td>
<td>Nonhomologous end-joining</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>PTM</td>
<td>Protein modification</td>
</tr>
<tr>
<td>QOL</td>
<td>Quality of life</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma gene</td>
</tr>
<tr>
<td>RIPa</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPPA</td>
<td>Reverse phase protein array</td>
</tr>
<tr>
<td>shMACROD2</td>
<td>shRNA MACROD2 gene knockdown</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short-hairpin RNA</td>
</tr>
<tr>
<td>siMACROD2</td>
<td>siRNA MACROD2 gene knockdown</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small-interfering RNA</td>
</tr>
<tr>
<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>STI</td>
<td>Sexual transmitted infection</td>
</tr>
<tr>
<td>STR</td>
<td>Short tandem repeat</td>
</tr>
<tr>
<td>SV</td>
<td>Structural variants</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline and 1% tween</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TLM</td>
<td>Transoral Laser Microsurgery</td>
</tr>
<tr>
<td>TORS</td>
<td>Transoral Robotic Surgery</td>
</tr>
<tr>
<td>TRA</td>
<td>Translocation</td>
</tr>
<tr>
<td>VST</td>
<td>Variance stabilization transformation</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole Genome Sequencing</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>Phosphorylated histone variant H2AX at the Ser-139 residue</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Overview

The central objective of this thesis is to uncover the molecular basis of treatment resistance in human papillomavirus (HPV) associated head and neck squamous cell carcinoma (HNSCC). Specifically, we sought to explore MACROD2 as a potentially useful biomarker for treatment resistance in HPV+ HNSCC patients experiencing tumour recurrence after concurrent chemotherapy and radiation. In this introductory chapter, details of HNSCC, HPV infection, current treatment options, and predictive biomarkers for treatment resistance are discussed. Further, a review of the current literature on MACROD2 was completed to explore how it may be related to cancer progression and an aggressive phenotype in HPV+ HNSCC. Finally, the hypothesis is discussed.

1.2 Cancer

Cancer is a disease caused by the dysregulation of normal cell growth, cell differentiation, and tissue organization resulting in aberrant cell survival\(^1\). Cancer driven tumours can arise in almost any human tissue resulting in carcinomas (in epithelial cells), sarcomas (in supporting tissues), leukemias (in blood), and lymphomas (in lymphocytes)\(^1\). Of these, carcinomas represent approximately 90% of cancer cases\(^1\). In approximately ¾ of the earth’s population, cancer is either the first or second cause of death in adults aged 30-69\(^2\).

In 2000, six hallmarks of cancer were proposed as the main sources of cancer development and metastasis (Fig. 1)\(^3\). The 6 hallmarks of cancer are: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing or accessing vasculature, and activating invasion and metastasis\(^3\). Over a decade later, the authors suggested 4 other significant mechanisms of cancer development – including two enabling characteristics and two emerging hallmarks – that contribute to cancer acquisition and transformation\(^4\). The emerging hallmarks include deregulating cellular metabolism and avoiding
immune destruction⁴. All of these hallmarks of cancer are supported by a further two enabling mechanisms which drive the development of disease. The first enabling characteristic is genomic instability and mutation, where the accumulation of gene mutations in a cell provide a selective advantage for its growth⁴. The other enabling factor, tumour-promoting inflammation, suggests tumours contain immune cells that promote tumour progression and survival by activating an inflammatory state⁴. Overall, cancer is a devastating disease that functions by perturbing normal cell growth patterns in favor of tumour survival, proliferation, and dissemination⁵.
Figure 1: The Hallmarks of Cancer
The various hallmarks of cancer postulated by Hanahan and Weinberg as the key mechanisms leading to cancer development and progression. The current paradigm consists of 8 hallmarks and 2 enabling characteristics of cancer which provide a foundation for our current understanding of oncobiology. Adapted from Hanahan, *Cancer Discov*, 2022.
1.3 Head and Neck Cancer

Head and neck cancers consist of a heterogenous group of malignancies that arise from the mucosal epithelium of the upper aerodigestive tract. Areas include various anatomical sites in the oral cavity, pharynx, larynx, nasal cavity, paranasal sinuses, and salivary glands. The majority of head and neck cancers however, arise in the oral cavity, pharynx, and larynx (Fig. 2). Together they are referred to as head and neck squamous cell carcinomas (HNSCC). In 2018, HNSCC was responsible for 888 000 new diagnoses globally and this number is expected to rise in the future. Specifically, HNSCC cases of the oropharynx caused by human papillomavirus (HPV) infection increased from 16.3% to 70% between the periods of 1984-1989 and 2000-2004, respectively. As a result, HNSCC is currently the sixth most common cancer worldwide, and cases in the oropharynx are now the most common HPV-associated cancer surpassing cervical cancer. Over the past three decades, the survival rate for HNSCC has improved, due in part to the increased incidence of HPV related disease which has a markedly better prognosis than HPV- HNSCC. Between the 1980’s and the early 2000’s, the 5-year survival rate for all HNSCC cases improved from 53% to 66% respectively. Further, a randomized control trial revealed the 3-year survival rate of HPV+ HNSCC patients was 82% compared to 57% in HPV- HNSCC patients. Consequently, HNSCC is a devasting disease with increased incidence posing a risk for current and future populations.
Figure 2: Anatomical site of Head and Neck Cancer
Cancer arises in the mucosal epithelium of the oral cavity (lips, tongue, hard palate, gums, and floor of mouth), the pharynx (nasopharynx, oropharynx, hypopharynx, soft palate, uvula, base of tongue, tonsils, tonsillar pillars, and oropharyngeal wall), the larynx (supraglottic, infraglottic, and vocal cords), the salivary glands, the nasal cavity, and the paranasal sinuses. Adapted from Johnson DE, et al., Nat Rev Dis Prim, 2020.
1.3.1 HNSCC Risk Factors

The most common risk factors for developing HNSCC are associated with environmental insults such as excessive tobacco and alcohol use\(^{13}\). Other documented causes of HNSCC include areca nut chewing – prominent in south-central Asia\(^{14}\) – as well as diets low in fruits and vegetables, previous exposure to radiation, and genetic susceptibility\(^{15}\). In recent years however, there has been an emerging subset of HNSCC cases associated with the sexually transmitted disease HPV\(^{13}\). Prevalence of HPV-associated HNSCC has increased in both Canada\(^{16}\) and internationally\(^2\). As a result, multiple sexual partners and sexual activity including oral-genital contact, are now the primary risk factors for HNSCC\(^7\). These HPV-associated HNSCC are also more common amongst men of a higher socioeconomic status\(^9\).

1.3.2 HNSCC Screening and Diagnosis

Unfortunately, HNSCC can go undiagnosed until later stages due to unambiguous clinical presentation and a lack of screening protocols\(^{15}\). Where women at risk for cervical cancers associated with HPV infection can be screened by a pap smear or high-risk HPV DNA testing, similar screening methods are not currently possible for HNSCC\(^7\). This is mainly due to the fact that the oropharyngeal mucosa is harder to assess histologically for dysplastic lesions with the potential to progress to carcinomas, particularly within the lymphatic tissue in the palatine and lingual tonsils where the majority of these cancers arise\(^7\). Further, detection of oral HPV infection doesn’t necessarily confirm a potential HNSCC case, as most patients will clear the infection by the immune system\(^7\).

Generally, HNSCC malignancies arise in adults with a median age of 60 and are more common amongst men\(^{14}\). Depending on the primary site and type, HNSCC often presents as a neck mass along with mouth sores or ulcers \(^6\). Patients often seek intervention with their primary physician or dentist after experiencing symptoms of dysphagia (difficulty eating), dysarthria (difficulty speaking), otalgia (ear pain), voice hoarseness and mouth pain\(^{17}\). A large proportion of patients will present with small primary tumour stage T1 or T2\(^9\). Diagnoses of HNSCC differs between countries, but generally involves imaging (CT, MRI or PET CT) and a biopsy of the primary tumour site or an involved neck lymph node\(^{15}\). Confirmation of disease is followed by
further testing to elucidate if the disease is related to HPV infection, which is determined by immunohistochemical (IHC) staining for p16, HPV specific detection by PCR, in situ hybridization, or other means\textsuperscript{18}.

1.4 HPV

The unique clinical and molecular characteristics that define HPV-associated HNSCC has now differentiated head and neck cancers into two subsets: HPV+ and HPV-.\textsuperscript{9} HPV+ HNSCC have distinct mutation, expression, and immune profiles compared to HPV- negative disease\textsuperscript{6}. Furthermore, HPV+ HNSCC diagnoses have increased in recent years, and is expected to continue rising due to increasing rates of oral HPV infection, low vaccination rates and the delay between infection and presentation\textsuperscript{16}. Consequently, it is important to discuss HPV in terms of its prevalence, oncogenic potential, and current worldwide prevention measures.

1.4.1 HPV Epidemiology

HPV is a sexually transmitted infection (STI) with ubiquitous transmission throughout society. Approximately 75-80% of sexually active adults will have an HPV infection at some point in their life, making HPV the most common STI worldwide\textsuperscript{19}. HPV transmission is commonly the result of sexual contact, where oral-genital contact leads to HPV infection of the oral cavity\textsuperscript{7}. The prevalence of genital HPV infection is similar between men and women\textsuperscript{20}. Interestingly, men have higher rates of oropharyngeal HPV infection than women\textsuperscript{7}, which correlates with increased incidence of men diagnosed with HPV+ HNSCC\textsuperscript{6}. The reason for the increased rates of HPV infection in men is not fully elucidated, however, various causes have been postulated. Primarily, men report greater sexual partners than women – which is a primary risk factor for HNSCC\textsuperscript{9}. Secondly, it is suggested that female genital mucosa is more susceptible to HPV infection than male genitalia and therefore, women contain a higher viral load of HPV\textsuperscript{7,20}. As a result, men performing oral sex on female genitalia have greater exposure to HPV\textsuperscript{7}. Lastly, it is hypothesized that women have a greater immune response after primary
genital HPV infection than men, therefore, after oral re-infection, women are able to clear the virus faster\textsuperscript{20}.

1.4.2 HPV Genome

HPV is a small non-enveloped DNA virus consisting of a double-stranded circular genome of approximately 8 kb\textsuperscript{21}. The viral genome is divided into three main segments: Long Control Region (LCR), Early Region (E), and Late Region (L)\textsuperscript{21}. The LCR functions in viral gene expression, while the E and L genes make up approximately 90\% of the viral genome and perform most of the biological functions\textsuperscript{21}. The E region encodes 6 viral proteins – E1, E2, E4, E5, E6, and E7\textsuperscript{21}. This region functions to regulate viral transcription and replication\textsuperscript{21}. The L region is composed of two genes, L1 and L2, which are responsible for encoding capsid proteins and packaging DNA into virions\textsuperscript{21,22}. The most important genes of the HPV genome include E6 and E7 which are defined as oncogenes primarily involved in tissue transformation and inducing genomic instability\textsuperscript{21,23}. Together, E6 and E7 oncoproteins are responsible for viral entry into the host epithelium and immortalization of human keratinocytes\textsuperscript{9,23}. The E6 oncoprotein targets and degrades p53 and the E7 oncoprotein binds and degrades various members of the retinoblastoma (Rb) gene family\textsuperscript{21}. By disrupting cell cycle checkpoints p53 and pRB, HPV infected cells are able to maintain proliferative capacity leading to DNA damage and the accumulation of gene mutations\textsuperscript{21,23}. Through the action of tissue transformation by E6 and E7 oncogenes and the subsequent tissue mutation accumulation, this ultimately leads to cancer progression\textsuperscript{23}.

1.4.3 HPV Oncogenesis

Papillomaviruses such as HPV are infectious agents that infect the basal layer of human keratinocytes resulting in benign warts or tumours (known as papillomas)\textsuperscript{21,24}. The virus can infect various mucosal epithelial tissue including those found in the cervix, anus, penis, vulva, vagina, and oral cavity\textsuperscript{7}. After HPV infects its host, the HPV genome can either integrate into the host genome, reside episomally in the cell, or as human-viral episomal hybrids\textsuperscript{25}. Integrated HPV’s have increased stability and growth advantages compared to those that remain
Researchers found HNSCC patients with integrated viral DNA have shorter overall survival than patients whose HPV has not integrated into the genome.

