

**PROFILING THE IMMUNE AND GENOMIC
LANDSCAPE OF ANAL SQUAMOUS CELL
CARCINOMA AND ESTABLISHING PRECLINICAL
MODELS TO EXPLORE NEW THERAPEUTIC OPTIONS**

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Submitted November 2019 in total fulfilment of the requirements of the degree

Doctor of Philosophy

Surgical Oncology / Differentiation and Transcription Laboratories

Cancer Immunology Research

Sir Peter MacCallum Department of Oncology

Faculty of Medicine, Dentistry and Health Sciences

The University of Melbourne

And

Division of Cancer Surgery

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Abstract

Anal SCC is a rare disease that has increased significantly in both incidence and mortality over the last forty years. Definitive chemoradiotherapy is the primary modality of treatment, offering a 5-year overall survival rate of 65%. For patients with locally persistent or recurrent disease, salvage surgery is an option with a 5-year overall survival of 50%. However, for those patients with un-resectable locoregional or metastatic disease, there are limited treatment options, and patients face a dismal outcome. Progress in identifying new treatment options for patients with anal cancer has been hampered by a deficiency in understanding the underpinnings of the disease and a lack of appropriate preclinical models.

This thesis has focussed on addressing both of these deficiencies in addition to assessing the success of salvage surgery at a quaternary centre in Australia. Firstly, an attempt has been made to further our understanding of the biology of Anal SCC. This was undertaken by exploring the immune and genomic landscape of ASCC, to identify potential prognostic and therapeutic biomarkers. This has provided insight into the prognostic power of assessing the CD8+ immune infiltrate in Anal SCC. It has also identified PI3K aberrations as a frequent genomic event that may serve as a future therapeutic target.

Secondly, it has led to the establishment of both human and mouse preclinical models of this disease. This includes the world's first panel of human anal SCC cells lines and a syngeneic mouse model. Both of these pre-clinical models have been validated and characterised, with features closely resembling the human disease. These models can now act as a platform to further explore and facilitate investigation into potential new therapeutic options in this disease.

Publications

Publications directly related to these works

- 1) **Guerra GR**, Kong JC, Bernardi MP, Ramsay RG, Phillips WA, Warrier SK, Lynch AC, Ngan SY, Heriot AG. **Salvage Surgery for Locoregional Failure in Anal Squamous Cell Carcinoma.** *Dis Colon Rectum*. 2018. Feb; 61(2): 179-186. doi: 10.1097/DCR.0000000000001010
- 2) JC Kong, **GR. Guerra**, RM Millen, S Roth, H Xu, PJ Neeson, PK Darcy, MH Kershaw, S Sampurno, J Malaterre, DSH Liu, TD Pham, V Narasimhan, M Wang, Y-K Huang, K Visvanathan, J McCormick, AC Lynch, S Warrier, M Michael, J Desai, W Murray, C Mitchell, SK Ngan, WA Phillips, AG Heriot and RG Ramsay. **Tumor Infiltrating Lymphocyte Function Predicts Response to Neoadjuvant Chemoradiotherapy in Locally Advanced Rectal Cancer.** *JCO-PO*. Nov 2018. doi: 10.1200/PO.18.00075

Other publications completed during research fellowship

- 3) JC Kong, **GR. Guerra**, A Lee, SK Warrier, AC Lynch, AG Heriot. **Long-term Outcomes of Locally Advanced Rectal Cancer after Neoadjuvant Chemoradiotherapy: A Bi-national Colorectal Cancer Audit Study.** *World J Colorectal Surg*. Oct 2019. doi:10.4103/WJCS.WJCS_16_19
- 4) Teh J, Duncan C, Qu L, **Guerra G**, Narasimhan V, Pham T, Lawrentschuk N. **Inguinal lymph node dissection for penile cancer: a contemporary review.** *Trans Androl Urol* 26 Aug 2019. doi:10.21037/tau.2019.08.37

- 5) Marian L. Burr, Christina E. Sparbier, Kah Lok Chan, Yih-Chih Chan, Ariena Kersbergen, Enid Y.N. Lam, Elizabeth Azidis-Yates, Dane Vassiliadis, Charles C. Bell, Omer Gilan, Susan Jackson, Lavinia Tan, Stephen Q. Wong, Sebastian Hollizeck, Ewa M. Michalak, Hannah V. Siddle, Michael T. McCabe, Rab K. Prinjha, **Glen R. Guerra**, Benjamin J. Solomon, Shahneen Sandhu, Sarah-Jane Dawson, Paul A. Beavis, Richard W. Tothill, Carleen Cullinane, Paul J. Lehner, Kate D. Sutherland and Mark A. Dawson. **An Evolutionarily Conserved Function of Polycomb Silences the MHC Class I Antigen Presentation Pathway and Enables Immune Evasion in Cancer.** *Cancer Cell.* 26 Sep 2019. doi.org/10.1016/j.ccell.2019.08.008.

- 6) JC Kong, **GR. Guerra**, T Pham, C Mitchell, AC Lynch, SK Warriar, RG. Ramsay, AG. Heriot. **Prognostic Impact of Tumor Infiltrating Lymphocytes in Primary and Metastatic Colorectal Cancer: A Systematic Review and Meta-Analysis.** *Dis Colon Rectum.* 2019, Apr; 62(4): 498-508. doi: 10.1097/DCR.0000000000001332.

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- 8) Pham T, Narasimhan V, **Guerra GR**, Kong JC, Desai J, Heriot AG, Ramsay RG. **Wither Surgical Oncology?** *ANZ J Surg* June 2018. doi:10.1111/ans.14758.

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- 16) Stewart M, **Guerra GR**, Sutherland T, Elmer S. **Abdominal wall metastasis following open nephroureterectomy for upper tract urothelial carcinoma in a patient with Lynch Syndrome.** *BMJ Case Rep*. 31 Mar 2016. doi:10.1136/bcr-2016-214940

Presentations

2019

Establishment and Characterisation of a Panel Of Human Anal SCC Cell Lines

- RACS Annual Scientific Congress, Bangkok, Thailand. May

2018

Deriving A Panel of Cell Lines from Human Anal SCC

- RACS Surgical Research Society Annual Meeting, Sydney. Nov

Developing And Characterising A Mouse Model Of Anal SCC

- RACS Surgical Research Society Annual Meeting, Sydney. Nov

2017

The Immune Infiltrate in Anal SCC: Prediction and Preclinical Models

- ACPGBI Annual Meeting, Bournemouth, UK. July

Exploring the Predictive Power of the Immune Infiltrate in Anal SCC

- ACPGBI Annual Meeting, Bournemouth, UK. July

Salvage Surgery for Anal Squamous Cell Carcinoma – A 30 Year Experience.

- Tripartite Conference, Washington State Convention Centre, Seattle, USA. June

Exploring the Immune Landscape in Anal SCC

- ASC, Adelaide. May

Developing Preclinical Models to Explore New Therapies in Lower GI Cancer

- ASC, Adelaide, May

2016

The Immune Infiltrate in Anal SCC: Prediction and Preclinical Models

- Surgical Research Society, Melbourne. Nov

Grants, Scholarships and Prizes

Research Grants

CSSANZ Foundation Research Grant, CSSANZ

- Selected on merit and proposal

Scholarships

Covidien Colorectal Research Scholarship, CSSANZ / Covidien

- Awarded to support dedicated research of a year's duration

Foundation for Surgery Research Scholarship, RACS

- Selected on merit to support research towards a higher degree

ANZ Journal of Surgery Research Scholarship, RACS

- Selected on merit to support research towards a higher degree

NHMRC Postgraduate Research Scholarship

- Selected on merit to support research towards a higher degree

Foundation for Surgery Tour de Cure Cancer Research Scholarship, RACS

- Selected on merit to support research towards a higher degree

Cancer Therapeutics Top-Up Scholarship, Cancer Therapeutics CRC

- Selected on merit and proposal

Australian Postgraduate Award

- Selected on merit

Prizes

2019

Mark Killingback Prize, RACS

- Best presentation at RACS Annual Scientific Congress

2018

CSSANZ Foundation Award for Most Publishable Research Project

- Awarded for most publishable ANZTBCRS fellow's research project

2017

University of Melbourne Department of Surgery Annual Research Forum

- Best Oral Presentation

Association of Coloproctology of Great Britain and Ireland Travelling Fellowship

- Visits to Colorectal centres throughout the UK and Ireland and presentation at the annual meeting

2016

CSSANZ Foundation Second Year Research Award

- Awarded for most publishable second year ANZTBCRS fellow's research project

2015

Philip Douglas Education Award, CSSANZ 2015

- Best presentation at the ANZ Colorectal Fellows weekend

Declaration

This is to certify that

- i. The thesis comprises only original work towards the Doctor of Philosophy degree
- ii. Due acknowledgement has been made in the text to all other material used
- iii. The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Glen R Guerra

Nov 2019

Acknowledgments

I first contemplated a PhD in 2006 following encouragement by Prof Robert Thomas, the then Director of Cancer Surgery at Peter MacCallum Cancer Centre. In his words, while surgery provides great reward in managing patients on a day to day basis, as your career progresses, research becomes the key factor to maintain your motivation and interest in work through keeping the mind active. Despite such insights, it took a further 10 years of discussions and much deliberation before finally deciding to embark upon the pathway towards a PhD. There were several mentors who I certainly must thank, as they were all instrumental in this decision, most notably Prof Michael Henderson, Prof Mark Shackelton and Prof Alexander (Sandy) Heriot, all of whom were honest in colouring it as a hard but rewarding journey.

Beginning a basic science PhD was much more challenging than I had first anticipated, and the journey may have come to an abrupt stop had it not been for the guidance and support of my three supervisors. Prof Wayne Phillips, Prof Rob Ramsay and Prof Sandy Heriot have provided the critical advice and guidance that has allowed me to complete this body of work.

I thank Prof Heriot for his gentle guidance and supreme ability in attempting to keep me to a timeline, tempering my desire to explore tangents. Sandy has been an incredible clinical and research mentor who I will continue to seek guidance and advice from, as I move forward with my career. I thank Prof Phillips for his steadfast input, support and belief in a project that had more than its fair share of hiccoughs along the way. I thank Prof Ramsay for his reassurance of my project's progress and validation of becoming a clinician-researcher. The critical mentorship I have received from both Wayne and Rob, has forged a relationship

that I will continue to draw upon as I look towards incorporating science research into my future career.

To my colleagues in both Phillips and Ramsay labs, it has been a great joy to have spent four years working so closely together. I have been privileged to work in such a pleasant, jovial and supportive environment, which was critical to the success of my project. I hope the friendships I have made are ongoing. I must give Shienny Sampurno and Karen Montgomery a special mention, as they were instrumental in ensuring the integrity of my experiments and the constant port of call when troubleshooting in the lab. I also must thank my clinician colleagues Matt Read and David Liu who provided guidance and reassurance particularly during the challenging teething phase of my project.

I must acknowledge the input of Sara Roth, whose robust research integrity and extensive knowledge in immunology and mice was essential to many aspects of my project. Your sharp wit also provided a light-hearted and collegial atmosphere in the lab. I must also single out a special thank-you to my closest research colleagues Joseph Kong and Rosie Millen. It was a pleasure to work so closely alongside the both of you and the collaboration and friendship we forged during the endless tissue culture marathons into the wee hours of the night, was critical in establishing our novel assay. I look forward to working together again in the future.

My project also had critical input and support from many departments within both the clinical and research divisions of the Peter MacCallum Cancer Centre. This included the staff and resources of the Animal Facility, the Centre for Advanced Microscopy and Histology, the Victorian Centre for Functional Genomics, the Bioinformatics Core and the Flow Cytometry Facility. I must mention a thanks to Dr Catherine Mitchell, who was ever obliging in providing

assistance for histology. A thank-you must also go to Prof Stephen Fox and So-Young Moon for facilitating collaboration with the Cancer 2015 project. Thanks also to Dr Ken Doig for assistance with variant calling; Dr Maria Doyle for advice with galaxy, Richard Lupat for assistance with the whole exome sequencing analysis and Huiling Xu for her assistance and help on so many fronts. I must also thank Caroline Owen, for everything administrative and her tireless efforts in ensuring that I am always aware and prepared for looming deadlines.

I must specially thank Prof Mark Shackelton, with whom I have shared many conversations (and the occasional beer) both prior to and during the course of my PhD. You have been an incredible mentor over the years, providing encouragement, guidance and inspiration across all aspects of both work and life, particularly when things were looking a little downcast at the beginning of my PhD. Even though our formal catchups are now less frequent, I know your advice is only a phone call away.

I also must acknowledge the very generous financial support I received, both in scholarships and grants. The Colorectal Surgical Society of Australia and New Zealand Foundation provided funding for my laboratory research. The Royal Australasian College of Surgeons, the Foundation for Surgery, Tour de Cure, The National Health and Medical Research Council and Cancer Therapeutics all provided scholarship support, allowing me to concentrate on my laboratory work. My PhD would not have been possible without this support, and for that I am incredibly grateful.

I am most grateful to my parents, friends and surgical colleagues for their support and encouragement to complete my research. I lastly and most importantly must acknowledge the role my amazing wife and family have played, for without their tireless support, this journey would not have been

possible. Penny has been the hidden stalwart throughout my prolonged surgical training and higher degree, shouldering the load of raising four children and maintaining a functioning family unit that would otherwise have descended into chaos. She has done all of this while maintaining her ever-present sunny disposition, providing comic relief and an ear for catharsis at times of despondency. You are an incredible person who supports me without question, and I look forward to the next chapter in life together.

List of Abbreviations

Abbreviation	Definition
2DRT	Two-Dimensional Radiotherapy
3DCRT	Three-Dimensional Conformal Radiotherapy
4-OHT	4-Hydroxy-Tamoxifen
5FU	5-Fluorouracil
18F-FDG	[¹⁸ F]fluorodeoxyglucose
ACK	Ammonium Chloride Potassium
ASCC	Anal Squamous Cell Carcinoma
AQIS	Australian Quarantine and Inspection Service
BSA	Bovine Serum Albumin
CFS	Colostomy Free Survival
CIM	Cell Invasion Migration
Cis	Cisplatin
CL	Cell Line
COSMIC	Catalogue of Somatic Mutations in Cancer
CR	Complete Response
Cre	Cre Recombinase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRTx	Chemoradiotherapy
CTx	Chemotherapy
CT	Computed Tomography
CTV	Clinical target Volume
DARE	Digital AnoRectal Examination
DFS	Disease Free Survival
DMF	Dimethylformamide
DMBA	7/12-Dimethybenzanthracene

DMSO	Dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
EDTA	EthyleneDiamineTetraAcetic acid
EUA	Examination under Anaesthesia
FACS	Fluorescent Associated Cell Sorting
FCS	Foetal Calf Serum
F/Up	Follow Up
FVS	Fixed Viability Stain
Fwd	Forward
GFP	Green Fluorescent Protein
Gy	Gray
HD	High Dose
H & E	Haematoxylin & Eosin
HIV	Human Immunodeficiency Virus
HNSCC	Head & Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
HS	Human Serum
HSIL	High Grade Squamous Intraepithelial Neoplasia
ICC	Immunocytochemistry
IFN- γ	Interferon-gamma
IHC	Immunohistochemistry
IMRT	Intensity Modulated Radiation Therapy
IU	Infectious Unit
K5	Keratin 5
Kb	Kilobyte
KM	Kaplan Meier
LN	Lymph Node
Ln	Natural Log

LRC	Locoregional Control
LRF	Locoregional Failure
LSIL	Low Grade Squamous Intraepithelial Neoplasia
LVI	Lymphovascular Invasion
MDSC	Myeloid Derived Suppressor Cells
MFI	Mean Fluorescence Intensity
MHC I	Major Histocompatibility Complex I
Mins	Minutes
MMC	Mitomycin C
MOI	Multiplicity of Infection
MRI	Magnetic Resonance Imaging
MSM	Men who have sex with men
MQ	MilliQ
NBF	Neutral Buffered Formalin
NLRD	Notifiable Low Risk Dealing
NK	Natural Killer
NKT	Natural Killer T Cells
NSG	Nod-SCID-IL2R γ ^{null}
Nude	Athymic Mice
O ₂	Oxygen
OCT	Optimal Cutting Temperature
OS	Overall Survival
pac	Puromycin N-Acetyl-Transferase gene
PBS -/-	Phosphate Buffered Saline (without Ca ²⁺ /Mg ²⁺)
PBMC	Peripheral Blood Mononucleocytes
PDL1	Programmed Cell Death Ligand 1
PDTX	Patient Derived Tumour Xenograft
PFA	Paraformaldehyde

PFS	Progression Free Survival
pH	Potential Hydrogen
PI	Propidium Iodide
PMCC	Peter MacCallum Cancer Centre
PNI	Perineural Invasion
PTEN	Phosphatase and Tensin Homolog
QC	Quality Control
R0	Resection Margin Clear
R1	Resection Margin Microscopically Positive
R2	Resection Margin Macroscopically Positive
RBC	Red Blood Cell
rcf	Relative Centrifugal Field
RPMI	Roswell Park Memorial Institute
rpm	Revolutions Per Minute
RT	Room Temperature
RTCA	Real Time Cell Analysis
RTx / RT	Radiotherapy
Rev	Reverse
SCC	Squamous Cell Carcinoma
ScEM	Scanning Electron Microscopy
SCNA	Somatic Copy Number Analysis
SD	Standard Dose
SEER	Surveillance, Epidemiology and End Results
SEM	Standard Error of the Mean
SF2	SF2
SM	Staining Media
STR	Short Tandem Repeat
TAE	Tris Acetate Ethylenediaminetetraacetic acid EDTA

TCGA	The Cancer Genome Atlas
TILs	Tumour Infiltrating Lymphocytes
TNM	Tumour Node Metastasis
Treg	Regulatory T cell
UBC	Ubiquitin C
US	Unstained
VCS	Victorian Cytology Service
VR	Vicryl rapide
Vcf	Variant Calling Format
WES	Whole Exome Sequencing
wt	Wildtype
X-Gal	5-bromo-4-chloro-3-indolyl-B-D-galactoside
yr	Year

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Chapter 1: Introduction

1.1 Anal Squamous Cell Carcinoma

1.1.1 Demographics, Incidence and Epidemiology

Anal squamous cell carcinoma (ASCC) is the most common malignancy to affect the anal canal, accounting for the majority of all histological types. It is a rare disease, comprising less than 5% of all carcinomas of the lower gastrointestinal tract, with the annual incidence rate of ASCC ranging from 1 to 1.9 per 100,000 population in western countries.¹ It is most frequently diagnosed in the 5th and 6th decade of life, and has a female predominance at 1.5 times the incidence of males.²

The incidence of ASCC has risen dramatically and more than doubled over the last thirty years, both in Australia and overseas. In Australia in 1982, the incidence rate was 0.6 per 100,000 population, which has now tripled to 1.8 per 100,000 in 2014 as demonstrated in Figure 1-1.³ This equates to approximately 400 new cases being diagnosed each year in Australia. These findings have been identified elsewhere in the world, predominantly within western countries.^{1,4}

Despite the rising incidence in ASCC across the globe, anal cancer survival rates have improved little over this period, with the SEER database reporting that the current 5-year overall survival remains at around 67.4%, and over 1100 deaths per year in the United States of America.² From an Australian perspective, the data demonstrates a similar trend to the incidence, with the mortality rate of anal cancer having increased four-fold since 1968, to approximately 90 deaths per year in Australia (Figure 1-1).

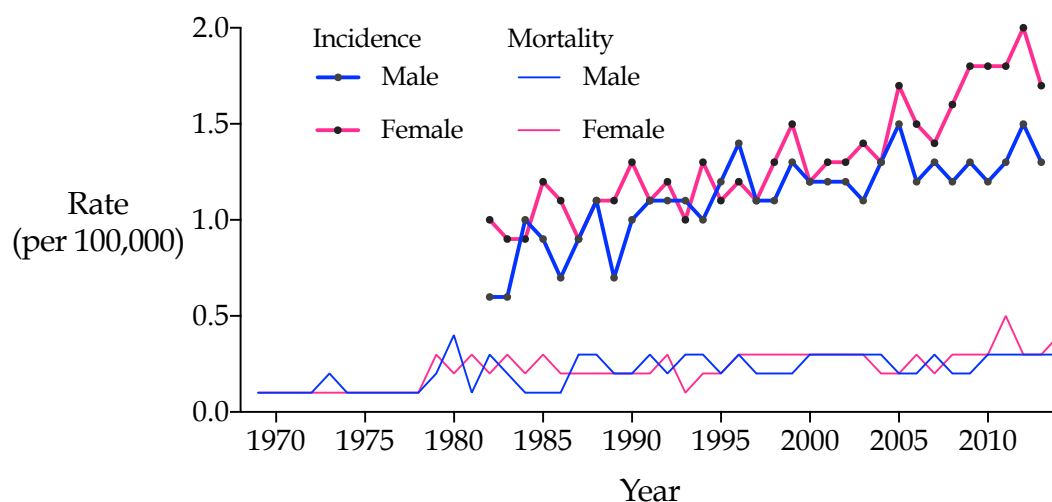


Figure 1-1 Incidence and Mortality of anal SCC in Australia

Age-standardised rate per 100,000 persons over time.

1.1.2 Aetiology, Risk Factors and Pathogenesis

Oncogenic human papilloma virus (HPV) infection is associated with approximately 90% of ASCC cases and the precursor anal squamous intraepithelial lesions, both high and low grade (HSIL, LSIL). Other risk factors appear to relate to an increased risk of HPV infection or modulation of the immune system's ability to effectively deal with the virus. Such factors include human immunodeficiency virus (HIV; incidence > 100 per 100,000), men having sex with men (MSM; incidence > 35 per 100,000), anoreceptive intercourse, high number of sexual partners, immunosuppression (chronic steroid use, organ transplantation), autoimmune disease, increasing age, other HPV related cancers, smoking and low socioeconomic status.^{4, 5} While the cause of the increasing rate of ASCC is not well defined, it is postulated that changing sexual practices have led to an increased prevalence of oncogenic HPV in the anal epithelium and that an increasing number of individuals living with HIV has contributed.⁶⁻¹³

Biologically, anal cancer is quite homologous to cervical cancer with a strong association with HPV and its likely origin from pre-malignant changes with progression from low to high-grade intraepithelial lesions (HSIL) and

subsequently to invasive cancer. This also offers some explanation behind the high number of affected individuals being female, with a perineal field change secondary to HPV infection, often involving the cervix, vagina, vulva and anus. Evidence suggests that similar to the pre-malignant changes in the cervix, up to 10-11% of high-grade pre-malignant anal cases progress to invasive anal cancer as reported by Watson et al.¹⁴ This also highlights the importance of prevention by way of HPV vaccination, given its impact across all HPV driven cancers. Furthermore, HPV-associated ASCCs are more responsive to concurrent chemoradiotherapy (CRTx), providing some insight into why females have a higher incidence of ASCC yet better prognosis than men.

1.1.3 Histological Classification

The anal canal is an area of anatomical complexity secondary to it marking the embryological junction of the hindgut with ingrowth of the proctodeum. Consequently, the upper half is of endodermal origin and lined by columnar epithelium and the lower half is ectodermal in origin with non-keratinising stratified squamous epithelium. An anal transitional zone or squamocolumnar junction exists at the level of their convergence, indicated approximately by the irregular dentate line. The anal verge or ano-cutaneous line is where the distal end of the anal canal meets with perianal skin, characterised by keratinising squamous epithelium with skin appendages.¹⁵ This junction approximately corresponds to the inter-sphincteric groove. The anal margin is the surrounding 5cm of skin from the anal verge characterised by darker pigmentation.¹⁶

Given the complexity of this region, there are multiple histological subtypes of anal cancer as listed in Table 1-1, of which squamous cell carcinoma is the most common.¹⁷ Squamous tumours are graded from well through moderately to poorly differentiated, with significant intra-tumour heterogeneity in larger

tumours. Histological grade has not been confirmed as a prognostic factor in multivariate analysis.¹⁶ Furthermore, while historical subclassifications of basaloid, transitional, spheroidal, cloacogenic and keratinising were utilised, there is no evidence based prognostication or treatment implication. Consequently, this terminology has been abandoned in recent guidelines.^{16, 18} Mixed histology tumours are also rarely encountered including adenosquamous carcinomas.

Table 1-1 Histological Subtypes of Anal Cancer

Histology	Frequency (%)
Epithelial	96
Squamous Cell Carcinoma	83
Adenocarcinoma	12
Neuroendocrine Carcinoma	1
Melanoma	1.6
Lymphoma	<1
Gastrointestinal Stromal Tumour	<1
Secondary Tumours	<1

1.1.4 Anatomical Classification

While SCC is the predominant histological type of anal cancer, there are two anatomical subtypes of anal carcinoma. They are distinguished by their location in relation to the anal verge and have important prognostic and treatment implications. Anal margin SCC's which account for 25%, are located in the anal margin skin distal to the anal verge and have a more favourable prognosis. They usually present at an early stage, are often well differentiated, more commonly found in men and are generally managed with wide excision similar to cutaneous SCC's. The exception is when they are locally advanced and invading adjacent structures, most frequently the anal sphincters.^{16, 19}

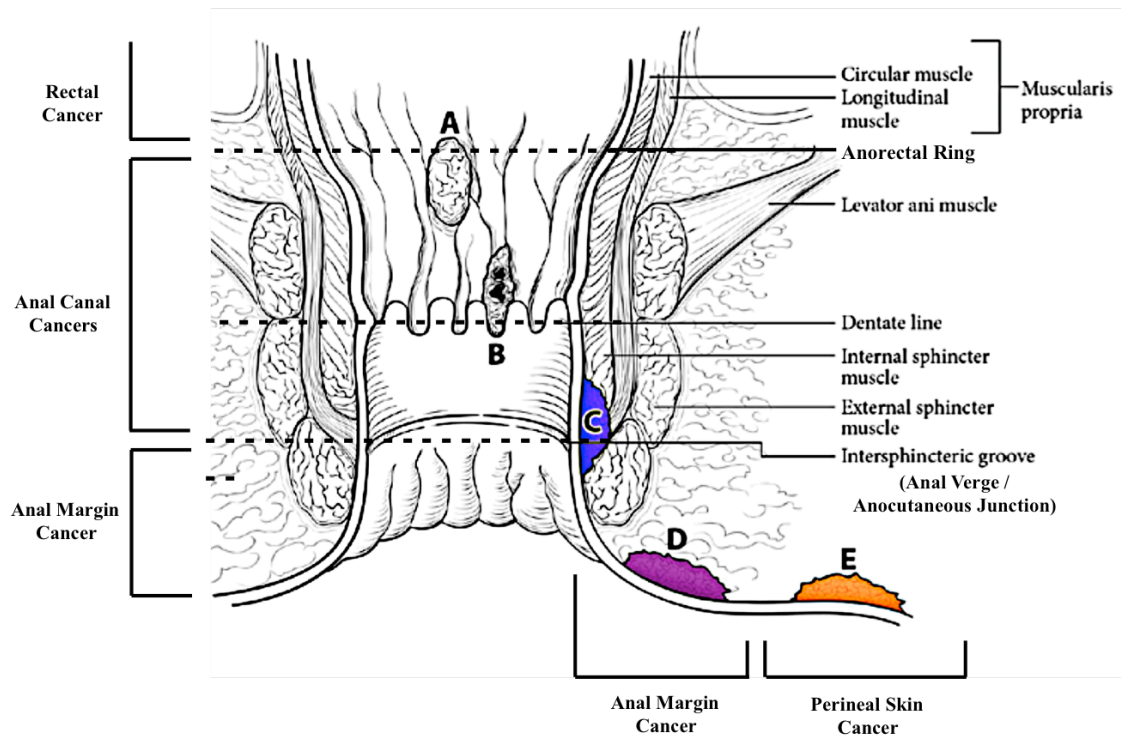


Figure 1-2 Anatomy of the Anal Canal

Sagittal cross-section demonstrating the complex anatomy of the anal canal with corresponding anatomical zones affected by anal canal (A-C), anal margin (D), skin (E) and rectal cancers. Adapted from Amin et al ²⁰

Anal canal SCC's on the contrary have an epicentre based somewhere between the anorectal ring (proximal end of the anal canal) and the anal verge (distal end of the anal canal), and account for the majority of ASCCs.^{16, 21} SCCs that arise outside 5cm from the anal verge are considered to be cutaneous SCCs. SCCs that occur proximal to the anorectal ring are considered to be rectal in origin and occur at an even lower frequency than anal. While this classification system is appropriate, in practice, large tumours often cross anatomical boundaries, which can create difficulty in identifying the point of origin as rectal, anal canal or margin. In these cases, most are managed as anal canal SCCs, with definitive chemoradiotherapy.

1.1.5 Clinical Presentation

Symptoms of anal cancer most frequently include bleeding, pain, and a palpable mass or ulcer (Figure 1-3). These typically mimic other diseases including haemorrhoids and fissures that are more common and benign in nature. Owing to these non-specific symptoms and with up to 20% of patients being asymptomatic, a diagnosis of anal cancer is often delayed.¹⁸ Less commonly, patients can present with tenesmus, change in bowel habit, faecal incontinence, pruritis, and in advanced disease fistulae or inguinal lymphadenopathy. Incidental findings of early anal cancer can occur after the removal of anal skin tags or haemorrhoids and in the setting of surveillance biopsies for HSIL.¹⁶

A comprehensive history and physical examination including a digital ano-rectal examination (DARE) is required whenever patients present with the aforementioned symptoms that raise the need to exclude an insidious cause. Definitive diagnosis is obtained by way of an Examination Under Anaesthesia (EUA) with a biopsy for histological confirmation.



Figure 1-3 Locally Advanced Anal Canal SCCs

An ulcerated (left) and large fungating (right) anal canal SCC involving anal margin skin and sphincters (Consent obtained from respective patients for publication of clinical photographs)

1.1.6 Staging

Anal cancer is staged prior to treatment in accordance with the American Joint Committee on Cancer (AJCC) Tumour Node Metastasis (TNM) cancer staging system.²⁰ This staging system is predictive of treatment response and prognostic of outcome.²² Consequently, accurate pre-treatment staging informs appropriate treatment decisions. Staging is undertaken by combining the findings of the EUA with imaging. Several imaging modalities are often employed to improve the accuracy for each component of the TNM system.

1.1.6.1 Tumour Stage

Tumour stage is an independent predictive and prognostic factor influencing the treatment response, risk of local relapse and disease free and overall survival in patients treated with curative intent.²³ The stage of anal cancer at diagnosis and the 5-year overall survival based on stage at presentation derived from the SEER database is represented in Figure 1-4.

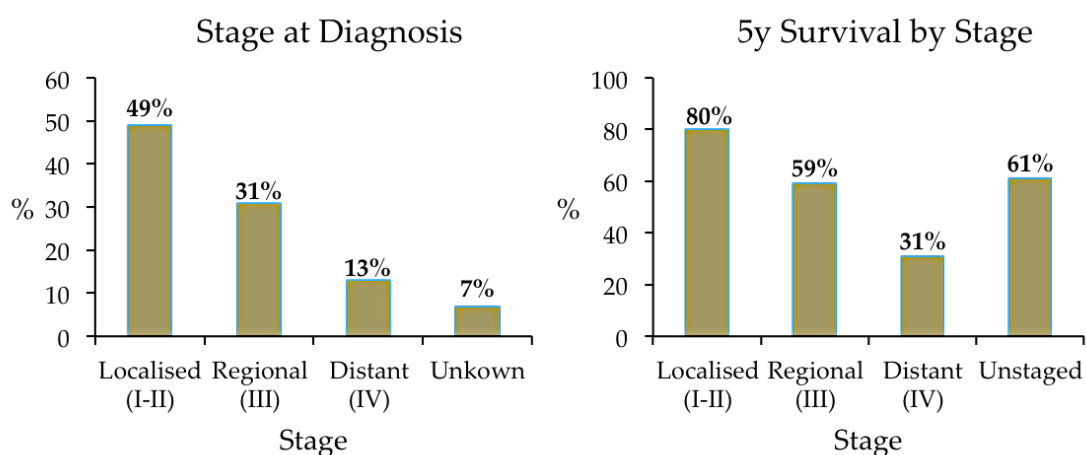


Figure 1-4 Stage at diagnosis and 5-year overall survival based on stage

AJCC stage of anal cancer at diagnosis and the 5yr overall survival for all anal cancer patients based on data derived from the SEER database

Examination under anaesthesia not only provides an opportunity to obtain biopsy tissue for a histological diagnosis, but importantly facilitates the assessment of the primary tumour. This includes determining its position in relation to anatomical landmarks to allow the delineation as an anal canal or margin tumour. It also enables assessment of the size of the tumour and whether there is evidence of invasion (degree of mobility / fixation) into other organs to define the T stage.