Of the over one-hundred HPV types, there are only a small subset of high-risk types associated with HNSCC development and progression. These include HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-68, and HPV-73, and HPV-82. Each of these high-risk HPV types have the capacity to encode oncoproteins E6 and E7 leading to carcinogenesis. However, HPV-16 is the most common cause of HNSCC, being responsible for approximately 90% of all oropharyngeal HNSCC.

HPV resides in a latent life cycle, meaning viral proliferation ceases yet the viral genome can continue to replicate along with its host. Therefore, HPV infections may persist for years before causing a productive infection. People with HPV infections that persist and do not clear by the immune system have a higher chance of developing HPV-associated cancers. Approximately 70-90% of HPV infections are cleared by the immune system making those with delayed viral clearance more susceptible to carcinogenesis.

1.4.4 HPV Vaccines

The introduction of HPV vaccines began in 2006 when the Gardasil® HPV vaccine was approved by the FDA to protect against 4 HPV types: HPV-6, HPV-11, HPV-16, and HPV-18. Since then, three other HPV vaccines have been developed including Cervarix (targeting HPV-16 and HPV-18), Gardasil®9 (targeting HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-45, HPV-52, and HPV-58), and Cecolin® (targeting HPV-16 and HPV-18). These vaccines are designed against the viral capsid L1 which is expressed during initial HPV infection and are safe and effective at mitigating HPV infection.

HPV vaccination was recommended by The Advisory Committee on Immunization Practices for females aged 13-26 to prevent initial infections in 2006. By 2011, vaccination was also recommended for boys age 11-26. An estimated 70-90% of all HPV-associated cancers are preventable by prophylactic HPV vaccination. Although vaccines do protect against the initial infection of HPV, it does not protect the previously infected population and their capacity to
develop cancer due to the long latency period associated with HPV+ HNSCC. As a result, long term results on vaccine efficacy in HNSCC may not be available for multiple years as there are a large number of individuals with latent HPV infections who will later develop cancer. Nonetheless, researchers found a decrease in HPV infection prevalence by 38% between 2009-2010 and 2015-2016 in men in the US. Interestingly, this decrease was associated with vaccine-type HPV’s, suggesting herd protection from increased female vaccination. Herd immunity against HPV has not yet been established however, as the current global vaccination rate according to the World Health Organization (WHO) is only 29-60% and herd protection is reached at approximately 80%.

Currently in Canada, Gardasil®9 is being administered on a two or three-dose regimen to protect against 7 high-risk carcinogenic HPV types and 2 low-risk HPV’s causing genital warts. Interestingly, vaccination hesitancy is higher amongst men despite HPV-associated HNSCC being one of the fastest rising cancers today in men. This ideology is the result of inadequate public health education on HPV+ HNSCC and the notion that vaccination of girls will be protection enough against HPV infection. However, vaccination of both males and females is optimal at nearing herd immunity and protecting against HPV-infections and their associated carcinogenic sequelae.

1.5 Treatment

The most common treatment for HPV+ HNSCC is concurrent chemotherapy and radiation. The chemotherapeutic agent used for HNSCC is the platinum-based drug cisplatin which is typically administered at a dose of 100 mg/m² every 3 weeks. Radiation therapy administration involves 2 Gy fractions 5 days a week for a total dose of 66-70 Gy. Although chemoradiation is the standard treatment for local/locoregional disease, the treatment regime can differ depending on the primary site and stage, HPV status, treatment response, comorbidities, and patient preferences. For when patients fail standard treatment, alternate treatment options include three targeted therapies: cetuximab (targeting epidermal growth factor receptor (EGFR)), pembrolizumab, and nivolumab (both targeting programmed
cell death protein 1 (PD-1))\(^6\). Cetuximab is administered to HPV-negative HNSCC patients and those who are cisplatin resistant as a radiation sensitizer\(^6\). Nonetheless, two clinical trials have shown cetuximab is less effective as a substitute for cisplatin in HPV+ HNSCC patients\(^{31,32}\). Additionally, pembrolizumab is used in patients with unresectable or metastatic disease\(^6\). Pembrolizumab and nivolumab can also be used for HNSCC patients expressing the PD-L1 marker who exhibit tumour recurrence and metastasis or do not respond well to cisplatin\(^6,33\). However, various clinical trials integrating immunotherapy with chemoradiation have all failed at proving improved outcomes\(^{34,35}\).

Primary surgery is an option for selected patients, particularly with the development of transoral robotic surgery (TORS) and transoral laser microsurgery (TLM) techniques\(^9\). Studies on TORS efficacy as a primary treatment have shown similar quality of life (QOL) outcomes compared to primary radiation, however, varying results have been returned regarding using TORS for treatment de-escalation efforts as they carry a risk of life threatening complications\(^{33,36-39}\). Nonetheless, TORS remains a minimally invasive option for patients with early-stage oropharyngeal SCC or as a potential deintensification option\(^{22}\). Various clinical trials are currently underway to evaluate the efficacy of immunotherapies as treatment de-intensifiers in HNSCC\(^{15}\). Therapies such as abelumab, atezolizumab, and pembrolizumab are being studied however, no definitive conclusions have yet been reached when compared to the standard of care\(^{15}\). Other novel therapeutic options that are the focus for various researchers and clinical trialists include immunotherapeutic vaccines, cell based approaches, and immunomodulators\(^{22}\).

1.5.1 Treatment Toxicities

Radiation with concurrent high-dose chemotherapy carries significant toxicities for patients. Treatment associated toxicities include swallowing/voice issues, oral dysfunction, nausea, ototoxicity, and nephrotoxicity\(^{6,33}\). Damage can persist for years after radiation as approximately 70% of HNSCC patients report speech impairment even 10 years post-intervention\(^6\). Other severe late toxicities include feeding tube dependance and decreased overall quality of life (QOL)\(^{40}\). To note, patients experiencing more severe toxicities were commonly older, had an elevated T-stage, and cancer arising in the larynx or pharynx\(^{40}\). Interestingly, HPV+
HNSCC patients respond better to standard therapy than those with HPV- HNSCC, suggesting HPV+ patients may undergo treatment de-escalation in order to optimize both survival outcomes and QOL measures\(^{30}\). As the standard of care paradigm was established in a HPV-negative population, research into reducing the adverse effects associated with treatment will save HPV+ patients from toxicities in the long term\(^{30}\).

Treatment de-escalation efforts have put an emphasis on surgical management, reduced radiation dosing, and alternate systemic therapies such as immunotherapy\(^{15,41}\). To date, numerous clinical trials have explored the therapeutic benefit of treatment deintensification in HNSCC\(^{9,40}\). Decreasing radiation exposure is key in de-escalation strategies as radiation is the main driver of severe toxicities\(^{33}\). Studies have shown reducing adjuvant radiation dosages to 30-36 Gy improves swallowing capacity and QOL while maintaining progression-free survival (PFS) and overall survival (OS)\(^{42}\). To date, clinical trials exploring immunotherapy efficacy in HNSCC have not confirmed their effectiveness at reducing toxicities while maintaining high survivorship\(^{6}\). Therefore, there continues to be a need to identify alternative therapeutic options for patients who respond well to therapy but want to decrease the toxicities associated with chemoradiation. However, no definitive clinical trial to date has proven a safe de-escalation strategy and concurrent cisplatin and radiation remains the standard of care.

1.5.2 Treatment Resistance

Amongst HNSCC patients, tumour recurrence and metastasis can often persist after treatment\(^{15}\). Of HPV+ HNSCC, 10-25% of patients exhibit an aggressive phenotype associated with treatment resistance, recurrent disease, and distant metastasis\(^{9,41}\). Frequently, these patients are not eligible for adjuvant surgery or radiotherapy and must rely on systemic therapies alone to treat the disease\(^{15}\). Therefore, there is an unmet need to identify molecular markers successful at stratifying patients based on tumour risk so patients at high risk can have their treatment intensified, while those that are treatment sensitive can undergo treatment de-escalation.
1.6 HNSCC Biomarkers

Research on biomarkers in HNSCC have the capacity to prognosticate premalignant tumours, uncover therapeutic targets, predict treatment response, and stratify patients for treatment de-escalation⁶,⁹. Although cancer progression can be understood as a multifactorial event, there is considerable evidence that suggests specific somatic tumour mutations with oncogenic potential drive the development of numerous malignancies, including those with a propensity for tumour recurrence and metastasis⁴³. Therefore, exploration of DNA and transcriptomic changes seen during tumour recurrence in HPV+ HNSCC can begin to explain how genetic aberrations confer resistance to chemoradiation treatment.

1.6.1 Biomarkers for Treatment Resistance in HPV+ HNSCC

Through exploration of the mutational landscape of HNSCC, various biomarkers have been identified as key regulators of HNSCC tumour biology that may be utilized as stratification biomarkers for tumour resistance in the HPV+ subtype⁴¹. One meta-analysis found markers for tumour hypoxia and poor immunological response are predictive of an aggressive phenotype in HPV+ HNSCC⁴¹. Other studies found that recurrent HPV+ HNSCC tumours had mutational landscapes similar to primary HPV- tumours which typically have worse prognosis⁴⁴,⁴⁵. Unfortunately, most of the studies attempting to characterize genes associated with treatment resistance in HPV+ HNSCC were limited by their sample size and a lack of paired pre- and post-treatment sample sets⁴⁴-⁴⁷. Consequently, due to the lack of significant findings from genomic discovery studies, no novel biomarkers for treatment stratification have been approved for HNSCC⁴⁴.

Considering the paucity of molecular profiling in treatment resistant HPV+ HNSCC, our laboratory sought to elucidate genes potentially associated with tumour recurrence and metastasis. Our laboratory has prospectively collected pre-treatment and recurrent tumour samples as well as matched blood, referred to as ‘triplet sets’, from an expanding cohort of HPV+ HNSCC patients⁴⁸. Comparison of recurrent samples with matched pre-treatment tumour samples provides an opportunity to identify somatic tumour mutations highly associated with recurrence. With this resource, whole genome sequencing (WGS) and transcriptomic profiling
was performed to uncover target genes putatively involved in resistance to chemoradiation. From the WGS results, significant structural variations including gene duplications, deletions, inversions, and translocations were identified in genic and regulatory regions (Fig. 3). Specifically, the most frequently deleted gene in the recurrent dataset, LRP1B, was deleted in approximately 36% of tumours and was previously investigated in our laboratory as a mutation associated with treatment 48. Other mutated genes with copy number losses in the recurrent dataset include FARP2, CSMD1, TRAF3, and MACROD2. One gene of interest, MACROD2, was deleted in 17% (3/18) of recurrent tumours and has been implicated as a common mutation associated with tumour recurrence in other cancers 49–52. As a result, we sought to complete further downstream functional validation studies to confirm MACROD2’s involvement in treatment resistance in HPV+ HNSCC.
Figure 3: Whole Genome Sequencing (WGS) results uncover significant mutations associated with treatment resistance in HPV+ HNSCC

WGS from 18 HPV+ HNSCC patients was completed from both primary and recurrent tumours matched to normal blood DNA. Structural variations were located in both genic and regulatory regions including duplications (DUP), deletions (DEL), inversions (INV), and translocations (TRA). One gene in particular, MACROD2, seen significant gene deletions in tumours samples from 3/18 patients (approximately 17% of patients) all of which were treatment failures (100%). Adapted from Shaikh MH, et al. Under Revision in Oral Oncol, 2023.
1.6.2 Mono-ADP ribosylhydrolase 2 (MACROD2)

1.6.2.1 Member of the PARP Family

Mono-adenosine diphosphate (ADP) ribosylhydrolase 2 (MACROD2) is a highly conserved enzymatic macrodomain, a part of the poly(ADP-ribose) polymerase (PARP) family of proteins\textsuperscript{53}. The PARP family has a wide variety of biological functions\textsuperscript{53}. In particular, PARP can localize to DNA lesions and use nicotinamide adenine dinucleotide (NAD+) to recruit DNA repair factors\textsuperscript{54}. Repairing single and double strand breaks (SSB/DSB) by PARP involves adding and removing ADP-ribose units onto substrate proteins using various enzymes\textsuperscript{54}. One of these enzymes, MACROD2, is capable of removing a single terminal ADP moiety at sites of DNA damage to assist in the repair process\textsuperscript{54}. As a result, deletion of MACROD2 may result in deregulation of the PARP signalling pathway leading to DNA damage.