Magnetic Resonance Imaging (MRI) of the pelvis is also frequently employed as an adjunct that allows accurate definition of the local anatomy and assists with the delineation of the T stage.^{24, 25} The advent of diffusion-weighted imaging has also provided the ability for MRI to be used in the assessment of treatment response.²⁶ However, it is contraindicated in a proportion of patients (metallic device, claustrophobia etc), expensive and not freely available at all centres. Endoanal ultrasound is utilized in some centres as a means of improving the accuracy of T staging. However, the quality of this assessment is highly operator dependent, and combined with an inability to accurately stage the nodal disease, it has had limited uptake across the globe.²⁷

[¹⁸F]Fluorodeoxyglucose - Positron Emission Tomography (18F-FDG-PET) allows assessment of the metabolic activity of the primary tumour, with the majority of ASCC's being [¹⁸F]Fluorodeoxyglucose (18-FDG) - avid.^{28, 29} In a similar manner to MRI, this functional imaging modality can also be used in the assessment of response following the completion of treatment.²⁶

Table 1-2 AJCC and UICC TNM 8th edition staging classification of ASCC

Primary Tumour (T)			
<i>Tx</i>	Primary tumour cannot be assessed		
<i>T0</i>	No evidence of primary tumour		
<i>Tis</i>	High grade squamous intraepithelial lesion (HSIL) [#]		
<i>T1</i>	Tumour ≤ 2cm		
<i>T2</i>	Tumour > 2cm but ≤ 5cm		
<i>T3</i>	Tumour > 5cm		
<i>T4</i>	Tumour of any size invading adjacent organ(s)		
Regional lymph Nodes (N)*			
<i>Nx</i>	Regional lymph nodes cannot be assessed		
<i>N0</i>	No regional lymph node metastasis		
<i>N1</i>	Metastasis in regional lymph nodes		
<i>N1a</i>	Metastasis in inguinal, mesorectal or internal iliac nodes		
<i>N1b</i>	Metastasis in external iliac lymph nodes		
<i>N1c</i>	Metastasis in external iliac with any N1a nodes		
Distant Metastases (M)			
<i>Mx</i>	Distant metastasis cannot be assessed		
<i>M0</i>	No Metastases		
<i>M1</i>	Metastases		
Stage	T	N	M
<i>I</i>	T1	N0	M0
<i>II</i> <i>A</i>	T2	N0	M0
<i>B</i>	T3	N0	M0
<i>III</i> <i>A</i>	T1-2	N1	M0
<i>B</i>	T4	N0	M0
<i>C</i>	T3-4	N1	M0
<i>IV</i>	T any	N any	M1

[#]HSIL – previously termed carcinoma in situ, Bowens disease, anal intraepithelial neoplasia II-III, high grade anal intraepithelial neoplasia; *N1, N2, N3 have been re-classified in the 8th edition to N1a, 1b, 1c

1.1.6.2 Nodal Stage

The nodal stage is critical not only in its influence on prognosis, but most importantly in treatment planning, by allowing the tailoring of appropriate radiotherapy fields and doses based on the nodal basins involved.²³

The lymphatic drainage of the anal canal is bi-directional secondary to its embryological origin. The proximal half drains to perirectal nodes and subsequently to higher echelon nodes along the inferior mesenteric artery. The distal half drains to the internal pudendal and internal iliac nodes as well as inguinal, femoral and external iliac nodes (Figure 1-5). Consequently, diagnosis and treatment of locoregional disease is based on the assessment of all of these lymph node basins.²⁰

Nodal involvement is frequently identified as one of the most significant prognostic variables after T stage to affect overall survival and locoregional failure in ASCC. However, assessing nodal spread in ASCC is challenging, given difficulties in distinguishing benign reactive lymphadenopathy from malignant nodes. It is reported that up to 35% of ASCC patients have nodal involvement, with 15% to 25% affecting the inguinal nodal basins.³⁰⁻³² This group of patients requires targeting of the inguinal and other nodal basins by radiotherapy, to ensure adequate treatment to all detectable disease including a selective boost to affected nodal basins.²⁸ While clinically palpable inguinal nodes are easily assessed as involved, non-palpable inguinal lymph nodes and other nodal basins aren't clinically assessable, and present a more difficult challenge. The identification of their involvement is key to reduce the under and over-treatment of patients.

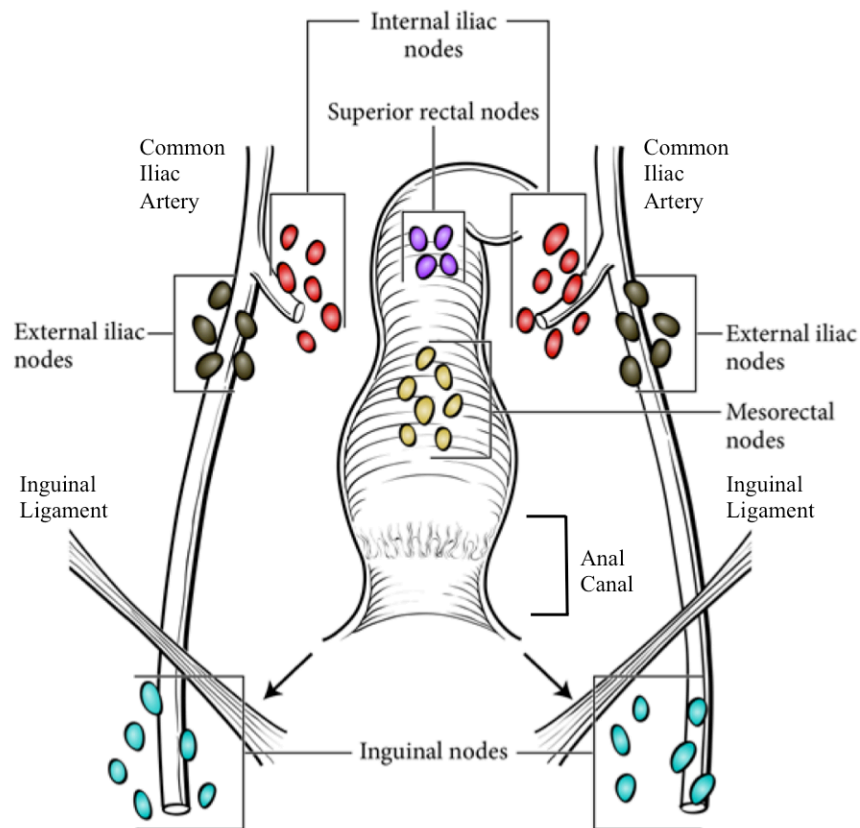


Figure 1-5 Lymph node stations affected in anal cancer:

Lymph node drainage from the anal canal occurs via the inguinal nodes to the higher echelon iliac node basins and via the mesorectal nodes to the superior rectal and more proximal mesenteric lymph node basins. Adapted from Amin et al ²⁰

Computed Tomography (CT) and MRI are two frequently employed imaging modalities to assist with the delineation of nodal stage. Given the inaccuracy (overestimation) of relying on size criteria, high resolution MRI has improved nodal discrimination by evaluating morphological characteristics that are associated with metastatic infiltration.²⁶

18-FDG-PET has also been utilised for determining nodal involvement, by comparing the metabolic activity of the primary tumour with the possible sites of nodal disease. The greatest limitation with detection of involved nodes with this modality is a nodal size >8mm. A systematic review and meta-analysis of the current literature revealed that utilising 18-FDG-PET led to altered nodal

staging in 28% of patients when compared to conventional imaging modalities of CT and MRI. Furthermore, when limited to studies assessing the more recent advancement of combination 18-FDG-PET/CT, the TNM staging was altered in 41% of patients.³³⁻³⁶ In light of these findings, routine pre-treatment staging with 18-FDG-PET/CT was recommended. Adding weight to this argument, is the further utility of both MRI and 18-FDGPET/CT to assess the treatment response when comparing pre and post-treatment scans.

For suspicious nodal involvement, a fine needle aspirate or core biopsy can also be employed to confirm the diagnosis, given the impact on treatment decisions of such critical information.²⁶

The utility of sentinel lymph node biopsy (SLNB) in staging the regional disease has also been explored in limited studies. This facilitates the identification of inguinal nodal metastases when otherwise not clinically evident, and relies on there being an inherently significant false negative rate in nodal staging via imaging alone. It also relies on the tenet that radiation to the groin has significant toxicity, and by improving nodal staging, this can be avoided in a select cohort of node negative patients, including 30% of patients with T3/4 tumours.³⁷ Patients with ASCC that do not receive prophylactic inguinal irradiation will develop inguinal recurrence at a rate of 0-12% for T1-2 and 19-30% for T3-4 disease.³⁸⁻⁴⁰ Consequently, elective irradiation is the current standard of care for all but T1N0 tumours, with a failure rate under 5%.^{31, 41}

The literature on SLNB is quite heterogenous, with an early publication quoting a complication rate of 24%, with metachronous inguinal metastases in 14% of the SLN negative group on follow up, highlighting under-treatment due to a significant false negative rate.⁴² More recent studies have demonstrated a much lower false negative rate, with a review of the literature summing to a rate of

3.7%.³⁷ In the most recent publication, SLNB improved upon PET based nodal staging, allowing for the selection of true negative patients that could avoid inguinal irradiation.³⁸

Adding weight to this discussion, a recent publication has also highlighted the challenges with nodal staging, by demonstrating the fact that with the adoption of new imaging modalities including MRI and PET, there has been a significant increase in the lymph node positivity rate (LNP) without a change in survival, from 17%-20% for trials undertaken in the 90s (RTOG87-04⁴³, ACT I⁴⁴) to 27%-35% for trials in the 2000s (RTOG 98-11⁴⁵, ACT II⁴⁶). This has been postulated to lead to nodal stage migration, which subsequently reduces prognostic discrimination and theoretically results in patient overtreatment.²²

Nonetheless, there have been significant improvements in the delivery of radiotherapy over the last 40 years, with a consequent improvement in toxicity. This must be weighed up against the risks of undergoing an invasive procedure in SLNB. While SLNB is not currently part of routine practice, it continues to be evaluated. With significant false negative rates for both imaging and SLNB, elective inguinal irradiation remains the current recommendation in all but T1 tumours, in the most up to date published guidelines.^{16, 18} This may change in the future, with the current Personalising Anal cancer radioTherapy dOse (PLATO) trial assessing the effectiveness of modifying the dose of chemoradiotherapy delivered based on the tumour and nodal staging.

1.1.6.3 Metastasis Staging

The identification of metastatic disease is crucial for treatment decision making in the current era, with novel and multimodality management holding the potential hope of cure in a historically palliative cohort of patients.⁴⁷

A CT of the chest, abdomen and pelvis is routinely undertaken to assess for the presence of distant metastatic disease. PET/CT has also been shown to be a useful adjunct in assessing metastatic disease, by comparing the metabolic activity of the primary with the suspected metastatic lesions.^{33, 35} In the setting where the diagnosis of metastatic disease remains unclear, a fine needle aspirate or core biopsy can also be employed to confirm the diagnosis, given the importance of this information for subsequent management.

The most frequent sites affected by metastatic disease in ASCC are para-aortic nodes, liver and lung and infrequently the brain, bones and skin.

1.1.7 Prevention

While a nine valent vaccine has been developed and is currently administered to all school aged girls and boys in Australia, its role is in the prevention of HPV associated cancers. However, this vaccine does not currently have a role in established ASCC. Furthermore, given the lag period between the initial HPV infection and the development of ASCC being approximately 40 years, the vaccine will take approximately four decades before we see a significant decline in HPV driven ASCC incidence.⁴⁸

1.2 Treatment

Various treatments have historically been utilized to treat ASCC. Prior to the mid-1970s, surgery in the form of an abdominoperineal resection with removal of the entire anal canal was the preferred management for patients with ASCC.⁴⁹ This procedure offered reasonable survival outcomes but was associated with significant morbidity and impacted on the patient's quality of life, due to the necessity of a colostomy bag. Furthermore, given the magnitude of the operation,

the poor health of some patients and the advanced nature of some cancers, precluded surgery as an option.⁵⁰

In 1972, Dr. Norman Nigro pioneered the “Nigro Protocol” which involved combination chemoradiotherapy to manage ASCC. Patients were administered 5-fluorouracil and mitomycin-C in conjunction with 3000 rads of external beam radiotherapy.^{51, 52} While this was initially followed by surgery, pathological evaluation identified that this pre-operative regimen was able to achieve complete tumour regression in over 80% of patients. Consequently, chemoradiotherapy as a definitive treatment was further explored, and found to offer superior results to surgical resection alone and without the associated morbidity.^{53, 54} The Nigro protocol established itself as the gold standard in the primary treatment of ASCC, offering locoregional control and improved preservation of anal function and quality of life.

1.2.1 Primary Chemoradiotherapy

There have only been six phase III randomised controlled trials undertaken in ASCC over the last thirty years, with the findings dictating current primary treatment. This has included significant evolution and refinement to Nigro’s original protocol. The details of these trials are presented in Table 1-3, with the summary of the findings below.

1.2.1.1 Chemoradiotherapy superior to Radiotherapy

The United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Anal Cancer Trial (ACT) 1 compared definitive treatment with radiotherapy alone (45Gy + 15Gy boost after 6 weeks) to radiotherapy combined with 5FU (1000mg/m²/ day, days 1-4 & 29-32) and MMC (12mg/m² on day 1). The combined modality arm resulted in a 25% improvement in locoregional control

and 10% increase in colostomy free survival at 5 years. In the European Organisation for Research and Treatment of Cancer (EORTC) – 22861 trial, radiotherapy consisted of 45Gy + 20/15Gy boost (partial / complete response) after 6 weeks compared with combined chemoradiation with 5FU (750mg/m² daily, days 1-5 & 29-33) + MMC (15mg/m² on day 1). This revealed a 26% increase in the complete response rate, an 18% reduction in locoregional failure and a 32% improvement in colostomy free survival at 5 years.

1.2.1.2 Concurrent 5FU / MMC superior to 5FU

Radiation Therapy Oncology Group (RTOG) 87-04 assessed the addition of MMC to 5FU and radiotherapy. This identified that the addition of MMC was beneficial, with a 12% improvement in CFS and 22% in DFS.

1.2.1.3 5FU / Cisplatin not superior to 5FU / MMC

RTOG 98-11 and UKCCCR ACT II assessed the replacement of MMC with Cisplatin, given the toxicities associated with MMC. This demonstrated an improved CFS rate of 7% in the MMC arm of RTOG 98-11, with no statistically significant difference identified in ACT II, but a trend towards also favouring MMC for survival outcomes. 5FU / MMC remains the preferred chemotherapeutic options in the definitive chemotherapeutic regimen.

1.2.1.4 No benefit with neoadjuvant or adjuvant chemotherapy

Induction chemotherapy (CTx) was investigated with 5FU / Cisplatin in RTOG 98-11 and ACCORD-03, with both trials identifying no benefit in terms of locoregional control or survival outcomes. Maintenance chemotherapy was investigated in ACT II for both 5FU / Cisplatin and 5FU / MMC, with no impact on survival outcomes.

Table 1-3 Randomised Phase III Clinical Trials in ASCC

Trial	<i>n</i>	Eligible	Comparison Arms	F/Up# (Med)	CR (%)	LRC (%)	CFS (%)	DFS / PFS (%)	OS (%)	Independent Prognostic Variables
UKCCCR ACT I^{55, 56} (1987-1994)	585	T1-4 N0-3	RT* alone RT + 5-FU/MMC	13y	30 39	43 (5y) 68	37 (5y) 47	- -	53 (5y) 28 (12y) 58 33	Male Gender Clinically + LNs High WBC count
EORTC 22861⁵⁷ (1987-1994)	110	T3/4 N+	RT* only RT + 5-FU/MMC	3.5y	54 80	50 (5y) 68	40 (5y) 72	- -	56 (5y, combined)	Male Gender Nodal Status Cutaneous ulceration
RTOG 87-04⁴³ (1988-1991)	310	T1-4 N0-3	RT* + 5-FU RT +5-FU/MMC	3y	85 92	66 (4y) 84	59 (4y) 71	51% (4y) 73%	67 (4y) 76	<i>Not evaluated</i>
RTOG 98-11^{45, 58} (1998-2005)	682	T2-4 N0-3	Induction 5-FU/Cis → RT* + 5-FU/Cis RT + 5-FU/MMC	2.5y	- -	67 (5y) 75	65 (5y) 72	<i>54 (5y)</i> <i>60</i>	70 (5y) 75	Male Gender Clinically + LNs Tumour > 5cm
UKCCCR ACT II⁴⁶ (2001 – 2008)	940	T1-4 N0-3	RT +5-FU/MMC (maintenance) RT +5-FU/MMC (no maintenance) RT+5-FU/Cis (maintenance) RT+5-FU/Cis (no maintenance)	5y	91 90	- -	68 MMC (5y) 67 Cis 69 Main (5y) 66 No Main	<i>69 MMC (5y)</i> <i>69 Cis</i> <i>70 Maint (5y)</i> <i>69 No Maint</i>	79 MMC (5y) 77 Cis 76 Maint (5y) 79 No Maint	Male Gender T-stage Baseline Haemoglobin
UNICANCER ACCORD-03^{59, 60} (1999-2005)	307	>40mm +/or N+	Induction 5-FU/Cis → RT + 5-FU/Cis + SD boost Induction 5-FU/Cis → RT + 5-FU/Cis + HD boost RT + 5-FU/Cis + SD boost RT + 5-FU/Cis + HD boost	4.2y	79% (2m)	72 (5y) 88 84 78	<i>70 (5y)</i> 82 77 73	<i>64 (5y)</i> 78 67 62	74 (5y) 71	Age > 55y Total RTx dose > 60Gy Skin ulceration Inguinal Node involvement Circumferential tumour spread > 1/3

ACT I: 45Gy + 15Gy boost after 6-week gap. EORTC 22861: 45Gy + 20Gy/15Gy (partial/complete response) after 6 weeks; RTOG98-11: Induction – 2 cycles Cisplatin / 5FU, 45Gy / 25F, T3/4 or residual T2 – boost to 54Gy. ACT II: 50.4Gy / 28F, Phase I 30.6Gy, Phase II 19.8 Gy, no gap; ACCORD-03: 45Gy / 25F, 3wk gap boost to 15Gy vs 20-25Gy; Primary endpoints are italicised. Statistically significant results are in bold face print. # F/Up – Follow-Up; Med – Median; CR – Complete Response, LRC – Locoregional Control; CFS – Colostomy Free Survival; DFS / PFS – Disease Free Survival / Progression Free Survival; OS – Overall Survival; 5FU – 5 Fluorouracil; MMC - Mitomycin C; Cis – Cisplatin; RT / RTx – Radiotherapy; Maint – Maintenance; HD – High dose; SD – Standard dose; N – Node; LN – Lymph Node;

1.2.1.5 Evolution of Radiotherapy

There has been significant evolution in the delivery and dose of external beam radiotherapy since its acceptance as a component of primary treatment in ASCC.

1.2.1.6 Radiotherapy Dose

Nigro originally utilised low dose radiation at a cumulative dose of 30Gy. However, higher doses have demonstrated reduced recurrence, with the standard dose now a total of 50.4 (T1/2N0) to 54Gy (T3/4N0/1) in 30 fractions to the gross disease with 45Gy to elective nodal basins.⁶¹ Intensification of the radiation dose above this level is associated with greater toxicity and the requirement for treatment breaks, which have been linked with poorer outcomes and are a predictor of disease recurrence.^{62, 63} RTOG 92-08 assessed dose escalation to 59.6Gy, with a mandatory 2-week break, which only 12% of patients required in RTOG 87-04. This resulted in a colostomy rate of 30% at 2 years compared with 7% in RTOG 87-04, demonstrating no improvement in local control secondary to the need for a treatment break.⁶⁴ ACCORD-03 also assessed the efficacy of a radiation boost (20-25Gy vs 15Gy), with no statistically significant difference identified. There was however a trend towards an effect, and this may have been masked by the structure of the trial and low number of patients receiving a radiotherapy boost.

This question is again being addressed with a Phase II/III clinical trial (ACT 5) as part of the Personalising Anal cancer radioTherapy dOse (PLATO) trial. This will assess higher dose chemoradiotherapy (58.8Gy/28# and 61.6Gy/28#) in locally advanced disease utilising a more targeted delivery with IMRT than what has previously been evaluated, to determine if it reduces the risk of cancer recurrence without excessive side effects. On the flipside, ACT 3 is looking at the

elimination or minimisation of radiotherapy in small anal tumours that have been excised and ACT 4 is assessing reduced dose chemoradiotherapy (41.4Gy/23#) for intermediate-risk disease.

1.2.1.7 Radiotherapy Targeting and Contouring

The Clinical Target Volume (CTV) is used to tailor the delivery of radiotherapy to the tumour, surrounding tissue and nodal basins that harbour macroscopic or may be involved with microscopic disease. This includes the anal canal and surrounding tissue, mesorectum and the obturator, common iliac, external iliac, internal iliac, pre-sacral and inguinal nodal basins. The exception is early T1N0 tumours, where inguinal and high pelvic nodal basins are often excluded from the CTV.⁶¹ It is important to contour nodal volumes, by understanding areas of highest risk through anatomic landmarks and the use of PET/CT to improve the accuracy of planning treatment volumes. To assist in this regard, two high resolution contouring atlases with practical planning guidelines have been developed and published.⁶⁵⁻⁶⁷ While this now facilitates more accurate targeting of the tumour with avoidance of radiation to normal surrounding structures and consequently reduced toxicity, it has necessitated significant evolution in the delivery of external beam radiation.⁶¹

1.2.1.8 Radiotherapy Delivery

The delivery of radiotherapy has evolved over time since the historical two-dimensional technique (2DRT) used by Nigro with parallel-opposed fields. Three-dimensional conformal radiation therapy (3DCRT) involves the incorporation of CT imaging data to improve targeting and minimise radiation to bystander normal tissue. Intensity Modulated Radiation Therapy (IMRT) has further optimised the delivery beyond 3DCRT, achieving a highly conformal

approach of targeting the tumour and nodal basins while avoiding surrounding dose sensitive structures by utilising the published contouring atlases.^{62, 66}

The IMRT approach facilitates improved dose homogeneity above that offered with 3DCRT, minimising hot and cold spots within the CTV. This most accurately targets the primary and often complex anatomy of the nodal basins in the pelvis whilst minimising exposure to at risk organs including small intestine, bladder, the hip joints and genitalia. RTOG 05-29, a phase II trial explored the use of dose-painted (DP)-IMRT, achieving a 92% complete response rate, with reduced treatment breaks secondary to a reduction in G2+ haematological and G3+ dermatological and gastrointestinal toxicity compared to the conventional 2DRT regimen in RTOG 98-11.^{63, 68, 69} The most recent development has been Volumetric Modulated Arc Therapy (VMAT), which is effectually IMRT delivered from a rotating source compared with the fixed field IMRT. This further optimises the accuracy of dose delivery to the CTV, with early reports demonstrating acceptable toxicity.^{70, 71}

1.2.1.9 Elective Nodal Irradiation

Inguinal nodal irradiation is effective in reducing treatment failure in the inguinal region. In the Trans-Tasman Radiation Oncology Group (TROG) 99.02 prospective trial, omission of prophylactic inguinal radiotherapy for T1-2 tumours led to a 22.5% inguinal failure rate at 44 months of follow-up, with isolated inguinal disease in 12.5%.³⁹ In the CORS-03 study, the 5-year cumulative inguinal recurrence rate was 2% in the prophylactically irradiated cohort and 16% in the group without inguinal irradiation, with a 5-year failure rate of 12% for T1/2 and 30% for T3/4 tumours.⁷² While OS and DFS remained similar between the groups despite the above differences, no significant lower limb toxicity was observed. In an Australian study, lack of prophylactic inguinal

irradiation resulted in a failure rate of 1.9% for T1 and 12.5% for T2 tumours.⁴⁰ Given these findings, most guidelines currently recommend prophylactic inguinal irradiation except for T1N0 disease.

1.2.2 Novel Therapies

The majority of the clinical trials undertaken over the last 30 years have focussed on fine-tuning the regimen of chemoradiotherapy as a primary modality in anal cancer. However, there have been a limited number of other drugs and alterations to radiotherapy that have been explored in the primary treatment setting as listed in Table 1-4.

Capecitabine has been investigated as a replacement for infusional 5FU after demonstrating efficacy in clinical trials for other cancers. The EXTRA study demonstrated that capecitabine was well tolerated, with minimal toxicity and equivalent response rates, with similar findings published in multiple retrospective cohort studies and another Phase II trial by Oliveira et al, with a locoregional control rate of 86% at 6 months.⁷³⁻⁷⁶ A systematic review and meta-analysis further validated these findings across 6 studies and 218 patients, with a CRR of 91% and the conclusion that capecitabine is an acceptable and convenient alternative to 5-FU, with similar response rates to those reported in the Phase III trials.⁷⁷

Oxaliplatin has also been investigated as an adjunct to primary treatment, with a Phase II study XELOX-XRT investigating the safety and efficacy of capecitabine with oxaliplatin plus radiotherapy as definitive treatment in twenty patients. This demonstrated a 94% complete response rate and acceptable toxicity, however, no further updates of this trial or other series have been published since 2009.⁷⁸

Paclitaxel, Capecitabine, Mitomycin C and radiotherapy has also been investigated as a primary treatment regimen. The phase II study revealed a CR rate of 86.8% at 26 weeks with a 2-year OS and PFS of 93.6% and 83.9% respectively and G3/4 toxicity in 60.5%. Despite the high toxicity, the authors concluded that it may be beneficial as primary therapy in locally advanced ASCC.^{79, 80}

EGFR inhibitors have also been investigated in ASCC, given the high prevalence of increased EGFR expression in this malignancy.⁸¹⁻⁸³ Both phase I and II trials (ACCORD 16) evaluating the utility of cetuximab as an adjunct to primary treatment revealed significant toxicity requiring early trial closure.^{84, 85} Two further phase II trials E2305 and AMC045 evaluated 106 patients, again demonstrating a G4 toxicity rate of 29% and 4% treatment-associated mortality, despite an improved LRF rate, confirming cetuximab as an unacceptable option for definitive treatment.⁸⁶⁻⁸⁸ This has been further emphasised by the long-term results of ACCORD 16, where after a median follow-up of 4.6 years, the 4-year CFS rate was 53%, OS 73% and DFS 53%, all of which are below the reported rates on long term follow up of RTOG 98-11.⁸⁹

Panitumumab has also been investigated in phase I and II clinical trials.⁹⁰⁻⁹² The Phase I trial required the lowest dose of Panitumumab in conjunction with only one dose of MMC given toxicity. The phase II (FFCD 0904) trial following on from this phase I identified a CR rate of 66.7%, well below that expected, and precluding progression to a phase III study.⁹² The phase II VITAL study (GEMCAD 09-02) recruited 58 patients, with 91% developing G3/4 adverse events but no deaths. 3 year OS was 78.4% and DFS 61.1%, offering no benefit over standard treatment when compared to the Phase III trials.⁹³

Immunotherapy has also been explored in the primary treatment setting of ASCC. ADXS11-001 Lm-LLO immunotherapy involves a live attenuated *Listeria monocytogenes* (Lm) which has been bioengineered to produce an HPV-16-E7 fusion protein which can be delivered as a treatment as opposed to preventive (Gardasil) vaccine to stimulate specific T cell immunity to HPV positive tumours. This has been studied as an adjunct to standard CRTx in a phase II trial of 10 patients.⁹⁴ This demonstrated an 89% DFS at 3 years, with acceptable toxicity.⁹⁵ This provides encouraging data to further explore the role of this therapy in the primary treatment setting.

Table 1-4 Publications on Novel Therapies explored in the primary treatment of ASCC

Articles	Pts	Types of patients	Type of Study	Study	Outcomes	OS
Sparano et al. ⁸⁸ (AMC045, 2017)	45	HIV associated Anal Ca	Phase II	Cetuximab + CRTx (5FU, Cisplatin)	LRF: 20% (3y); PFS 72% (3y) G4 Toxicity: 26%; Rx mortality: 4%	79% (3y)
Garg et al. ⁸⁶ (E3205, 2017)	61	Immunocompetent + Anal Ca	Phase II	Cetuximab + CRTx (5FU, Cisplatin)	LRF: 21% (3y); PFS: 68% (3y); G4 Toxicity: 32%; Rx mortality: 5%	83% (3y)
Yordanov et al. ⁷⁰ (2017)	21	Anal Ca	Cohort	VMAT + CTx	LRC: 81%	91% (2y)
Aparicio et al. ⁹² (2019)	45	Anal Ca	Phase II	Panitumumab + 5-FU/MMC + RTx	CR: 66.7%	94.6%(16m)
Mitra et al. ⁶⁸ (RTOG 0529, 2017)	99	Anal Ca	Phase II	DP-IMRT + 5FU/MMC	CCR: 92% (49m); PFS: 76% (4y) G3+ Toxicity: 20%	86% (4y)
Gordeyev et al. ⁷⁹ (2016)	38	Anal SCC	Phase II	Capecitabine, MMC + Paclitaxel + RTx	G3+ Toxicity: 61%; CR: 87%; 2yr PFS: 84%	94% (2y)
Oliviera et al. ⁷⁶ (2016)	43	Localized anal SCC	Phase II	Capecitabine in place of 5FU	CR: 86%; PR: 7%, LRC: 86% (6m); PD: 23.2% PFS: 21.8 (m)	97.7 %
Perez et al. ⁹⁶ (2015)	10	Anal cancer	Phase I/II	ADXS11-001 + standard CRTx	Complete response with no recurrence	NR
Leon et al. ⁹⁷ (2015)	13	LA Anal Ca	Phase I	Cetuximab + 5-FU/MMC + RTx I	NR	88% (2y)
Olivatto et al. ⁸⁴ (2013)	14	LA Anal Ca	Phase I	Cetuximab + 5FU/Cis + RTx	DFS: 4/14; LRC: 64.2%	2/21 died
Deutsch et al. ⁸⁵ (2013) (ACCORD 16)	16	LA anal Ca	Phase II	Cetuximab + RT + 5FU/Cis	CR: 55%; PR: 45%; PFS: 62% (1y) Recurrence: 38%	92% (1y)
Chakravarthy et al. ⁹⁸ (ECOG E4292, 2011)	33	Anal cancer	Phase II	High dose RTx + 5Fu and Cisplatin	CR: 78%; PFS: 55% (5y)	69% (5y)
Allison et al. ⁹⁹ (2010)	8	Anal Cancer	Cohort	Photodynamic therapy	No local failure, no local or pelvic recurrence	NR
Matzinger et al. ¹⁰⁰ (2009)	80	LA Anal SCC	Phase II	MMC/5FU/RTx vs MMC/CDDP/RTx	ORR 80% vs 92%	NR
Eng et al. ⁷⁸ (XELOX-XRT, 2019)	20	Anal SCC	Phase II	Capecitabine + Oxaliplatin + RTx	CR: 94%; CFS: 100% (2y)	100% (2y)
Glynn-Jones et al. ⁷⁵ (EXTRA, 2009)	31	Anal SCC	Phase II	Capecitabine + MMC + RTx	CR: 77%;	

Konski et al. ¹⁰¹ (RTOG 9208, 2008)	66	Anal Cancer	Phase II	5Fu/MMC + RTx (59,4 Gy) planned treatment breaks	LRF (5y): 29% (Arm A), 15% (Arm B)	NR
Kouloulis et al. ¹⁰² (2005)	49	Anal cancer	Phase II RCT	CRTx + Intracavitary Hyperthermia (RTx+5-FU/MMC +/- ICHT)	LF: 0% (Arm A), 4% (Arm B) LR: 4% (Arm A), 32% (Arm B)	>90% (NS*)
Waternberg et al. ¹⁰³ (1996)	10	Elderly high risk localized Anal Ca	Cohort	CO2 laser	NR	8 y (mean)
Kapp et al. ¹⁰⁴ (1993)	14	Anal cancer	Phase I/II	Interstitial hyperthermia and high dose rate brachytherapy	Recurrence: 78.5%	26 months

OS – Overall Survival; LRF – Locoregional Failure; PFS – Progression Free Survival; G4 – Grade 4; CRTx – Chemoradiotherapy; Rx – Treatment; CR – Complete Response, LRC – Locoregional Control; DFS – Disease Free Survival; CFS – Colostomy Free Survival; PD – Progressive Disease; CCR – Complete Clinical Response; ORR – Overall Response Rate; LF – Local Failure; LR – Local Recurrence; 5FU – 5 Fluorouracil; MMC – Mitomycin C; Cis/CDDP – Cisplatin; RT / RTx – Radiotherapy; VMAT – Volumetric Modulated Arc Therapy; IMRT – Intensity Modulated Radio Therapy; DP – Dose Painted; ICHT – Intracavitary Hyperthermia; N – Node; LN – Lymph Node; LA – Locally Advanced;

1.2.3 Assessment of Treatment Response

Assessment of treatment response in ASCC patients post completion of CRT usually involves a clinical assessment including a digital rectal examination. It also usually entails imaging, dependent on the pre-treatment modalities employed, with CT, MRI and PET all having an element of demonstrated use in this setting. Where any concerns remain, an examination under anaesthesia and biopsy of a suspicious lesion, ulcer or node is mandated to exclude persistent disease.⁴⁶

The timing of assessment of response is equally as important as the means with which treatment response is assessed. Follow-up is normally undertaken at 2/3-monthly intervals for the first year and 6-monthly in the second to fifth years. A recent re-analysis of the ACT II trial has identified that some ASCC patients post treatment do not obtain a complete response for up to 26 weeks.¹⁰⁵ This adds support to the concept that patients should be observed closely with repeat response assessments out to this time-point, if they are not demonstrating definite evidence of disease progression. This provides the patient with the greatest possibility of avoiding an unnecessary operation.

1.2.4 Prognostic Factors

Clinical independent prognostic factors have been identified in five of the six Phase III randomised controlled trials (Table 1-3). Male gender was consistently found to be a negative prognosticator in four of the five trials, with a hazard ratio of 1.57 (males) for overall survival (OS) identified on long-term follow-up in Radiation Therapy Oncology Group (RTOG) 98-11,¹⁰⁶ 1.56 in ACT1 for OS^{55, 56} and 1.56 in ACTII for colostomy free survival (CFS)¹⁰⁷ with EORTC not listing a hazard ratio. In keeping with this finding, females have been identified to have

improved survival and reduced relapse rates in cohort studies within Australia.^{11,}

⁴⁰ An explanation as to why females have a higher incidence of ASCC yet better prognosis than men, may be secondary to a higher proportion of HPV-associated ASCCs in women, which are innately more responsive to concurrent chemoradiotherapy, however, this is purely speculative.