MACROD2 is capable of reversible post-translation modification (PTM) in many cellular settings resulting in the regulation of various biological functions\textsuperscript{54}. For instance, ADP-ribosylation has been implicated in regulation of gene transcription, chromatin structure, metabolism, apoptosis, and cellular growth\textsuperscript{55,56}. Specifically, studies show the reversible ADP-ribosylation activity of MACROD2 enhances the glycogen synthase kinase 3 beta (GSK3β) pathway involved in regulating β-catenin accumulation and downstream Wnt signaling\textsuperscript{55}. Other studies show MACROD2 is exported from the nucleus upon DNA damage dependent on the ataxia-telangiectasia mutated (ATM) protein kinase responsible for organizing the cellular response to DNA damage\textsuperscript{57}. As a result, MACROD2 has a regulatory role in the DNA repair process – as well as other cellular mechanisms – which can result in several disease states such as neurogenerative disorders, inflammation, and cancer\textsuperscript{58}.

1.6.2.2 MACROD2 in Cancer

MACROD2, located at a cancer-specific fragile site at chromosome 20p12.1\textsuperscript{59}, has also been implicated as a key regulator of carcinogenesis in various tumour types. Specifically, copy number losses in MACROD2 have been identified in colorectal cancer suggesting a role as a novel tumour suppressor gene\textsuperscript{49}. Functional studies show that the loss of function mutation of
MACROD2 impairs DNA-damage response through regulation of the PARP signalling pathway leading to chromosome instability (CI) and cancer evolution\textsuperscript{49,60}. Functionally, MACROD2 deletions cause an increase in the mono-ADP-ribosylation of PARP1 proteins and therefore, can inhibit PARP1 transferase activity leading to an increased sensitivity to genotoxic stress caused by DNA damage\textsuperscript{49}. Dysregulation of DNA repair mechanisms and an increased susceptibility of cells to genotoxic stress ultimately leads to tumorigenesis in colorectal cancer\textsuperscript{49}. Copy number losses of MACROD2 is also associated with poor disease-free survival (DFS) in stage III colorectal cancer patients treated with adjuvant 5-fluorouracil (5-FU) chemotherapy\textsuperscript{50}. Furthermore, the loss of function mutation of MACROD2 in hepatocellular carcinoma (HCC) was associated with poor overall survival (OS), tumor recurrence, and metastasis through regulation of the GSK3β pathway\textsuperscript{51}. The inhibition of MACROD2 lead to phosphorylation of GSK3β causing upregulation of β-catenin expression and development of HCC\textsuperscript{51}. Additionally, WGS of a small panel of patients with gastric and esophageal squamous cell carcinoma (ESCC) revealed MACROD2 was deleted in approximately 14% of tumours\textsuperscript{52}. In contrast, MACROD2 was found overexpressed in metastatic breast cancer tumours conferring resistance to the antiestrogen drug tamoxifen\textsuperscript{59}. These findings are paradoxical considering copy number data from The Cancer Genome Atlas (TCGA) suggests deletions of MACROD2 is common in colorectal, stomach, cervical, esophageal, uterine, lung, liver, and thyroid cancers\textsuperscript{49}.

MACROD2 has also been found as a common site for HPV integration in cancers associated with the virus\textsuperscript{61,62} leading to loss of expression of MACROD2. In cervical cancer, MACROD2 was the most frequent site of HPV integration based on an analysis of 272 cervical cancer patients\textsuperscript{61}. Similarly, MACROD2 was the fourth most common site of HPV integration in a panel of HNSCC patients\textsuperscript{62}. Integration of a viral genome into a host genome can cause epigenetic changes resulting in mutations in the host, including deletion of a gene. Therefore, this may explain copy number losses of MACROD2 in cervical and oral tissue that may be the genesis for cancer progression.

Based on the literature and our genomic data, MACROD2 has been implicated in various cancers including those that are associated with HPV infection. It can be postulated that deletion of MACROD2 in HPV+ HNSCC may be responsible for the constitutive reversible ADP-
ribosylation of various regulatory processes, such as the PARP1 and GSK3β pathways, leading to an aggressive phenotype in tumours and resistance to treatment.

1.7 Hypothesis

Inhibition of MACROD2 drives resistance to cisplatin chemotherapy and radiation in HPV+ HNSCC.
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Chapter 2

2. MACROD2 is a biomarker of radiation resistance in HPV+ head and neck squamous cell carcinoma

2.1 Abstract

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide. Infection by human papillomavirus (HPV) has caused a rise in HNSCC cases. Unfortunately, a cohort of HPV+ HNSCC patients exhibit treatment resistance. At present, no known treatment resistant biomarkers have been identified. We completed genomic characterization of a local HPV+ HNSCC cohort and observed MACROD2 deletions in the treatment failure dataset. Functional validation revealed siRNA and shRNA MACROD2 knockdown in HNSCC cells led to elevated cell viability and colony formation. Interestingly, shMACROD2 caused no change in cisplatin sensitivity, but a significant increase in radiation resistance in HPV+ HNSCC. Transcriptomic, proteomic, and immunofluorescence studies demonstrated that MACROD2 depleted HPV+ HNSCC cells displayed elevated hypoxia and altered DNA damage response signatures suggestive of possible mechanisms of radioresistance. Further work is needed to understand how to overcome these molecular mechanisms of radiation resistance in MACROD2 deficient HPV+ HNSCC.

2.2 Introduction

Head and neck squamous cell carcinoma (HNSCC) includes a group of malignancies arising in the oral cavity, pharynx, and larynx. Together, HNSCC are responsible for an estimated 453,000 deaths worldwide every year. The risk factors associated with HNSCC have historically been associated with environmental stimulants such as excessive smoking and drinking. However, in recent years there has been an increase in HNSCC cases due to oral infection by the sexually transmitted disease human papillomavirus (HPV). As a result, HNSCC cases can now either be characterized as HPV-positive or HPV-negative. Patients of HPV-related HNSCC are typically Caucasian men with a high socioeconomic status. Interestingly, HPV-related HNSCC has improved the overall survival rate of HNSCC due in part
to patients with HPV-related disease having a better prognosis than their HPV-negative counterparts\textsuperscript{5}.

The most common treatment for HPV+ HNSCC is concurrent chemotherapy and high dose radiation\textsuperscript{6}. This treatment strategy is effective in curing the majority of patients, however, it carries significant toxicities including difficulty swallowing with a risk of permanent feeding tube dependence, hearing loss, kidney injury and carotid artery narrowing among others\textsuperscript{6,7}. As a result, there is interest in treatment de-escalation for HNSCC patients who respond well to therapy to optimize survival outcomes as well as quality of life\textsuperscript{8}. However, approximately 10-25\% of patients experience treatment failure leading to tumour recurrence and metastatic spread\textsuperscript{4}. For patients with this aggressive phenotype, little is known about the cause of their treatment resistance. Biomarkers are needed to stratify patients based on tumour risk so those responsive to treatment can undergo treatment de-intensification while those resistant to treatment can consider alternate therapies.

Several studies have attempted to characterize the molecular landscape underlying HPV+ HNSCC carcinogenesis and identify biomarkers of treatment outcome\textsuperscript{9-12}. While some promising mutations (TP53, NOTCH1) and transcriptomic signatures have been identified\textsuperscript{9,12,13} most require further validation before making it to the clinic. Therefore, there is an unmet need to uncover the molecular basis of treatment resistance in HPV+ HNSCC. Our lab sought out to identify treatment resistant genes by prospectively collecting pre-treatment and recurrent tumour samples as well as matched blood from a cohort of HPV+ HNSCC patients. Whole genome sequencing (WGS) identified a number of genes that were enriched or occurred exclusively in patients that failed treatment\textsuperscript{14}. Of these, MACROD2 was found to be deleted in 17\% of recurrent tumours. Interestingly, MACROD2 has been implicated in various cancers as novel tumour suppressor gene\textsuperscript{15-17} and as a common site for HPV integration in both cervical and HNSCC\textsuperscript{18,19}. These observations suggest that genomic aberrations in MACROD2 may play a key role in HPV+ HNSCC resistance to chemoradiation therapy. In this study, in vitro functional validation and mechanism of action studies were completed to uncover the importance of MACROD2 loss of function mutations in HPV+ HNSCC.
2.3 Materials and Methods

2.3.1 Cell Culture

Cell lines were cultured and maintained for in vitro experimentation as described in Supp. Table 1. This includes HPV+ HNSCC cell lines, HPV-negative HNSCC cell lines, the human embryonic kidney cell line HEK293T for lentivirus production, as well as breast cancer cell line MCF7 used as a control. All cell lines were cultured in DMEM/F12 media (WISENT inc., Quebec, Canada) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic. Cells were maintained in a 37°C and 5% CO₂ incubator. The identity of all cell lines was confirmed by short tandem repeat (STR) profiling (The Centre for Applied Genomics, Genetic Analysis Facility, Toronto) shown in Supp. Table 2.

2.3.2 MACROD2 inhibition

2.3.2.1 Small-interfering RNA (siRNA)

To introduce transient MACROD2 knockdown by siRNA, 4 predesigned MACROD2 guides pooled into a single siRNA (Dharmacon, Waltham, MA, USA, ON-TARGETplus Human MACROD2 siRNA SMARTPool, Catalog ID:L-015258-00-0005), a scramble control siRNA (Invitrogen™, Silencer™ Select Negative Control No. 1 siRNA, Catalog ID:4390844), and Lipofectamine® RNAiMAX reagent (Invitrogen™, Catalog ID:13778150) were used to transfect cells. Prior to each functional experiment, cells were seeded at appropriate densities and MACROD2 was knocked down according to the Lipofectamine® RNAiMAX transfection protocol in OPTI-MEM® (Invitrogen™, Catalog ID: 31985070).

2.3.2.2 Short-hairpin RNA (shRNA)

2.3.2.2.1 shRNA Plasmid Amplification

For generation of stable MACROD2 knockdown cell lines, an shRNA plasmid kit containing 4 predesigned shRNA plasmids (A-D) against MACROD2, and one plasmid control were used (Origene, Catalogue ID: TL314332). shRNA plasmid DNA for each guide was
amplified by bacterial transformation in E. coli MAX Efficiency® DH5α™ competent cells (Invitrogen, Catalogue ID: 18258012) and DNA was collected using the QIAprep® Spin Miniprep Kit (Qiagen, Catalogue ID: 27104). To ensure the plasmids were successfully amplified, DNA from the 4 MACROD2 plasmids and control plasmids was verified by Sanger Sequencing (London Regional Genomics Centre).

2.3.2.2.2 shRNA Lentiviral Packaging

shRNA plasmids were packaged into lenti-viral particles for easier transduction into cells using the Lenti-vpak packing kit (Origene, Catalogue ID: TR30037) following manufacturer’s instructions. HEK293T cells were seeded in a 6-well plate at 5x10^5 cells/well and incubated overnight at 37°C. The following day, 1 µg of shRNA plasmid DNA and 1.2 µg of packaging plasmid was diluted in Opti-MEM (Invitrogen™, Catalog ID: 31985070) and combined with TurboFectin transfection reagent (Origene, Catalogue ID: TR30037) and added to cells. Two batches of viral supernatant were then collected from each MACROD2 shRNA (A-D) and control shRNA and filtered through a 0.45 µM filter for storage.

2.3.2.2.3 shRNA Lentivirus Titer Calculation

The viral titer of the generated lenti-viral particles for each shRNA MACROD2 guides (A-D) and the shRNA control was calculated using the qPCR lentivirus titer kit (abmgood, Catalogue ID: LV900). qPCR was performed in technical duplicate in 20 µl reactions containing: 10 µl BlasTaq™ qPCR Titer MasterMix, 2 µl of the provided Primer Mix, and 2 µl of lentiviral sample. Results of the qPCR were compared to a standard curve generated by five 10-fold serial dilutions of the standard control DNA provided. Thermal cycling conditions were: 95°C for 10 minutes followed by 30 cycles of 95°C for 15 seconds, 60°C for 1 seconds. Calculations of lenti-viral titers were done using the qPCR Lentivirus Titer Calculation Form on the abmgood website.
2.3.2.2.4  shRNA Transduction

To perform the shRNA transduction, cells were seeded in a 24-well plate at 5x10^4 cells/well and incubated overnight at 37°C. The following day, cells were transduced with shRNA MACROD2 lentivirus and the shRNA control (Origene, Catalogue ID: TL314332) at a Multiplicity of Infection (MOI) of 0.3, 1, 10, 30, and 50 depending on the guide and lentiviral titer. In each experiment, a well was left un-transduced as a control for puromycin selection. Polybrene was added to each well at a concentration of 8 µg/ml and cells were incubated for 18-20 hours at 37°C. Puromycin selection media was then applied for 72 hours to isolate cells containing the shRNA plasmid guides. Finally, cells were expanded for further downstream in vitro studies.