Tumour stage and skin ulceration have also been identified as important prognostic factors in four of the five RCTs. Tumour diameter >5cm had a HR of 1.62 on OS in RTOG 9811, with a reduced overall survival in ACT II of 54% vs 79%. Skin ulceration was found to have a HR of 1.57 on CFS in ACCORD03 (HR not listed in EORTC).⁶⁰ Nodal status has also been identified as a strong predictor of outcome, with a HR of 1.74 on OS for palpable lymph nodes identified in ACT 1 and 2.09 on OS in RTOG 98-11 (HR not listed in EORTC). Inguinal node involvement was identified to have a HR of 1.98 on CFS in ACCORD-03. When both tumour stage and nodal involvement are combined, the 4 yr overall survival for T3/4N+ was 48% compared with 81% for T1/2N- in RTOG 98-11.¹⁰⁶

Age was also identified as an independent prognosticator for colostomy free survival in ACCORD-03, with a favourable HR of 0.62 for those >55 years old. Radiotherapy dose was also identified as an independent prognosticator, with a HR of 0.37 for doses of 60-65 Gy. A high WBC count (ACT I), circumferential tumour spread (ACCORD-03) and low baseline haemoglobin (ACT II), have been identified as other independent prognosticators.

1.2.5 Patterns of Treatment Failure

Post completion of chemoradiotherapy, treatment failure can manifest as locoregional and / or distant disease. All patients identified with relapsed disease should undergo discussion at an MDT within a quaternary setting experienced

with the management of this complex cohort in order to maximise outcomes in this rare subset of patients.

1.2.5.1 Locoregional Disease

The most frequent pattern of treatment failure is locoregional disease. This can manifest as persistent disease following the completion of treatment, or the development of recurrence at least 6 months after the completion of treatment, which generally manifests within the first 3 years.^{108, 109} Locoregional failure generally encompasses disease at the primary site and / or inguinal and / or pelvic nodal sites. The long term follow up data from RTOG 98-11 reports a 5-year LRF rate of 20%, similar to that reported in other large retrospective cohort series.¹¹⁰

1.2.5.2 Metastatic Disease

At initial diagnosis, only 15-20% of patients present with extra-pelvic metastases with a further 10-20% of locally advanced patients developing metastases after primary treatment. Patients with HPV and/or p16 negative ASCC have poorer treatment response and overall survival, however, with the overall predominance of HPV positive disease, the majority of patients with disease relapse are viral positive.¹¹¹

There are two distinct patterns of distant failure, oligometastatic disease, and widely disseminated diffuse metastases. Currently, there is no standard care for metastatic ASCC, however the options are partly dictated by the pattern of disease. For patients with oligometastatic disease, multimodality treatment, in the form of chemoradiotherapy or surgery at the metastatic site is appropriate, while with disseminated disease, systemic therapy is the only option.^{47, 112}

1.2.6 Salvage Surgery

In the setting of locoregional relapse, salvage surgery is the only viable treatment option that offers a hope of cure.¹¹³ En bloc resection of the tumour with wide margins including at the level of the previously irradiated perineal skin and anal sphincter complex is required. Depending on the state of the disease in the pelvis, this may include an extended resection with removal of adjacent viscera or less frequently a pelvic exenteration. Flap closure is more frequent in the current era in an attempt to reduce the high complication and wound breakdown rate.

There have been multiple cohort studies published on the role of salvage surgery as summarised in Table 1-5. The 5-year OS from the published studies ranged from 23% to 69% and independent prognostic factors included the resection-margin status, nodal disease, tumour size and perineural / lymphovascular invasion. The largest cohort series identified that if three of the above factors, resection margin, nodal disease and LVI / PNI were negative, 5-year survival post salvage surgery was 55% compared with 0.03% for patients with positive findings. A recent systematic review of salvage APR in ASCC has reported that the median 5-year DFS was 44% with a median locoregional re-recurrence of 23.5% and 9% for metastatic disease with wide inter-quartile ranges for both.¹¹⁴ This highlights the lack of uniformity in selecting patients for salvage surgery across the globe, re-affirming the need to standardise follow-up and surveillance and ensure patients are discussed at a dedicated and experienced MDT. It has been proposed that more than 60% of patients with relapse should be offered salvage surgery, with the audit standard 5-year OS greater than 40%.¹¹³

Colostomy free survival (CFS) has been used as a surrogate for the success of CRT. However, while salvage surgery is the main reason for patients to receive

a colostomy, the treatment course can lead to anorectal complications and side effects that can result in a stoma with or without an APR. This is consequently a less robust marker for treatment failure. In a large Australian retrospective cohort, 5-year CFS was reported as 73% with the breakdown of reasons being 71% due to treatment failure, 16% pre-treatment and 5% due to late toxicity.⁴⁰

For isolated inguinal nodal recurrence, if the groins were included in the radiotherapy fields with the delivery of primary treatment, a formal inguinal lymphadenectomy is appropriate. Furthermore, the use of SLNB may also have a role in patients undergoing salvage surgery, to better define the need to perform an inguinal lymphadenectomy.

Table 1-5 Systematic Review of Salvage Surgery in ASCC (Studies > 20 pts)

Studies	<i>n</i> (Trial Period)	Median F/Up# (Months)	5y OS (%)	Independent Prognostic Variables
Hagemans et al. (2018) ¹¹⁵	47 (1990-2016)	80	42	Tumour Size; Nodal Disease; R0 status
Guerra et al. (2018) ¹⁰⁸	41 (1983-2015)	20	51	T stage; R0 status
Pedersen (2018) ¹¹⁶	47 (2011-2016)	20	50	R1 status; Nodal disease
Pesi et al. (2017) ¹¹⁷	20 (1988-2012)	NR	37	NR
Alamri et al. (2016) ¹¹⁸	27 (1996-2015)	NR	78	Not identified
Severino et al. (2015) ¹¹⁹	36 (1992-2012)	24	46 (3y)	AJCC stage
Hallemeier et al. (2014) ¹¹²	32 (1993-2012)	19	23	Recurrent vs Persistent; R0; Viable disease in resection specimen
Lefevre et al. (2012) ¹²⁰	105 (1996-2009)	33	61	T3-4 status; R0; Metastatic Disease
Correa et al. (2012) ¹²¹	111 (1982-2011)	16	25	Nodal disease; R0; LVI/PNI
Eeson et al. (2011) ¹²²	51 (1998-2006)	34	29	Resection margin status, age, gender, tumour size, nodal status at time of recurrence, and failure type, presence or absence of disease
Sunesen et al. (2009) ¹²³	49 (1997-2006)	25.5	61	NR
Mariani et al. (2008) ¹²⁴	83 (1969 – 2003)	104	57	Age > 55yrs, Nodal disease, T3-4 tumour, Local extent
Schiller et al. (2007) ¹²⁵	40 (1987-2006)	18	39	Charlson comorbidity score; size of resected tumour; Sex (male)
Stewart et al. (2007) ¹²⁶	22 (1985–2001)	15	26	Tumour differentiation, R0
Mullen et al. (2006) ¹²⁷	31 (1990-2002)	29	64	Nodal disease; <55Gy RTx
Renahan et al. (2005) ¹⁰⁹	73 (1988-2000)	45	40	Resection margin; age; nodal involvement.
Ghouti et al. (2005) ¹²⁸	36 (1987-2002)	67	69	NR
Akbari et al. (2004) ¹²⁹	47 (1980-2001)	NR	40	Persistent disease; pN+ resected specimen
Nilsson et al. (2002) ¹³⁰	35 (1985-2000)	33	52	Type of locoregional failure, tumour stage, nodal status, age
Smith et al. (2001) ¹³¹	22 (1978-1989)	30	33	NR
Allal et al. (1999) ¹³²	26 (1976-1996)	22	45	NR
Pocard et al. (1998) ¹³³	21 (1986-1995)	56	33	NR
Ellenhorn et al. (1994) ¹³⁴	38 (1980-1992)	47	44	Age, gender, initial response to chemoradiation, histologic levator muscle involvement, perirectal lymph nodes nodal status, and extent of lymphadenectomy
Zelnick et al. (1992) ¹³⁵	20 (1981-1990)	35	24	Nodal status

F/Up - Follow Up; OS – Overall Survival; pN+ - Pathological node positive; NR – Not Recorded; R0 – Clear margin; R1 – Microscopic positive margin; LVI – Lymphovascular Invasion; PNI – Perineural Invasion;

1.2.7 Treatment of Unresectable Locoregional Relapse and Metastatic Disease

Recruiting for trials of patients suffering relapsed disease poses difficulties, given the rarity of ASCC in conjunction with a response rate in two out of three patients undergoing primary treatment. As a result, there have been few advances in the treatment options for this population over recent decades. A summary of the publications on novel treatments in this cohort is presented in Table 1-6.

A major development in the last 12 months has been the Phase II randomised InterAACT trial, with provisional results presented at ASCO in 2018.¹³⁶ A total of 91 patients were enrolled with inoperable locally recurrent (12%) or metastatic treatment naïve (88%) ASCC. This compared the response rate to 5FU + cisplatin (57.1%) vs carboplatin and paclitaxel (59%), revealing improved median PFS / OS of 8.1 / 20 months with carboplatin plus paclitaxel compared to 5.7 / 12.3 months (HR 2.0, p=0.014) for 5FU / cisplatin and with reduced toxicity. These results now place carboplatin and paclitaxel as the new standard of care for first line treatment in unresectable local relapse and metastatic disease.¹³⁷

The other significant development is the encouraging results of using immunotherapy for inoperable and metastatic ASCC. Nivolumab an anti-PD1 inhibitor was assessed in a phase II study of 37 patients with previously treated unresectable metastatic anal cancer. This identified that 9 patients (24%) had a response with two patients achieving a complete response without any serious adverse events.¹³⁸ The second study was the phase 1b Keynote-028 trial, which assessed Pembrolizumab (Anti-PDL1) in PDL1 positive ASCC.¹³⁹ Of the patients screened, 68% were found to be PDL1 + and all patients were heavily pre-treated for their relapsed disease. The overall response rate was 20% (5 / 25) including 1 complete response with a manageable safety profile.

These trials have made significant inroads into the treatment of this otherwise palliated group of patients. Further molecular and immune characterisation of this disease is now required to improve the selection and stratification of patients by identifying therapeutic and predictive biomarkers to optimise outcomes and minimise side effects.

Table 1-6 Publications on novel therapies explored in metastatic and relapsed anal SCC

Articles	Pts	Types of patients	Type of Study	Study	Disease control	OS
Morris et al ¹³⁸ (NCI9673, 2017)	37	Unresectable Metastatic Anal Ca	Phase II	Nivolumab (3mg/kg)	Disease control: 27%; Stable 47%; CR: 2/37; PR: 7/37; PFS: 4.1 mths	11.5 months; 1-year OS (48%)
Deeken et al ¹⁴⁰ (2015)	22	Advanced solid tumour malignancies	Phase 1	Cetuximab and Lapatinib	Disease control: 50% PR: 17%	17%
Le Tourneau et al ¹⁴¹ (2015)	741	Advanced Ca	Phase II RCT	Molecularly targeted therapy vs conventional therapy (SHIVA)	PFS: 2-3 months (Exp); 2-0 months (control)	NR
Rogers et al ¹⁴² (2015)	1	Metastatic ASCC	Case report	Cetuximab with cisplatin and 5FU	Improvement in overall Sx 58% disease control;	NR
Ott et al ¹³⁹ (2017)	43	Recurrent Anal Ca	KEYNOTE 028 Phase Ib.	Safety and antitumor activity of pembrolizumab (anti PD1 Ab)	42% stable, PR: 17% ORR: 17% (95% CI 5-37%)	NR
Kim et al ¹⁴³ (2013)	8	Recurrent advanced Anal SCC	Phase II	Docetaxel, cisplatin, and 5-FU	CR: 50%; PFS: 19-88m	62.5% (1y)
Abbas et al ¹⁴⁴ (2011)	7	Advanced anal cancer	Case series	Paclitaxel (2nd line)	ORR: 57%; PFS: 2-8m	5-14 (m)
Ruter et al ¹⁴⁵ (2010)	27	Advanced solid tumors	Phase 1	Immune modulation weekly dosing agonist CD40 antibody	Stable disease: 26%	NR
Alcindor ¹⁴⁶ (2008)	5	Metastatic anal SCC	Case Series	Paclitaxel (1st and 2nd line)	ORR: 60%; PFS: 3-8m	4-20 (m)
Jhawer et al ¹⁴⁷ (ECOG E7282, 2006)	20	Advanced Anal Ca	Phase II	MMC, Adriamycin, Cisplatin + Bleomycin-CCNU	ORR: 60% PFS: 8m	15 (m)
Wieder et al ¹⁴⁸ (2002)	18	Squamous cell carcinoma	Phase I/II	Fenretamide (Accutane) as a potentiator of IV carboplatin and paclitaxel	Stable disease 13.3 %; CR: 6.7%; PR: 20%; PD: 60%	NR
Hainsworth et al ¹⁴⁹ (2001)	60 (4)	Advanced carcinomas	Phase II	Docetaxel, cisplatin, and 5-FU (max = 4 cycles)	CR: 25%; ORR: 65%; PFS: 26m	NR

Faivre et al ¹⁵⁰ (1999)	18	Metastatic anal SCC	Cohort	5-FU + Cisplatin	CR: 15%; ORR 65%; PFS: 4m	NR
Ajani et al ¹⁵¹ (1989)	3	Anal canal carcinoma	Case series	5-FU + cisplatin	PFS: 17m	NR
Wilking et al ¹⁵² (1985)	15	Advanced anal canal SCC	Phase II	Vincristine, bleomycin, and high-dose methotrexate	ORR: 25%; PFS: 2m	NR

OS – Overall Survival; PFS – Progression Free Survival; CR – Complete Response; PR – Partial Response; PD – Progressive Disease; ORR – Overall Response Rate; 5FU – 5 Fluorouracil; MMC - Mitomycin C; N – Node; LN – Lymph Node; LA – Locally Advanced; Mths/m - Months; Sx - Symptoms

1.3 Molecular Biology and Pathogenesis

1.3.1 Introduction

Much of the basic science and translational research into ASCC has been focussed towards the identification of predictive and prognostic biomarkers. While the TNM staging system provides reasonably consistent prognostication in terms of survival, it is not perfect, with heterogeneity in outcome for individual patients in the same stage of disease. This is secondary to the underlying biology of each tumour irrespective of the disease stage at diagnosis, influencing both innate tumour factors and response to chemoradiotherapy. Consequently, delineating these key factors will assist in the personalisation of treatment, both in the primary and relapsed setting. This is of critical importance to improve treatment response and outcomes including the minimisation of toxic side effects of treatment.

1.3.2 Human Papilloma Virus

It is widely accepted that approximately 85-95% of ASCCs are associated with HPV infection, with evidence from epidemiological and experimental studies identifying it as an aetiological factor.^{153, 154} This is in similarity with other HPV driven cancers, notably head and neck and cervical SCC.

When exposed to the HPV virus, the circular double stranded DNA of the virus is able to enter the stratified squamous epithelial cells. The genome can remain episomal or integrate into the host genome, with this integration event more frequently observed in HSIL and invasive lesions, but not essential for the development of carcinoma. In excess of 150 subtypes of HPV have been identified, however, most are deemed to have low risk of inducing carcinogenesis. The highest risk subtypes linked with the development of

carcinoma include HPV 16, 18, 31, 33, 45, 52, 58, of which HPV 16 is the highest risk and most frequently associated with ASCC.

The HPV genome encodes for early (E1, E2, E3, E4, E5, E6, E7) and late (L1, L2) structural genes, which are involved in viral replication. The oncogenic effect of the HPV virus is attributed to the expression of the E6 and E7 oncogenes, with the consequent oncoproteins having an effect on cell cycle regulating proteins. This is particularly with the E6 and E7 oncogenes of the HPV 16 virus subtype, which have been implicated in the immortalisation of cells and is postulated to be a key event in driving the cell towards genomic instability and on the pathway towards carcinogenesis.¹⁵⁵

These two oncogenes, E6 and E7, inactivate the products of the tumour suppressor genes p53 and pRb respectively.¹⁵⁶ E6 functions by binding p53, promoting its degradation and thereby preventing its ability to induce growth arrest and apoptosis.^{157, 158} E7 binds pRb, which allows the release of the E2F transcription factor, which facilitates progression through the G1 to S phase of the cell cycle, with unchecked cell proliferation.¹⁵⁹ With the action of these two oncoproteins likened to a tumour-initiating event, it is hypothesised that further genomic events are facilitated that promote and subsequently lead to the development of ASCC.¹⁵⁶

Anal squamous intra-epithelial lesions (SILs) are areas of dysplasia from which ASCC can arise. Risk factors for both low grade SIL (LSIL) and high grade (HSIL) are the same as for ASCC, with the prevalence low in the general population and highest in the HIV, immunosuppressed and MSM populations. Progression from LSIL to HSIL and to invasive SCC is low outside these risk groups.

Despite the high prevalence of HPV subtypes in precursor lesions, the rate of progression is relatively low, with the highest rate being in those who are immunocompromised.¹⁴ The reason behind which patients affected with HPV will ultimately develop carcinoma remains unclear across all sites of HPV related cancer. Most immunocompetent individuals mount a cell-mediated immune response with subsequent clearance of the HPV infection.¹⁶⁰ Consequently, approximately 50% of infections are reduced to undetectable levels by 12 months and more than 90% within a few years, with those with persistent infection having a raised probability of progression to invasive cancer.¹⁶⁰

Despite the significant rates of viral clearance, HPV can evade the immune system through a variety of mechanisms. The HPV 16 virus has the ability to inhibit production of the anti-viral protein IFN via the action of E6 and E7.¹⁶¹ Furthermore, E6 can reduce E-cadherin expression, reducing antigen presentation by minimising the interaction with antigen presentation cells mediated by this ligand.¹⁶² E7 can also interfere with antigen presentation by reducing the expression of transporter associated antigen protein 1 (TAP1).¹⁵⁶ These all provide avenues through which the virus can escape the immune system within human keratinocytes and eventually lead to cellular transformation.

There is an increased risk of progression to anal SCC from HPV precursors in HIV patients, with invasive disease historically developing up to two decades earlier in this population. Persistence of HPV infection is considered to be a major contributing factor in this pathway, with patients with low CD4+ counts having the highest risk. It is likely that immune evasion and a deficient immune response are the factors underlying this increased predisposition.¹⁶³⁻¹⁶⁵

The detection of HPV alone however may misclassify some tumours as HPV-associated, since it does not assess for the over-expression of viral oncogenes and therefore of HPV induced transformation. Furthermore, the identification of HPV is limited by firstly the sensitivity of the test employed and secondly the number of HPV subtypes assessed. Most frequently, cyclin dependent kinase inhibitor 2A (*CDKN2A*), best known as p16^{INK4a} (p16) is employed as a surrogate marker of HPV infection. This is secondary to pRb being a negative regulator of p16, with the consequent upregulation of p16 in the absence of pRb due to the effect of E7.

While p16 overexpression is frequently used as a surrogate for HPV infection, it is not entirely restricted to HPV-associated transformation. A proportion of HPV negative tumours are p16 positive and conversely, a number of HPV positive tumours are p16 negative. This was highlighted by the study published by Baricevic et al, demonstrating that 95% of anal cancers are HPV related when assessed by high sensitivity genotyping and that 89% of the HPV genotypes were HPV 16.¹⁵⁴

In a publication by Meulendijks in 2015,¹⁶⁶ it was determined that in a cohort of 107 patient samples, 13% were HPV negative using a line probe assay (LiPA) for 20 high risk and 8 low risk subtypes. This revealed that of the 14 HPV negative samples, 71% (10) were p16 negative with 29% (4) over-expressing p16 despite being HPV negative. This identified a significantly worse 3-year LRC (15%) for the HPV-/p16- group (15%) compared with HPV+/p16+ (82%) and HPV +/p16- (75%) patients. The same was identified for OS (35% vs 87% vs 75%) respectively.

A systematic review of the literature reveals that HPV positive ASCC has been associated with improved overall survival and reduced locoregional and systemic failure across 10 publications.¹⁶⁷ In the multivariate analyses, HPV16

and p16 were reported to be independently predictive of improved OS and PFS in 7 studies^{154, 168-173}, with two studies^{166, 174} identifying HPV and p16 together but not in isolation to be predictive of OS and PFS. In cervical, oropharyngeal and ASCC, HPV positivity and/or p16 expression are associated with improved response to chemoradiotherapy and good prognosis.^{168-170, 172, 174-177} However, for it to be utilised in routine clinical practice, prospective validation in a clinical trial is required.

Increased mutant p53 expression has been identified to be an independent predictor of inferior LRC and OS after CRT and is inversely related to HPV.^{169 166} Sequencing data has confirmed negligible rates of p53 mutations in HPV positive tumours, with the effect of E6 on p53 likely an early event in this cohort.¹⁶⁶ This finding of *TP53* having a negative prognostic impact has also reached in other studies.¹⁷⁸⁻¹⁸⁰ Consequently, p53 inactivation or suppression is likely an early driver of tumourigenesis in most ASCCs.

The improved responsiveness of HPV positive ASCC to CRT is postulated to be secondary to the maintenance of apoptotic mechanisms. While E6 and E7 suppress the cell cycle checkpoints p53 and pRb, this is theoretically overcome in the face of extensive DNA damage from CRT.^{181, 182} Mutant p53 in the viral negative group on the other hand precludes the commitment of cells to apoptosis in the face of DNA damage, creating an inherently radioresistant tumour.

Nonetheless, by examining the prognostic value of p16 in Head and Neck SCC (HNSCC), it is clear that there is still a survival advantage even in those patients treated with surgery alone.¹⁸³ This certainly suggests that other innate factors are involved in the survival of this cohort. HPV 16-E6 and E7 specific T cells have been identified in HPV-driven oropharyngeal cancer patients as part of an

adaptive immune response, and this may be a contributing factor in improved survival for the viral positive cohort.¹⁸⁴

1.3.3 Genomic Profile of Anal SCC

While there has been some research undertaken over the last two decades to define the molecular biology underlying ASCC, the genomic landscape has only begun to be explored in detail within the last 2 years, with limited data available at the commencement of my PhD.

A systematic review of 21 studies published in 2010,¹⁸⁵ reported on 29 prognostic biomarkers in anal cancer, with 13 demonstrating an association with outcome (Table 1-7). However, only two (p53 and p21) demonstrated prognostic significance in more than one study, with their value not collectively supported by the literature. The studies were particularly limited by low sample numbers, and varying techniques employed to assess each biomarker. This highlights the difficulty with undertaking exploratory studies in a rare disease with limited technology. Following the rapid progress in new investigative platforms including the advent of Next Generation Sequencing (NGS), progress in this area has begun to rapidly accelerate.

A publication in 2015 by Smaglo et al¹⁸⁶ performed targeted sequencing (NGS) identifying 33% of samples (28/86) to harbour a *PIK3CA* mutation and 15% (8./54) a *TP53* mutation. The first publication to perform a more in depth exploration of the genomics of ASCC was by Chung et al in March 2016.¹⁸⁷ This consisted of a cohort of 70 patients, with comprehensive genomic profiling using next generation sequencing taking DNA from FFPE specimens. This involved assessing 3769 exons across 236 cancer related genes, and 47 introns from 19 genes commonly rearranged in cancer. *PI3K* aberrations were identified in 63%

ASCC cases. There was a mean of 3.5 genomic aberrations (GA) per sample, with 91% of tumours having at least one GA, and 76% at least one clinically relevant GA. Copy number variations (CNV) accounted for 40.7% of aberrations.

Table 1-7 Molecular Biomarkers in Anal SCC

Prognostic Significance	
Tumour suppressor gene	
p53 p21	p53 expression associated with reduced local tumour control ¹⁷⁶ and reduced DFS ^{178, 179} Absent p21 expression associated with reduced OS ¹⁸⁸ and increased LRF ¹⁸⁹
EGFR (TK protein)	
EGFR/HER-1	HER-1 Highly expressed, no correlation with DFS ^{190, 191}
Regulator of apoptosis	
NF-κB	High expression associated with reduced DFS ¹⁹¹
Bcl-2	Expression associated with improved local tumour control and DFS ¹⁷⁹
M30	Expression associated with reduced local tumour control and DFS ¹⁷⁹
Cyclins	
Cyclin A	High expression associated with increased tumour-specific & OS ¹⁸⁹
Proliferation & Invasion	
Ki67/MiB1	Ki67 independent predictor of DFS ¹⁹¹
Ki67/MiB1	High Ki67/Mib1 index associated with increased CFS ¹⁹²
index MCM7	High expression associated with improved RFS and CSS ¹⁹³
Tumour-specific markers	
SCC Antigen	High expression associated with reduced tumour-free survival and OS ¹⁹⁴
Hedgehog signalling	
SHH	Overexpression of both associated with reduced DFS ¹⁹¹
Gli-1	Overexpression of both associated with reduced DFS ¹⁹¹

Abbreviations: TK – Tyrosine kinase; NF-κB - Nuclear factor-κB; MCM7 - Minichromosome maintenance protein 7; RFS - relapse-free survival; CSS - cancer-specific survival; VEGF - Vascular endothelial growth factor; SHH - Sonic hedgehog;

The most frequently altered genes were *PIK3CA* (40%), *MLL2* (16%), *PTEN* (14%), *SOX2* (14%), *FBXW7* (13%), *TP53* (11%) and *MYC* (10%). Amplifications accounted for 21% of *PIK3CA* alterations, with missense mutations in 78.8% of alterations. Recurrent GA's were observed in other genes involved in the *PI3K/AKT/mTOR* pathway. *KRAS* mutations were identified in only 4.3% of samples with *NRAS* mutations in 1.4%. Of the HPV negative samples (13%), a predominance of *p53* (66.7%) and *CDKN2A* (55.6%) mutations were identified, with 3.3% and 0% identified respectively in HPV positive samples. *PIK3CA* mutations again were found in 22.2% and *PI3K* pathway alteration in 77.8% of samples in this HPV negative cohort compared with 60.7% of HPV positive samples.

Other GA's associated with clinical responses to targeted therapies included alterations in genes involved in the DNA repair pathway, RAS signalling (10%), fibroblast growth factor receptor (FGFR) signalling (7%), chromatin remodelling (4%) and ERBB receptor signalling (4%), which were identified cumulatively in 30% of cases. EGFR alterations were identified at a low frequency despite previous studies suggesting EGFR overexpression by IHC at rates close to 90%.⁸¹

PI3K pathway aberrations were not associated with disease stage, suggesting that such alterations are early events in oncogenesis. PI3K pathway alterations have been associated with clinical responses to targeted therapies in other HPV-associated malignancies. In patients with cervical and head and neck SCC with *PIK3CA* or *PTEN* altered tumours, treatment with *PI3K/AKT/mTOR* targeted therapy resulted in durable responses and improved PFS.^{195, 196} This therapeutic approach could benefit relapsed and treatment refractory ASCC patients, given that GAs in this pathway are identified in the majority of ASCC patients.

A publication by Mouw et al¹⁹⁷ also published in 2016, explored the genomics of ASCC by performing the first reported series of whole exome sequencing in ASCC on 31 primary and 30 recurrent samples. This identified that the mutational burden was not different between pre and post chemoradiotherapy tumours, with a mean somatic mutational burden of 5.7 mutations/Mb (0.8-35 mutations/Mb). It revealed that *PIK3CA* (32%) was the most commonly mutated gene among both primary and recurrent tumours, followed by *FBXW7* (19%) and *TP53* (4.7%). Of the HPV negative tumours, 67% harboured a *TP53* mutation. It also again identified amplification of chromosome 3q, which includes the genes *PIK3CA*, *SOX2* and *TP63*.

When assessing the genomic evolution between matched primary and recurrent tumours, the mutations and CNV were shared in addition to some unique events in either sample. Furthermore, there was a trend towards increased non-synonymous mutations in recurrent compared to primary tumours (7.1 vs 3.7 mutations / Mb). In an isolated case, a patient with an average mutational burden in the primary, developed a high mutational burden of 10 mutations / Mb in their relapsed disease (metastatic nodes), progressing after 6 cycles of FOLFOX, and subsequently demonstrating an exceptional and durable clinical response to Anti-PD1 therapy that persisted at 27 months of follow up. This paper also identified that HPV negative tumours were enriched among the recurrent cases.

A further publication by Cacheux¹⁹⁸ also in 2016, performed sequencing for 8 specific genes, again identifying *PIK3CA* as the most common genomic aberration in ASCC. This assessed 148 patients with ASCC and identified that 20.3% of samples harboured a *PIK3CA* mutation. It identified that 6.1% harboured a *FBXW7* mutation, 4.7% a *TP53* mutation and only 2% a *KRAS* mutation. No patients harboured a *HRAS*, *NRAS*, *BRAF* or *MET* mutation. Of importance, this study identified that *TP53* mutations were restricted to

recurrence samples with 13.5% vs 0% in the treatment naïve group. However, this finding is likely explained by the fact that the treatment naïve cohort had 95 HPV + and only one HPV – sample. The tumour recurrence cohort had a much higher incidence of HPV –ve samples (5/52), demonstrating that despite being much less prevalent, they have a more treatment resistant biology. *TP53* mutations were strongly associated with HPV16-negative tumours (23.5% in HPV – vs 2.3% HPV +), and this goes some way to explaining the treatment resistance of this group, with *TP53* mutations frequently associated with radio-resistance as detailed above (section 1.3.2).

In the recurrence cohort of 38 patients, *PIK3CA* was identified as the most frequent genomic aberration in 28.9% of patients, followed by *FBXW7* at 13.2% and *TP53* at 10.5%. Multivariate analysis of this cohort identified that *PIK3CA* was an independent prognostic factor on overall survival. Furthermore, it has been identified that *PIK3CA* mutations are associated with poor outcome after salvage abdomino-perineal resection.¹⁹⁸

A further publication by Cacheux in 2018¹⁹⁹ reported on WES of 20 treatment naïve ASCC samples, revealing *PIK3CA* to be mutated in 25%, *FBXW7* in 15%, with *FAT1* and *TRIP12* also mutated in 15%. Copy number gains of chromosome 3q (*PIK3CA* gene) were again identified in 90% of samples, with alterations in the *PI3K/AKT/mTOR* pathway in 60%, and in chromatin remodelling in 45% and ubiquitin mediated proteolysis in 35%. No microsatellite instability was observed in any of the 20 samples. 45% of tumours had deletion of 10q (*PTEN* locus), with one also harbouring a *PTEN* mutation. 3 of the 20 samples analysed in this study demonstrated a mutational load >200 somatic mutations. Cacheux also published a further article in 2018²⁰⁰ utilising array comparative genomic hybridization in 49 ASCC cases. This identified that the most frequent minimal regions of deletion encompassed 11q22.3 (*ATM*, 55%) and the most frequent

minimal regions of gain 3q36.32 (*PIK3CA*, 57%). Again, the *PI3K/AKT/mTOR* pathway was the most extensively affected, particularly in recurrences compared with treatment naïve tumours (64 vs 30%)

Given the poor outcomes of HPV negative ASCC patients with standard therapy, there is a need for alternative treatment approaches. With the high incidence of *PI3K/AKT/mTOR* pathway alterations, the potential for a targeted therapy approach is present. There is a need for further preclinical work to investigate the differences and the importance of the *PI3K* pathway in both HPV +ve and –ve disease, to better inform targeted approaches.

1.3.4 Immune Profile of Anal SCC

The role of the immune system in the control of cancer has been under investigation for well over a century, garnering great momentum in the last decade. In a similar fashion to the genomics of ASCC, limited work had been undertaken at the commencement of my PhD, with several publications ensuing.

The immune profile in ASCC was first reported by Grabenbauer in 2006.²⁰¹ This evaluated the presence of TILs in 38 pre-treatment patient samples using IHC for the markers CD3, CD4, CD8, CD68 and FOXP3. This identified that patients with high numbers of CD3+ and CD4+ TILs had improved 3-year disease-free survival (89% vs 54% and 95% vs 48% respectively). They also identified that granzyme B+ cytotoxic TILs had a significant negative prognostic effect ($p=0.0008$). They however did not find an association with CD8+ cells. Assessment was undertaken using a tissue microarray with 2mm needle cores. Consequently, there may have been some sampling bias, in being unable to identify areas of highest TIL infiltrate.

In a study by Hu et al, a high tumoural infiltration of CD8+ T Cells was associated with well-differentiated tumours, early stage disease and better prognosis in contrast to the study by Grabenbauer.²⁰² Only CD8+ cells were assessed across 40 patient samples in this project, and this was undertaken on both the intra-tumoural and peri-tumoural regions in areas of highest TIL infiltrate. The correlations were identified for high infiltration across both regions on the tissue. Furthermore, they were identified in both HIV positive and negative cohorts. This has been further delineated in a study, which demonstrated that the immune profile within the tumour microenvironment of ASCC's in HIV positive patients is similar to those without HIV.²⁰³ This supports the investigation of immune therapies in all ASCC patients irrespective of HIV status.

Gilbert et al published on the largest study assessing TIL infiltrates in 284 pre-treatment ASCC patients combined from a UK and Danish cohort. This study utilised Haematoxylin & Eosin (H & E) rather than IHC slides and the expertise of a pathologist to quantify the TIL infiltrate. They demonstrated that ASCCs with a high TIL count were much less likely to suffer from relapse.¹¹¹ Furthermore, a high TIL infiltrate was found to retain independent prognostic value over and above p16 in a multivariate analysis.

PDL1 expression has also become of great interest over recent times, given the emergence of immune checkpoint therapies. A study of 41 ASCC samples identified PDL1 expression in 56%, with an association with worse recurrence and survival.²⁰⁴ it is known that PDL1 expression falls under the control of oncogenic activation of the AKT-mTOR pathway in NSCLC.²⁰⁵ Given the frequency of activating aberrations in the PI3K pathway in ASCC, this may suggest that PDL1 has a role to play in those treatment resistant patients with a *PIK3CA* or other PI3K oncogenic mutation, by promoting immune escape through driving the expression of PDL1.

As with p16 and HPV status, the immune infiltrate appears to have significant predictive and prognostic value, which in turn can inform treatment decisions. This is by way of increasing radiotherapy doses or utilising novel agents in poor risk cases and de-escalating treatment in good prognosis patients to minimise toxicity. Nonetheless, these findings require external validation in a larger cohort or prospectively in a clinical trial before assuming a role in clinical practice. Furthermore, as our understanding of the immune subsets in cancer evolves, there is further scope to unravel the importance and prognostic power of the host response in ASCC.

1.4 Preclinical Models of ASCC

There is a significant paucity of preclinical studies and models in ASCC, which has hindered progress in understanding the biology of this disease and exploring the effectiveness of new therapies, particularly for patients with relapsed and metastatic disease.

1.4.1 Anal SCC Cell Line

Human tumour cell lines facilitate the study of cell growth, differentiation, apoptosis and metastasis, both in general as well as specifically to the cancer from which they were derived when compared with other cancer-type cell lines. Most importantly, they also allow the assessment of therapeutics, both *in vitro*, as well as *in vivo* following the establishment of cell line tumour xenografts in immunocompromised mice. They consequently represent an efficient and cost-effective means of investigating tumour response to potential novel therapeutics.

A single publication from 2009, by Takeda et al, reports on the establishment and characterisation of an HPV negative ASCC cell line derived from a lymph node

metastasis.²⁰⁶ This paper describes characterisation with rt-PCR, IHC, chemosensitivity to 5FU and Cisplatin, and tumourigenicity studies in immunodeficient mice. The line has never been banked with any of the major cell repositories and the authors, university and hospital at which the line was developed are uncontactable. Consequently, this purported first *in vitro* model has been unable to be utilised for further study. A further publication in 2018 reported HPV-16 transformation of a human anal epithelial line, that demonstrated some in vitro features suggestive of neoplasia, however it was not tumourigenic in mice (Nod-SCID-IL2R^{NULL} – NSG) and not representative of ASCC.²⁰⁷

1.4.2 Mouse Models of Anal SCC

There are only two previously published mouse models of ASCC. The first model was published in 2010 by Stelzer et al.²⁰⁸ This model is based upon a Keratin-14 Cre-recombinase E6/E7 transgenic mouse of FVB/N genetic background. The E6/7 transgenes are the two main oncogenic drivers of the HPV 16 virus and having them linked to a K14 Cre allows for targeted expression in stratified epithelium, which is a great foundation for exploring HPV in the development of anal cancer. However, this model does not develop spontaneous anal tumours in ageing mice, with the requirement for further carcinogenic exposure to DMBA. The target of DMBA is the *ras* gene, most frequently *H-ras*. While at the time of this publication, there was little data on the genomic landscape of human ASCC, recent literature has revealed *ras* mutations to be infrequent in ASCC (<2%).^{81, 187, 209} This creates significant limitations within this model, given the lack of similarity with the genetic profile of human ASCC.

The second model was published in 2013 by Sun et al. This model is based upon a transgenic mouse with a combined deletion of *Tgfb β 1* and *Pten* linked to an

inducible K14-Cre (K14-CreER^{tam}.*Tgfb β 1*^{fl/fl}Pten^{fl/fl}) on a mixed genetic background (C57Bl/6, FVBN, CD1, 129).²¹⁰ This model reportedly develops spontaneous ASCC after exposure to oral tamoxifen. However, when the H & E images of the blocked anal canal are examined, it is clear, that the anal canal is intact and normal, with the SCC appearing to be more likely anal margin or gluteal in origin. Furthermore, the mice required euthanasia because of the development of tumours within the head and neck, limiting the ability to utilise the model for the study of anal cancer. This is further emphasised by the publication of the same mouse model for head and neck SCC in 2012, with the subsequent anal cancer publication arising from the same head and neck group.²¹¹

Despite these deficiencies, this model demonstrated activation of the *Akt* pathway, which is a frequent observation in human anal cancer. Furthermore, the authors were able to demonstrate that an mTOR inhibitor (Rapamycin) significantly delayed the onset of ASCC tumours, with decreased pAkt and pS6.

The other complicating factor with both models is the genetic background of the mice. The ideal genetic background for immune based studies in mice is the C57Bl/6, with more publications of this nature based around C57Bl/6 mice than other backgrounds. This is related to the initial development of this mouse strain with the goal to assist in the study of cancer and immune responses.²¹²

1.5 Conclusion

There has been limited progress in the development of new therapies for ASCC since the establishment of chemoradiotherapy as definitive treatment over four decades ago. Salvage surgery now acts as the only option offering a significant

survival benefit in patients with disease relapse. While the molecular underpinnings of ASCC are beginning to be unravelled, further progress has been hampered by the rarity of this disease coupled with an absence of appropriate pre-clinical models. In an era that has seen the dawn of immunotherapy and the continuing evolution of personalised medicine, there is a need to deepen our understanding of the genomic and immune aspects of this disease and establish models on which to explore new therapeutics, particularly for patients suffering treatment failure.

1.6 Aims and Hypothesis

This thesis aimed to expand the knowledge base surrounding the treatment and biology of anal SCC and develop preclinical models of the disease through the following aims:

Aim 1: To explore the current role of salvage surgery in patients with locoregional relapse

Hypothesis

That salvage surgery offers a survival benefit to appropriately selected patients with locoregional relapse in an Australian quaternary referral centre.

Summary

By analysing the experience of treating anal SCC at a quaternary referral centre in Australia, locoregional relapse was identified as the most frequent pattern of treatment failure, independently predicted by the pre-treatment T stage. Most patients with locoregional treatment failure can be salvaged, however, survival was entirely dependent on a clear resection margin. Consequently, the need for

extensive preoperative evaluation and careful patient selection is paramount in offering surgery, which not infrequently involves an extended resection.

Aim 2: To profile the immune and genomic landscape of ASCC

Hypothesis

The immune and genomic landscape of Anal SCC is predictive of treatment response and outcome

Summary

With limited research published on the genetic and immune underpinnings of Anal SCC, this knowledge base has been expanded. CD8+ TILs in pre-treatment biopsies were identified to be predictive of treatment response and stratified patients above and beyond the TNM staging system for 5-year overall survival. The identification of other immune subsets, including myeloid derived suppressor cells, which appear to be present in large numbers in ASCC, has further unravelled the complex tumour – immune microenvironment. The identification of *PIK3CA* mutations and PI3K pathway aberrations as a frequent event in ASCC, is in keeping with the small volume of published data, adding impetus to the assessment of both as a possible therapeutic target.

Aim 3: To establish and validate preclinical models of ASCC

Hypothesis

In vitro and *in vivo* preclinical models of ASCC can be developed using human anal cancer tissue and a transgenic mouse with a *pik3ca* mutation.

Summary

The overarching aim of research in this thesis was to develop preclinical models of ASCC that recapitulate the human disease. The rarity of this disease creates difficulties in performing clinical trials, making preclinical investigation key to identify potential therapies. Furthermore, there has been limited progress in understanding the pathogenesis of this disease. The development, validation and characterisation of human and murine *in vitro* and *in vivo* preclinical models, has addressed this hiatus, creating platforms to deepen our knowledge in the biology of ASCC and assess novel therapeutics.

Chapter 2: Materials and Methods

2.1 Cohort Description

The anal cancer database is a retrospective cohort series updated bi-annually on all patients treated at Peter MacCallum Cancer Centre including all peripheral satellite centres. The anal cancer tissue bank allows fresh tumour biopsy specimens to be prospectively obtained following informed consent of patients. Both projects have been approved following review by the Peter MacCallum Human Research Ethics Committee HREC/16/PMCC/101 (16/78) and HREC/16/PMCC/100 (16/77) respectively.

Germline DNA was obtained from peripheral blood mononuclear cells, harvested at the time of the patient's operation.

2.2 Tissue Culture

Unless otherwise indicated, all reagents were of analytical grade and purchased from standard commercial sources.

2.2.1 Tumour Processing

Once obtained, the tissue (Human ASCC or PDTX (Patient Derived Tumour Xenograft)) was immediately placed in RPMI tumour wash [RPMI 1640 + 2.5mmol L-glutamine (Gibco, Thermofisher Scientific, #11875-084) containing 100U/ml Penicillin + 100µg/ml Streptomycin (Gibco #15140122), Nystatin, 10µg/ml (Sigma, #N6261), Gentamicin (Pfizer #A502) and 0.05µg/ml Amphotericin B (Gibco, #15290018)] in a Falcon™ 50ml conical centrifuge tube on ice for transportation back to the laboratory. The fresh tumour tissue was then taken to a biosafety containment hood and processed with aseptic technique using sterile equipment. Solid pieces of the tumour were washed three times with 50mls of ice-cold RPMI tumour wash in a Falcon™ 50ml conical centrifuge tube over a period of 15 mins. The tumour was then processed within a 10cm tissue

culture petri dish (Falcon) using a size-23 scalpel blade and forceps to macroscopically dissect the tumour in a small volume (100µl) of RPMI + 20% Foetal Calf Serum (FCS, Gibco #1603007). The tumour pieces are then allocated as per Figure 2-1 with segments of the tumour snap frozen, cryopreserved and formalin fixed followed by paraffin embedded.

Formalin fixation involved placing one or more tumour segments into 10% Neutral Buffered Formalin (NBF) for 24-48 hours of fixation, with subsequent paraffin embedding. Snap freezing was undertaken by placing further tumour segments into appropriately labelled cryovial/s which were then dropped into liquid nitrogen contained in a cryogenic storage dewar before subsequent transfer to a -80°C freezer. For cryopreservation tumour pieces were placed in appropriately labelled cryovials/s with 1.5mls of 10% DMSO / 90% FCS freezing mix per cryovial. The cryovials/s were then transferred to a Mr Frosty™ cryogenic container (Thermo Scientific) with isopropanol and placed in a -80°C freezer for 24 hours. The cryopreserved vials were then transferred to liquid nitrogen vapour phase storage. Any remaining tumour tissue was allocated to other applications as required.

2.2.2 Explant Cell Line Development

Tumour pieces allocated for cell line development were further dissected macroscopically into a fine slurry with many small fragments, the largest measuring less than 0.5mm in longest dimension. The resultant tumour aggregates were then aspirated into a P1000 pipette tip with further small aliquots of media and transferred into a 24 well 'Explant' plate containing 2ml of RPMI 1640 + 2.5mml L-Glutamine + 20% FCS + 100 U/ml Penicillin and 100µg/ml Streptomycin per well. Gentle pipetting of the plated wells ensured an even

distribution of the tumour aggregates across the surface. The plate was subsequently transferred into a 37°C 5% CO₂ incubator.

The plate was not moved for a minimum of four days to facilitate adherence of the tumour explants to the plastic of the 24 well plate. Half the media was then changed, with subsequent changes three times a week for the first three weeks. This involved gentle removal and instillation of fresh media against the wall of the well to minimise explant disruption. Following adherence of the tumour explant, fibroblast outgrowth occurred over a period of 7-10 days, after which epithelial cells grew out circumferentially from the tumour explant onto the bed of fibroblast cells.

Frequent visualisation under the microscope was undertaken prior to each media change to determine the success of explant adherence and outgrowth and exclude contamination. Wells with successful explantation were marked for ongoing maintenance. Once the epithelial outgrowth had begun, all media was changed regularly three times a week. When the epithelial component occupied at least 70% of the well surface, the first passage was undertaken. This involved removal of the media, a wash step with PBS $-/-$ (Without Ca²⁺/Mg²⁺) and re-aspiration, followed by utilisation of 1ml/well TrypLE Express (Gibco #12604021), to facilitate detachment, occurring over a period of 3-5 mins (monitored with microscopy). The cells were then transferred to a 15ml Falcon tube and centrifuged at 1500rpm for 5 mins at RT. They were then re-suspended in RPMI 1640 + 2.5mmol L-glutamine + 20% FCS + Y27632 (ROCK-inhibitor, Sigma #Y0503-5MG) and seeded in a 12 or 6 well plate dependent on the number of cells. The time period between initial plating of the explants and first passage was up to 3 months and dependent upon the rate of epithelial cell expansion.

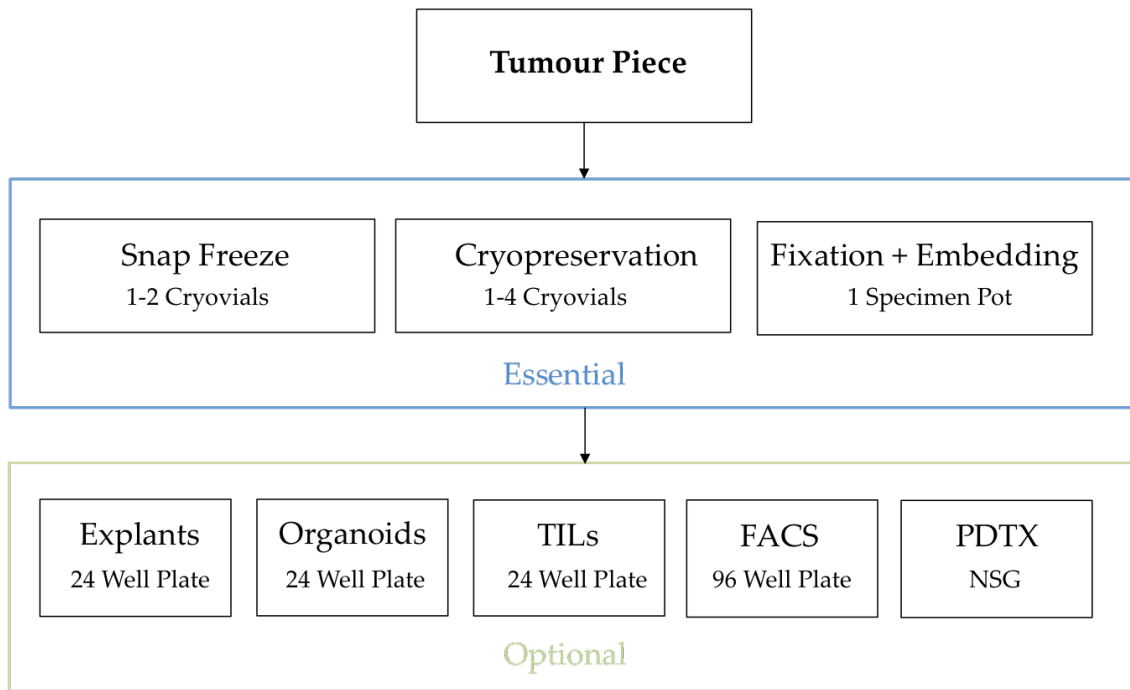


Figure 2-1 Processing of Tumour

2.2.2.1 Cell Line Passaging

Once the cell lines were robust, generally by passage 10-15, the cells were passaged when they reached 70% confluency in the tissue culture flask. Media was aspirated and cells were washed with PBS +/- for 3 mins. After removal of the PBS, TrypLE Express was added and the flask incubated at 37°C until the cells detached from the surface, over a time period of for 3-8 mins, dependent on the cell line. TrypLE was then inactivated with RPMI + 10% FCS and the cell suspension aliquoted into a Falcon tube for centrifugation at 500 relative centrifugal field (rcf) for 5 mins. Supernatant was aspirated and the cell pellet resuspended in 5mls of RPMI + 10% FCS. A count was then performed utilising 0.4% Trypan Blue (Gibco, Thermofisher Scientific) and a Neubauer haemocytometer. Cells were then appropriately split into new culture flasks to achieve exponential growth, and utilised for other applications (cryopreservation, snap frozen) as required. For T175 flasks, P1, P2 & P3 were

seeded at 5 million cells, LRF1 at 1 million cells, LRF2 at 2 million cells and Anal1 and Anal1 E6/7 at 0.5 million cells.

2.2.2.2 Cryopreservation and Resurrection

Cells were cryopreserved following the lifting and counting of cells at the time of passaging. After aliquoting and pelleting the desired cell number for cryopreservation, the supernatant was aspirated, and the pellet was resuspended in 1.5ml of 10% dimethyl sulfoxide (DMSO, Sigma Aldrich) and 90% FCS and transferred to an appropriately labelled cryovial. The cryovials underwent controlled freezing at 1°C / minute by placing them within a Mr Frosty Container with 100% Isopropanol into a -80°C freezer overnight. They were then transferred to vapour phase liquid nitrogen for long-term storage.

Resurrection of cells involved rapid thawing in a 37°C waterbath. The contents were then transferred into a 50ml Falcon tube containing RPMI-1640 +10% FCS, which was then centrifuged at 500rpm, 5 mins. Supernatant was aspirated and the cells then resuspended in RPMI + 10% for counting and subsequent seeding.

2.2.3 Tumouroid Establishment

2.2.3.1 Primary Tumour and Cell Line Derived Tumouroids

The tumour portion was macroscopically dissected in a similar fashion to the technique for explants. The resultant slurry was then placed in a 6 well tissue culture plate with 5ml of Tumour Digestion Solution (10ml / gram tissue of Advanced DMEM/F12 (Gibco, #12634028) + 2.5% FCS + 50U/ml Penicillin + 50µg/ml Streptomycin + 100U/ml Collagenase IV (CLS-4 Worthington #LS004188) + 1mg/ml Dispase II (Gibco #17105-041) + 50µg/ml Hyaluronidase (Sigma Aldrich #H3506) + 100µg/ml DNase Type I (Sigma Aldrich #D4263-1VL)

and 10 μ M Y27632) sealed with parafilmTM and placed in a rocking incubator at 37°C for 30-45 minutes. The incubation time for digestion was dependent on the amount of tissue and its character, with large and / or fibrotic tumours requiring longer incubation. The enzymatic solution was then neutralised with RPMI-1640 + 10% FCS. The resultant mix was then filtered through a 100 μ M filter into a 50ml conical Falcon tube facilitated by a 3ml syringe plunger to compress the tissue through the filter membrane and optimise cell cluster recovery. The filtered mixture was then centrifuged at 1500 rpm for 5mins, and an ammonium-chloride-potassium (ACK) lysis step utilised if the pellet was found to harbour a significant red blood cell (RBC) component. This involved re-suspension of the pellet in 2ml of ACK lysis buffer (150mM ammonium chloride (Millipore #12125029) + 10nM potassium bicarbonate (Merck #104854) + 0.1mM EDTA (Amresco #0105) + HCl to adjust pH to 7.2-7.4) at RT for 3 mins, followed by dilution to 50mls with PBS and re-centrifugation. The pellet was then resuspended in growth factor reduced Matrigel (Corning) while being kept on ice to ensure maintenance of its liquid state.

Cell line derived tumouroids were established by re-suspending a pellet of cells in Matrigel in a similar fashion to the primary tissue cell pellet. Assessment of the cell density of the Matrigel cell suspension was undertaken by visualising a small aliquot under light microscopy. Additional Matrigel was added as required to achieve an optimal seeding density. For primary tissue tumouroids, the optimal density was high at 1-10 $\times 10^4$ cells / well. For cell line tumouroids and passaging of all established tumouroids, the optimal density was much lower, at 5 - 50 $\times 10^2$ cells / well. The suspension was then plated out in 40 μ l aliquots per well of a pre-warmed 24-well plate (Corning). The plate was placed into a cell culture incubator at 37°C and 5% CO₂ for 30 mins to allow polymerisation of each droplet. Tumouroid complete media (Primary

Tumouroids) or RPMI-1640 + 20% FCS (Cell Line Derived Tumouroids) was then added at 500ul per well.

2.2.3.2 Tumouroid Maintenance and Passaging

The culture was replenished with fresh media every 3-4 days during tumouroid growth. Dense cultures with tumouroids ranging in size from 200 to 500µm were passaged weekly. After removal of media, 1ml of pre-warmed TrypLE Express was vigorously added with a P1000 pipette tip to each well to dislodge the droplet from the plastic and partly disrupt it facilitating transfer to a 15ml conical Falcon tube for placement in a water bath at 37°C for 5 mins. Trituration with a P1000 tip was utilised to assist with the fragmentation of the tumouroids. The resultant digested mix of cell clusters and single cells was then washed with media and 10% FCS and centrifuged before re-suspension in Matrigel and replated. For the primary tumouroids, at passage 2 to 3, bio-banking was undertaken with 10% DMSO freezing solution as per section 2.2.2.2, utilising two wells of tumouroids per 1.5ml of cryopreservation solution in appropriately labelled cryovials. Cultures were screened for mycoplasma by PCR and confirmed negative as per section 2.3.6 before being utilised for experimental assays.

2.2.4 Tumour Infiltrating Lymphocyte Expansion

Tumour Infiltrating Lymphocytes (TILs) were expanded from viable human tumour. The tumour segment allocated to TIL expansion was macroscopically dissected under sterile conditions into small pieces (1mm maximum dimension), with each placed into a well of a 24-well plate with 2ml of TIL culture media (RPMI-1640 + 10% Human AB serum (Valley Biomedical #HS1017) + 1M HEPES (Gibco #15630080) + 50U/ml Penicillin + 50µg/ml Streptomycin + 10µg/ml Gentamicin (Pfizer, #A502) + 0.05µg/ml Amphotericin B (Gibco, #15290018) 2mM

GlutaMAX (Gibco #35050061)) supplemented with recombinant human Interleukin-2 (rhIL-2, NCI, Charles River Laboratories) at 6,000 IU/ml. Half media changes were undertaken twice weekly, with fresh TIL culture media supplemented with 12,000 IU/ml. Once confluent, cells were utilised in a cytotoxic assay and/or flow sorted.

2.2.5 TIL-Tumouroid Cytotoxic Assay

2.2.5.1 Tumouroid Preparation

Tumouroids utilised in both human and mouse cytotoxic assays were of cell line origin. Passaging of the tumouroids to the 96-well black optical μ -plate (Ibidi #89626) was performed 3 – 5 days prior to performing the assay, with a minimum size of 50 μ m and viable appearance under light microscopy on the day of the assay. The passaging process outlined above was utilised, with aliquots of 10 μ l per well and a total volume of 350 μ l of media.

2.2.5.2 TIL Preparation

Expanded TILs from confluent wells of the 24-well plate were harvested and filtered through a 70 μ M filter into a 50ml conical Falcon tube. Following a wash step with RPMI-1640 + 10% Human Serum, centrifugation and re-suspension in 2mls of TIL media, a cell count was performed using a Neubauer haemocytometer. The Effector : Target (E:T) ratio of TILs : Tumouroids was determined by counting the average number of tumouroids per well. A concentration of 5000 TILs : 1 tumouroid was utilised with a maximum of 300,000 TILs / well.

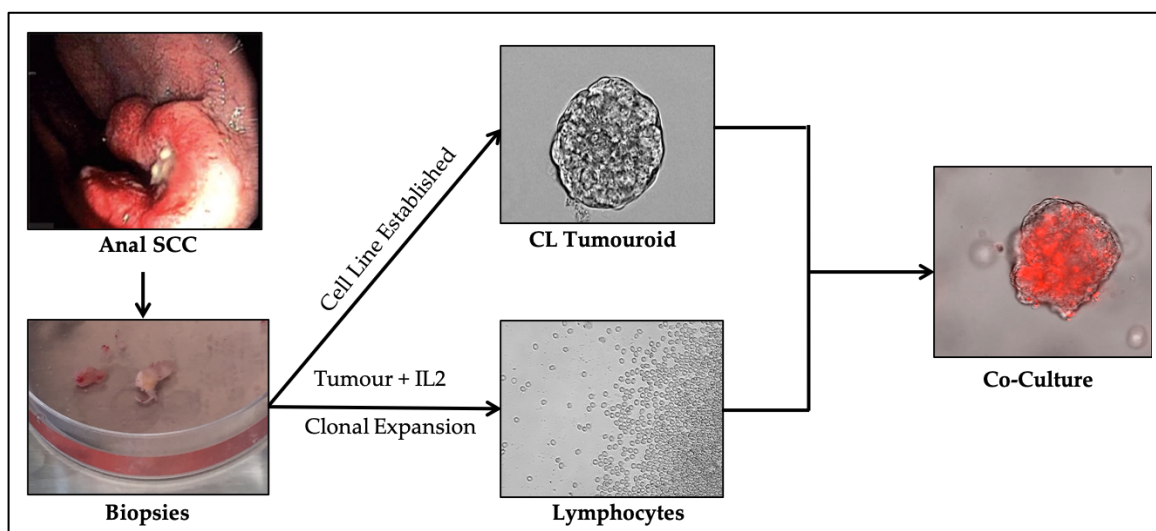


Figure 2-2 Process of performing the TIL-Tumouroid Co-Culture Cytotoxic Assay (Consent obtained for publication of clinical photograph)

2.2.5.3 Media Preparation

The number of wells required for the cytotoxic assay was determined and multiplied by 344µl of RPMI + 10% FCS. The appropriate number of TILs were re-suspended in this media to create the appropriate TIL concentration for the experimental assay. Propidium iodide (PI, Sigma-Aldrich) was then added to a final concentration of 2µg/ml (6µl per well). Media was removed from the experimental wells of the 96 well µ-plate and the TIL-media suspension was then added to the wells for each pre-defined condition in duplicate.

2.2.5.4 Human Experimental Conditions

Conditions assessed for the human cell line derived tumouroids included: tumouroids alone (negative control), tumouroids + TILs, tumouroids + TILS + anti-PD1 (Keytruda, Merck). For negative control wells, 350µl of RPMI-1640 + 10% FCS + 6µl PI was added. The TIL suspension was added to the remaining wells and supplemented with 6,000IU/ml rhIL-2. Anti-PD1 was added to respective wells at a final concentration of 50µg/ml.

2.2.5.5 Mouse Experimental Conditions

Conditions assessed for the mouse cell line derived tumouroids included: tumouroids alone (negative control), tumouroids + TILs, tumouroids + TILs + mAnti-PD1 (RMPI-14, BioXCell #BE0146), tumouroids + TILs + mAnti-CTLA4 (9H10A, BioXCell #BE0131), tumouroids + TILs + mAnti-PD1 isotype control (2A3, BioXCell #BE0089).

2.2.5.6 Image Acquisition and Analysis

The 96 well μ -plate was mounted on the stage of an IX83 fluorescence capable inverted microscope (Olympus) within an atmosphere-controlled chamber (The Brick, Ibidi). Conditions were maintained at 37°C and 5% CO₂. The 10x (numerical aperture 0.5) air objective was selected to identify 10 tumouroids per well at the periphery of the matrigel using the brightfield setting. The cellSens software (Olympus) was utilised to automate the sequential imaging and optical capture of the selected tumouroids at 2 hourly time-points in both brightfield and PI channels (excitation 493nm) for 48 hours. Analysis was undertaken using ImageJ software (NIH) for the series of acquired images. The Mean Fluorescence Intensity (MFI) of PI uptake in tumouroids over time was calculated with the mean of the 10 tumouroids per well representative of the kinetics of TIL-induced tumouroid killing over time.

2.2.6 Peripheral Blood Mononucleocytes (PBMCs)

Blood was harvested from matched patients undergoing an ASCC biopsy, (obtaining between 15 and 30mls in EDTA blood collection tubes) for processing to obtain plasma and PBMCs. Tubes were briefly centrifuged to collect plasma, which was stored at -80°C. To obtain PBMCs, density gradient SepMate™-50 tubes (Stemcell Technologies, Vancouver, BC) containing Ficoll-Paque PLUS (GE Healthcare Life Sciences, Mississauga, ON) were used.

Blood was diluted 1:2 with PBS and layered onto SepMate™ tubes, which were centrifuged at 1200g for 10 mins with the break off. Mononuclear cells in the top layer were poured into a new 50ml conical Falcon tube, washed with PBS and re-centrifuged. An ACK lysis was undertaken for 3 minutes to remove residual red blood cells, followed by a further wash and re-centrifugation. Cells were then suspended in RPMI-1640 supplemented with 10% FCS and counted in 0.4% Trypan Blue using a Neubauer haemocytometer and cryopreserved (2.2.2.2).

2.2.7 Flow Cytometry Immune Phenotyping

2.2.7.1 Tissue Processing for Flow Cytometry

The human tumour specimen allocated to flow Cytometry was finely dissected using a size 11 scalpel blade. The resultant tissue slurry was then digested enzymatically with 5mls DMEM containing 75U/ml collagenase IV (Worthington) and 0.1µg/ml DNase (Sigma Aldrich) in a 6 well plate secured with parafilm and placed on a shaker at 37°C incubator at 100 rpm for 45 mins. The resultant digestion mix was then filtered through a 100µm filter into a conical 50ml Falcon tube. The plunger of a 3ml syringe was utilised to assist with grinding residual tissue aggregates through the filter. The filter was then washed with RPM-1640 + 10% FCS to neutralise the enzymatic reaction and the resultant mix re-filtered through a 70µm followed by 40µm filter. Cells were centrifuged at 330g for 10 mins at 4°C. The supernatant was aspirated and if a significant RBC component was present, an ACK lysis for 3 minutes at RT was undertaken. The 50ml Falcon was then filled to 50ml with PBS and centrifuged at 300g for 10 mins at 4°C. Supernatant was aspirated and cells were suspended in 2mls of RPMI-1640 + 5% FCS for counting with 0.4% trypan blue using a Nebauer haemocytometer. Cells were maintained on ice at 4°C until ready for staining for flow Cytometry.

2.2.7.2 *Surface Staining for Flow Cytometry*

Following tumour digestion, cells were divided into 4 wells of a 96-well v-bottom plate (Nunc, Thermo Scientific). For Unstained (US), Fixable Viability Stain (FVS) and Isotype controls, 0.5×10^6 cells were added to respective wells. For stained samples, $0.5 - 1 \times 10^6$ cells were added. Wells were washed with PBS twice and centrifuged at 400g for 4 minutes at 4°C. Fixable Viability Stain 575 (BD Biosciences) was thawed on ice and diluted 1:1000 with PBS to create the appropriate viability staining solution. The viability stain was added (200µl) to FVS and isotype controls and the stained sample, with the unstained control resuspended in PBS. Cells were incubated at room temperature for 10 mins in the dark. The plate was then centrifuged at 400g for 4 mins at 4°C, supernatant discarded, and the cells washed with PBS. Human Fc Block (BD Pharmingen™) was diluted 1:10 in Brilliant Stain Buffer (BD Pharmingen™) and for human samples, the stain and isotype control samples were resuspended in 50µl of this blocking medium. Unstained and FVS controls were suspended in RPMI, 4% FCS, 100U/ml penicillin and 100µg/ml streptomycin subsequently referred to as Staining Media (SM) and incubated for 10 mins at 4°C in the dark. Subsequently, 100µl of the antibody cocktail was added to stain and isotype-control wells, SM was added to unstained and FVS controls and samples incubated for 30 mins at 4°C in the dark. Samples were then washed twice with SM and fixed with 100µl 1% paraformaldehyde (PFA) in SM. Alternatively for intracellular staining, the cells were then fixed with 100µl IX Cytofix/Cytoperm (BD Pharmingen™) solution and incubated for 20 minutes at 4°C in the dark. The plate was then centrifuged, the supernatant discarded, and the cells washed with 1x Perm/Wash™ (BD Pharmingen™) solution and centrifuged at 350g for 4 mins at 4°C. The supernatant was discarded, and the isotype and stain samples were suspended in 100µl of the relevant antibody diluted in Perm Wash solution for

45 mins at 4°C in the dark. A further 100µl of Perm Wash was then added and the plate centrifuged at 350g for 4 mins at 4°C, followed by a further Perm Wash and re-centrifugation. Cells were then resuspended in 200µl SM and transferred to FACS tubes and acquired on the BD LSRFortessa™ X-20 within 48 hours. Samples were stored at 4°C protected from light during this period until acquired.

2.2.7.3 *Flow Cytometry Antibodies*

Antibodies used for human tumours were all mouse anti-human, with antibodies used for mouse tumours being rat anti-mouse unless otherwise stipulated. Surface antigen specific mAbs included those specific for: Human Tumour Panel: CD25 (M-A251, BD Biosciences), CD107a (H4A3, BD Biosciences), CD45RO (UCHL1, BD Biosciences), CD45 (HI30, BD Biosciences), CD3 (UCHT1, BD Biosciences), CD4 (SK3, BD Biosciences), CD8α (SK1, BD Biosciences), CD56 (NCAM16.2, BD Biosciences), HLA-DR (G46-6, BD Biosciences), CD11b (ICRF44, BD Biosciences), CD33 (WM53, BD Biosciences), CD279/PD1 (MIH4, BD Biosciences), CD274/PD-L1 (MIH1, BD Biosciences) and the intracellular antibody Foxp3 (259D/C7, BD Biosciences), Mouse Tumour Panel: FOXP3 (FJK-16, eBioscience), CD25 (PC61, BioLegend), CD8a (53-6.7 BioLegend), CD4 (GK1.5, BioLegend), CD69 (11/41, eBioscience), CTLA-4 (UC10-4F10-11, BD Biosciences, Hamster), NK1.1 (PK136, eBioscience, Mouse), CD3 (17A2, eBioscience), CD45.2 (104, eBioscience, Mouse), CD44 (IM7, BioLegend), H-2Kb (AF6-88.5, BD Biosciences), TCRb (H57-597, eBioscience), PD1 (29F.1A12, BioLegend), CD62L (MEL-14, eBioscience), Ly6G (1A8, BD Biosciences), CD11b (M1/70, BioLegend), CD11c (N418, BioLegend, Armenian Hamster), F4/80 (BM8, eBioscience), CD19 (1D3, eBioscience), CD103 (2E7, BioLegend, Armenian Hamster), PDL1 (eBioscience), Ly6C (HK1.4, BioLegend), MHC II (M5/114.15.2, eBioscience). IFN-

γ Stimulation: Human HLA Class I A,B,C (BioLegend, W6/32, mouse), PDL1 (CD274, APC-R700 BD Biosciences, Mouse)

2.3 Nucleic Acid Extraction

2.3.1 DNA Extraction

Genomic DNA was extracted from cell lines, PBMCs and tumour tissue using the Qiagen DNeasy Blood and Tissue kit. Cells were washed with PBS -/- and then lifted with TrypLE Express when 70% confluent as described in 2.2.2.1. Cells were then centrifuged, resuspended and washed in PBS -/- and then 2×10^6 pelleted in an eppendorf at 500 rcf for 5 mins. Cells and PBMCs (refer 2.2.6) were treated in a similar fashion with 2mg/ml Proteinase K digestion. Tumour tissue (10-15mg) was macroscopically dissected with a 23 blade into small pieces (maximum 0.5mm in longest dimension) and transferred to an eppendorf for a prolonged digestion and homogenisation with 2mg/ml Proteinase K in ATL buffer (proprietary kit). The eppendorf was sealed with parafilm and placed in a thermomixer at 56°C and 900rpm until all macroscopic tumour pieces were dissolved (6-12 hours). All cells from the three separate sources (cell lines, PBMCs, tumour) were then treated with the addition of RNase A to a concentration of 2µg/µl for 2 mins at RT. All samples were then processed for DNA extraction utilising a DNeasy spin column in the same manner in accordance with the manufacturer's protocol. DNA was eluted in two 50µl aliquots. DNA yield was checked with the Nanodrop and Qubit prior to storage at -20°C.