2.3.3  Validation of MACROD2 knockdown

To validate siMACROD2 knockdown by both qPCR and western blot, cells were seeded overnight in 6 well dishes at 5x10^5 cells/well. The following day, cells were transfected with either the siMACROD2 pooled guide or the siControl guide and incubated. Seventy-two hours post transfection, a cell pellet was collected for either RNA extraction or protein extraction.

2.3.3.1  Quantitative Polymerase Chain Reaction (qPCR)

MACROD2 expression was determined by collecting total RNA from cell lines using the Monarch® Total RNA Miniprep Kit (NEB, Catalogue ID: T2010S). RNA was reverse transcribed to complementary DNA (cDNA) using the LunaScript® RT SuperMix (NEB, Catalogue ID: E3010L). qPCR was performed in technical and biological triplicates in 20 µl reactions containing: 250 nM of both forward and reverse primers, 10 µl of Luna® Universal qPCR Master Mix (NEB, Catalogue ID: M3003L), and 40 ng or 400 ng of cDNA (for GAPDH or MACROD2 measurement, respectively). Thermal cycling conditions were: 95°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and then a plate read. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene, the normalized gene expression fold change was calculated with the 2^−ΔΔCt method. The following primers
were synthesized by IDT (5’-3’): MACROD2 (F – 5’ TCATCTCTGAAGCTCGTGAAG 3’, R – 5’ CTTAATGGTGTGGGCAATG 3’) and GAPDH (F – 5’ GTCTCCTCTGACTTCAACAGCG 3’, R – 5’ ACCACCCCTGTGCTGAGCCAA 3’).

2.3.3.2 Immunoblotting

For measurement of MACROD2 protein expression, whole cell lysates were collected in Radioimmunoprecipitation Assay (RIPA) lysis buffer. Primarily, RIPA buffer + PMSF was added to cell lysates and placed on ice for 20 minutes, with intermittent vortexing every 5 minutes. Samples were then centrifuged for 20 minutes at 4°C at 14,000 rpm and supernatant collected in separate microcentrifuge tubes. Protein concentration was then quantified by Bradford assay (Biorad, Catalogue ID: 5000006). 30 µg of protein were separated on a NuPAGE™ 4 to 12%, Bis-Tris polyacrylamide gel (Invitrogen™, Catalogue ID: NP0321BOX) and transferred to a PVDF membrane (GE Healthcare). Membranes were then blocked with 5% milk blocking buffer, washed with Tris Buffered Saline containing 1% Tween (TBST), and incubated with an anti-MACROD2 primary antibody (1:1000 in 5% milk, generous gift from Dr. Gyula Timinszky, Institute of Genetics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary) overnight at 4°C. The following day, a goat anti-rabbit secondary antibody (1:5000) was applied for 1 hour and antibody binding was detected in 1-2 ml enhanced chemiluminescence (ECL) detection Immobilon® Forte Western HRP Substrate (Millipore, Catalogue ID: WBLUF0100). Similarly, membranes were blotted with anti-α-tubulin as a loading control (Cell Signaling Technology, #2125, 1:1000). Images were obtained using a Bio-Rad Gel Doc™ XR+ and Image Lab™ software.

2.3.4 Functional studies of MACROD2 knockdown

2.3.4.1 Discrepancies between siRNA and shRNA functional experiments

Due to the transient nature of siRNA biology, an siRNA transfection preceded each functional experiment (cell viability, colony forming, cell migration, cisplatin sensitivity, and
radiation sensitivity). As a result, there is a slight discrepancy between how the siRNA and the shRNA functional studies were carried out. Mainly, in siRNA studies the cells of interest were plated at appropriate densities, and the following day cells were transfected with either siMACROD2 or siControl. For cell viability, cells were read with PrestoBlue® Reagent (Invitrogen™, MA, USA, Catalogue ID: A13262) 72 hours after siRNA transfection. For colony forming, cell migration, and radiation sensitivity experiments, cells were siRNA transfected in 6-well plates and then replated 24 hours later according to each respective protocol. For cisplatin sensitivity, transfected cells were incubated for 48 hours, then treated with varying doses of cisplatin (40 – 0.08 μM) and incubated for a further 48 hours before a plate read with PrestoBlue® Reagent (Invitrogen™, MA, USA, Catalogue ID: A13262).

2.3.4.2 Cell Viability Assay

Cells were seeded in a 96-well plate at 5000 cells/well and incubated overnight at 37°C. After 72 hours of incubation, PrestoBlue® Reagent (Invitrogen™, MA, USA, Catalogue ID: A13262) was added to cells and left to incubate for 1 hour at which point the number of viable cells was determined by a Synergy™ H4 Hybrid microplate reader (BioTek, VT, USA) with 560 nm excitation and 590 nm emission wavelengths. Results were graphed and statistical analysis performed using two-tailed t-test in Prism® 9 GraphPad Software.

2.3.4.3 Colony Forming Assay

Cells were seeded at low density (500-1000 cells/well) and incubated at 37°C for 7-14 days or until visible colonies were formed. Plates were then washed with PBS, fixed with 10% neutral buffered formalin (NBF), and stained with 1% crystal violet (Fisher Scientific, PA, USA). The stain was rinsed away with water and plates left to air-dry at which point they were scanned by the Bio-Rad Gel Doc™ XR+ and imaged using Image Lab™ software. Colonies with greater than 50 cells/colony were counted using Fiji software. Results were graphed and statistical analysis performed using two-tailed t-test in Prism® 9 GraphPad Software.
2.3.4.4 Migration Assay

Cells were prepared in serum free media and seeded at $1 \times 10^4$ cells/well in migration trays provided from the CytoSelect™ Migration and Invasion Assay Kit in 96-well format (Cell Biolabs, CA, USA, Catalogue ID: CBA-106). Migration trays containing cells were inserted into feeder trays containing complete culture media (10% FBS) and cells were allowed to migrate over a 24-hour period at 37°C. Cells were then lysed and quantified using CyQuant® GR fluorescent dye and the number of migratory cells was determined by a Synergy™ H4 Hybrid microplate reader (BioTek, VT, USA) with 480 nm excitation and 520 nm emission wavelengths. Results were graphed and statistical analysis performed using two-tailed t-test in Prism® 9 GraphPad Software.

2.3.4.5 Cisplatin Sensitivity Assay

Cells were seeded in a 96-well plate at 5000 cells/well and incubated overnight at 37°C. Cells were then treated with a range of cisplatin doses (40 – 0.08 μM) and incubated for 72 hours. PrestoBlue® Reagent (Invitrogen™, Catalogue ID: A13262) was then added to cells and left to incubate for 1 hour at which point the number of viable cells was determined by a Synergy™ H4 Hybrid microplate reader (BioTek) with 560 nm excitation and 590 nm emission wavelengths. Cisplatin half maximal inhibitory concentration (IC50) values for shControl and shMACROD2 dose-response curves were calculated in Prism® 9 GraphPad Software. Averages were calculated from 3 biological and 3 technical replicates.

2.3.4.6 Radiation Sensitivity Assay

Cells were seeded in 6-well dishes at varying densities (250 – 2000 cells/well). After 1 hour, plates were either left untreated or irradiated at a range of doses: 250 cell/well plate received 0 Gy radiation, 500 cell/well plate received 2 Gy radiation, 1000 cell/well plate received 4 Gy radiation, and 2000 cell/well plate received 6 Gy radiation. Cells were then incubated at 37°C for approximately 7-21 days. Once visible colonies were formed, plates were washed with PBS, fixed with 10% neutral buffered formalin (NBF), and stained with 1% crystal violet (Fisher
Scientific, PA, USA). The stain was rinsed away with water and plates left to air dry at which point they were scanned by Bio-Rad Gel Doc™ XR+ and imaged using Image Lab™ software. Colonies with greater than 50 cells/colony were counted using Fiji software. Linear-quadratic cell survival curves were generated using the ‘CFAssay’ package (v. 1.32.0) and statistical analysis was performed by two-way ANOVA using the ‘cfa2way’ function in R software (v. 4.2.2).

2.3.5 Mechanistic studies of MACROD2 knockdown

2.3.5.1 RNA Sequencing

RNA was collected from shControl and shMACROD2 cells in triplicate using the Monarch® Total RNA Miniprep Kit (NEB, Catalogue ID: T2010S). Samples with an optical density (OD) 260/280 ratio of ≥1.9 and a 260/230 ratio of ≥1.8 were prepared for shipment. A minimum of 1 µg of RNA was prepared in 10 µl and shipped on dry ice to GeneSeeq (Toronto, ON) for sequencing with a target read coverage of 5 million reads per sample.

FastQC (v. 0.12.1) was used to confirm the quality of the raw reads. After quality and adapter trimming using Trim Galore! (v. 0.6.7), transcript abundance was estimated using Salmon22 (v. 1.10.1). Subsequently, the resultant transcript-level quantifications were collapsed to gene-level quantifications using the ‘tximport’23 R package (v. 1.24.0). Differential expression analysis was performed using the ‘DESeq2’24 R package (v. 1.36.0). A gene was considered differentially expressed by the Wald test when the adjusted p-value ≤ 0.05 and the Log2 Fold Change (LFC) ≥ 0.5. The Benjamini-Hochberg method was employed to adjust for multiple comparisons. Volcano plots were generated using the 'Enhancedvolcano’ package (v. 1.16.0), and the enrichment analysis was performed using Metascape25. R software (v. 4.2.0) was used for all of the relevant steps.
2.3.5.2 Hypoxia Estimation

The hypoxia scores for three HPV+ HNSCC cells lines generated with MACROD2 knockdown were calculated using five validated hypoxia signatures (Buffa, Eustace, Ragnum, Sørensen, and Winter). To calculate the hypoxia score, data was normalized using variance stabilization transformation (VST) and then the geometric mean was calculated based on the VST expression value for each gene in the given signature. Statistical significance was determined using a two-tailed t-test and p-values for each gene signature were combined using the Fisher’s method in R software (v. 4.2.0).

2.3.5.3 Reverse Phase Protein Array (RPPA)

Cells from both shControl and shMACROD2 generated lines were seeded in triplicate in 6-well dishes at 5x10^5 cells/well and incubated for 24 hours at 37°C. Cells were then washed twice with 1x PBS and lysed with freshly prepared RPPA lysis buffer containing: 1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na₃VO₄, 10% glycerol, and protease and phosphatase inhibitors (Roche Applied Science Catalogue ID: 05056489001 and 04906837001, respectively). 100 ul of lysis buffer was added to each plate and incubated on ice for 20 minutes with occasional shaking every 5 minutes. Cells were collected in microcentrifuge tubes and centrifuged for 10 minutes at 14,000 rpm and then supernatant collected. Protein concentration was then determined by Bradford assay (Biorad, Catalogue ID: 5000006) and diluted to 1.5 μg/ul using RPPA lysis buffer. Samples were combined with freshly prepared 4X SDS sample buffer containing: 40% Glycerol, 8% SDS, 0.25M Tris-HCL, pH 6.8 and Beta-mercaptothion at 1/10 of the volume added just before use. Samples were then boiled for 5 minutes and stored in -80°C freezer until sample submission.

Samples were sent to MD Anderson’s Functional Proteomics RPPA Core Facility to be processed. Firstly, protein samples were serially diluted and spotted onto nitrocellulose-coated slides. The sample set was then probed with 496 validated antibodies and visualized using a DAB colorimetric reaction. Slides were scanned using a Huron TissueScope scanner and spot intensities determined using Array-Pro Analyzer software. Relative protein levels were
determined, and samples were normalized for protein loading. The resultant log2-transformed, normalized, and batch effect corrected data was analyzed using a two-tailed t-test to determine significant differences in protein abundances between shControl and shMACROD2 samples. The Benjamini-Hochberg method was employed to adjust for multiple comparisons. A protein-protein interaction network was constructed using STRING to query the interactions and Cytoscape to construct the network.