2.3.2 RNA Extraction

Total RNA was extracted from the mouse cell lines using the Qiagen RNeasy kit. Cells were washed with PBS -/- and then lifted with TrypLE Express when 70% confluent as described in 2.2.2.1. Cells were then centrifuged, resuspended and

washed in PBS -/- and then 2×10^6 pelleted in an eppendorf at 500 rcf for 5 mins. Cells were then suspended in a lysis buffer of RLT (600 μ l) and β -mercaptoethanol (6 μ l) and vortexed for 1 minute to facilitate cell disruption and homogenisation. Ethanol (600 μ l) is then added to the sample, and it is transferred to a RNeasy spin column with extraction performed in accordance with the manufacturer's protocol. An on-column DNase step was performed using the Qiagen RNase-Free DNase Set. RNA was eluted in a volume of 50 μ l RNase-free water, and samples were assessed for yield with the Nanodrop Spectrophotometer. This value was used to calculate the required 1 μ g of total RNA for reverse transcription.

2.3.3 Reverse Transcription and cDNA Synthesis

cDNA was generated using Invitrogen SuperScript™ III according to the manufacturer's protocols. This involved utilising 1 μ g of total RNA in 9.5 μ l of RNase free H₂O, to which 0.5 μ l random primers (500ng/ μ l; Promega) and 2 μ l of 5mM dNTPs. This was incubated at 65°C for 5 minutes and then for at least 1 minute on ice. After brief centrifugation, 4 μ l of 5X First-Strand Buffer, 1 μ l 0.1M DTT, 1 μ l RNasin and 1 μ l of SuperScript™ III Reverse Transcriptase (200U/ μ l) is added to the mix and pipetted gently prior to being left for 10 mins at RT. Incubation at 50°C for 60 mins is undertaken before inactivation of the reaction at 70°C for 15 mins. The cDNA was stored at -20°C.

2.3.4 PCR and Electrophoresis

A solution of 50 x TAE buffer (242g of tris-base + 57.1ml of 100% acetic acid + 100ml 0.5M sodium EDTA + MQ H₂O to a volume of 1 litre), was diluted to 1 x TAE with MQ H₂O. A 1.5% agarose gel was created by adding 1.5g agarose powder (Sigma #A9539) to 100mls 1 x TAE Buffer. After heating for a minute and once the agarose was completely dissolved, 6 μ l Midori Green Advance nucleic

acid staining solution (Nippon Genetics #MG04) was added to the gel solution. A gel tray was prepared and fit with an appropriate well comb (11 or 21 lanes) to suit the desired number of wells. The gel solution was poured into the tray and allowed to set in the casting tray at RT for 30 mins. Once set, the comb was removed without disrupting the gel, following which the gel was gently removed en bloc and transferred across to the electrophoresis chamber. The gel and wells were then covered in 1 x TAE buffer. The DNA ladder, samples and controls were then added to the loading wells, and the stipulated current applied for the defined time (both current and time were sample dependent). The gel was then transferred onto the platform of the Gel Doc™ XR Imager (BioRad, Gladesville, Australia). It was imaged with Transwhite for orientation and then TransUV with alteration of the contrast to maximise the image quality and subsequent annotation of the saved images.

2.3.5 HPV Assessment

Assessment of HPV subtypes present in the cell lines was undertaken via a collaboration with the Victorian Cytology Service (VCS). Multiple platforms were utilised as outlined below to delineate all subtypes. Five of the platforms (BD Onclarity™ HPV Assay, Cobas® 4800 & Cobas 6800® HPV Test, Seegene Anyplex™ II HPV HR and Cepheid Xpert® HPV Test) assessed for HPV 16, 18 and 12 other high risk genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68). The Linear Array® HPV Genotyping assay assessed for a total of 37 genotypes, including 13 high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and 24 low risk (6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, CP6108) subtypes.

2.3.6 *Mycoplasma* PCR Assessment

DNA was extracted from cell pellets containing 10,000 cells, which had been grown in culture without passage for a minimum of two days. After centrifuging the cells in a 1.5ml eppendorf tube at 500 rcf and aspirating the media, the pellet was processed for the extraction of DNA using 100µl of QuickExtract™ (QE) DNA Extraction Solution (EpiCentre, #QE09050) followed by dilution with 1.1ml of MQ H₂O. A mastermix solution was produced (5µl of 5x MangoTaq Buffer + 1.5µl 25mM MgCl₂ + 0.5µl 10 mM dNTPs + 2µl Myco Fwd + Rev Primers + 0.5µl Cyto Fwd + Rev Primers + 14.25µl MQ H₂O + 0.25µl Mango Taq 5U/µl) and 1µl of DNA added to a total volume of 25µl. The primers (Table 2-1) were centrifuged and then constituted to 100µM with the addition of ultrafiltered H₂O. The PCR reaction was performed by the thermocycler and involved 95°C for 5 minutes followed by 43 cycles of: 95°C for 30 seconds, 53°C for 30 seconds and 72°C for 30 seconds. This was followed by one cycle of 72°C for 5 minutes and 14°C hold. Cytochrome B was used as the loading control, to confirm the presence of gDNA in each lane with this band identified at 375bp. The mycoplasma band was at 520bp, with a 1kb ladder used as the reference. All samples were assessed in triplicate.

2.3.7 *Mutant PIK3CA* Validation

2.3.7.1 *PIK3CA H1047R Allele Specific PCR*

The *PIK3CA* mutation was assessed in the mouse cell lines derived from the transgenic mice. 23µl of Mastermix (5µl of Promega 5x Green GoTaq Buffer + 2.5 µl 25mM MgCl₂ + 1 µl 5 mM dNTPs + 2µl Pik Fwd + Rev (Wt or Mut) Primers (refer to Table 2-1) + 13.3µl MQ H₂O + 0.2µl 5U/µl Promega GoTaq® DNA Polymerase) is added to the PCR tube with 2µl of specimen cDNA. The primers utilised were specific for wildtype and the mutant exon 20, with silent mutations engineered into the mutant exon to distinguish it from the wildtype exon.²¹³ The

PCR reaction was performed by the thermocycler and involved 94°C for 3 minutes followed by 30 cycles of: 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. This was followed by one cycle of 72°C for 10 minutes and 14°C hold. The electrophoresis is performed on a 1.5% agarose gel at 100V for 45 mins. The wildtype and mutant bands are both 250bp long. Both wildtype and mutant positive controls were used in addition to water as a negative control. A 1 kilobyte (kb) ladder was used in end lanes to identify the band size.

Table 2-1 PCR Oligonucleotides

Type	Sequence
Mycoplasma Forward	5'-YGCCTGVGTAGTAYRYWCGC-3'
Mycoplasma Reverse	5'-GCGGTGTGTACAARMCCCGA-3'
Cytochrome B Forward	5'-AAAAAGCTTCCATCCAACATCTCAGCATGA-3'
Cytochrome B Reverse	5'-AAACTGCAGCCCCTCAGAATGATATTTGTC-3'
<i>Pik3ca</i> cDNA Forward (<i>P1F</i>)	5'-CAAGAGTACACCAAGACCAGAGAGTT-3'
<i>Pik3ca</i> cDNA wt Reverse (<i>P2Rwt</i>)	5'-TGTCGTCCATCCACCATGATGT-3'
<i>Pik3ca</i> cDNA mut Reverse (<i>P3Rmt</i>)	5'-TGTCGTCCACCCTCCGTGCCTA-3'
<i>Pten</i> Forward (<i>P1F</i>)	5'-TCCTGCAGAAAGACTTGAAGGT -3'
<i>Pten</i> Reverse (<i>P2R</i>)	5'-ACATGAACTTGTCTCCCCGC-3'
HPV E6/7 Forward	5'-TTGCAGATCATCAAGAACACGTAGA-3'
HPV E6/7 Reverse	5' CAGTAGAGATCAGTTGTCTCTGGTTGC-3'

2.3.8 *PTEN* PCR

The *PTEN* knockout was assessed in the mouse cell lines derived from the transgenic mice. 24µl of Mastermix (5µl of Promega 5x Green GoTaq® PCR Buffer + 1.5µl 25mM MgCl₂ + 0.5µl 10 mM dNTPs + 1.25µl *PTEN* Fwd + Rev Primers (refer to Table 2-1) + 15.5µl MQ H₂O + 0.25µl 5U/µl Promega GoTaq® DNA Polymerase) is added to the PCR tube with 1µl of specimen gDNA. The thermocycler conditions were 94°C for 15 minutes followed by 35 cycles of: 94°C for 1 min, 60°C for 2 mins and 72°C for 1 min. This was followed by one cycle of 72°C for 2 minutes and 14°C hold. Electrophoresis was performed on 1.5%

agarose gel at 135V for 45 mins. The mutant gene product is 368bp compared with the wildtype gene product of 608bp.

2.3.9 HPV E6/7 PCR

The sequence of the HPV E6 (highlighted yellow) and E7 (highlighted green) genes (single transcript) is listed in Figure 2-3 with the purple highlight denoting the starting three nucleotides of each gene. The position of the primers (forward and reverse) for identifying the E6 and E7 oncogenes is highlighted in red. 23µl of Mastermix (5µl of Promega 5x Green GoTaq® PCR Buffer + 2.5 µl 25mM MgCl₂ + 1 µl 5 mM dNTPs + 2µl HPV E6/7 Fwd + Rev Primers (refer to Table 2-1) + 13.3µl MQ H₂O + 0.2µl 5U/µl Promega GoTaq® DNA Polymerase) is added to the PCR tube with 2µl of specimen gDNA. The PCR reaction was performed by the thermocycler and then electrophoresis undertaken as described in 2.3.7.1. The gene product utilising these primers is 112bp.

ATGCACCAAAAGAGAACTGCAATGTTTCAGGACCCACAGGAGCGACCCAGAAAGTTACCACAGT
TATGCACAGAGCTGCAAACAACTATACATGATATAATATTAGAATGTGTGTACTGCAAGCAACAG
 TTAGTGGACGTGAGGTATATGACTTTGCTTTTCGGGATTTATGCATAGTATATAGAGATGGGAAT
 CCATATGCTGTATGTGATAAATGTTTAAAGTTTTATTCTAAAATTAGTGAGTATAGACATTATTGTT
 ATAGTTTGTATGGAACAACATTAGAACAGCAATACAACAAACCGTTGTGTGATTTGTTAATTAGGT
 GTATTAAGTGTCAAAAGCCACTGTGTCTGAAGAAAAGCAAAGACATCTGGACAAAAAGCAAAG
 ATTCCATAATATAAGGGGTCGGTGGACCGGTCGATGTATGTCTTGTTGCAGATCATCAAGAACAC
GTAGAGAAACCCAGCTGTAATCATGCATGGAGATACACCTACATTGCATGAATATATGTTAGATT
TCCAACCAGAGACAACTGATCTCTACTGTTATGAGCAATTAAATGACAGCTCAGAGGAGGAGGAT
 GAAATAGATGGTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATTACAATATTGTAACCTTTTG
 TTGCAAGTGTGACTCTACGCTTCGGTTGTGCGTACAAAGCACACACGTAGACATTTCGTACTTTGGA
 AGACCTGTTAATGGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCATAA

Figure 2-3 HPV 16 E6/7 Transgene

The HPV 16 E6/7 transgene sequence which mirrors that from the HPV16 virus. Starting three nucleotides of each oncogene are underlined; Blue – E6 oncogene; Red– E7 Oncogene; Forward and reverse primers for identifying the E6/7 oncogenes on gDNA by PCR are highlighted in green.

2.4 Immunohistochemistry and Immunocytochemistry

2.4.1 Tumour specimen fixation, embedding and sectioning

A portion of the tumour sample taken derived from a patient, PDX or syngeneic model was placed in a tissue specimen container with a minimum of 10x volume of 10% NBF for a period of 24-48 hours for fixation. The specimen was then further dissected and orientated within a paraffin cassette before being placed in 70% ethanol to begin the dehydration process. The dehydration process was then continued via an automated system within the Centre for Advanced Histology and Microscopy (CAHM) at Peter MacCallum Cancer Centre.

Sections were cut at 4µm onto Superfrost™ uncoated for H & E staining or Superfrost™ Plus for immunohistochemistry staining. Slides were incubated at 37°C overnight to facilitate adherence.

2.4.2 Superfrost™ Plus Slide Processing

Positive and negative controls including a slide where the primary antibody was omitted, were processed with the study samples to confirm appropriate antibody specificity.

The selected tumour Superfrost™ Plus slides were baked in an incubator at 60°C for 45-60 minutes to melt the paraffin. The slides were then dewaxed and rehydrated in the Leica Jung Autostainer (CAHM at PMCC) utilising the dewax protocol. Antigen retrieval was performed by placing the slides in an alkaline (EDTA, 1mM, pH8; Dako High pH, pH 9.5) or acidic buffer (Citrate, 10mM, pH 6) in a Dako pressure cooker at 125°C for 3 minutes, and then allowed to cool to 90°C for 10 seconds. The slides were cooled for a further 20 minutes before washing with MilliQ H₂O. Slides were then rinsed in Tris-Buffered Saline with

0.05% Tween (0.05% TBS-T, pH7.6). A Pap pen was used to circle the tumour section on the slide.

Table 2-2 Human and Mouse Immunohisto/cytochemistry Antibodies

Antibody	Company / Cat #	Antigen Retrieval (Histo slides)	Secondary Antibody
Human			
p63	Abcam, #ab97865	Dako High pH Buffer	ImPRESS Rabbit
CK5	BioLegend, PRB-160P	10mM Na Citrate Buffer, pH6	ImPRESS Rabbit
p16	Roche, CINtec #9517	10mM Na Citrate Buffer, pH6	ImPRESS Mouse
Ki67	Abcam, #ab16667	10mM Na Citrate Buffer, pH6	ImPRESS Rabbit
Anti-Human	Millipore, #MAB1273	Dako High pH Buffer	ImPRESS Mouse
p53	Leica, NCL-p53-D07	10mM Na Citrate Buffer, pH6	ImPRESS Mouse
CD8	Abcam, SP16 #ab101500	10mM Na Citrate Buffer, pH6	ImPRESS Rabbit
PDL1	Ventana (SP263)	1mM EDTA Buffer, pH 8.0	ImPRESS Rabbit
MHC-1	Abcam, #ab70328	1mM EDTA Buffer, pH 8.0	ImPRESS Mouse
Mouse			
p63	Abcam, #ab97865	Dako High pH Buffer	ImPRESS Rabbit
CK5	BioLegend, PRB-160P	10mM Na Citrate Buffer, pH6	ImPRESS Rabbit
Ki67	Abcam, #ab16667	10mM Na Citrate Buffer, pH6	ImPRESS Rabbit
pten	Cell Signaling #9559	10mM Na Citrate Buffer, pH6	ImPRESS Rabbit
pS6	Cell Signaling #2211	10mM Na Citrate Buffer, pH6	ImPRESS Rabbit
CD3	Abcam (SP7) #ab16669	10mM Na Citrate Buffer, pH6	ImPRESS Rabbit
PDL1	Cell Signaling #64988	1mM EDTA Buffer, pH 8.0	ImPRESS Rabbit

2.4.3 Cell Fixation for Immunocytochemistry

Cells were lifted with TrypLE™ Express from tissue culture flasks seeded a day prior. After washing with 10% RPMI and centrifugation, the cells were resuspended in a 15ml Falcon tube, and counted to allow appropriate seeding into an 8-well Nunc Lab-Tek II Chamber slide. Each chamber was filled with a

total of 300µl media. The chamber slide was then transferred to a 37°C 5% CO₂ incubator to allow the cells to settle and adhere overnight.

The following day, ensuring all cells were appropriately 40-70% confluent, the media was aspirated, and the wells were washed with 500µl PBS. After aspiration of the PBS, gentle fixation was performed with 500µl 4% PFA for 20 mins at room temperature. The PFA was then removed, with a further 2 x washes with 500µl PBS. Permeabilisation was then undertaken with 500µl of 0.2% TritonX for 10 mins at room temperature. Two washes with 0.05% TBS-T were performed, following which the immunocytochemistry staining was undertaken as outlined in the immunohistochemistry protocol.

2.4.4 Blocking and Staining

Superfrost™ Plus & Chamber Slides were processed in a similar fashion, with the exception of reagents being added to each well of the chamber slide in contrast to the Pap marked area or whole slide.

Endogenous peroxidase blocking was undertaken with a quenching step of 3% H₂O₂, at room temperature for 10 mins on a rocker. A further 2 x 5-minute washes with 0.05% TBST were then performed on a rocker. Further blocking was then undertaken with 10% Bovine Serum Albumin (BSA) for 1 hour in a humidified chamber at room temperature to reduce non-specific binding. A further 2 x 5-minute washes were then undertaken with 0.05% TBST on a rocker. Following the completion of the blocking steps, the primary antibody was added at an optimised concentration. The slides were then incubated in a humidified chamber at 4°C overnight.

The following day, slides were washed 3 times with 0.05% TBS-T on a rocker for 5 mins. The appropriate secondary antibody was then added to the Pap pen marked sections and incubated in a humidified chamber for 30 minutes at room temperature. A further series of 3 x washes was undertaken with 0.05% TBS-T on the rocker for 5 mins. The slides were then developed with the Dako Liquid DAB + Substrate-Chromogen beginning with the positive control to determine the duration of exposure for subsequent slides. Slides were then placed in MQ H₂O, before being counterstained with haematoxylin and sequentially dehydrated in ethanol and placed in histolene by utilising the Leica Jung Autostainer. MM24 (Leica Biosystems) mountant was then applied and the slides cover slipped. Slides were scanned when required including to aid with computer quantitation for IHC using a VS120 Virtual Slide Microscope.

2.4.5 Haematoxylin and Eosin Staining

Slides were de-waxed in Histolene for 4 minutes, followed by rehydration in reducing concentrations of ethanol. The slides were then placed in deionised water followed by staining with haematoxylin for 2 minutes. They were then blued with Scott's tapwater for 30 seconds. Slides were then placed in Eosin for counter staining, followed by sequential dehydration with ethanol. Slides were rinsed in histolene and cover-slipped with MM24 mountant.

2.5 IFN- γ Gamma modulation of MHCI and PDL1

2.5.1 Cell Lines

Modulation of the immune relevant cellular markers Major Histocompatibility Complex I (MHC I) and Programmed Cell Death Ligand 1 (PDL1) was assessed at baseline and following exposure to Interferon- γ (IFN- γ). This provides some indication of the ability for the tumour cells to evade a T-helper 1 immune

response (Th1), which is the major adaptive arm of the anti-tumour immune response.

Cells were seeded in a 12 well plate (P1, 2, 3 – 2×10^5 , LRF1, 2 – 1×10^5) in triplicate for each condition with 1.5ml of RPMI-1640 + 10% FCS and allowed to adhere overnight. The following day, media was removed and replaced with fresh media alone or media with 100ng/ml IFN- γ (BS Pharmingen #554617). The plates were returned to the 37°C 5% CO₂ incubator for 48 hours. The plates were then assessed under an inverted microscope for the effect on the cell line colony size (qualitative) and number (quantitative). Cells were then lifted with TrypLE Express and washed with PBS, pelleted and transferred to a 96 well plate for FACS staining with anti-PDL1 and anti-MHC I antibodies as outlined in 2.2.7.2. Flow cytometric assessment was undertaken with the LSRFortessa platform, with the Mean Fluorescent Intensity (MFI) used as a marker across the isotype, control and stimulation conditions. Data was analysed with Graphpad Prism, assessing the log-fold change in the MFI.

2.5.2 Cell Line Tumouroids

The IFN- γ stimulation was also undertaken on cell line tumouroids in a similar fashion to the experiment undertaken with the cell lines. Established cell line tumouroids (>50 μ m) were plated (>10/well) without disruption in fresh matrigel in a 24-well plate with 1ml of RPMI-1640 + 10% FCS and returned to the incubator overnight. The media was replaced the following day with fresh media alone or media supplemented with 100ng/ml IFN- γ and the plates returned to the incubator for 48 hours. The cell line tumouroids were then retrieved, fixed with 4% PFA and washed before embedding in Histogel™. Sections were then stained with immunohistochemistry for anti-MHC-I (Abcam, #ab70328 EMR8-5; EDTA

Buffer) and anti-PDL1 (Ventana, SP263, #790-4905; EDTA Buffer), with representative images taken with the Olympus BX61 for qualitative assessment.

2.6 Drug Cytotoxicity and Cell Viability Assays

Cells were seeded in a flat bottom 96 well plate 24 hours prior to treatment. They were seeded at variable densities depending on the cell line used. Cells were plated in a volume of 200µl per well, with all experimental conditions performed in triplicate and on two separate occasions. Cytotoxic drugs were assessed across 10 concentrations including a negative control of media alone. Edge wells were filled with PBS to reduce evaporative and edge effects. After 24 hours, the plates were checked under the inverted microscope to ensure adequate cell adherence with a confluency of 20 - 30%. The media was then aspirated from the wells, and the appropriate drug concentration, vehicle control or media alone added. Plates were returned to the incubator for 96 hours.

Table 2-3 Cytotoxic Drugs

Drug	Supplier	[Stock]	Vehicle	[Working]
5-Fluoro Uracil	Pfizer	384.7mM	H ₂ O	0.01 - 10000µM
Mitomycin C	Bristol-Myers Squibb	1.5mM	H ₂ O	0.01 - 100µM
BYL719 (Alpelisib)	Novartis	10mM	DMSO	0.01 - 50µM
TP-3654	Tolero Pharmaceuticals	10mM	DMSO	0.01 - 50µM

Following the 96-hour incubation, a Resazurin (AlamarBlue®) assay was undertaken to assess cell viability as an inverse marker of cytotoxicity. This assay quantifies the conversion of non-fluorescent resazurin (Sigma #R7017-1G) to fluorescent resorufin by mitochondrial metabolic activity. To each well, 20µl of 20% v/v Resazurin solution was added without removing pre-existing media and the plate returned to the incubator for 2 - 6 hours. A 20% v/v Resazurin (AlamarBlue®) solution is produced by dissolving sequentially 75mg of

Resazurin, 12.5mg of Methylene Blue (Sigma #MB-1), 164.5mg of potassium hexacyanoferrate (III) (Sigma #P8131) and 211mg of potassium hexacyanoferrate (II) trihydrate (Sigma #P9387) in 500ml of sterile PBS within the confines of a Tissue Culture Hood.

The plate was then loaded into a FLUOstar OPTIMA microplate reader (BMG labtech, Mornington, Australia) for fluorescence measurement. Parameters were set for an excitation of 540 nm and an emission of 590 nm. The percentage of viable cells was calculated as follows: $(\bar{x}_D - \bar{x}_M) / (\bar{x}_V - \bar{x}_M) \times 100$, where $\bar{x}_D$: mean fluorescence of drug-treated wells, $\bar{x}_M$: mean fluorescence of media only wells, and $\bar{x}_V$: mean fluorescence of vehicle-treated control wells. Dose-response curves and IC50 values were generated using Graphpad Prism.

2.7 Cellular Proliferation Assay

The xCELLigence RTCA-DP (ACEA biosciences, Noble Park North, Australia) E-plate platform measures impedance across small electrodes on the flat surface of a purpose designed culture plate at serial time-points while the plate is contained within the confines of an incubator. When cells are seeded to the wells, they initially settle, adhere and then spread, before undergoing proliferation. The increased contact of cells across the plate increases the impedance to current flow, which is converted into a real time surrogate measure of cellular proliferation by the xCELLigence software, designated the cell index (Figure 2-4). The E-plate has a 2 x 8 flat bottom well configuration.

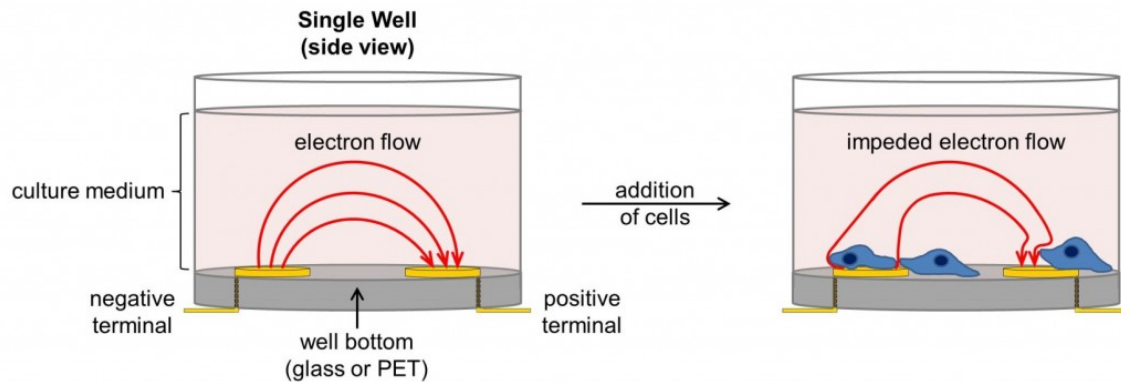


Figure 2-4 xCELLigence System: Impedance as a measure of cell proliferation

Measurement of cell proliferation by assessing the impedance across the surface of the well bottom. (ACEA biosciences, copyright permission granted)

The proliferation protocol as published by ACEA biosciences was followed strictly. Following equilibration and assessment of the background impedance with media alone, cells were seeded into the wells of an E-plate in duplicate and mounted in the xCELLigence RTCA platform within an incubator at 37°C and 5% CO₂. Seeding densities for each cell line were 2×10^4 (P1, P2, P3), 1×10^4 (LRF1, LRF2), 1×10^3 (Ana1, Ana1 E6/7). Timepoints were set at 15 minutes for the first 6 hours followed by hourly out to 150 hours.

2.8 Migration Assay

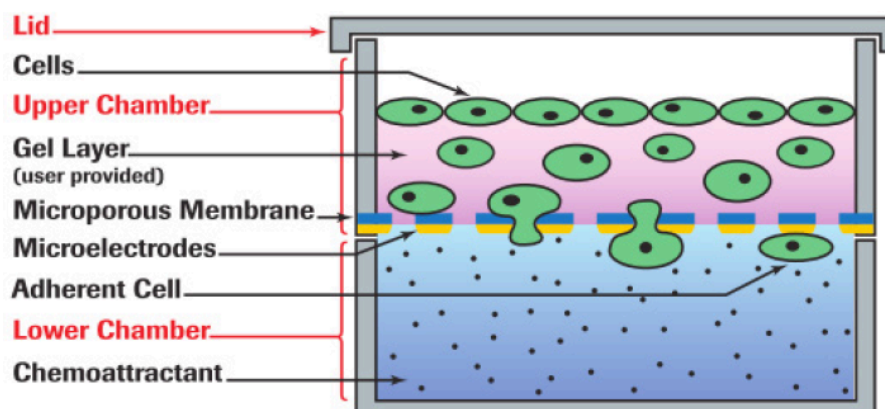


Figure 2-5 xCELLigence CIM Plate configuration

Measurement of impedance on the underside of the membrane for migrated cells (ACEA biosciences, copyright permission granted).

The Cell Invasion Migration (CIM) plate was used for undertaking the migration assay. This plate differs from the E-plate in having both an upper and lower chamber, with electrodes located on the underside of the microporous membrane, which is a component of the upper chamber (Figure 2-5). The migration protocol as published by ACEA biosciences was followed strictly. RPMI + 20% FCS was added to the bottom chamber well as a chemoattractant in both assays. Cells were seeded in RPMI without FCS into the upper chamber at densities of 8×10^5 (P1, P2, P3) 4×10^5 (LRF1 and 2) and 2×10^5 (Anal1, Anal1 E6/7). Measurements were made at 15-minute intervals for a period of 24 hours. The experiment was abbreviated at this point, in order to remove the potential of proliferation contributing to the cell index reading. At the conclusion of the experiment, the plate was removed and disassembled, and the bottom of the upper chamber stained with methanol (Sigma) + 0.5% w/v crystal violet (Sigma #C3886), rinsed in water and air dried, before imaging to correlate with the cell index results.

2.9 Radiotherapy Clonogenic Survival

To investigate the effects of radiotherapy on the clonogenic survival of the cell lines, cells were seeded in 6 well plates and allowed to adhere for 12 hours before treatment with radiotherapy doses of 2, 4, 6, 8 or 10 Gray (Gy). The seeding density was optimised and dependent on the radiotherapy dose being delivered as outlined in Table 5-5 and Table 6-3 for each cell line. The optimisation was undertaken by plating out a minimum of three cell densities for each line and radiotherapy dose. The density that resulted in a colony count of 25-150 was utilised to determine the surviving fraction. Cell colonies were fixed with 3 mls of 0.5% w/v crystal violet in 25% methanol for 5 mins, rinsed with water and allowed to air-dry. Discrete colonies of ≥ 50 cells were counted manually under an inverted microscope with the aid of a grid. The colony count was expressed

as a percentage of the untreated group. Graphpad Prism was used to analyse the data using a linear quadratic equation, which takes into account both the alpha (linear) and beta (quadratic) aspects of different cell responses to radiotherapy treatment.

2.10 Puromycin Killing Curve

Puromycin dihydrochloride (Gibco #A1113802) is an aminonucleoside antibiotic that causes rapid cell death by translational inhibition. Resistance can be conferred by the *Streptomyces alboniger* puromycin N-acetyltransferase (pac) gene. Adherent mammalian cells are generally sensitive to concentrations of puromycin from 2 to 5µg/ml. A puromycin killing curve determines the concentration of puromycin that will kill all cells of a specified cell line without the pac gene. The stock solution of 10mg/ml is diluted in MQ H₂O to the appropriate working concentration.

Cells were plated into a 96 well plate at 2×10^3 cells/well and allowed to adhere overnight in standard RPMI + 10% FCS media. Media was aspirated the following day, and RPMI + 10% FCS + puromycin dihydrochloride (Gibco, Thermofisher Scientific)) instilled at concentrations of 0.5, 1, 2, 4 and 8µg/ml in triplicate, with positive (no cells) and negative controls (cells with media alone + vehicle) in triplicate. After 72 hours of incubation in a 37°C 5% CO₂ incubator, 20µl AlamarBlue® was added to each well without removing the existing media and incubated for 3 hours.

The plate was then loaded into the FLUOstar OPTIMA microplate reader for fluorescence measurement with an excitation of 540 nm and emission of 590 nm. The percentage of viable cells was calculated as follows: $(\bar{x}_P - \bar{x}_M) / (\bar{x}_V - \bar{x}_M) \times 100$, where $\bar{x}_P$: mean fluorescence of puromycin-treated wells, $\bar{x}_M$: mean fluorescence of media only wells, and $\bar{x}_V$: mean fluorescence of vehicle-treated

control wells. A puromycin dose-response curve was generated using Graphpad Prism. Macroscopic assessment under the microscope was used to validate the concentrations at which all cells were dead.

2.11 Viral Transduction

Transductions were undertaken in accordance with the Australian Governments notification of Notifiable Low Risk Dealing (NLRD) physical containment level 2 (PC2), NLRD PC2 05 2014.

2.11.1 GFP-Reporter Lentiviral Control Transduction

Lentiviruses are used to transduce both dividing and non-dividing cells by actively passing through nuclear membranes and integrating into the host genome, with the main limitation being the insert size.

The optimal Multiplicity Of Infection (MOI) was determined by using a GFP-reporter lentivirus in the presence of Polybrene (Sigma, #TR-1003). Polybrene was prepared from the stock solution of 10mg/ml to a working concentration of 0.8mg/ml in MQ H₂O. Cells were plated out in a 6-well plate at 3×10^5 cells / well and the cells allowed to adhere overnight in standard RPMI + 10% FCS. This resulted in a confluency of approximately 50% by the following morning. The GFP-reported lentivirus was rapidly thawed in a 37°C water bath. The lentivirus was added to the 6-well plate at 2ml/well in the presence of 4µg/ml polybrene to improve the transduction efficiency of the cells, with a MOI of 0.5, 1 and 2 only assessed.

After lentiviral incubation overnight, the following morning, without removing media, fresh RPMI + 10% FCS was added 1:1. After a further 24 hours of incubation, the viral supernatant was aspirated, and fresh media again added for

a further 24 hours. After a total time of 72 hours, the cells were subcultured into a new 6-well plate for each MOI, with the remainder passaged into a T175 flask for expansion. After incubation for 24 hours in the 6 well plates, the adherent and surviving cells were assessed under Fluorescent capable phase contrast microscopy (Evos® FL Imaging System, Thermofisher). Quantitation was performed to determine the transduction efficiency using both the transmitted light and GFP (Excitation 470/22nm, Emission 510/42 nm) fluorescent channel to identify cells. A grid was utilised on the base of the 6-well plate to facilitate counting. When the T175 flask had 70% confluency, the cells were lifted with TrypLE Express and FACS sorted for the GFP positive cells. These cells were subsequently cultured for two further passages and cryopreserved as Anal1-GFP cell line.

2.11.2 HVP 16 E6/7 Lentiviral Transduction

The lentivirus was imported through Australian Customs with an AQIS (Australian Quarantine and Inspection Service) Import Permit (Lenti-HPV-16 E6/7 Virus, #G268, 10⁶ Infectious Unit/ml (IU/ml), 10mls, abm®). As demonstrated in Figure 6-11, it contained the full sequence of the HPV E6/7 oncogenes between a CMV and SV40 promoter and with a puromycin selection gene within its construct.

The transduction was performed in the same fashion as that for the GFP lentivirus (refer 2.11.1), with cells plated in a 6-well plate and subsequently exposed to 2ml/well HPV E6/7 lentivirus in the presence of 4µg/ml polybrene. The MOI used was 1, to aim for a transduction rate of 10%. Following 72 hours of incubation, the cells were sub-cultured into a new 6-well plate and allowed to adhere overnight. The puromycin killing curve (refer 2.10) identified that 2µg/ml was adequate to kill all non-transduced cells. The following morning the media

was changed with the addition of puromycin at a concentration of 2µg/ml. Cells were cultured in puromycin for a minimum of 4 days prior to passaging, and this exposure / passaging process was repeated three times prior to assessment of the resistant cells for the HPV E6/7 oncogenes via PCR.

2.12 LacZ Staining

Fixation was performed in ice cold 2% formaldehyde, 0.2% glutaraldehyde in PBS, and left at this temperature on ice for 6 hours. The formaldehyde solution was then replaced with 20% sucrose solution in PBS. The solution was changed 4 times in 28 hours. The anal canal tissue is then bisected in both sagittal and axial planes and placed in a cryomould for embedding in OCT media on dry ice, and then placed in the -80°C freezer. Sections were cut at 15µm in thickness on the cryotome.

The yellow staining solution was prepared by adding 0.1g MgCl, 0.48g potassium ferricyanide and 0.64g potassium ferrocyanide to 500mls PBS +/- 400µl 5% X-gal (Promega) in dimethylformamide is added to 50ml of yellow staining solution. The slides were incubated in this solution overnight in the dark at 37°C. They were then washed three times in PBS, counterstained with Nuclear Fast Red for 1 minute, rinsed for 5 mins in H₂O, dehydrated and cover-slipped.

2.13 Scanning Electron Microscopy

Cell line tumouroids were plated and maintained in a 24 well plate as described in 2.2.3.2. Once established, media was aspirated from the wells and the tumouroids retrieved from the matrigel without significant disruption utilising Cell Recovery Solution (Corning™). The tumouroids were then plated in RPMI 1640 + 10% FCS onto a purpose cut segment of thin acryl plastic, which lay at the base of the well in a 24-well plate. The acryl plastic was sterilised with 90%

ethanol prior and allowed to dry before being placed with a sterile technique into the well. The plate was returned to the incubator and allowed to sit for 48 hours during which time the tumouroids adhered to the acryl plastic. The media was then removed and the tumouroids fixed in 2% PFA, 2.5% glutaraldehyde in 0.1 sodium cacodylate buffer at 4°C. The tumouroids were then washed in 0.1M sodium cacodylate buffer before being stored in 0.08M sodium cacodylate buffer with 5% sucrose at 4°C.

The Leica EM CPD300 critical point drier was used to dehydrate tissue samples. The tumouroids adherent to the acryl plastic were then mounted on a scanning electron microscopy stub (agar scientific) and gold sputter coated with an Emscope SC500 before being imaged with the Jeol JCM-6000PLUS Neoscope Benchtop scanning electron microscope. Images were acquired at 15kV under high vacuum.

2.14 Genomic and Transcriptomic Sequencing

2.14.1 Targeted Cancer Panel

Through a collaboration with the Cancer 2015 study at Peter MacCallum Cancer Centre, 43 patient samples were utilised both retrospectively and prospectively to have tissue sequenced for a targeted cancer panel.

FFPE pre-treatment tumour blocks were obtained from Peter Mac Pathology or external pathology services where necessary. Ten sections at 5µm were cut from each respective block. One section was stained with H & E for histological review and nine sections were stained with methyl green to allow macroscopic visualization of tissue. The H&E section was reviewed by a qualified pathologist

to mark the tumour and assess its cellularity. The tumour area was macro-dissected manually or via an automated computer-guided system.

Table 2-4 Somatic Panel v2 Gene Information

Gene	Exons covered	Hotspot Codons
<i>AKT1</i>	3	17
<i>ALK</i>	20, 22, 23, 24, 25	1174, 1245, 1275
<i>BRAF</i>	11, 15	469, 600
<i>CDKN2A</i>	p16 – 1, 2	58, 80
<i>EGFR</i>	18, 19, 20, 21	Multiple
<i>FGFR2</i>	7, 9, 12	252, 382, 549
<i>FGFR3</i>	7, 10, 16	249, 380, 716
<i>HER2 (ERBB2)</i>	8, 17, 19, 20, 21, 22, 27	310, 615, 678, 755, 842
<i>KIT</i>	9, 11, 13, 17	Multiple
<i>KRAS</i>	2, 3, 4	Multiple
<i>MAP2K1</i>	2, 3	-
<i>MET</i>	14	Splice site mutations
<i>NRAS</i>	2, 3, 4	Multiple
<i>PDGFRα</i>	12, 14, 18	Multiple
<i>PIK3CA</i>	10, 21	542, 545, 1047
<i>PTEN</i>	Multiple	Multiple
<i>RAC1</i>	3	-
<i>RNF43</i>	3, 9	-
<i>TP53</i>	Multiple	Multiple

The macro-dissected tissue underwent genomic DNA extraction using the Qiagen DNA extraction kit. The genomic DNA was then screened for mutations in selected exons of 19 cancer-related genes (refer Table 2-4) using an amplicon-

based next generation sequencing (NGS) assay developed at Peter MacCallum Cancer Centre. NGS was performed on an Illumina MiSeq platform, using 2 x 150bp reads. Seqliner v0.7 was used to generate aligned reads and call variants against the GRCh37 (hg19) human reference genome assembly. Variants were described according to HGVS nomenclature version 2 (<http://varnomen.hgvs.org/>). Synonymous changes and intronic variants outside of splice-sites were not reported.

2.14.2 Whole Exome Sequencing

WES was undertaken for the panel of human ASCC cell lines. Analysis was performed on paired DNA samples of cell line and matched normal (PBMCs) or corresponding parent tumour and matched normal (PBMCs). Capture of the coding sequences from individual libraries for each sample was performed using the SureSelect Whole Exome Sequencing Kit v6(Agilent Technologies). Sequencing of the captured libraries was then performed using the Novaseq 6000 Genome Analyser (Illumina) with a mean target coverage of 124X achieved and a mean fragment length of 225bp. Quality control was performed with FastQC (v0.11.7, Babraham Institute) and the sequenced reads were aligned to the GRCh37 human reference genome using Burrows-Wheeler Aligner (BWA, v0.6.2).²¹⁴

2.14.2.1 Detection of somatic mutations

Quality filters were applied to the samples for the selection of both single nucleotide variants (SNVs) and small insertions/deletions, predicted as present in the cell lines/tumours and not in their matched PBMC's (normal control). The variants that were included met all of the following quality criteria: (1) Two independent callers for variants from MuTect, MuTect2 (v4.0.8.0)²¹⁵ and VarDict;²¹⁶ (2) gnomad population allele frequency missing or <1%; (3) on the

canonical transcript; (4) with a total depth read depth of 10 or more in the samples, with at least 5 supporting reads (allele depth 5) coming from both forward and backward strands.

The Oncoplot summary of the most frequent variants identified in the cohort of ASCC lines and the mutational load of the cohort compared to other TCGA (The Cancer Genome Atlas, <https://www.cancer.gov/tcga>) listed cohorts were generated by Maftools (Bioconductor). The R-library upSet was used to identify the overlap of shared variants between cell lines and parent tumour.

2.14.2.2 Detection of mutational trinucleotide signatures

To generate the mutational trinucleotide signature plots for each sample, the computational framework of Alexnadrov et al²¹⁷ and the R Package deconstructSigs (R Core Team, 2015) was utilised.²¹⁸ An iterative approach was employed to determine the weights to assign to each signature, with the raw trinucleotide counts normalised by the number of times each trinucleotide context is observed in the human exome. The mutation signatures (v2, 2015) are derived from the COSMIC database.²¹⁹

2.14.2.3 Detection of somatic copy number alteration

The somatic copy number alterations in the parent tumour and in the cell lines compared to the matched PBMC's were characterised using FACETs (v0.5.0).²²⁰ The copy number frequency plots were generated for all genes and the cutoff of the average log ratio was 0.3. The mean log ratio in a chromosome region between 0.3 and 1.0 was classified as genomic gain, more than 1.0 as focal amplification, less than -0.3 as heterozygous deletion, and less than -1.0 as homozygous deletion.

The cytoband copy number plot was constructed by segmenting the genome into bins of 10000Mbp size, and each bin/box coloured by integer copy number.

2.14.3 RNA Sequencing Analysis

RNA sequencing data previously extracted and sequenced on a cohort of 11 ASCC patients was retrieved. These patients had inadequate tissue or blocks remaining from which to obtain DNA that could be utilised as part of the Cancer 2015 Study. Consequently, the RNA data was utilised to assess for genomic variants of clinical significance to complement and expand the dataset achieved through Cancer 2015.

The RNA sequencing raw reads were aligned to reference genome HG38 with bowtie v2.2.9²²¹ and Tophat v2.1.1²²². Integrated Genomics viewer (Version 2.3.55) was utilised to visualise the resulting BAM files. Analysis was performed with Galaxy Peter Mac (Genomics Virtual Laboratory 4.0.0, Galaxy version 17.01).²²³ Variant calling was undertaken by generating variant calling format (vcf) files using MPileup and Varscan 2 on the Galaxy platform. A list of candidate genes and BAM files were sliced (using SAMtools slice) to include only genes of interest. MPileup (SAMtools) was undertaken using human reference genome (HG38) to generate a pileup file.²²⁴ Raw variants were filtered using PathOS to detect significant variants.²²⁵ PathOS is an in-house variant calling pipeline (Peter MacCallum Cancer Centre, Department of Pathology).

2.15 Murine

2.15.1 Animal Husbandry

2.15.1.1 Ethics

All procedures were undertaken in accordance with the Prevention of Cruelty to Animals Act 1986 and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Ethics approval was obtained from the Peter MacCallum Cancer Centre, Animal Experimentation Ethics Committee (AEEC), Approval numbers E497, E512, E582, E584, E601.

2.15.1.2 Housing and Maintenance

Mice were housed in individually ventilated cages with a maximum of 6 mice per cage. They were fed standard chow and had unlimited access to chow and water. The facility was temperature controlled with a set point of 25°C, and exposed to a 12-hour day-night cycle.

The following strains of mice were bred at the Peter MacCallum Cancer Centre. Nod-SCID-IL2R γ ^{NULL}(NSG) mice are the most immunocompromised strain available and a useful resource for establishing PDTXs. UBC-Cre^{ERT2}C57Bl/6 mice, have a tamoxifen inducible *Cre recombinase* that is linked to a ubiquitin promoter present within all cells.²¹³ C57Bl/6.*Pik3ca*^{H1047R} mice have a latent Cre-inducible exon 20 kinase domain activating mutation (H1047R) of the *pik3ca* gene.²¹³ C57Bl/6.*pten*^{fl/fl} mice have exon 5 floxed from both alleles of the *pten* gene.^{213, 226} K5Cre.C57Bl/6, mice have a keratin-5 specific promoter which is active in the basal epithelial layer of stratified epithelium.²²⁷ C57Bl/6.*Pik3ca*^{H1047R}.ROSA26 mice were available from previously crossing

C57Bl/6.*Pik3ca*^{H1047R} and C57Bl/6.ROSA26 mice. These mice have a ubiquitously expressed *LacZ* transgene, which allows localisation of transgene activation by performing X-gal staining on the fixed tissues of interest.²²⁸

Wild-type C57Bl/6 and athymic (Nude) mice were bred externally at the Walter and Eliza Hall Institute (Melbourne, Australia) and transported in as required. Wild-type C57Bl/6 mice were utilised in assessing the tumourigenicity of a C57/Bl/6 syngeneic model. Nude mice are the most partially immunocompetent mice, and a useful model for assessing tumourigenicity of cell lines and PDTXs as well as more robust than NSG mice when assessing chemo and targeted therapies *in vivo*.

Crossing of [UBC-Cre^{ERT2}C57Bl/6 and C57Bl/6.*Pik3ca*^{H1047R}] and [K5Cre.C57Bl/6 and C57Bl/6.*Pik3ca*^{H1047R}.ROSA26] mice was undertaken at the Peter MacCallum Cancer Centre (PMCC) animal breeding facility, with breeding pairs established at 3 weeks of age. The offspring had DNA extracted from tail clips for genotyping, with a portion of the offspring being UBC-Cre^{ERT2}C57Bl/6.*Pik3ca*^{H1047R} and K5Cre.C57Bl/6.*Pik3ca*^{H1047R}.ROSA26 mice. UBC-Cre^{ERT2}C57Bl/6.*Pik3ca*^{H1047R} offspring were then crossed with C57Bl/6.*pten*^{fl/fl} mice multiple times to establish UBC-Cre^{ERT2}C57Bl/6.*Pik3ca*^{H1047R}.*pten*^{fl/fl} mice confirmed on genotyping.

2.15.1.3 Mouse Genotyping

Genotyping was undertaken on tail clippings of the litter pups to identify those with the appropriate transgenes.

Tail clips taken from the C57Bl/6 offspring were digested in 800µl of lysis buffer (50mM Tris, 1% SDS, 100mM NaCl, 100nM EDTA and 250µg/ml of Proteinase K) in an Eppendorf tube and incubated at 56°C overnight. The following morning,

300µl of NaCl was added and samples were shaken for 5 minutes and then placed on ice for a further 5 minutes. The tube was then centrifuged at 13,000rpm for 10 mins at room temperature. The supernatant was transferred to a new Eppendorf tube with cold isopropanolol, and the tube centrifuged again at 13,000rpm for 5 minutes at 4°C. The supernatant was discarded, and the pellet rinsed with 75% ethanol, air dried and then re-suspended in 100µl of RNase free H₂O (for long term storage, samples are kept at -20°C).

2.15.1.4 PCR Genotyping

The DNA obtained from the mouse tail clippings was used as a template for PCR amplification of the *pik3ca*, *pten*, *Rosa* and *Cre recombinase* genes to confirm the genotype of each mouse. The PCR reaction was performed in 5x GoTaq Polymerase Buffer. The final primer concentrations for the primer sets were 0.5µM. Amplification was conducted on a BioRad Thermal Cycler for each primer set using optimal PCR conditions. A negative water control containing all solutions except the DNA template was included to confirm the absence of contamination and positive / heterozygous and negative / wild type controls were also used in all PCR reactions. The PCR products were held at 14°C until removed from the machine and stored at 4°C or run immediately on agarose gels (1.5% agarose with Midori Green at 150V for 30 mins) in 1 x TAE buffer alongside a 100bp DNA ladder. The gels were then visualised and photographed on a Gel DocTM XR Imager.

Table 2-5 Primers for genotyping and PCR conditions for each primer set

Name	Sequence (5' - 3')	Amplicon		Denaturing	Cycle	Cycles	Extension
			Size (bp)	94°C (min)	Conditions	(n)	72°C
<i>UBC-Cre</i>	R: GGGATTGCTTATAACACCCTGTTACG	WT	Blank	3'	94 °C 30''; 60 °C 30''; 72 °C 30''	30	10'
	R: TATTCGGATCATCAGCTACACCAGAG	HET	213				
<i>Pik3ca</i>	F1: TTGGTTCCAGCCTGAATAAAGC	WT	553	10'	94 °C 30''; 60 °C 30''; 72 °C 45''	35	10'
	F2: GTCCAAGGCTAGAGTCTTTTCGG	HET	372, 553, 601				
	R1: TCCACACCATCAAGCAGCA	HOM	372, 601				
<i>pten</i>	F: CAAGCACTCTGCGAACTGAG	WT	156	2'	94 °C 20''; 60 °C 15''; 72 °C 10''	30	2'
	R: AAGTTTTTTGAAGGCAAGATGC	HET	156, 328				
		HOM	328				
<i>ROSA26</i>	F: GTCTGCCGCGCTGTTCTCCTCTTC	WT	blank	10'	94 °C 30''; 64 °C 30''; 72 °C 30''	35	2'
	R: CTTGCGCCAATAGCAGCCAGTCC	HET	317				
<i>K5Cre</i>	F: GGACATGTTTCAGGGATCGCCAGGCG	WT	Blank	5'	94 °C 1'; 64 °C 1'; 72 °C 1'	28	2'
	R: CGACGATGAAGCATGTTTAGCTG	HET	300bp				

2.15.2 *Mouse General Anaesthesia*

Anaesthesia was delivered to mice for all procedures except for tumour measurement and mouse weights. The techniques and recovery of the mice are detailed below. Mice were marked with ear clippers for identification while under anaesthesia.

2.15.2.1 *General Considerations*

The development of hypothermia during and after mouse procedures was reduced by employing a number of measures. This included the routine use of a PhysioSuite heatpad (Kent Scientific) for recovery, and use of a Nesco infra-red heat lamp (Nesco Medlab) during procedures that took in excess of 5 mins to complete. Risk of ocular injury was mitigated by routinely utilising Poly Visc Eye Ointment (Clifford Hallam Healthcare) for all procedures.

2.15.2.2 *Intraperitoneal Anaesthesia*

Each mouse was weighed and a mixture of Xylazine (10mg/ml) and Ketamine (2mg/ml) administered intraperitoneally at 80µl per 10g of body weight. The technique involved scruffing the mice and positioning them head down and right side up to facilitate injection into the right lower abdominal wall minimising the risk of injection into the bowel lumen.

2.15.2.3 *Volatile Anaesthesia*

Prior to commencement, checks were undertaken to ensure “The Stinger” small animal anaesthetic machine and TEC 3 ISO Vaporiser (Advanced Anaesthesia Specialists) was functioning correctly and that sufficient Isoflurane (Abbott Laboratories) and oxygen (Air Liquide Healthcare) were available. The mouse was then placed into the induction chamber with Oxygen at 0.8l/min.

Anaesthetic induction was then commenced with Isoflurane 4% and oxygen (0.8l/min). The time required for surgical anaesthesia was 2-5 mins depending on the mouse strain and age. Once anaesthetised, they were removed from the induction chamber and placed on the operating space with a nose cone to deliver ongoing Isoflurane 2.5% with 0.8l/min oxygen for the duration of the procedure. At this point, Polyvisc ointment was applied and the mouse was depilated if required.

2.15.2.4 Recovery

Mice were recovered in a pre-warmed individually ventilated box with both food pellets and nectar glucose jelly in petri dishes. The mouse boxes are placed in a dedicated recovery room on a heat-mat overnight.

2.15.3 Tumourigenicity in Mice

To confirm tumourigenicity of human and mouse cell lines, 5×10^6 cells (passage 20 – 30) were suspended in 100 μ l of a 1:1 Matrigel (BD Biosciences, USA) PBS mix at 4°C. Subcutaneous injections were performed into the right leg of Nude (4-6 weeks), NSG (6-8 weeks) or wtC57Bl/6 mice (6-8 weeks). NSG and C57Bl/6 mice were shaved over the area of injection immediately prior. Injection was performed under sterile conditions in a laminar flow operating room and 2% (v/v) chlorhexidine gluconate / 70% (v/v) isopropyl alcohol applied and allowed to dry for skin asepsis prior to injection. Mice and tumours were monitored, with tumour measurements twice weekly. Mice were culled when ethical limits or study endpoints were reached or at the first signs of discomfort. Resection of the tumour was then performed with subsequent fixation (10% NBF) and paraffin embedding for H & E staining, pathological examination and IHC.

2.15.4 Patient Derived Tumour Xenograft & Mouse Derived Tumour Syngraft

Established PDTXs and primary mouse tumours were resected with sterile instruments after prepping the mouse and tumour with a 2% chlorhexidine and 70% ethanol mix. The tumours and human anal cancer biopsies were then dissected to 1mm³ size and placed in 50µl of liquid growth factor reduced Matrigel at a 1:1 ratio on ice at 4°C until implantation. Mice (NSG or C57Bl6^{wt}) were appropriately anaesthetised with volatile anaesthetic, prepped with 2% chlorhexidine gluconate / 70% ethanol and a vertical incision made along the dorsal midline of the mouse. The subcutaneous plane was dissected on the right side to expose the area, and a 4/0 Vicryl Rapide™ (VR) suture placed into the paravertebral musculature to facilitate creation of an intra-muscular pocket with dissecting tenotomy scissors. The Matrigel was taken off ice and allowed to reach a semisolid state facilitating implantation into the intra-muscular pocket. The muscular pocket was then closed with the pre-placed suture, followed by skin closure over the top with 4/0 VR. The mouse was recovered and subsequently monitored as outlined previously (refer to 2.15.1.2)

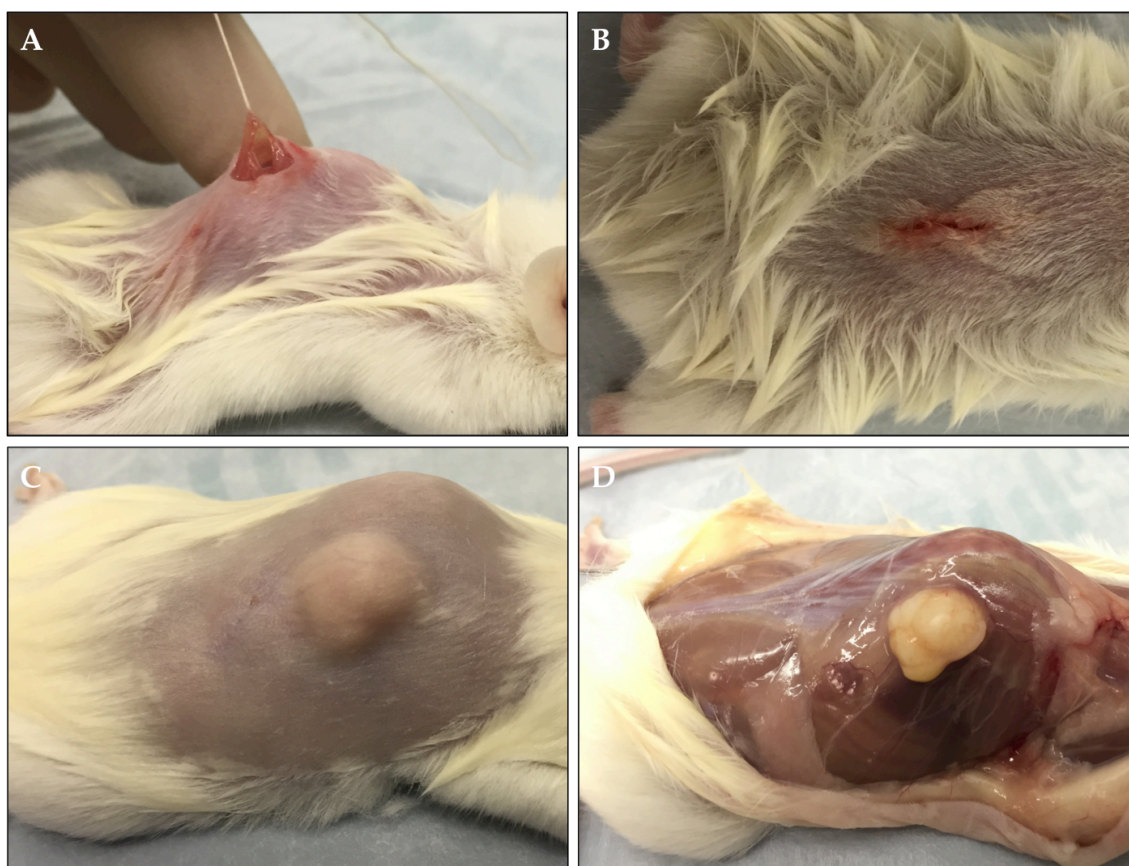


Figure 2-6 Patient Derived Tumour Xenograft and Mouse Syngraft Technique

The technique for establishing a patient derived tumour xenograft / mouse Syngraft is demonstrated. This involved the anaesthetisation of recipient mice, followed by preparation of the skin overlying the area for implantation. A skin incision was then made and a submuscular pocket developed and tented open with a suture to facilitate implantation of the tumour piece coated in Matrigel (A). The wound was then closed in layers and the mouse recovered (B). Successful engraftment is depicted on the right flank of a NSG mouse in (C) and following removal of the skin with preparation for resection (D).

2.15.5 Tamoxifen Administration

For conditional activation in the transgenic mice with a Tamoxifen inducible Cre, 4-OHT was utilised. This was made up to a concentration of 2mg/ml (300 μ M) in DMSO, which can be stored at -20°C for up to 1 year.

Chapter 3: Salvage Surgery in ASCC

Published Manuscript

Salvage Surgery for Locoregional Failure in Anal Squamous Cell
Carcinoma

3.1 Abstract

Background

Anal squamous cell carcinoma (ASCC) is a rare cancer with a high cure rate, making research into the treatment of locoregional failure difficult.

Objective

The purpose of this study was to examine factors related to local treatment failure and determine the outcomes of patients undergoing local salvage resection.

Design

This was a retrospective cohort study.

Setting

This study was conducted at a quaternary referral centre.

Patients

Patients with ASCC treated with chemoradiotherapy between January 1983 and December 2015 were included.

Main Outcome Measures

The influence of patient, tumour and treatment related factors on the primary outcome measures of locoregional failure, overall and disease free survival were investigated.

Results

Of 467 ASCC patients, 63 suffered locoregional failure with 41 undergoing salvage resection. Twenty seven patients (38%) suffered persistent disease and 36 (62%) developed locoregional recurrence. Multivariate analysis identified tumour stage (HR 3.16 $p < 0.002$) as an independent predictor of locoregional failure. Thirty abdominoperineal resections and 11 pelvic exenterations were undertaken with no surgical mortality. At a median follow up of 20 months (range 4 - 150 months), 5-year overall and disease free survival for the salvage cohort was 51% and 47% respectively. Margin positivity was an independent predictor for relapse post salvage surgery on multivariate analysis (HR 20.1 $p 0.027$). Nineteen patients (48%) developed further relapse, which included all 10 patients with a positive resection margin, 3 of which underwent re-resection. Of the 19 patients with relapse, 3 remain alive, 2 with persistent disease.

Limitations

Limitations include the retrospective nature of the database, the prolonged time period of the study and episodes of incomplete data.

Conclusions

Advanced T stage is an independent predictor of local failure in anal SCC. Most patients can be salvaged, with a positive resection margin being a strong predictor of further relapse and poor outcome.

Keywords: Anal Cancer; Squamous Cell Carcinoma; Chemoradiotherapy; Locoregional Failure; Salvage Surgery

3.2 Background

Anal cancer is a rare disease accounting for 2.5% of all gastrointestinal cancers with squamous cell carcinoma (SCC) the most common histological subtype.^{7, 16} Definitive chemoradiotherapy remains the primary management, following the seminal work of Norman Nigro in the 1970's.⁵⁰ Subsequent phase III randomised controlled trials confirmed oncological equivalence of this regimen, relegating surgery to a salvage role. With sequential modifications over the ensuing 4 decades, this regimen offers 5-year survival rates of 65-90% with local control in 70-90%.^{43, 44, 57, 229-232}

Local failure in ASCC remains a problem in 10-30% of patients, portending a poor prognosis and presenting a difficult management problem for symptoms of locally progressive disease. This cohort comprises both patients with persistent disease following CRT and patients with recurrent disease after initially achieving a complete clinical response.^{112, 113, 124, 125, 127, 130, 233} Salvage surgery is appropriate in select patients with local failure, in an attempt to provide durable local control and possible cure. Abdominoperineal resection (APR) is the traditional salvage operation for patients with local failure, however, as is the nature with other advanced perineal and pelvic tumours, extended resections and multivisceral exenterations with autologous flap reconstructions are often required. Overall survival for patients with local failure has ranged extensively on review of the literature, from 23% up to 78%, with improved survival where management has included more radical surgical approaches.^{40, 118, 122, 125-127, 129, 133, 234-237} Consequently, prediction of success is foremost in the selection of appropriate patients for salvage surgery.

The aim of the study is to assess the long-term outcomes associated with local salvage surgery for ASCC, and the modified approaches employed at a

quaternary centre. Predictive variables of local failure and the success of salvage surgery are examined, to guide future patient selection and management decisions.

3.3 Methods

3.3.1 Cohort Selection

A search of a retrospective anal cancer database was performed. This contains information on all anal cancer patients treated at a single quaternary referral centre over a 33-year period (January 1983 – December 2015). The database was interrogated for patients diagnosed with squamous cell carcinoma of the anal canal or margin and treated with curative intent radical CRT over this period with a standardised protocol. Patients with metastatic disease, those managed with radiotherapy alone, and patients without adequate follow up were excluded. Although the radiotherapy technique (2-D radiotherapy, 3-D conformal radiotherapy, intensity modulated radiotherapy) has evolved over time, ninety percent of patients received a total dose of 50.4-54 Gy in conjunction with chemotherapy in the form of 5-Fluorouracil (5FU) and Mitomycin C (MMC). Data collection and extraction was approved by the Peter MacCallum Human Research Ethics Committee.

3.3.2 Patient Evaluation

Pertinent demographic and clinical data were extracted. Tumour staging was undertaken in accordance with the American Joint Committee on Cancer (AJCC) 2010 TNM staging system for anal cancer, involving a combination of clinical, radiological and histological means.²³⁸ Response to primary treatment was confirmed in a similar manner. In the earlier years of this cohort, patients were predominantly assessed by clinical (Digital Ano-Rectal (DARE) and nodal basin

examination) and histological means in conjunction with a CT scan for exclusion of distant disease. This compares with more recently, where patients are assessed on clinical and radiological grounds given the improved adjuncts of PET/CT and MRI for regional and distant disease staging. Nonetheless, histological confirmation has remained the mainstay of clarifying equivocal clinical or radiological findings.

Assessment of response was undertaken in the period of 6 weeks to 6 months post the completion of primary therapy. Patients were then followed up clinically every 3 months for the first three years, 6 monthly out to five years, and annually thereafter. DARE and nodal examination was the mainstay of assessment, with adjunctive scans performed at the clinician's discretion once a complete clinical response had been confirmed, to exclude locoregional relapse and distant disease. Time to disease relapse following completion of CRT was recorded from the date of response assessment, with local relapse patients classified as harbouring persistent or recurrent disease. Persistent disease was defined as an incomplete response to primary treatment. Recurrent disease was defined as evidence of tumour relapse at least 6 months following primary therapy and after achieving an initial complete response.

3.3.3 Salvage Surgery

Surgical treatment details were recorded including the specifics of extended resections and the type of flap reconstruction when utilised. Tumour characteristics were recorded in addition to resection margin status. Postop patients were reviewed clinically within the first month, followed by 3 monthly in the first year and 4-6 monthly subsequently. Imaging (PET/CT, MRI) was used to assist assessment and biopsies were taken as deemed appropriate. Time and site(s) of relapse were also recorded.

3.3.4 End Points

The primary endpoints included OS and disease free survival (DFS) measured from the date of diagnosis. Time to Locoregional failure (LRF) and time to re-recurrence were also examined, from the date of response assessment to last follow-up or event. Wound complication rates were examined in the salvage cohort.

3.3.5 Statistical Analysis

Time to event analyses were undertaken for all anal SCC patients and the subsets of LRF and salvage surgery by Kaplan-Meier analysis. This included time to initial local recurrence, re-recurrence post salvage surgery and DFS and OS for all cohorts. The Log Rank test was used to assess the difference between groups. The cumulative incidence method was used to analyse patient, tumour and treatment characteristics. Univariate analysis was performed to identify significant predictors of LRF in the entire cohort and of re-recurrence in the salvage surgery subset. Fisher's exact or Pearson Chi-square tests were performed for categorical data and a Student's t-test for continuous data. Multivariate analysis with a Cox proportional hazards model via stepwise selection was used to identify independent predictive factors for time to event outcomes. A p-value of < 0.05 was accepted to demonstrate statistical significance. Follow-up data was recorded through to December 2016. Statistical analysis was performed using Graphpad Prism 7 (La Jolla California USA) and IBM SPSS Statistics for Windows software (IBM Corp, Version 23 Armonk, New York, USA).

3.4 Results

3.4.1 Patient Characteristics

Four hundred and eighty three ASCC patients were identified, of which 467 received curative intent CRT and were included for analysis. Baseline patient and tumour characteristics and subsequent treatment and outcomes are displayed in Table 3-1 and Figure 3-1. Follow up data was available over a median of 5 years (range 0-21 years). Eighty eight patients were identified to have failed primary therapy, 25 with distant disease, and 63 with LRF. Of those with LRF, 41 underwent local salvage surgery with curative intent. The rarity of HIV positive patients in this cohort reflects the referral patterns in Melbourne, with a dedicated HIV service co-located with another radiation oncology centre.

3.4.2 Primary Treatment Characteristics

Curative intent definitive CRT was administered to a median dose of 54Gy including the inguinal nodal basins for those patients with involved nodes. From the mid 1990's onwards, patients with T2-4N0 disease were managed with prophylactic irradiation (36Gy) to the inguinal region. Twenty-four patients were unable to complete their radiotherapy treatment, with a further 36 having modification of their chemotherapy regimen. Median time to assessment of response post completion of CRT was 73 days (Range 27 – 235). Median survival of the entire cohort was 19 years, with a 5 year OS of 83% (95% CI 78% - 86%) and 5 year DFS of 80% (95% CI 76% - 83%).