2.3.5.4 Immunofluorescence Microscopy

Cells from both shControl and shMACROD2 generated cell lines were seeded on sterilized glass coverslips in 24-well plates at 3x10^5 cell/well and incubated overnight at 37°C. Cells were irradiated at 4 Gy and incubated for 30 minutes. Cells were then washed with 1x PBS 0.1% Tween 20 (PBST) and then fixed to slides using 4% paraformaldehyde in PBS for 20 minutes. Slides were washed three times with ice-cold PBST and permeabilized with 0.25% Triton X-100 for 10 minutes. Cells were incubated with 10% donkey serum in PBST for 30 minutes and then labelled with rabbit anti-Phospho-Histone H2A.X (Ser139) (20E3) mAb (Cell Signaling Technology, Catalogue ID: 9718) for 1 hour at room temperature. Samples were then incubated for 1 hour protected from light with the Alexa Fluor-conjugated secondary antibody (Molecular Probes, Alexa 488 anti-rabbit) and counterstained using DAPI for 10 minutes. Coverslips were mounted on slides using a 10% glycerol solution, sealed, and stored in the dark at 4°C. Slides were viewed under fluorescent microscopy using the Nikon ECLIPSE Ti2 confocal microscope using 60x/1.4 NA oil immersion objective.

2.4 Results

2.4.1 Validation of MACROD2 Knockdown

To understand the baseline expression of MACROD2 across our selected HNSCC cell lines, qPCR analysis was performed to detect mRNA expression. Results show baseline MACROD2 expression varied drastically across the seven HPV+ HNSCC cell lines (Fig. 4a).
After preliminary analysis, siRNA knockdown was determined to be most effective 72 hours after transfection (Supp. Fig. 1). Therefore, siMACROD2 knockdown validation studies by qPCR and immunoblotting were completed 72 hours after gene knockdown. Fold change mRNA gene expression was quantified by qPCR where MACROD2 transcript abundance was relative to control treated cells in each cell line. Across the seven HPV+ HNSCC cell lines studied, siMACROD2 knockdown caused an average 46 ± 17% knockdown of MACROD2 (Fig. 4b, Supp. Table 3). Further, the constitutive knockdown of MACROD2 by shRNA was validated by both qPCR and western blot. Across the four HPV+ HNSCC cell lines studied, shMACROD2 knockdown caused an average 73 ± 14% knockdown of MACROD2 (Fig. 5a, Supp. Table 3). In the HPV- cell line UWO8, shRNA knockdown caused an approximate 60 ± 12% knockdown of the gene (Fig. 5a, Supp. Table 3).

Various manufacturing and distribution issues associated with procuring a primary antibody for MACROD2 from a commercial distributor led to acquisition of an antibody from another laboratory. Dr. Gyula Timinszky’s laboratory (Institute of Genetics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary) manufactured a MACROD2 antibody and provided us with an aliquot with the understanding it was both a low expressing and non-specific antibody. Previous studies on MACROD2 have also had difficulty immunoblotting for the protein, often relying on generating the antibody in-house. Consequently, siMACROD2 knockdown measured by western blotting in the seven HPV+ HNSCC cell lines (Fig. 4c) was only detected in cell lines with high MACROD2 mRNA abundance at baseline (Fig. 4a). Similarly, shMACROD2 knockdown was measured by western blot in the four HPV+ HNSCC cell lines and one HPV- HNSCC cell line (Fig. 5b). Knockdown of the gene can be detected by western blot in two HPV+ HNSCC cell lines (UWO37 and UMSCC47) while no knockdown is present in the HPV- HNSCC cell line (UWO8) (Fig. 5b). Due to the inconsistent and unreliable detection of MACROD2 by western blot in previous studies as well as our own, we primarily relied on qPCR to validate gene knockdown.
Figure 4: siRNA knockdown of MACROD2 in HPV+ HNSCC cell lines

**a.** qPCR analysis of MACROD2 expression at baseline across seven HPV+ HNSCC cell lines

**b.** siMACROD2 knockdown as confirmed by qPCR in seven HPV+ HNSCC cell lines (n = 3, average ± SD)

**c.** siMACROD2 knockdown cells show decreased MACROD2 protein expression compared to the control cells by western blot in the four HPV+ HNSCC cell lines with the highest MACROD2 mRNA expression.
Figure 5: shRNA knockdown of MACROD2 in HNSCC cell lines

a. shMACROD2 knockdown as confirmed by qPCR in four HPV+ HNSCC cell lines \((n = 3, \text{ average } \pm \text{ SD})\). b. shMACROD2 knockdown cells show decreased protein expression compared to the shControl cells by western blot in two HPV+ HNSCC cell lines (UWO37 and UMSCC47) and no knockdown in the HPV- HNSCC cell line (UWO8).
2.4.2 Functional studies of *MACROD2* knockdown in HNSCC cell lines

Several functional assays were performed on HPV+ HNSCC cell lines to assess the impact of *MACROD2* knockdown on cell aggressiveness and chemoradiation resistance. In cellular viability assays with PrestoBlue™, siMACROD2 treated cells had a significant increase in cell viability (average +18 ± 9.7% across all seven HPV+ HNSCC cell lines) compared to the siControl (Fig. 6a). In shRNA experiments, shMACROD2 caused a significant increase in cellular viability (average +32 ± 25% in three out of the four HPV+ HNSCC cell lines (UWO37, UWO23, and 93VU147T)) while shMACROD2 led to a significant decrease in cellular viability (-5 ± 1.4%) in UMSCC47 cells when compared to the control (Fig. 7a).

In colony forming assays, siMACROD2 treated cells had a significant increase in clonogenic potential (average +53% ± 38% in four of the seven HPV+ HNSCC cell lines (UPCI:SCC090, UMSCC47, UWO23, UWO37)) when compared to the siControl (Fig. 6b). shMACROD2 treated cells had a significant increase in clonogenic potential (average +104% ± 30% in two of the HPV+ HNSCC cell lines (UWO37 and UWO23)) while there was a significant decrease in colony forming (-35% ± 1.9%) in 93VU147T cells compared to the shControl (Fig. 7b).

In cell migration assays, siMACROD2 treated cells show a significant decrease in cell migration (average -32 ± 29% in four of the seven HPV+ HNSCC cell lines (UPCI:SCC154, HMS001, UWO23, 93VU147T)) when compared to the siControl (Fig. 6c). shMACROD2 inhibition led to a significant decrease in migratory cells (-40% ± 4.3%) in 93VU147T cells compared to the shControl (Fig. 7c).

In chemoradiation sensitivity assays, siMACROD2 treated cell lines had a modest increase in resistance to cisplatin chemotherapy noted by the shift in half-maximal inhibitory concentration (IC50), however, none of these relationships were found to be significant (Fig. 8a). Comparable results were found in shMACROD2 treated cell where there was no detectable change in cisplatin sensitivity when compared to the shControl in both HPV+ and HPV- HNSCC cell lines (Fig. 9a).
Figure 6: Functional studies of siMACROD2 knockdown in HPV+ HNSCC cell lines

Knockdown of MACROD2 by siRNA led to significantly **a. increased cellular viability**, **b. increased colony forming potential** in 3/7 cell lines and **c. decreased cell migration** in 4/7 cell lines. (*n* = 3, Avg ± SD, two-tailed t-test, *p* ≤ 0.05, **p** ≤ 0.01, ***p** ≤ 0.001)
Knockdown of MACROD2 by shRNA led to:

a. a significant increase in cellular viability in 3/4 of the HPV+ HNSCC cell lines and in the HPV- cell line

b. a significant increase in colony forming potential in 2/4 HPV+ cell lines and in the HPV- cell line

c. a significant decrease in cell migration in one HPV+ cell lines and in the HPV- cell line. (n = 3, Avg ± SD, two-tailed t-test, * p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001).

Figure 7: Functional studies of shMACROD2 knockdown in HNSCC cell lines
Figure 8: Chemoradiation functional studies of siMACROD2 knockdown in HPV+ HNSCC cell lines

Transient siRNA knockdown of MACROD2 led to a. a modest but statistically insignificant increase in resistance to cisplatin chemotherapy (n = 3, two-tailed t-test, p > 0.05) and b. a modest but statistically insignificant increase in radiation sensitivity (n = 3, two-way ANOVA with ‘CFAssay’ package function ‘cfa2way’ in R software (v. 4.2.2)).
Figure 9: shMACROD2 knockdown in HPV+ HNSCC cells leads to radioresistance but not cisplatin resistance
Constitutive knockdown of MACROD2 by shRNA led to a. no significant changes in cisplatin chemotherapy sensitivity (n = 3, two-tailed t-test, p > 0.05) b. but a significant increase in radiation resistance in all HPV+ HNSCC cell lines (n = 3, two-way ANOVA with ‘CFAssay’ package function ‘cfa2way’ in R software (v. 4.2.2)). No change in radioresistance in HPV-shMACROD2 treated cells (depicted in blue) suggests radioresistance related to shMACROD2 knockdown is unique to HPV+ HNSCC models (depicted in red).
In radiation sensitivity assays, siMACROD2 treated cells had a moderate increase in resistance to radiation, however, none of these relationships were found to be significant (Fig 8b). Radiation assays could not be completed in the UPCI:SCC090 cell line due it being a slow growing cell line with an inability to form countable colonies, especially after harsh radiation treatment. The lack of significance in these functional assays may be due in part to the transient nature of siRNA experiments, and the longer experimental timeline of radiation sensitivity assays. Contrastingly however, cells with shMACROD2 inhibition had a significant increase in radiation resistance in all HPV+ HNSCC when compared to control cells (Fig. 9b). Interestingly, this appears to be an HPV+ specific affect, as there was no change in radiation resistance in the HPV- cell line UWO8 (Fig. 9b).

A summary of the findings from all the functional experiments performed (cell viability, colony forming, cell migration, cisplatin sensitivity, and radiation sensitivity) following MACROD2 knockdown by both siRNA and shRNA can be found in Supp. Table 3.

2.4.3 Mechanistic studies of MACROD2 knockdown in HNSCC cell lines

Little is known about the mechanism of action of MACROD2 inhibition in HPV+ HNSCC. As a result, we completed various assays to explore how inhibition of MACROD2 may lead to radiation resistance in vitro. Primarily, RNASeq analysis was performed on a panel of 3 HPV+ HNSCC cell lines (UWO37, UMSCC47, UWO23) in control and shRNA MACROD2 models. Volcano plots present genes that are differentially expressed in control and MACROD2 knockdown in the three cell lines (Fig 10a). In UWO37, UMSCC47, and UWO23 cell lines there was a total of 396, 2149, and 259 genes that were significantly differentially expressed, respectively (Supp. Table 4). A total of 99 genes were differentially expressed in the same direction in at least two cell lines (Supp. Table 5) as depicted by the Venn diagram (Fig 10b). An enrichment analysis of these 99 genes reveals MACROD2 deficient cells exhibit an elevated hypoxia and apoptosis signature when compared to the control (Fig 10c).

As hypoxic tumour environments have been implicated in radiation resistance, we sought to compare control and MACROD2 inhibited HPV+ HNSCC cell lines by several validated
hypoxia scores. Results indicate significantly higher hypoxia scores when \textit{MACROD2} was knocked down in all three cell lines studied (Fig 11).
Figure 10: MACROD2 deficiency is associated with genes enriched in hypoxia, apoptosis, and extracellular matrix organization.