Table 3-1 Predictive factors for locoregional failure

Univariate Analysis				
Variables	No Local Recurrence* (n = 406)	Local Recurrence [‡] (n = 61)	Salvage Cohort (n = 41)	p-value (LR [‡] v No LR*)
Sex				
Male	136 (33.7)	25 (39.7)	18 (44%)	0.393
Female	268 (66.3)	38 (60.3)	23 (56%)	
Age (SD)	61.3 (14.4)	61.6 (12.7)	60 (39 - 86)	0.877
HIV positive	11 (3%)	2 (3%)	2 (5%)	0.801
Site				
Margin	61 (15.1)	6 (9.5)	30 (73%)	0.490
Canal	254 (62.9)	42 (66.7)	4 (10%)	
Both	87 (21.5)	15 (23.8)	7 (17%)	
Differentiation				
Well	36 (8.9)	5 (7.9)	5 (12%)	0.779
Moderate	113 (28)	21 (33.3)	10 (24%)	
Poor	118 (29.2)	19 (30.2)	12 (29%)	
Not specified	137 (33.9)	18 (28.6)	14 (34%)	
Adjacent Organ Involvement				
No	357 (88.4)	44 (69.8)	28 (68 %)	<0.001
Yes	47 (11.6)	19 (30.2)	13 (31 %)	
T Stage				
TI	96 (23.9)	3 (4.8)	2 (5%)	<0.001
T2	204 (50.9)	19 (30.2)	13 (32%)	
T3	54 (13.5)	18 (28.6)	9 (22%)	
T4	47 (11.7)	23 (36.5)	17 (41%)	
N Stage				
N0	298 (73.7)	38 (60.3)	30 (73%)	0.005
N1	14 (3.5)	8 (12.7)	5 (12%)	
N2	46 (11.4)	6 (9.5)	3 (7%)	
N3	46 (11.4)	11 (17.5)	3 (7%)	
CRT Treatment				
Complete	357 (88%)	50 (79%)	32 (78%)	0.047
Incomplete	47 (12%)	13 (21%)	9 (22%)	
Cox Proportional Hazards Model				
Independent Predictors for LRF over Time				
Variable	HR	95% CI	p-value	
T stage	3.16	1.53 to 6.53	0.002	

LR – Local Recurrence; T – Tumour, N – Nodal; CRT – Chemoradiotherapy

HR – Hazard Ratio; CI – Confidence Interval

Univariate analysis of the entire cohort identified that T stage, N stage, adjacent organ involvement and incomplete primary treatment (but not treatment interruption) were predictors of LRF. Only T stage reached statistical significance as an independent predictor of local recurrence over time in a cox proportional hazards model, with a HR of 3.15 (95% CI 1.52-6.53, p=0.002) (Table 3-1). The 5-year LRF-free rate for T3/4 patients was 72% (95% CI 62% - 79%) compared with 92% (95% CI 88% - 95%, p<0.001) for T1/2 patients (Figure 3-2).

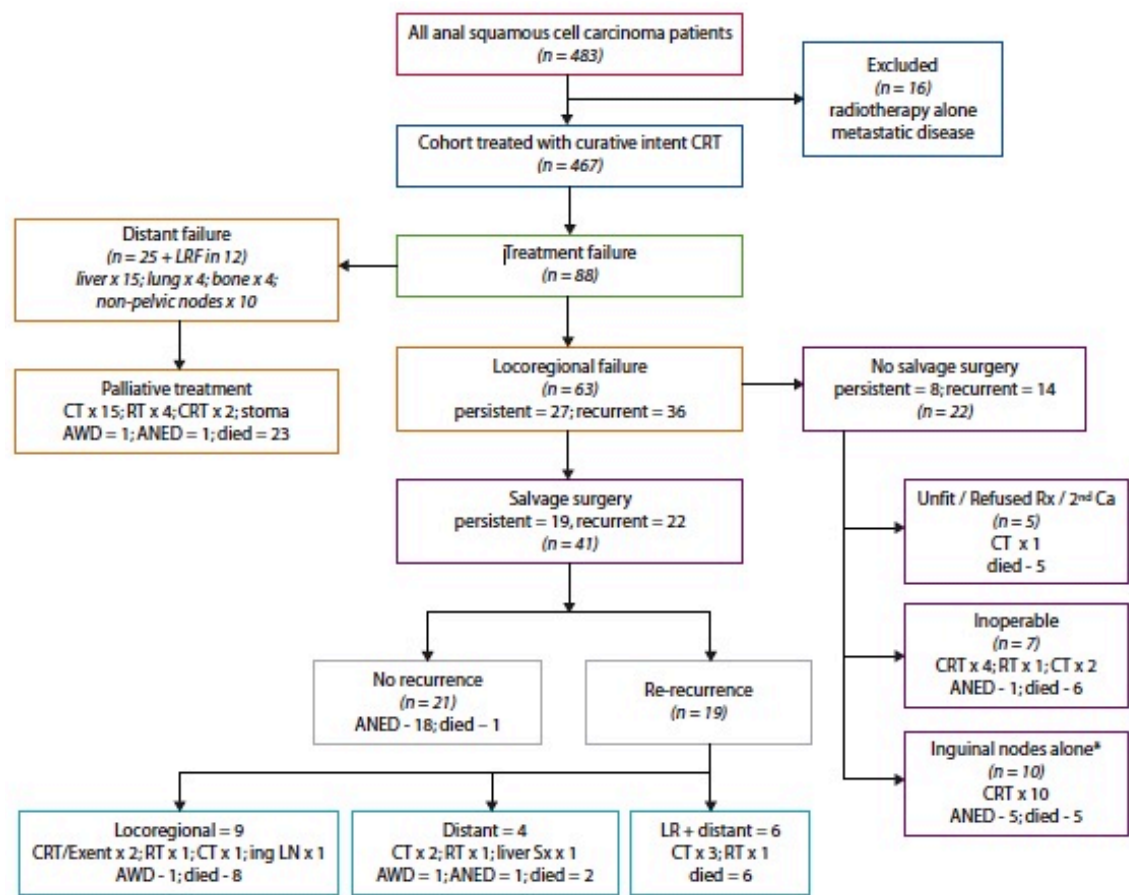


Figure 3-1 Cohort and subsets of ASCC patients, their treatment and subsequent outcomes

CRT – Chemoradiotherapy; LRF – Locoregional Failure; CT – Chemotherapy; RT – Radiotherapy; CRT – Chemoradiotherapy; AWD – Alive with disease; ANED – Alive no evidence of disease; Exent – Exenteration; Ing LN – Inguinal lymphadenectomy; Sx – Surgery

3.4.3 Locoregional Relapse and Salvage Surgery

At a median time to assessment of 83 days (range 32 – 235), 27 patients were confirmed to have persistent disease of which 19 underwent local salvage surgery. Thirty six patients subsequently developed local recurrence at a median duration of 15 months (range 6 - 111) following confirmation of a complete clinical response. Twenty-two of the local recurrence patients subsequently underwent salvage surgery.

The outcome for patients that had persistent or recurrent disease that did not undergo salvage surgery is displayed in Figure 3-1. Reasons for exclusion included inoperable disease, unfitness for surgery, patient refusal and the development of a 2nd incurable cancer. Ten node negative patients from the first decade of the cohort developed inguinal node only relapse, which was managed with CRT (given the basins had not been treated prophylactically).

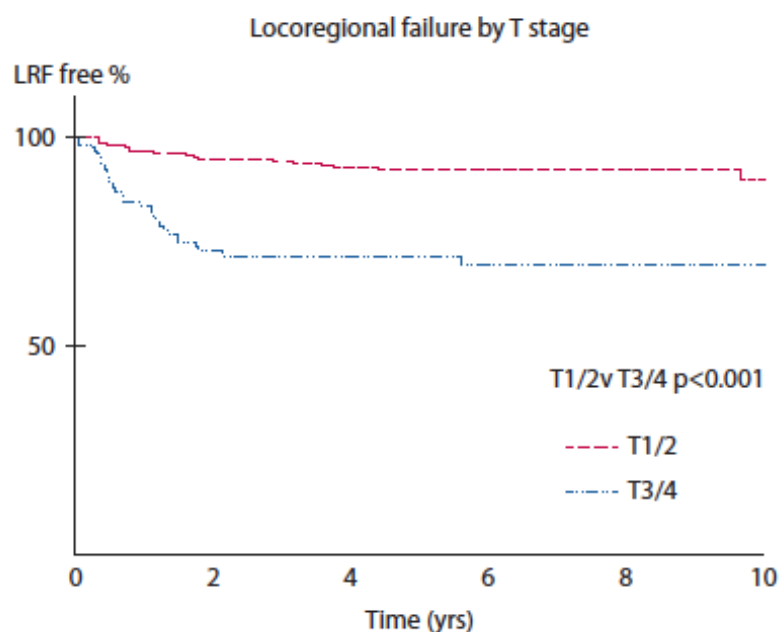


Figure 3-2 Locoregional Failure of the entire cohort stratified by T Stage

(from time of diagnosis)

Of the 41 patients who underwent salvage, 18 were male and 23 female with a median age of 60 years (range 39 - 86). Thirty patients underwent an APR with or without a posterior vaginectomy (8 patients). 11 patients underwent an extended resection or exenteration including sacrectomy (n=6), vaginectomy (n=5), lateral pelvic sidewall dissection (n=4), hysterectomy and salpingo-oophorectomy (n=4), cystoprostatectomy (n=2), cystectomy (n= 1), inguinal node dissection (n=1), vascular resection (n=1) and intra-operative radiotherapy (n=3). The majority of specimens demonstrated poor differentiation (66%) with lymphovascular or perineural invasion identified in 37%. Margins were clear in 71% of resections, and all specimens were found to be node negative. The location of the tumour and the exenterative procedure undertaken did not stratify for an increased likelihood of a positive margin, however the small numbers limit interpretation.

Of the patients undergoing salvage surgery, five year and median overall survival for the persistent cohort was 41% and 2.3 years, compared with 59% and 6.3 years for the recurrent group. This difference failed to reach statistical significance ($p=0.64$). Median time to recurrence did not predict whether patients underwent salvage surgery, with a similar time to failure between patients who subsequently underwent salvage or not (1.4 vs 1.2 years, $p=0.32$). Median time to recurrence was similar for patients who survived or succumb post salvage surgery (1.3 vs 1.8 years, $p=0.3$). Furthermore, persistence did not predict for an involved surgical margin compared with recurrence ($p=0.83$).

A total of 21 patients in our salvage cohort underwent flap repair of the perineum with complications recorded in 8 (38%, vacuum assisted closure n = 6, flap revision n = 2). In the non-flap salvage patients, 7 (35%) suffered wound breakdown with delayed healing. Two patients required a repair of a perineal hernia on follow up, both with an autologous flap. There was no statistically

significant difference identified in wound complications between the two groups ($p=0.84$).

3.4.4 Recurrence and Survival after Salvage Surgery

At a median follow up of 20 months (range 2 – 210 months) and no post-operative mortality, 5-year OS was 51% (95% CI 33% – 67%), DFS 47% (95% CI 31% - 64%) and local DFS 50% (95% CI 32% - 66%)(Figure 3-3). One patient was lost to follow up with no evidence of disease at the 2 month timepoint. There was no statistical difference in OS for the salvage patients based on the operation undertaken.

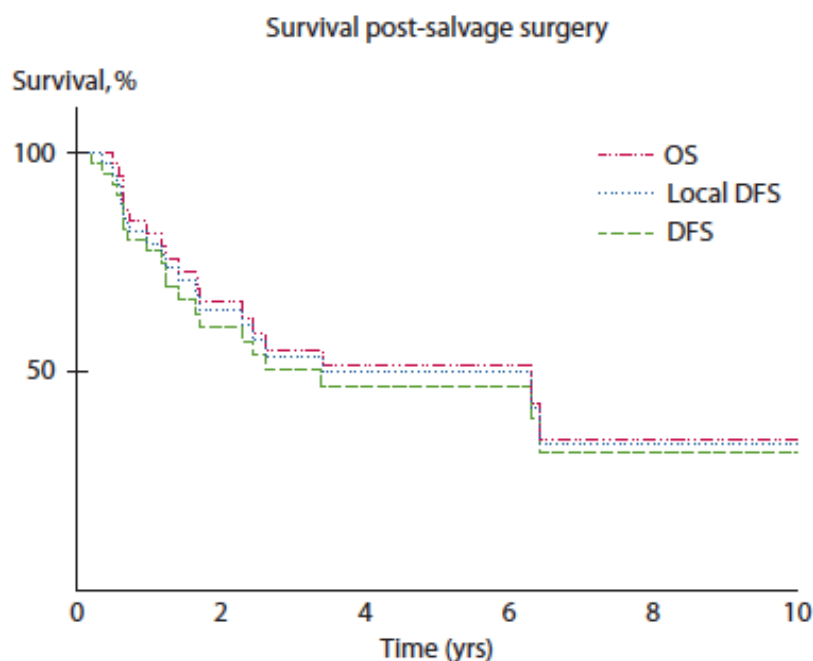


Figure 3-3 Overall (OS), disease free (DFS) and local DFS of the salvage cohort

(from time of surgery)

Nineteen patients developed relapse of disease post salvage surgery, with 16 patients succumbing to their disease and two alive with disease at the conclusion of the study (5 and 15 months follow up). The remaining relapse patient underwent a partial hepatectomy for an isolated liver metastasis, and is currently alive and disease free at 2.5 years follow-up.

The patterns of re-recurrence are detailed in Figure 3-1. This group included all 10 patients with a positive resection margin. Three of these with local recurrence post APR, underwent curative intent pelvic exenterations, two of which received preoperative chemotherapy and re-irradiation in addition to IORT. Despite the radical treatment, all three relapsed and succumbed to their disease.

Table 3-2 Predictive factors for local disease recurrence post salvage surgery

Univariate Analysis			
Variables	No Recurrence (n = 22)	Recurrence (n = 18)	p-value
Sex, n (%)			
Male	7 (30.4)	11 (61.1)	0.064
Female	16 (69.6)	7 (38.9)	
Age, y (SD)	61.9 (13.5)	58.9 (12)	0.462
Differentiation, n (%)			
Well	2 (11)	0 (0)	0.78
Moderate	7 (39)	4 (25)	
Poor	9 (50)	12 (75)	
Missing	4	2	
Tumour Size (SD)	2.59 (1.8)	5.25 (3.48)	0.046
Margin involvement			
R ₀	19 (95)	6 (40)	<0.001
R ₁ /R ₂	1 (5)	9 (60)	
Missing	3	3	
LVI/PNI			
No	9 (64.3)	4 (33.3)	0.238
Yes	5 (35.7)	8 (66.7)	
Missing	9	6	
Cox Proportional Hazards Model			
Independent predictors for re-recurrence post salvage surgery			
Variable	HR	95% CI	p-value
R status	20.4	1.41 to 296	0.027
LVI – Lymphovascular invasion; PNI – Perineural invasion; R ₀ – Clear, R ₁ – Microscopic involvement; R ₂ – Macroscopic involvement; HR – Hazard ratio			

To identify predictive variables, we analysed the relapse rate of the 41 patients who underwent curative intent salvage surgery. Univariate analysis identified tumour size and margin positivity with subsequent cox proportional hazards

testing identifying margin positivity alone as an independent predictor of further recurrence, with a HR of 20.38 (95% CI 1.41 - 296) (Table 3-2). Kaplan Meier analysis demonstrated a significant difference in 5 year OS of 66% (95% CI 41% - 80%) for R₀ vs 7% (95% CI 1% - 32%) for R_{1/2} (Figure 3-4).

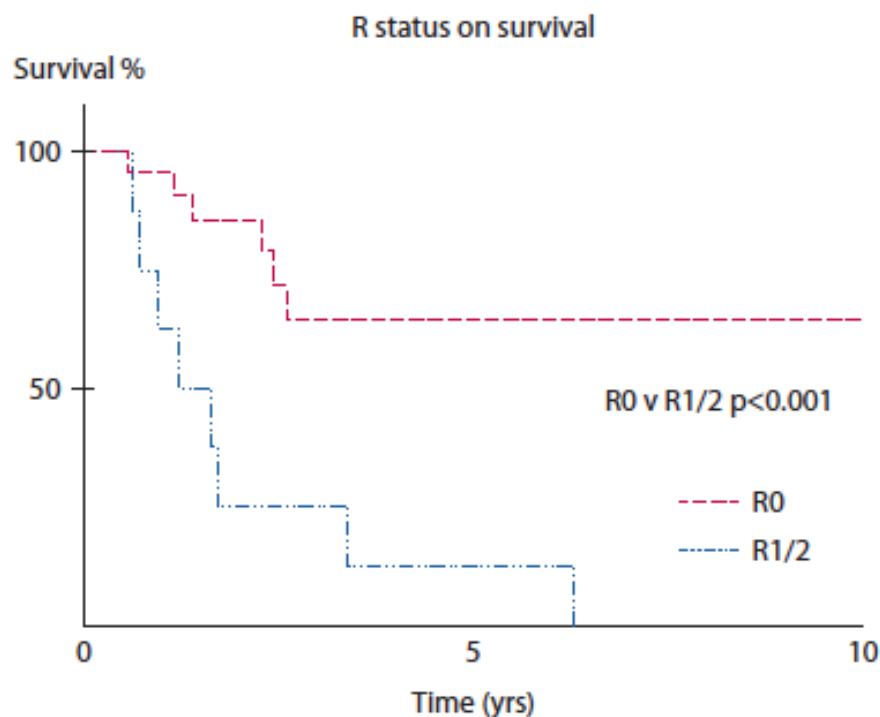


Figure 3-4 Overall survival of the salvage cohort stratified by R status
(from time of surgery)

3.5 Discussion

Definitive CRT offers a high rate of cure in ASCC. The rarity of this tumour and low failure rate after CRT makes research into the treatment of locoregional failure difficult. In our series of 467 patients, 63 patients suffered LRF translating to a 5 year LRF rate of 14% (95% CI 11-19%). This is consistent with RTOG 98-11 results (5-year LRF rate of 20%).¹¹⁰ When patient and tumour factors of the entire pre-treatment cohort were examined for prediction of LRF, only T stage reached

statistical significance on multivariate analysis. This is in contrast to the results of the EORTC trial, which also demonstrated that nodal involvement was an independent predictor of local recurrence over time, irrelevant of the site or extent of nodal disease.⁵⁷ Given that the majority of patients in our cohort are from the mid 1990's onwards, our results may reflect improvements in the delivery of treatment compared with the period of time over which the EORTC study was undertaken.

For the cohort of patients who suffer LRF, the majority can be salvaged with surgery offering an improved OS. In our cohort, 41 patients (65%) underwent curative intent local salvage surgery with a 30-day mortality rate of zero, and a 5-year OS and DFS of 51% and 47% respectively. The OS of our cohort sits midway within the range quoted in the literature of 23-78%.^{112, 118, 124, 125, 234-237} However, this approach often necessitates aggressive and complex surgery. While APR +/- posterior vaginectomy was the most common operation until the early 2000s, more than 50% of our salvage patients underwent an extended resection in the second half of our study period. This has provided the ability to offer a further line of treatment with an aim of cure, to patients who would otherwise face palliation.

Despite salvage surgery offering a curative second line of treatment, the magnitude of this form of surgery necessitates a patient who is medically fit to survive the taxing perioperative period. Consequently, patient selection and preoperative planning are of critical importance, but remain a challenge. On examining predictive factors of relapse post salvage surgery, only resection margin reached statistical significance on multivariate analysis. This is in keeping with the findings of previous studies and further emphasises the importance of achieving an R₀ resection given its impact on outcome.^{112, 124, 125, 233}

Our margin positivity rate of 29% is within the range reported in the literature of 5-41%.^{109, 125, 129, 133, 234}

For those with relapse post salvage surgery, the outcome is dismal, and consequently for most patients, there is only once chance to perform an adequate salvage operation. The most important factors to consider when evaluating patients with LRF for salvage surgery is the exclusion of metastatic disease and determining the likelihood of a margin negative resection. However, significant limitations still exist in identifying resectable patients, despite advances in imaging and exenterative surgery. The increased uptake of CT/PET and MRI as a modality for both pre and post treatment staging, has significantly improved the ability to more accurately determine resectability. Nonetheless, inflammatory change, post radiotherapy scarring and desmoplastic effects of the tumour at differing time points can all confound the ability to accurately predict a negative resection margin. Despite performing exenterative surgery in 11 patients, over a third (4 patients) had a positive margin. Three of these four were treated with intra-operative radiotherapy at the time of the resection, given the suspicion of microscopic involvement or confirmation on frozen section. All 4 patients went on to suffer relapse despite these measures. This is in keeping with previous results, where clear evidence of the benefit of intraoperative radiotherapy is lacking.^{233, 239}

There has been longstanding conjecture on what is the appropriate time period to wait before declaring a patient as having achieved a complete response or suffering persistent disease. This is not dissimilar to determining a post treatment response in rectal SCC or more recently in rectal adenocarcinoma, which has gained much attention since the advent of the novel watch and wait approach.^{240, 241} In our series, median time to assessment was 87 days in the LRF group, with a range of 27 – 235 days post treatment completion. A recent re-

analysis of the ACTII trial identified that a number of ASCC patients do not attain a complete response to primary treatment until the 6-month mark.¹⁰⁵ This delayed regression is of particular importance in ensuring patients are not salvaged prematurely, given the radical surgical approach and significant morbidity that accompanies it. Furthermore, delaying assessment of response in patients without clear progression also provides time for occult metastatic disease to become apparent, further reducing the incidence of futile surgery. Given our median assessment period was significantly less than that currently recommended, it may have increased the incidence of patients with persistent disease undergoing salvage surgery, and spuriously improved the survival of that subset.

Perineal wound healing in a radiotherapy-affected field is often poor, with flaps utilised in more recent times in an attempt to improve healing and reduce morbidity. Rectus abdominis flaps have dominated as the flap of choice at our institution up until 2014. Subsequently, the IGAM (Inferior Gluteal Artery Myocutaneous) flap has become the standard for reconstruction, given it preserves the anterior abdominal wall for stoma siting, and can be performed entirely following the resection in the prone position. However, despite the proposed benefits, of the 21 patients in our salvage cohort that underwent flap closure, 38% (8 patients) suffered a complication requiring a return to theatre, with two requiring a second flap. This is significantly higher than that reported previously, and similar to perineal wound complications in those with primary closure.^{234, 235, 242} However, the majority of these complications occurred early in the study period, with only one complication affecting the last 9 flaps performed, and none since instituting the IGAM flap (n=6).

While the salvage cohort has not achieved the same level of survival as primary therapy, it is likely that failure of chemoradiotherapy reflects a more biologically

aggressive tumour, rendering itself more difficult to salvage by any means. HPV positive tumours are traditionally more radiosensitive, a variable which was not captured in our dataset.¹⁶⁶ It would be of interest to identify whether p16 negative patients have a higher local failure rate and whether this may act as a marker of resistance.

There are several limitations to the conclusions that can be drawn from our results. Despite our institution being a major referral centre for anal cancer treatment, the infrequency of patients precludes a large cohort size, particularly when examining the salvage group. Furthermore, the data collection is retrospective, in some cases incomplete, and over a prolonged time period, during which there have been some changes in practice. These factors have limited the degree to which the data can be statistically analysed and interpretations made. Given the limited number of HIV patients in this cohort, inferences regarding the effectiveness of salvage surgery also cannot be generalised to this population. Nonetheless, this study has provided insight into the success of local salvage surgery in relapsed anal SCC patients, and reaffirmed it as currently the best available line of treatment for this patient group.

In the current era, patients who fail to respond to definitive CRT continue to present a treatment challenge. With the advent of new treatment modalities, there is the possibility of improved treatment options and response. The role of these therapies either in conjunction with neoadjuvant or as a second line of therapy, are however yet to be explored in anal cancer. Consequently, at present, salvage surgery remains the most successful course of treatment, offering long-term survival in over half of those salvaged for local relapse. Appropriate patient selection along with careful pre-operative planning and frequently extended resections are required to achieve a negative resection margin, which is critical to the success of this treatment pathway.

Chapter 4: Profiling the Immune and Genomic Landscape of ASCC

4.1 Introduction

Recent genomic profiling studies have begun to unravel the molecular landscape present in both HPV positive and negative ASCC. This has included the findings of the *PIK3CA* gene as the most frequently mutated in anal cancer and other aberrations in the PI3K pathway being detected in over 60% of ASCC tumours. However, secondary to the rarity of the disease, there has been a total of only just on 150 samples that have undergone sequencing. Consequently, there is still significant research required before a deeper understanding of the genomic underpinnings and pathways to carcinogenesis in ASCC are achieved and robust genomic biomarkers can be identified.

The immune profile of ASCC has also been explored to a lesser degree than the genetic side of this disease with only three publications on this topic. The two most recent of these publications have identified that a heavy TIL infiltrate prognosticates for an improved overall survival rate, including above and beyond the stratification provided by p16.^{111, 202} This however only touches the surface of what has become a rapidly progressing field of research in light of the evolving platforms to facilitate this research and the emergence of immunotherapy as a new pillar of cancer treatment. Consequently, this is an area requiring more attention, particularly with a view of identifying therapeutic biomarkers to facilitate the selection of patients for novel immune therapies.

4.2 Results

4.2.1 Profiling the Immune Landscape of ASCC

4.2.1.1 Patient Cohort

From the existing anal cancer tissue bank at Peter MacCallum Cancer Centre, as outlined in 2.1, a total of 42 patient FFPE samples were obtained for undertaking the immune profiling. This included 35 pre-treatment patient samples and 7 from patients with relapsed disease.

4.2.1.2 Immunohistochemistry and CD8+ Scoring

Sections from the FFPE blocks were cut onto Superfrost™ Plus slides and chromagenic immunohistochemistry undertaken for the CD8 marker as defined in section 2.4 on the cohort of 42 patient samples. The slides were scanned using the VS120 Virtual Slide Scanner in order to create digital images that were subsequently used for quantitation of the CD8+ infiltrate.

CD8+ TIL infiltrates were assessed using a methodology modified from that published by Jerome Galon in establishing the immunoscore and that published by Hu et al in quantifying the infiltrate in ASCC.^{202, 243} Four 250µm² areas were assessed in regions of high TIL infiltrate which included stroma and tumour (Figure 4-1). The median score of the entire group was calculated to determine the cut-off for classifying the CD8 immune infiltrate as high or low. This stratification then allowed for correlation with the treatment response and survival outcome.

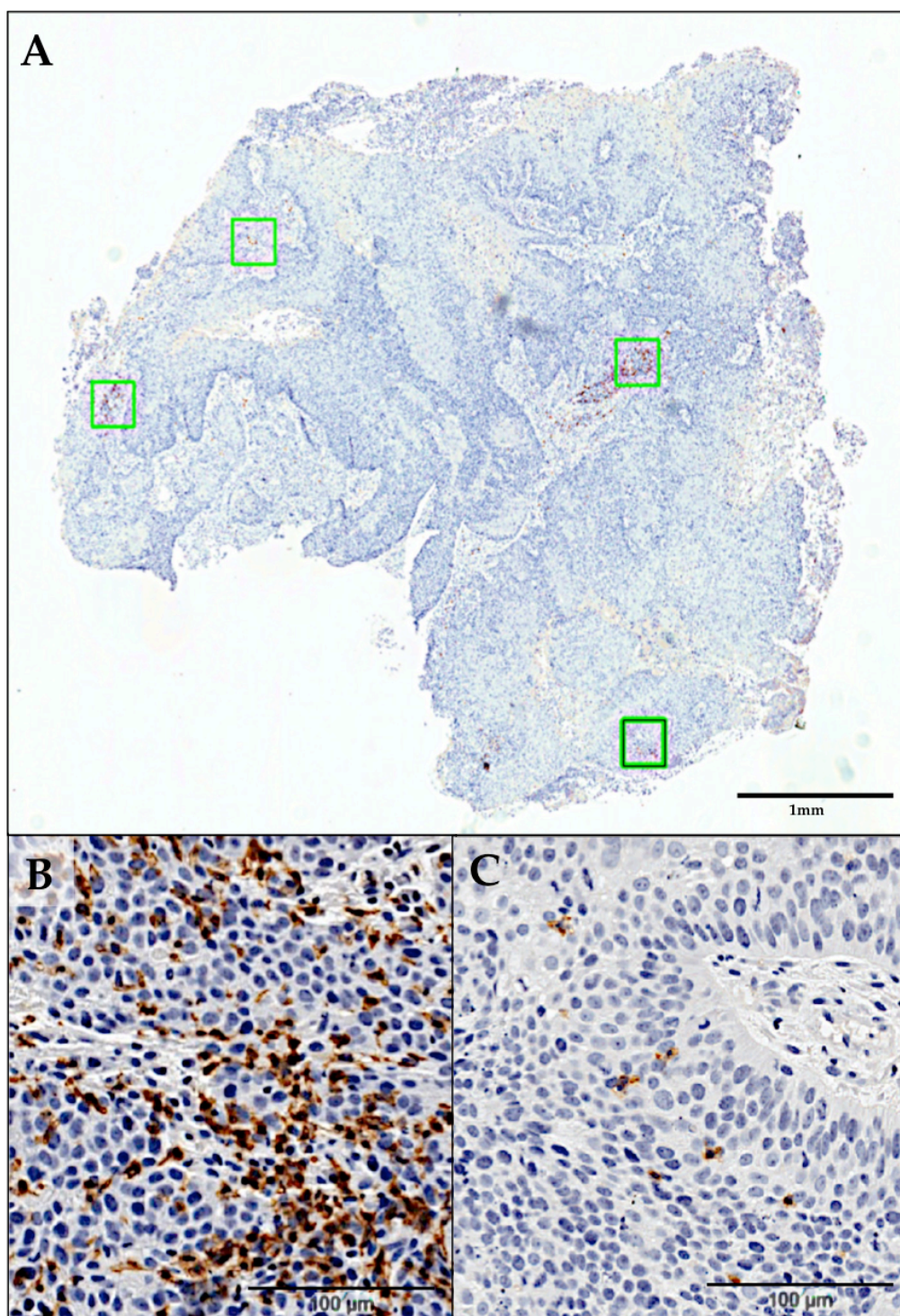


Figure 4-1 Technique for quantifying the CD8+ TIL infiltrate in human ASCC samples

A) Representative tissue biopsy section that underwent chromagenic IHC (brown chromagen) for CD8+ cells. The four green 250µm boxes are placed in regions of highest TIL infiltrate to capture images for subsequent CD8+ TIL quantitation and analysis. (Scale bar 1mm) B) 250µm square region from a patient sample with a high CD8+ TIL infiltrate. C) 250µm square region from the patient sample in image A (green / black box) with a low CD8+ TIL infiltrate (scale bar 100µm,).

4.2.1.3 FIJI Algorithm for Quantitation

The FIJI algorithm utilised for quantifying the TIL infiltrate is listed below. The algorithm was run on each of the four images per patient specimen, following which the average count was calculated to represent the CD8+ infiltrate for that patient.

Algorithm:

```
run("8-bit");
setAutoThreshold("Default");
//run("Threshold...");
//setThreshold(0, 161);
setOption("BlackBackground", true);
run("Convert to Mask");
run("Watershed");
setAutoThreshold("Default");
//run("Threshold...");
//setThreshold(0, 128);
run("Convert to Mask");
run("Convert to Mask");
run("Analyze Particles...", "size=25-500 summarize add");
```

4.2.1.4 Analysis

The median CD8+ of the entire population of patients was calculated as 130 CD8+ cells per 250µm field and used as the cut-off for classifying the samples as harbouring a high or low CD8+ TIL infiltrate. This stratification was then correlated with the treatment response of the pre-treatment patients (Table 4-1 A) and the 5-year overall survival outcomes for those patients with relapsed disease having undergone a salvage operation (Table 4-1 B). The contingency

tables were subjected to a Fisher's exact test with the resultant p-values demonstrated in Table 4-1. This demonstrated that for the pre-treatment cohort, the CD8+ TIL infiltrate reached statistical significance in predicting treatment response. However, in the salvage patient cohort, statistical significance was not reached, likely secondary to the low number of patients within this cohort.

Table 4-1 Predictive and Prognostic Value of the Immune Infiltrate in ASCC

A

CD8 Density	Response - 35 Pre-Rx Patients*	
	Complete	Failure
High	18 (85%)	3 (15%)
Low	5 (36%)	9 (64%)

* $p = 0.004$

B

CD8 Density	Outcome - 7 Salvage Patients [#]	
	Alive NED	Dead
High	1 (100%)	0 (0%)
Low	1 (17%)	5 (83%)

[#] $p = 0.286$; = NED – No Evidence of Disease

A high CD8+ TIL count in pre-treatment samples had a positive predictive value of 85% for determining the complete response to standard treatment in this study cohort. A low CD8+ TIL count however was not as insightful with 36% still obtaining a complete response to treatment in this cohort. This may be explained by the fact that patients are yet to receive chemoradiotherapy, which may increase the immunogenicity of the tumour, subsequently leading to a treatment response. This is further exemplified when examining the relapsed cohort of seven patients, in which 86% had a low CD8+ TIL count.

4.2.1.5 Kaplan-Meier Analysis

A Kaplan-Meier (KM) analysis of 5-year overall survival was performed on 33 of the 35 patients from the pre-treatment cohort, who had robust survival data. The cohort was categorised by their pre-treatment TNM stage, with the two groups defined as stage I/II vs stage III. The KM analysis revealed that there was evidence of stratification, however it failed to reach statistical significance on log-rank testing, due to the low numbers (n=8) in the stage I/II group Figure 4-2 A.

A KM 5-year OS analysis was also performed on the same cohort of 33 patient samples with categorisation based on the high vs low CD8+ immune infiltrate as defined and calculated in 4.2.1.2 and 4.2.1.4. This demonstrated that there was clear stratification with the high CD8+ infiltrate group having statistically significantly improved survival on log-rank testing Figure 4-2 B. Furthermore, when a KM analysis of 5-year OS was undertaken on the Stage III patients alone, the CD8+ immune infiltrate groups have a similar statistically significant prognostic impact. This is of key importance, as it allows further prognostic discrimination above and beyond the TNM systems median 5-year OS of 59% (Figure 1-4) for the locally advanced stage III group.