RNA sequencing was performed on a panel of 3 HPV+ HNSCC cell lines (UWO37, UMSCC47, and UWO23) in both shControl and shMACROD2 knockdown models. a. Volcano plots show differential gene expression in each cell line depicted by Log₂ fold change (LFC) vs. -log₁₀(p.adj)-value). The Benjamini-Hochberg method was employed to adjust for multiple comparisons. b. Venn diagram shows a total of 99 genes were differentially expressed in the same direction in at least two datasets. c. Enrichment analysis reveals MACROD2 inhibition in HPV+ HNSCC cells exhibit an elevated hypoxia signature.
**Figure 11:** *MACROD2* inhibition is associated with an elevated hypoxia signature in HPV+ HNSCC cell lines

Five hypoxia scores (Buffa, Eustace, Ragnum, Sørensen, and Winter) were used to assess differences in hypoxia levels in control and *MACROD2* knockdown HPV+ HNSCC cell lines. Hypoxia scores were calculated from the geometric mean of the expression of genes present in each signature. Combined p-values depict significantly increased hypoxia scores in shMACROD2 cells compared to the shControl (*n* = 3, two-tailed t-test, Fisher’s method, combined p-value < 0.05).
To examine the signalling pathways associated with MACROD2 knockdown that could contribute to radiation resistance in HPV+ HNSCC, RPPA analysis was performed on a panel of three HPV+ HNSCC cell lines (UWO37, UMSCC47, and UWO23) (Supp. Fig 2). The differential abundance of MACROD2 knockdown proteins was compared to shControl (Supp. Table 6). 99 significant differently expressed genes in the protein-protein interaction network from the RNASeq analysis were generated (Supp. Fig. 2a). Comparison between the RPPA and RNAseq data, identified a number of differentially expressed transcript/protein pairs (Supp. Fig. 2b). Based on RPPA results, UMSCC47 MACROD2 knockdown cells were noted to have higher levels of γ-H2AX, which is a marker for apoptosis and DNA double strand breaks (DSB)35 (Supp. Fig. 2b). MACROD2 has been shown to be a key regulator of the PARP pathway15, which is involved in detecting DNA double strand breaks and enacting a repair response36. Thus, we carried out immunofluorescent staining of γ-H2AX in the same panel of HPV+ HNSCC cell lines (UWO37, UMSCC47, and UWO23) after irradiation at 4 Gy. This revealed that radiation of HPV+ HNSCC cells resulted in increased γ-H2AX foci in the nuclei of cells deficient in MACROD2 when compared to control cell lines (Fig 12). No γ-H2AX staining was identified in non-radiated cells.
Figure 12: MACROD2 inhibition in HPV+ HNSCC causes an altered DNA damage response following radiation as measured by γ-H2AX foci
Representative immunofluorescence images of γ-H2AX staining in three HPV+ HNSCC cell lines (UWO37, UMSCC47, and UWO23) and compared between control and MACROD2 knockdown models. Cells were treated with 4 Gy of radiation and 30-minutes following irradiation were stained with γ-H2AX (green) and DAPI (blue). Scale bar = 100 µm.
2.5 Discussion

The identification of biomarkers of disease resistance is a clinically important research task for patient treatment stratification measures based on tumour risk. Head and neck cancers are of growing concern due to the rising number of diagnoses associated with HPV infection. However, while the prognosis is generally favourable, a portion of patients are susceptible to disease recurrence due to treatment resistance. Therefore, identification, characterization, and implementation of biomarkers of treatment resistance have the possibility to improve survival outcome and quality of life in the HPV+ HNSCC patient population.

Cancer development is a multifactorial process yet is expedited by genomic instability and mutation accumulation\textsuperscript{37}. Specifically, certain somatic tumour mutations in oncogenic and tumour suppressing genes favor the clonal expansion of an aggressive phenotype\textsuperscript{38}. The most commonly described somatic gene mutations associated with tumorigenesis are structural variants (SV) which can include gene deletions that dysregulate gene expression resulting in tumorigenesis\textsuperscript{38}. In a previous study by our group\textsuperscript{14}, \textit{MACROD2} was identified as a gene with significant SV deletion mutations associated with treatment resistance in 17\% of patients in a local cohort of HPV+ HNSCC disease. Of these loss of function mutations of \textit{MACROD2}, 100\% of them were found in the coding regions of recurrent patients.

Mono-adenosine diphosphate (ADP) ribosylhydrolase 2 (\textit{MACROD2}) is a highly conserved enzymatic macrodomain a part of the poly(ADP-ribose) polymerase (PARP) family of proteins\textsuperscript{36}. PARP localizes to DNA lesions and recruits DNA repair factors such as \textit{MACROD2} which removes single ADP units from proteins in a process called mono-ADP-ribosylation\textsuperscript{39}. \textit{MACROD2} is capable of reversible post-translation protein modification (PTM) in many cellular settings\textsuperscript{40,41}. Specifically in cancer, \textit{MACROD2} has been implicated as a novel tumour suppressor gene in colorectal cancer through inhibition of the DNA damage response (DDR) leading to chromosome instability (CI)\textsuperscript{15}. Further, the loss of function mutation of \textit{MACROD2} in hepatocellular carcinoma (HCC) is associated with poor overall survival (OS), tumor recurrence, and metastasis\textsuperscript{16}. Additionally, WGS of a small cohort of patients with gastric and esophageal squamous cell carcinoma (ESCC) revealed \textit{MACROD2} was deleted in approximately 14\% of tumours\textsuperscript{17}. Interestingly, \textit{MACROD2} has also been found as a common site for HPV integration in both cervical cancer and HNSCC\textsuperscript{18,19}. Consequently, deletion of \textit{MACROD2} is responsible for
the constitutive reversible ADP-ribosylation of various regulatory processes potentially leading to an aggressive phenotype and resistance to treatment.

Through *in vitro* experimentation, our results indicate inhibition of *MACROD2* using either siRNA or shRNA models leads to elevated cell viability and clonogenic potential in the majority of HPV+ HNSCC cell lines. Several molecular pathways enriched in our RNASeq and RPPA experiments could begin to explain the aggressive phenotype seen in *MACROD2* deficient cell lines. Primarily, overexpression of *LICAM* at both the transcript and protein level in our study has been implicated in cancer metastasis and tumour aggressiveness. *LICAM* upregulation has also been previously implicated in oral cancer tumor progression. Furthermore, gene enrichment analysis (GEA) reveals cells with *MACROD2* inhibition have elevated extracellular matrix (ECM) organization, TGF-beta cellular signalling, and focal adhesion signatures. The ECM contains the structural components of the cell and assists in intercellular communication and regulation of various cellular growth processes including cell survival in cancer. As such, activation of genes in our dataset associated with ECM organization – such as *CAV1, ETS1, TGFB2* – may begin to explain the increased cellular viability and colony forming in *MACROD2* deficient cells. Specifically, upregulation of *CAV1* increases ECM organization and mediates cellular metabolism in cancer cells. Further, *ETS1* is a proto-oncogene responsible for regulating ECM remodeling factors such MMP’s. Additionally, activation of TGF-beta cellular signalling leads to dysregulation of the ECM through synthesis of collagen and fibroblasts and is linked to immunosuppression. Similarly, focal adhesions are proteins connecting the cytoskeleton and the ECM which functionally, promote cell growth and cell morphology maintenance. Upregulation of *CAV1, DST, FLNB* genes from our RNASeq analysis are associated with maintaining focal adhesion integrity. Specifically, *DST* has been found to regulate focal adhesion and cellular growth in related cancers and *FLNB* acts as a actin-binding protein responsible for cellular invasion. Taken together, enhanced organization of the ECM and activation of genes supporting tumour aggressiveness, such as *LICAM*, suggest these factors portend a more aggressive phenotype in *MACROD2* deficient HPV+ HNSCC cells.

Further, we discovered that HPV-associated HNSCC cells deficient in *MACROD2* exhibited a unique resistance to radiation therapy that is not seen in HPV-negative cell lines.
After exploration of the mechanistic role of MACROD2 knockdown in three HPV+ HNSCC cell lines, it was uncovered that MACROD2 deficiency is associated with genes enriched for hypoxia by both gene enrichment analysis (GEA) and an elevated hypoxia score determined by several validated hypoxia gene signatures\textsuperscript{26-31}. A hypoxic tumour microenvironment is known to be a mediator of radiation resistance in a variety of cancer types resulting in adverse survival outcomes\textsuperscript{30}. This is due to the free radicals produced from radiation treatment having no oxygen to react with to induce DNA damage\textsuperscript{52}. As a result, our data suggests an elevated tumour hypoxia works concurrently with MACROD2 inhibition to mediate radiation resistance in HPV+ HNSCC.

RNASEq and RPPA data revealed differential expression patterns of genes associated with hypoxia in MACROD2 deficient cell lines. Specifically, upregulation of TP53, MET, and CAV1 genes involved in hypoxia was consistent across RNASEq and RPPA analysis. MET is overexpressed during hypoxia inducing invasive growth\textsuperscript{53} and CAV1 is regulated by the hypoxia-inducible factor (HIF) leading to hypoxia-induced cell growth\textsuperscript{54}. Additionally, TP53 encodes the well-known p53 tumour suppressor protein\textsuperscript{37}, which regulates cell cycle and apoptosis factors during severe hypoxia\textsuperscript{55}. The intersection between HPV+ cancers, p53 expression, and hypoxia has been studied in the past due to the paradoxical activation of p53 in hypoxic HPV-related tumours despite the conventional degradation of p53 by the HPV oncogene E6\textsuperscript{56-58}. Essentially, cells with low oxygen induce p53 expression by uncoupling from the E6 oncoprotein\textsuperscript{56} resulting in E6 downregulation, avoidance of cellular senescence, and inhibition of cell proliferation\textsuperscript{57}. Interestingly, in MACROD2 deficient HPV+ HNSCC cell lines, hypoxia mediated cell survival and radiation resistance is also accompanied by maintenance of cell proliferation suggesting a useful role of MACROD2 as a tumour suppressor.

Elevated protein expression of H2AX based on RPPA analysis led us to perform immunofluorescent studies of phosphorylated H2AX (γ-H2AX) in samples deficient in MACROD2. All three HPV+ HNSCC cell lines studied (UWO37, UMSCC47, UWO23) also expressed elevated immunofluorescent staining of γ-H2AX. γ-H2AX is a well-documented early marker of DNA double strand breaks (DSB)\textsuperscript{59}. Therefore, upregulation of γ-H2AX staining in our samples suggests delayed clearance of DSB and an altered DDR. In our study, upregulation of DSB may be the result of multiple factors including impaired PARP signalling, HPV expression, and an elevated hypoxia signature. Primarily, loss of function of MACROD2
dysregulates the PARP-mediated DDR resulting in elevated DSB, as shown in colorectal cancer\textsuperscript{15}. Further, the HPV oncoproteins E6 and E7 are responsible for various pathways in the DDR including degradation of p53 and Rb expression which favor a damaged cellular state\textsuperscript{60}. Finally, hypoxic environments induce DSB upregulation through altered reduction of the enzyme responsible for nucleotide production having low functionality in hypoxic environments resulting in DNA damage\textsuperscript{42}. Overall, numerous mechanisms of genomic instability are associated with a delayed DDR and γ-H2AX expression in \textit{MACROD2} deficient, radiation resistant, HPV+ HNSCC which may explain their aggressive phenotype and radiation resistance.

The primary finding from this research is that \textit{MACROD2} deficiency promotes radiation resistance in the HPV+ HNSCC population. Future studies should, therefore, focus on exploring alternative treatment options – such as hypoxia modifiers and radiosensitizers – to improve survival outcomes. Nimorazole is the only hypoxia modifying drug currently accepted into clinical practice based on the results from the phase III clinical trial DAHANCA 5\textsuperscript{61}. In this trial, radiation was supplemented with nimorazole and was found to improve both loco-regional tumour control (49 vs 33\%) and disease-specific survival with mild side effects\textsuperscript{62}. However, its clinical utility has only been approved in Denmark and a second trial (NIMRAD, NCT01950689)\textsuperscript{63} is currently underway to confirm their findings for widespread use\textsuperscript{60,64}. Additionally, employing \textit{PARP} inhibitors as an alternative to therapy could also be beneficial due to \textit{MACROD2}'s involvement in \textit{PARP} signalling\textsuperscript{39}. One of the most commonly studied \textit{PARP} inhibitors, olaparib, has been found to increase radiosensitivity in HNSCC cells, but more so in HPV- rather than HPV+ disease\textsuperscript{65}. Currently, a phase I clinical trial (NCT02229656) is exploring olaparib as a radiation sensitizer in HPV-negative HNSCC patients\textsuperscript{66}. It is suggested inhibition of \textit{PARP} will not only increase tumor sensitivity to DSB but will also reduce hypoxia through increased tumour perfusion. Consequently, testing these hypoxia modifiers in their ability to restore normoxia in \textit{MACROD2} deficient HPV+ HNSCC cell \textit{in vitro} would begin to explain their utility in the patient population.
2.6 Conclusion

In this study, MACROD2 was identified as a useful biomarker of radiation resistance in HPV+ HNSCC. Furthermore, a possible mechanism of radiation resistance in MACROD2 deficient HPV+ HNSCC cells was identified as an elevation of cellular hypoxia and an altered DNA damage response (DDR). These findings provide an opportunity to stratify HPV+ HNSCC patients based on MACROD2 loss, which can potentially stratify treatment for this patient population. Further studies are required to understand how to overcome hypoxia in MACROD2 deficient tumors to restore radiosensitivity.


### 2.7 Supplementary Material

#### 2.7.1 Supplementary Tables

**Supplementary Table 1:** Cell lines used for *in vitro* experimentation

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## Supplementary Table 2: Short tandem repeat (STR) profiling of HNSCC cell lines

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<th>D21S11</th>
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Supplementary Table 3: Summary of results of *MACROD2* knockdown experiments (siRNA and shRNA) in HNSCC cell lines

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siRNA – small-interfering RNA  
shRNA – short-hairpin RNA  
HPV – Human papillomavirus  
MD2 Exp. – Relative *MACROD2* mRNA abundance where 1 is the highest mRNA abundance and 8 is the lowest mRNA abundance  
KD – Percent *MACROD2* gene knockdown  
Cell Viab. – Cell viability  
CF – Colony forming  
Cell Mig. – Cell migration  
Cis. Resist. – Cisplatin chemotherapy resistance  
Rad. Resist. – Radiation chemotherapy resistance
Supplementary Tables 4, 5, and 6: Differential gene expression of MACROD2 inhibited HPV+ HNSCC cell lines based on RNASeq and RPPA analysis
2.7.2 Supplementary Figures

Supplementary Figure 1: siMACROD2 knockdown is most effective 72 hours post siRNA transfection

Samples were collected from cell lines (MC47 and 93VU147T) at three-time intervals after siRNA transfection (24 hrs post, 48 hrs post, and 72 hrs post). As determined by qPCR, MACROD2 expression was inhibited the most 72 hours after siMACROD2 transfection.
Supplementary Figure 2: The protein-protein network and differential gene expression profiles associated with MACROD2 inhibition in HPV+ HNSCC

RPPA analysis was performed on a panel of 3 HPV+ HNSCC cell lines (UWO37, UMSCC47, and UWO23) in both shControl and shMACROD2 knockdown models. The protein-protein interaction network generated from the 99 differentially expressed genes generated from RNAsSeq analysis. The differential abundance of proteins between MACROD2 knockdown and shControl samples in the three cell lines that intersect with differential expression in the RNAsSeq dataset were determined by two-tailed t-test and the Benjamini-Hochberg method to adjust for multiple comparisons.
Chapter 3

3 Discussion

3.1 Overview

The characterization of biomarkers of disease resistance is a clinically important research task for implementing patient stratification measures based on tumour risk. Head and neck cancers are of growing concern due to rising diagnoses associated with HPV infection and a portion of patients being more susceptible to disease recurrence due to treatment resistance. Therefore, identification, characterization, and implementation of biomarkers of treatment resistance have the possibility to improve survival outcome and quality of life in the HPV+ HNSCC patient population. In this study, MACROD2 was identified as a biomarker of radiation resistance in HPV+ HNSCC. When MACROD2 was inhibited in HPV+ HNSCC cell lines, it was associated with increased cell viability, colony forming potential, and resistance to radiation treatment in vitro. Interestingly, when MACROD2 inhibition was assessed in an HPV-negative cell line, it did not confer radiation resistance suggesting this radiation intolerance phenomenon is unique to HPV-associated disease. Moreover, mechanistic studies of MACROD2 deficiency in HPV+ HNSCC suggests the aggressive phenotype is mediated by elevated organization of the extracellular matrix (ECM) and increased hypoxic environment conferring an altered response to DNA damage. As a result, using MACROD2 as a biomarker in the clinical setting could provide an opportunity to identify radiation non-responders in the HPV+ HNSCC patient population.

3.2 Elevated MACROD2 Genomic Aberrations in HPV+ HNSCC

Although carcinogenesis is a multistep process involving dysregulation of various biological pathways, expedited cancer development is guided by genomic instability and mutation accumulation\(^1\). Certain gene mutations enable a selective cell growth advantage which favors the continued expansion of potentially malignant neoplasms\(^1\). Additionally, some tumours are not therapeutically alleviated and acquire treatment resistance due to acquisition of genomic aberrations that favor tumour preservation\(^2\). As a result, various somatic mutations with
oncogenic and tumour suppressing capabilities help drive the development and progression of cancer as well as the resistance to cancer therapeutics. The most commonly described somatic gene mutations associated with tumorigenesis are structural variants (SV). SV’s include deletions, insertions, duplications, inversions, and translocations of the genome affecting at least 50 base pairs (bp). SV’s function by dysregulating gene expression resulting in altered cellular signalling and tumorogenesis. Thanks to high-throughput DNA sequencing efforts, identification of significant SV’s associated with aggressive cancer phenotypes has been made possible. Our laboratory identified MACROD2 as a gene with significant SV deletion mutations associated with treatment resistance in 17% of patients in a local cohort of HPV+ HNSCC disease. Of these loss of function mutations of MACROD2, 100% of them were found in the coding regions of recurrent patients. Previous studies have also identified MACROD2 as a novel tumour suppressor gene in colorectal, liver, stomach, and esophageal cancer. Upon analysis of The Cancer Genome Atlas (TCGA) PanCancer Atlas of 523 HNSCC samples, it was revealed only 3% of patients had mutations in MACROD2 and approximately 80% of these mutations were in the HPV-negative population. Of the HPV+ patients in the TCGA cohort, only one had an SV deletion mutation of MACROD2. Therefore, the profound quantity of MACROD2 deletions in the genic regions of our local cohort, suggests a specific biological event related to tumour recurrence may be present that is worth exploring.

3.3 Inhibition of MACROD2 resulted in an aggressive phenotype in HNSCC cells

Through in vitro experimentation, I demonstrated that inhibition of MACROD2 using either siRNA or shRNA models leads to elevated cell viability and clonogenic potential in the majority of HPV+ HNSCC cell lines. Interestingly, discordant results were found for cell migration, even though previous studies have found inhibition of MACROD2 increases cellular migration in other cancers. This may be due to technical errors with the migration kit, or MACROD2 may have no impact on migration in this cell type. More work is required to elucidate this question.
Several molecular pathways enriched in our RNASeq and RPPA experiments could begin to explain the aggressive phenotype seen in MACROD2 deficient cell lines. Primarily, overexpression of L1CAM at both the transcript and protein level in our study has been implicated in cancer metastasis and tumour aggressiveness9. Specifically, L1CAM upregulation has also been previously implicated in oral cancer tumor progression10. Furthermore, gene enrichment analysis (GEA) reveals cells with MACROD2 inhibition have elevated extracellular matrix (ECM) organization, TGF-beta cellular signalling, and focal adhesion signatures. The ECM contains the structural components of the cell and assists in intercellular communication and regulation of various cellular growth processes11 including cell survival in cancer12. As such, activation of genes in our dataset associated with ECM organization – such as CAV1, ETS1, TGFB2 – may begin to explain the increased cell viability and clonogenic potential in MACROD2 deficient cells. Specifically, upregulation of CAV1 increases ECM organization and mediates cellular metabolism in cancer cells13. Further, ETS1 is a proto-oncogene responsible for regulating ECM remodeling factors such MMP’s14. Additionally, activation of TGF-beta cellular signalling leads to dysregulation of the ECM through synthesis of collagen and fibroblasts15 and is linked to immunosuppression16. Similarly, focal adhesions refer to proteins connecting the cytoskeleton and the ECM which functionally, promote cell growth and cell morphology maintenance12. Upregulation of CAV1, DST, FLNB genes from our RNASeq analysis are associated with maintaining focal adhesion integrity. Specifically, DST has been found to regulate focal adhesion and cellular growth in related cancers17 and FLNB acts as a actin-binding protein responsible for cellular invasion18. Taken together, enhanced organization of the ECM and activation of genes supporting tumour aggressiveness, such as L1CAM, suggest these factors portend a more aggressive phenotype in MACROD2 deficient HPV+ HNSCC cells.

3.4 Hypoxia mediates radiation resistance in MACROD2 deficient HPV-positive HNSCC

Through in vitro experimentation, we discovered that HPV-associated HNSCC cells deficient in MACROD2 exhibited a unique resistance to radiation therapy that is not seen in HPV-negative cell lines. After exploration of the mechanistic role of MACROD2 knockdown in three HPV+ HNSCC cell lines, it was uncovered that MACROD2 deficiency is associated with
genes enriched for hypoxia by both gene enrichment analysis (GEA) and an elevated hypoxia score determined by several validated hypoxia gene signatures. Hypoxia is a well-documented mediator of radiation resistance in a variety of cancer types, suggesting it may work congruently with MACROD2 inhibition to mediate radiation resistance in HPV+ HNSCC.

3.4.1 Hypoxia in cancer

Hypoxia refers to a biological state of lowered oxygen supply and is commonly detected in solid tumours\(^\text{19}\). Tumours, similar to other tissues in the body, require a consistent blood supply for adequate oxygenation for the growing mass\(^\text{19}\). However, ectopic tumours must generate their own oxygen supply resulting in a fractured and irregular vasculature system. Due to inadequate tumour oxygenation, this ultimately results in a heterogenous tumour harboring various hypoxic domains\(^\text{19}\). Although tumour hypoxia has been well documented in various cancers, one of the most commonly studied hypoxic malignancies is HNSCC\(^\text{20}\). Furthermore, tumours low in oxygen have been shown to have a negative survival outcome\(^\text{21}\). As a result, hypoxia has been defined as an independent negative prognostic factor in HNSCC\(^\text{22}\).

3.4.2 MACROD2 deficiency associated with elevated hypoxia gene signature

Various approaches of hypoxia screening have been documented including measuring intratumoral oxygen by electrodes, image guided approaches, immunohistochemical staining, and single-gene biomarkers such as hypoxia inducible factor (HIF), carbonic anhydrase (Ca9), and glucose transporter 1 (Glut1)\(^\text{19,21,22}\). None of these methods, however, have been extensively validated for the clinical setting and reflect the complex cellular signalling in hypoxic tumors\(^\text{23}\). As a result, various gene expression signatures have been established that combine numerous genes associated with hypoxia to estimate a hypoxia score\(^\text{21–26}\). Those used in this study to assess tumour hypoxia related to MACROD2 knockdown include the Sørensen\(^\text{21}\), Winter\(^\text{22}\), Eustace\(^\text{23}\), Buffa\(^\text{24}\) and Ragnum\(^\text{25}\) signatures. Although there is some overlap of genes in each hypoxia signature, they all differ slightly depending on the tumour dataset they were established in.
Nonetheless, when used together, these gene signatures have the possibility to predict poor prognosis and response to treatment in various cancer models⁷⁷,²⁸. Our results show an elevated hypoxia signature in the three HPV+ HNSCC cell lines with \textit{MACROD2} inhibition based on a combined p-value of all hypoxia scores.

3.4.3 Hypoxia mediates radiation intolerance

Along with being implicated in cancer progression and prognosis, tumour hypoxia is also commonly associated with poor response to radiation treatment¹⁹. Functionally, radiation treatment generates highly reactive free radicals in the target tumour mass¹⁹. These free radicals then bind to oxygen particles which can rearrange the chemical structure of the tumour resulting in DNA damage¹⁹. When there is a decreased oxygen availability however, tumours can escape radiation-induced damage and maintain cellular growth. In HNSCC, studies have shown that HPV-positive and HPV-negative tumours have a similar degree of hypoxia²⁹. Interestingly, however, HPV-positive HNSCC cases have historically been documented as more radiosensitive than their HPV-negative counterparts³⁰. In our study, we show that HPV+ HNSCC cell lines with \textit{MACROD2} knocked down have an elevated hypoxic environment related to resistance to radiation treatment. Consequently, it can be suggested that \textit{MACROD2} inhibition leads to a tumour hypoxic microenvironment in HPV+ HNSCC which mediates radiation, but not chemotherapy, resistance.

In the present study, RNASeq and RPPA data revealed differential expression patterns of genes associated with hypoxia in \textit{MACROD2} deficient cell lines. Specifically, upregulation of \textit{TP53}, \textit{MET}, and \textit{CAV1} genes involved in hypoxia was consistent across RNASeq and RPPA analysis. \textit{MET} is overexpressed during hypoxia inducing invasive growth³¹ and \textit{CAV1} is regulated by the hypoxia-inducible factor (HIF) leading to hypoxia-induced cell growth³². Additionally, \textit{TP53} encodes the well-known p53 tumour suppressor protein¹, which regulates cell cycle and apoptosis factors during severe hypoxia³³. The intersection between HPV+ cancers, p53 expression, and hypoxia has been studied in the past due to the paradoxical activation of p53 in hypoxic HPV-related tumours despite the conventional degradation of p53 by the HPV oncogene E6³⁴–³⁶. Essentially, cells with low oxygen induce p53 expression by
uncoupling from the E6 oncoprotein\textsuperscript{34} resulting in E6 downregulation, avoidance of cellular senescence, and inhibition of cell proliferation\textsuperscript{35}. Interestingly, in \textit{MACROD2} deficient HPV+ HNSCC cell lines, hypoxia mediated cell survival and radiation resistance is also accompanied by maintenance of cell proliferation suggesting a useful role of \textit{MACROD2} as a tumour suppressor.

In congruence with these findings, the primary pathway generated from gene enrichment analysis (GEA) was elevated hypoxia in \textit{MACROD2} deficient cells. Similarly, validated hypoxia gene signatures confirmed all cell lines with \textit{MACROD2} deleted, had an elevated hypoxia signature when compared to the control. Therefore, the loss of function mutation of \textit{MACROD2} is associated with an elevated tumour hypoxia microenvironment in HPV+ HNSCC cell lines. Interestingly, \textit{MACROD2} inhibition did not correlate to a similar intolerance to chemotherapy, nor did it confer radiation resistance in an HPV-negative population. Consequently, radiation resistance in the \textit{MACROD2} deficient HPV-positive HNSCC population is a useful finding, suggesting \textit{MACROD2} as a biomarker for elevated hypoxia and radiation resistance.

### 3.5 Altered DNA Damage Response in \textit{MACROD2} deficient HPV+ HNSCC

Elevated protein expression of H2AX based on RPPA analysis and increased immunofluorescent staining of phosphorylated H2AX (\(\gamma\)-H2AX) in samples deficient in \textit{MACROD2} suggests these cells have an altered DNA damage response (DDR). \(\gamma\)-H2AX is a well-documented early marker of DNA double strand breaks (DSB) and is commonly used to detect radiation exposure\textsuperscript{37}. DSB’s are implicated in genomic instability due to changes to both strands of DNA that if not repaired, may lead to downstream genetic aberrations\textsuperscript{38}. Upon DNA damage, the H2AX histone variant senses DSB and phosphorylates at Ser139 allowing rapid binding of DNA damage repair elements\textsuperscript{37}. This ultimately results in the nuclear accumulation of \(\gamma\)-H2AX foci\textsuperscript{37}. DSB’s can be introduced to the genome in a multitude of ways including exogenous factors (such as radiation, chemicals, and heat), endogenous factors (such as hypoxia, apoptosis, and telomere shortening), and lifestyle factors (such as smoking, age, and race)\textsuperscript{37}. Interesting to this study, delayed clearance of \(\gamma\)-H2AX foci in \textit{MACROD2} deficient HPV+
HNSCC cell lines may be the result of a combination of these factors including impaired PARP signalling, HPV expression, and an elevated hypoxia signature.

3.5.1 Role of PARP signalling in DDR

Impairment of the PARP signalling pathway, such as the loss of function of MACROD2, will result in elevated DSB. One of the main roles of the PARP signalling pathway is to repair damaged DNA. Designated PARP proteins localize to sites of DSB and acquire DNA repair factors, such as MACROD2, to help in the repair process. MACROD2 aids in the PARP mediated DDR through reversible mono-ADP ribosylation. Although DSBs can be repaired by two mechanisms, homologous recombination (HR) and nonhomologous end-joining (NHEJ), the prime mechanism of PARP is HR-mediated DSB repair. HR uses a complementary DNA template strand to mediate error-free DNA repair in cells. Studies in related cancers found the loss of function of MACROD2 leads to a reduction in HR-mediated repair due to an inactivation of the PARP mediated DDR. Therefore, elevated γ-H2AX foci in MACROD2 deficient HPV+ HNSCC cell lines, is suggested to be a consequence of the inactivation of the PARP repair process resulting in elevated DSB.

3.5.2 Role of HPV in DDR

Previous studies have implicated a defective DSB repair response is unique to HPV-associated disease. Expression of HPV, and the associated upregulation of the HPV oncogenes E6 and E7, are responsible for various pathways in the DDR. Specifically, E6 reduces both the expression of p53 and the DNA damage signalling transducer SMG-1 and E7 inhibits Rb expression and alters ataxia-telangiectasia mutated (ATM) -mediated DDR. Similarly, E7 associated Rb degradation leads to overexpression of p16 which has been implicated in delayed DDR and impaired HR. As a result, alterations in the DDR can be a result of HPV oncogenic properties which favor a damaged state. This can explain why even at baseline, HPV+ control cell lines express the γH2AX marker at moderate intensities due to inherent HPV cellular signalling.
3.5.3 Role of Hypoxia in DDR

Hypoxic states can also initiate a delayed DDR resulting in the upregulation of γ-H2AX foci in affected tissue. This is a consequence of the enzyme responsible for nucleotide production having low functionality in low oxygen environments resulting in elevated DNA damage. Previous studies have found hypoxia induced DDR is mediated by the previously described ATM signalling as well as TM- and Rad3-Related (ATR) signalling. Similarly, hypoxic states express reduced RAD51 protein implicated in DNA repair and activation of p53 mediated apoptosis. As a result, hypoxia puts pressure on the DDR leading to delayed repair, elevated DSB and genetic instability.

3.5.4 γ-H2AX staining indicative of impaired DDR

Immunofluorescent staining of γ-H2AX in our study indicates elevated foci in the MACROD2 knockdown population. Interestingly, staining of γ-H2AX foci proved to be divergent depending on the cell line which could be the result of varied biological manifestations as described in the literature. Specifically, two populations of γ-H2AX foci have been postulated in which large punctate foci colocalize with DSB repair elements whereas small dim foci do not. Alternatively, one study suggests an overabundance of DSB in the nucleus is depicted as a diffuse halo of γ-H2AX staining as appose to distinct quantifiable foci. It is unclear why MACROD2 knockdown in UWO37 and UWO23 cell line exhibited staining of γ-H2AX as a combination of small and large foci whereas UMSCC47 cells primarily present with small diffuse staining. For these reason, immunofluorescent staining of γ-H2AX was analyzed qualitatively, as the accumulation of foci presented differently in the three cell lines and were not quantifiable. Nevertheless, overexpression of γ-H2AX was associated with cells deficient in MACROD2 suggesting a delayed clearance of DSB and an altered DDR.

Overall, numerous mechanisms of genomic instability are associated with a delayed DDR and γ-H2AX expression in MACROD2 deficient, radiation resistant, HPV+ HNSCC. As detailed, deletion of MACROD2 leads to attenuated PARP-mediated DDR leading to upregulated DSB. Similarly, an elevated hypoxia signature can alter cellular signalling resulting in DDR impairment. Further, expression of HPV oncogenes E6 and E7 encourages delayed DDR.
Ultimately, this leads to an impaired DDR in \textit{MACROD2} deficient cells that favors an aggressive phenotype suggesting a cause for elevated tumour growth and radiation resistance.

3.6 Limitations

Although our work proved to discover novel findings related to \textit{MACROD2} in HPV+ HNSCC, specific experimental constraints may have limited the outcomes of the study and moving forward, will provide an avenue for improvement in future work. Primarily, small-interfering RNA (siRNA) and short-hairpin RNA (shRNA) mediated gene inhibition was employed as opposed to gene deletion by the clustered regularly interspaced short palindromic repeats (CRISPR) system. CRISPR and its associated protein 9 (Cas9) employ a bacterial defense mechanism which has been repurposed for targeted genome editing\textsuperscript{46}. Often used in cancer research, the CRISPR-Cas9 system can target genes of interest, such as oncogenes or tumour suppressors, to explore a genes functional role in tumorigenesis. Contrastingly, siRNA and shRNA systems induce gene knockdown in a method called RNA interference (RNAi)\textsuperscript{47}. In this approach, double-stranded RNA (dsRNA) guides target and degrade genes of interest leading to either transient (siRNA) or constitutive (shRNA) gene knockdown\textsuperscript{47}. In the interest of time, gene knockdown approaches were used in this study of \textit{MACROD2} deficiency in HPV+ HNSCC as opposed to gene knockout as it was both efficient and straightforward. Further, due to shRNA’s ability to generate stable gene knockdown cell lines, as reflected in our gene knockdown validation and functional experiments, these cell lines were selected for further downstream mechanistic exploration. Nonetheless, CRISPR deletion is still the favored approach for tumour suppressor studies as it better reflects the patient population with deletion mutations and provides a more complete loss of function of the gene.

Additionally, we found \textit{MACROD2} deletions confer radiation resistance from WGS of recurrent tumour samples therefore, it may prove clinically difficult to use \textit{MACROD2} as a predictive biomarker. It would be unhelpful for the patient population if we can only identify significant \textit{MACROD2} deletions only after they have already failed radiation treatment. As a result, using \textit{MACROD2} as a stratification tool may rely on further sequencing efforts using targeted sequencing panels that reach a deeper genetic clarity. Then, \textit{MACROD2} deletions could
be identified in the subclonal population that favours tumour aggressiveness even before radiation treatment.

### 3.7 Future Directions

Future studies on MACROD2 deficiency in HPV+ HNSCC will continue to help patients living with the disease and its associated toxicities. Specifically with this work, further validation studies could be performed \textit{in vivo} and in the patient population in order to detect the impact of deleted MACROD2 in the HPV+ HNSCC cohort. Studies in mice provide a clear foundation for how patients with MACROD2 deletions may portend resistance to treatment. Previous studies have generated MACROD2 deficient mice and found inhibition of the gene correlates with advanced tumourigenesis\textsuperscript{5,6}. Our lab is familiar with mouse xenograft and syngeneic models and could be replicated using MACROD2 as the gene of interest. Moreover, MACROD2 expression could also be assessed in clinical samples to further understand the frequency of these gene mutations in the patient population.

The primary finding from this research is that MACROD2 deficiency promotes radiation resistance in the HPV+ HNSCC population. Future studies should, therefore, focus on exploring alternative treatment options – such as hypoxia modifiers and radiosensitizers – to improve survival outcomes. Nimorazole is the only hypoxia modifying drug currently accepted into clinical practice based on the results from the phase III clinical trial DAHANCA 5\textsuperscript{48}. In this trial, radiation was supplemented with nimorazole and was found to improve both loco-regional tumour control (49 vs 33\%) and disease-specific survival with mild side effects\textsuperscript{49}. However, its clinical utility has only been approved in Denmark. A second trail (NIMRAD, NCT01950689)\textsuperscript{50} is currently underway to confirm the DAHANCA 5 findings for widespread use\textsuperscript{30,51}. Secondly, employing \textit{PARP} inhibitors as an alternative to therapy could be beneficial due to MACROD2’s involvement in PARP signalling\textsuperscript{40}. One of the most commonly studied \textit{PARP} inhibitors, olaparib, has been found to increase radiosensitivity in HNSCC cells, but more so in HPV- rather than HPV+ disease\textsuperscript{41}. Specifically, a current phase I clinical trial (NCT02229656) is exploring olaparib as a radiation sensitizer in HPV-negative HNSCC patients\textsuperscript{52}. It is suggested inhibition of \textit{PARP} will not only increase tumor sensitivity to DSB but will also reduce hypoxia through
increased tumour perfusion. Consequently, testing these hypoxia modifiers in their ability to restore normoxia in MACROD2 deficient HPV+ HNSCC cell *in vitro* would begin to explain their utility in the patient population.

### 3.8 Conclusion

In this study, *MACROD2* was identified as a useful biomarker of radiation resistance in HPV+ HNSCC. Furthermore, a possible mechanism of radiation resistance in *MACROD2* deficient HPV+ HNSCC cells was identified as an elevation of cellular hypoxia and an altered DNA damage response (DDR). These findings provide an opportunity to stratify HPV+ HNSCC patients based on *MACROD2* expression which can ultimately lead to improved outcomes for this patient population.
References


3.9 Supplementary Material

3.9.1 Supplementary Figures

Supplementary Figure 3: Analysis of *MACROD2* mutations in the TCGA PanCancer Atlas HNSCC dataset

Using cBioPortal, mutations in *MACROD2* were queried in the 523-sample dataset of HNSCC cases provided by the TCGA PanCancer Atlas. *MACROD2* was altered in 17 (or 3%) of the patient population. Of the mutations in *MACROD2*, majority (~80%) were in the HPV-negative HNSCC cohort.
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