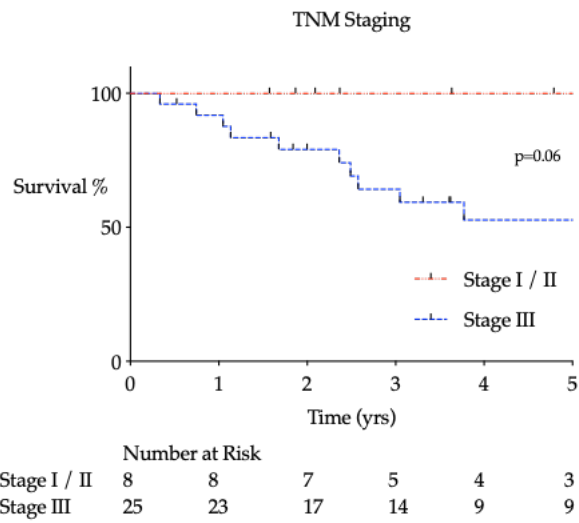
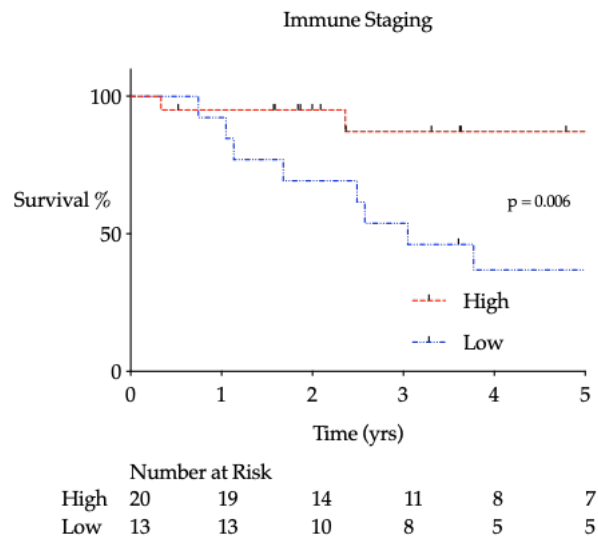
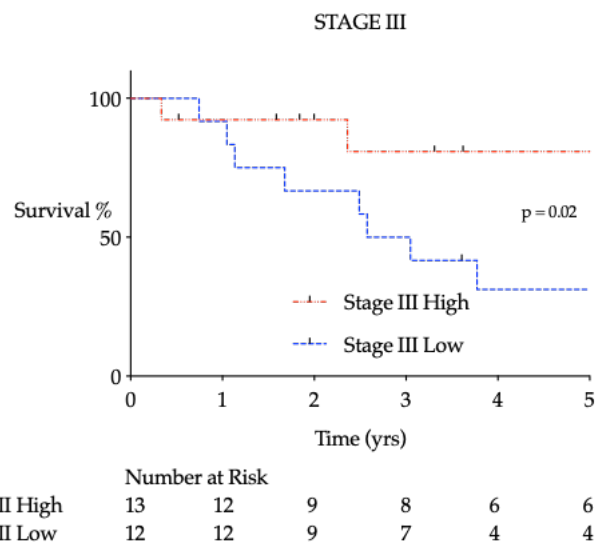
A**B****C**

Figure 4-2 Survival curves based on the pre-treatment TNM staging system and CD8+ infiltrate

A cohort of 33 ASCC patients with 5-year OS data were assessed with a KM survival analysis. A) Categorising patients based on their pre-treatment TNM stage (Stage I/II vs III) reveals stratification for 5-year OS, but without reaching statistical significance ($p=0.06$). B) Categorisation of the same cohort of patients based on their pre-treatment CD8+ immune infiltrate demonstrated statistically significant stratification ($p=0.006$) C) When assessing only those patients with locally advanced Stage III disease, stratification based on the CD8+ immune infiltrate remained statistically viable in predicting 5-year the ($p=0.02$).

4.2.1.6 Flow Cytometry

A total of four patient ASCC samples underwent successful flow cytometry to explore the phenotype of the immune landscape in individual tumours. Three of the samples were from primary pre-treatment patient tumours that subsequently achieved a complete response and one was from a relapsed patient who subsequently succumbed to their disease. A further three samples were processed, but with failure to extract an adequate number of cells to successfully perform staining, acquisition and a statistical analysis of the results. Given the challenge of obtaining an adequate amount of tumour tissue and the failure of three of seven samples, balanced against cost and time, a decision was made to cease this component of the project before reaching the goal of 10 patients.

The initial panel of immune markers was later expanded after the second sample to include a larger number of markers in order to better define the innate and CD45+CD3-CD56- populations (refer 2.2.7.3).

The gating strategy utilised for the phenotyping of the immune infiltrate was based on exploring both the adaptive and innate arms of the anti-tumour immune response. This is exemplified in the results of a single sample demonstrated in Figure 4-3, Figure 4-4, Figure 4-5 and

Figure 4-6. Figure 4-7 demonstrates the isotype gating applied as a negative control to ensure validity in assessing the immune marker expression.

For the adaptive arm of the immune response, gating was performed on CD45+CD3+ cells, assessing CD4+ and CD8+ populations and the PD1 and memory CD45R0 proportion of the CD3+ cells. These findings are represented in Figure 4-8. This revealed that there was a significant proportion of CD45+ cells (median 36.5%) with a smaller proportion of the CD45+ cells also being CD3+, with a median of 16.5%. There was a higher proportion of CD4+ (median 68%) than CD8+ cells (26.5%). When examining the CD4+ population, a median of 21% were found to express both CD25 and FOXP3, consistent with a regulatory CD4+ T cell (Treg).

The majority of the CD3+ cells expressed CD45R0 (median 81.5%) consistent with a memory phenotype. The median expression of PD1 was 14%, which may represent a small number of cells demonstrating evidence of activation and antigen recognition through to an exhausted phenotype. CD107a was used as another marker of recent activation and degranulation in the CD8+ population, however, it was found to be expressed no greater than isotype. Given the processing time before performing staining and fixation, this may not be an accurate representation of the true findings, with CD107a rapidly recycled back from the cytoplasmic membrane.

When assessing the innate arm of the anti-tumour immune response, there was only a small proportion of Natural Killer (NK – Median 2.5%) and Natural Killer T Cells (NKT – Median 1%). Given the small proportion and low number of events across the samples for these two populations, assessing the expression of the activation markers PD1 and CD107a was limited with inherent variability and a high possibility of inaccuracy. The data suggests PD1 expression on a small

proportion of NKT cells without CD107a expression and inversely significant CD107a expression without PD1 expression on NK cells.

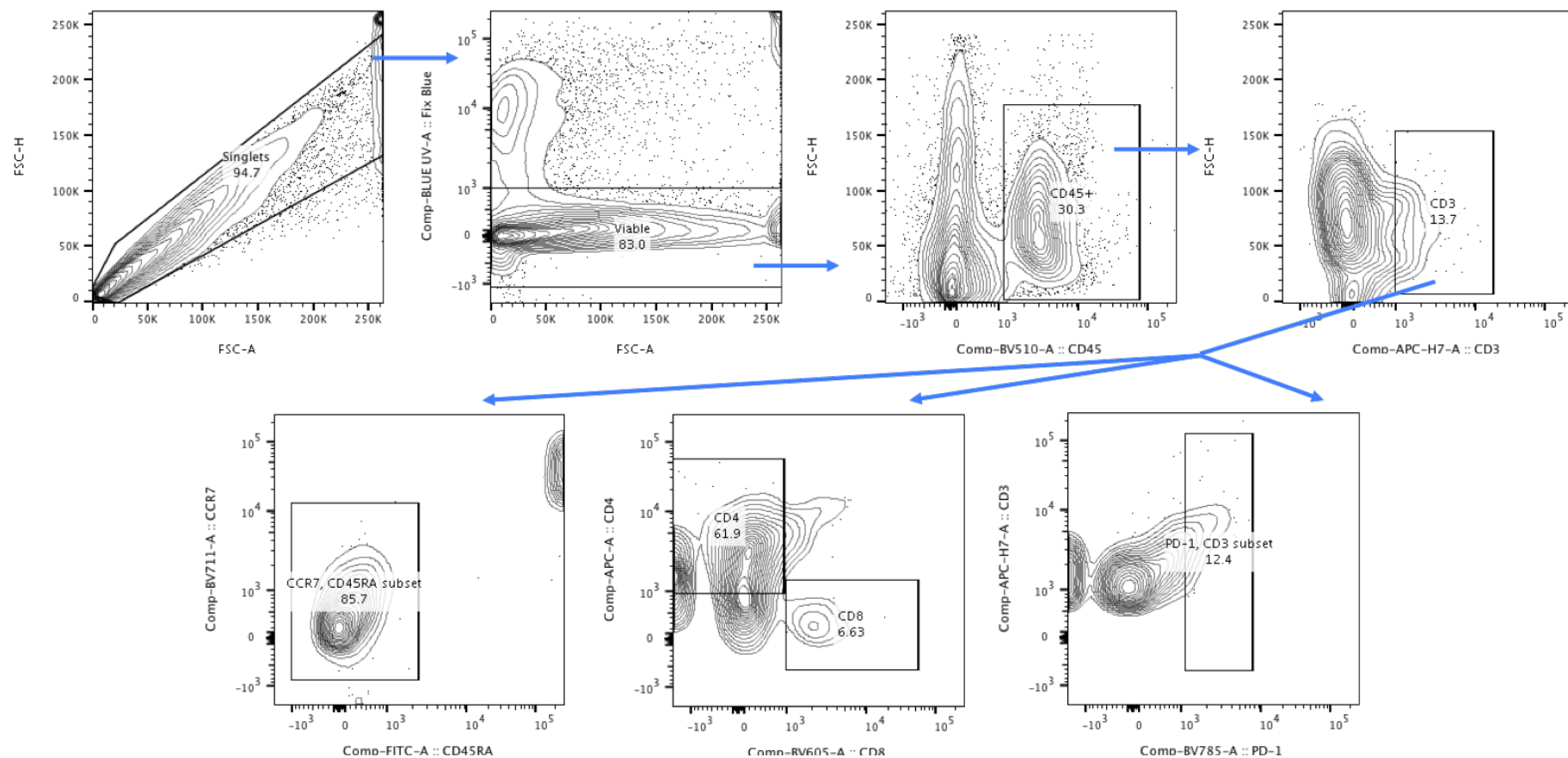


Figure 4-3 Original TIL Phenotype Gating Strategy

Flow Cytometric analysis was undertaken on processed and stained human tumour ASCC cells. Contour plots demonstrate the sequential gating on morphology, viability, CD45+ and CD3+ expression. CD3+ cells were then assessed for CD4+ and CD8+ subsets, PD1+ expression and effector memory phenotype (CD45RA-ve, CCR7-ve). Gates were placed based on isotype controls.

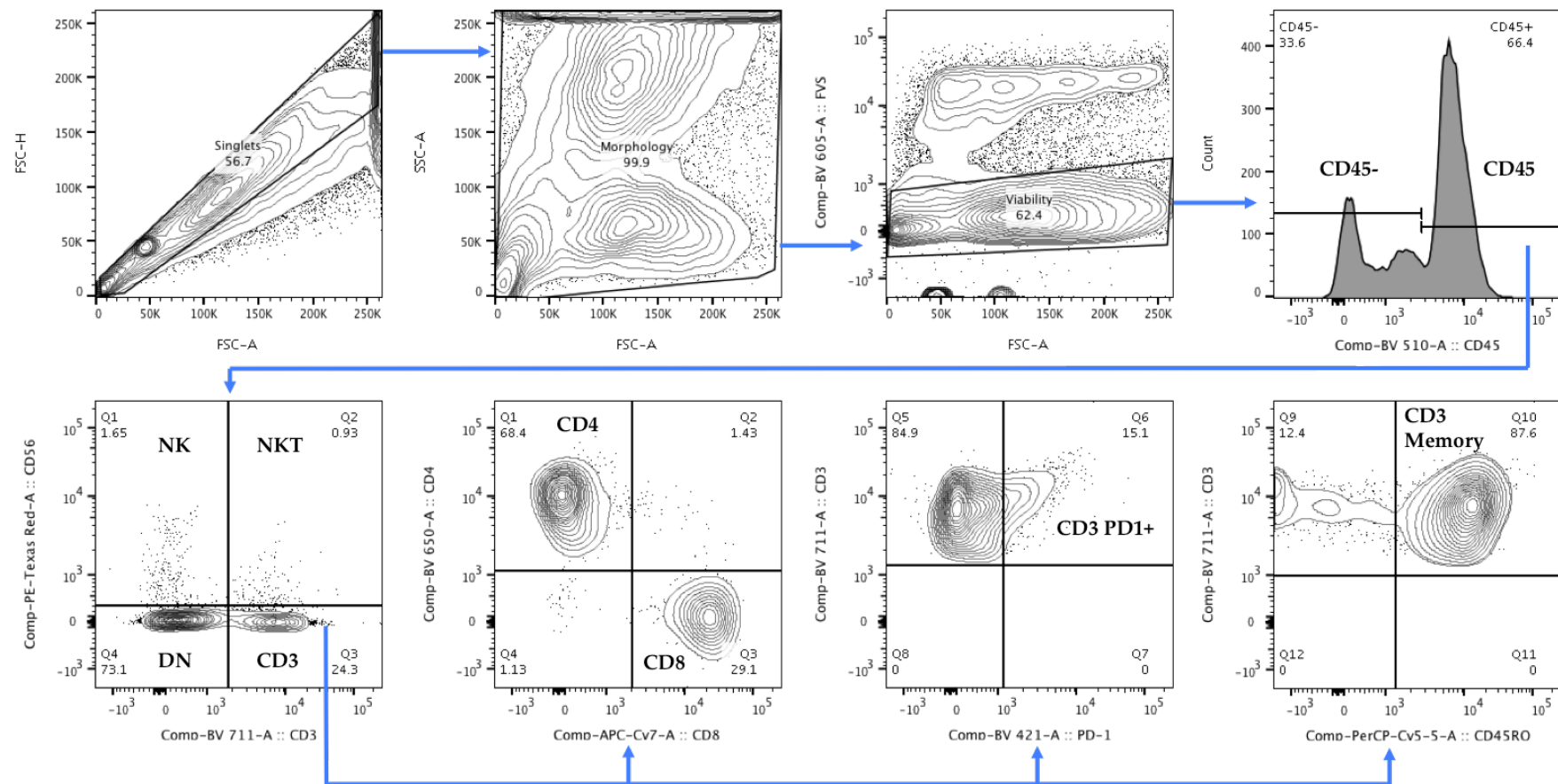


Figure 4-4 Expanded TIL Phenotype - Gating strategy for adaptive immune cells

Flow Cytometric analysis was undertaken on processed and stained human tumour ASCC cells. Contour plots demonstrate the sequential gating on morphology, viability, CD45+ and CD3+ expression. CD3+ cells were then assessed for CD4+ and CD8+ subsets, PD1+ expression and memory phenotype (CD45RO+). Gates were placed based on isotype controls.

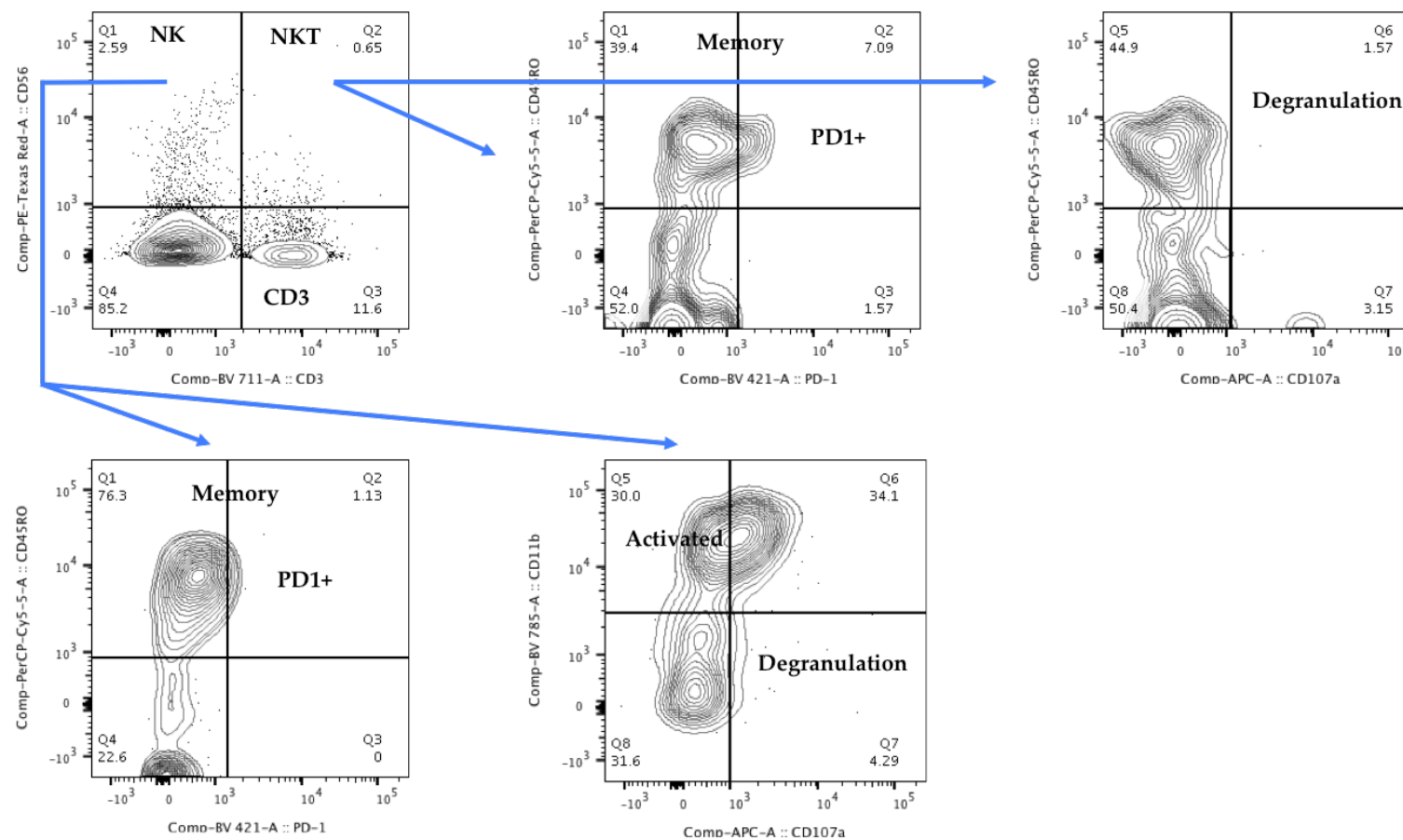


Figure 4-5 Expanded TIL Phenotype – Gating strategy for innate immune cells

Flow Cytometric analysis was undertaken on processed and stained human tumour ASCC cells. Gating was undertaken sequentially as demonstrated in Figure 4-4. Contour plots demonstrate the assessment of NK and NKT cells were then assessed for PD1+, memory phenotype (CD45RO), activation (CD11b) and degranulation (CD107a). Gates were placed based on isotype controls.

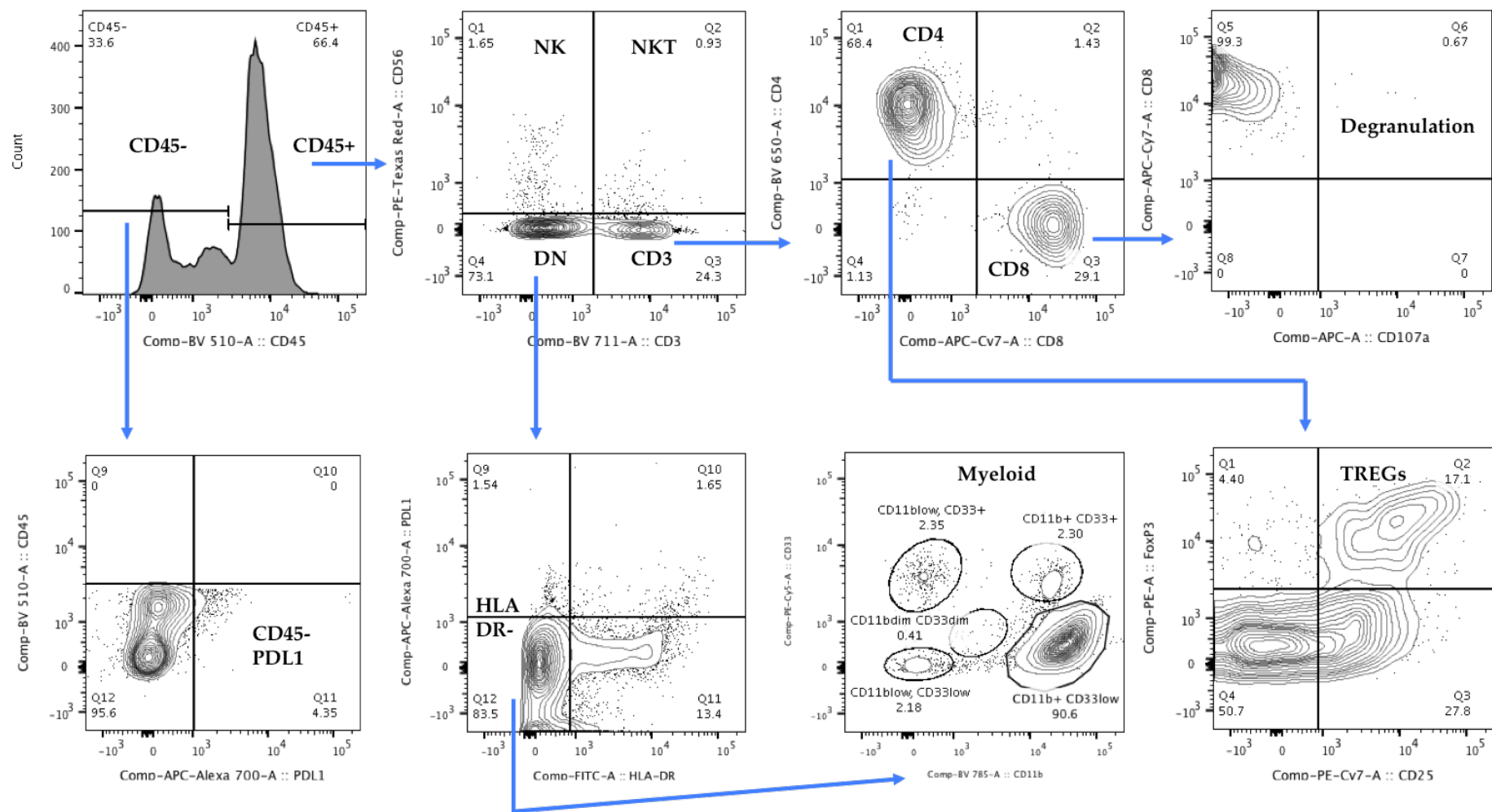


Figure 4-6 Expanded TIL Phenotype – Further gating strategy

Flow Cytometric analysis was undertaken on processed and stained human tumour ASCC cells. Gating was undertaken sequentially as demonstrated in Figure 4-4. Contour plots demonstrate the further assessment of both CD45+ and CD45- cells. The CD3+CD4+ population was assessed for TREG markers (CD25+, FOXP3+) and the CD3+CD8+ cells for degranulation (CD107a). The CD45- tumour cell population was assessed for PDL1 expression. The CD45+CD3-CD56- population was gated for HLA DR- myeloid cells. These were further defined into myeloid sub-populations by assessing CD33 and CD11b expression. Gates were placed based on isotype controls.

The four samples analysed had evidence of a high proportion of CD45+CD3-CD56- cells, with a median of 78.5% of all CD45+ cells. On further characterisation, these cells were identified to be predominantly HLA-DR-ve (median 77%), consistent with a myeloid population. This sub-population demonstrated PDL1 expression on a median of 9% of cells. The markers CD33 and CD11b were used to further delineate the myeloid subsets, with the great majority of cells found to be CD11b+CD33low (median 87%), consistent with myeloid derived suppressor cells (MDSC). Further markers, particularly CD14 and CD15 are required in order to further characterise this population into granulocytic and monocytic MDSCs²⁴⁴. Lastly, in assessing the CD45- population as a rough surrogate of the tumour cell population, the expression of PDL1 was identified on a median of 4.5% of cells.

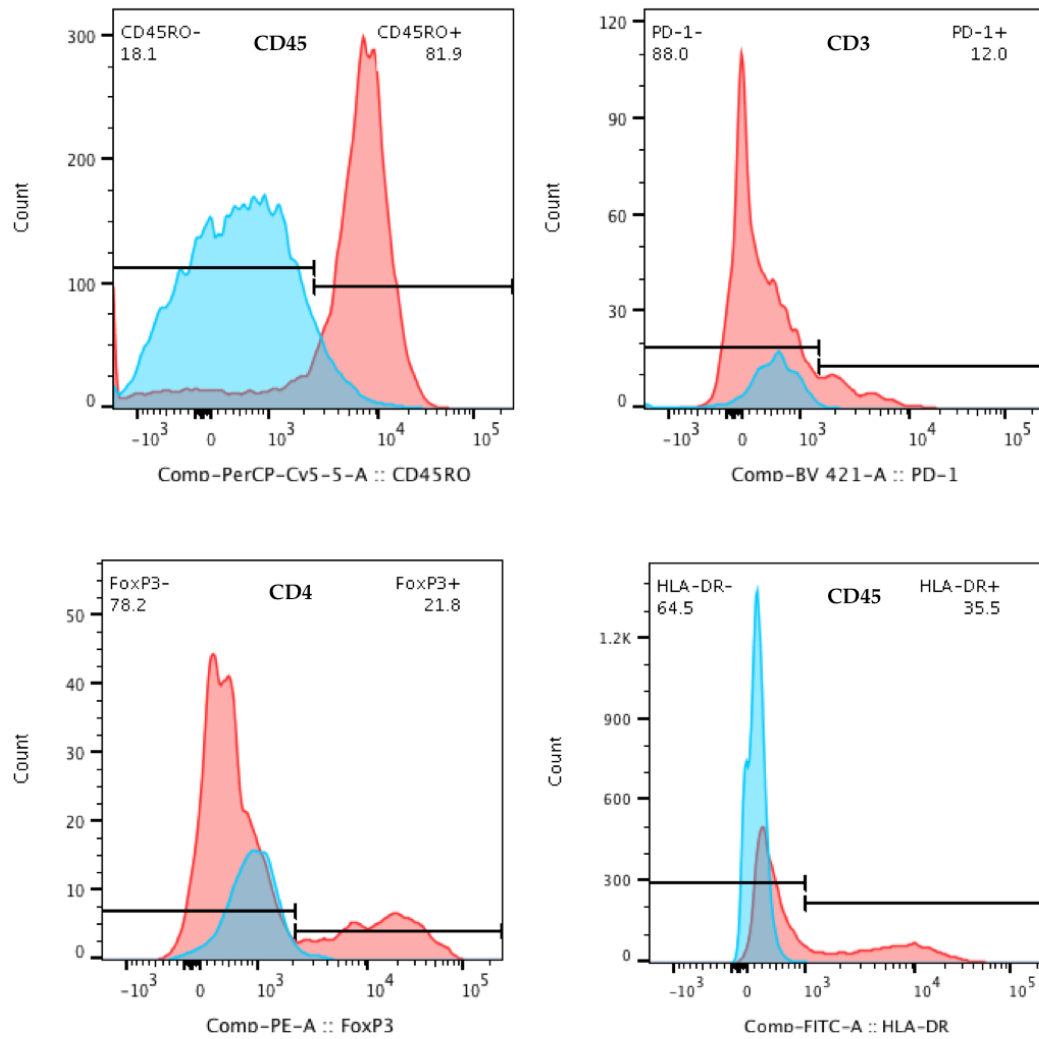


Figure 4-7 Isotype gating for accurate determination of marker expression

Flow Cytometric analysis was undertaken on processed and stained human tumour ASCC cells. By using an isotype control, the determination of an appropriate cut-off for defining a positive cell allowed for accurate gating to be undertaken. In each two-dimensional histogram, the placement of an isotype gate was based on the isotype sample (blue) compared with the human test sample (red).

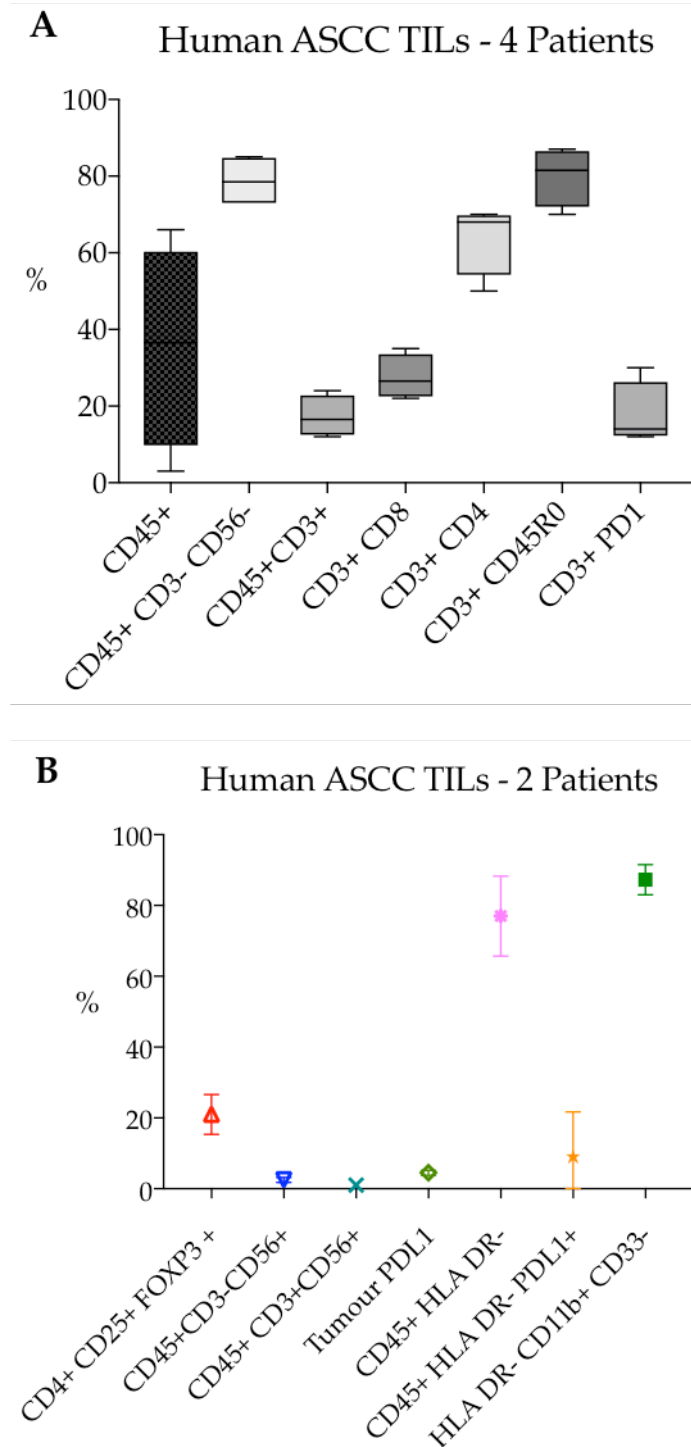


Figure 4-8 TIL phenotype across human ASCC

Box and whisker plots showing the proportion of each cell type based on their immune marker. (A) Plots based on the cumulative results of the four human samples with the markers shared across both flow cytometry panels. (B) Plots based on the two human samples processed with the new panel containing an extended panel of markers. (Boxes represent upper, median and lower quartile, whiskers represent minimum and maximum values).

4.2.2 Profiling the Genomic Landscape of ASCC

At the outset of my PhD, there had been limited exploration of the genomic underpinnings of ASCC. Most published studies had focussed on identifying specific mutations related to less than 5 genes. No study had been published on the use of Next Generation Sequencing (NGS) to better define the frequent genomic events in this disease.

Consequently, addressing this void in knowledge formed part of the initial focus of my PhD by utilising the established anal cancer biobank and database at Peter MacCallum Cancer Centre. By the completion of my PhD, a total of 46 patients had been recruited to the anal cancer tissue-bank and also enrolled in the Cancer 2015 study following approval by the Cancer 2015 Committee.

4.2.2.1 Cancer 2015 Collaboration

A total of 46 patient samples were included in the Cancer 2015 study. Unfortunately, only 22 samples were successfully sequenced by NGS. Of the remaining 24 samples, 21 samples failed DNA Quality Control (QC) and 3 failed NGS. The reason behind the high DNA QC failure rate, was the processing that was undertaken with the samples in the early phase of the anal cancer tissue bank. Samples were originally placed in RNA later to ensure the later downstream application of RNA sequencing was possible. While the RNA later is useful from the point of view of preservation for RNA, the remaining tissue was not rapidly processed for formalin fixing and embedding. Consequently, the remaining tissue was degraded and unable to be utilised.

Table 4-2 Somatic Mutations identified in Cancer 2015 Study

Gene	Frequency	Variant (22 patients)
<i>PIK3CA</i>	45%	E545K (x4), E542K (x2), Q546K (x1) Q546R (x1), M1043V (x1), H554N (x1)
<i>MET</i>	9%	T992I (x2)
<i>ERBB2</i>	4.5%	G1201Q (x1)
<i>PTEN</i>	4.5%	R142Q (x1)
<i>KRAS</i>	4.5%	G12V (x1)
<i>NF1</i>	4.5%	H389R (x1)
<i>TP53</i>	4.5%	A213* + G279T (x1 patient)
<i>CDKN2A</i>	4.5%	R80* (x1)
<i>FGFR2</i>	4.5%	C382R (x1)
<i>Nil</i>	36%	x 8

Of the 22 patients in this cohort, only 3 suffered locoregional failure and subsequently died of their disease. Two of these patients were HPV negative, with one harbouring two TP53 mutations and the other a CDKN2A mutation. The third patient had no mutation detected on the 19-gene cancer panel. Of significance, eight of the patients that successfully underwent sequencing with the 19-gene cancer panel were found to not harbour a mutation. This is consistent with low to moderate mutational burden in most ASCCs. Due to the limited number of patients sequenced and the small number of treatment failures in this cohort, no statistical analysis could be undertaken.

4.2.2.2 RNA Sequencing Data

For 11 of the samples that had failed DNA QC, RNA sequencing had been previously undertaken for a separate project. Through the bioinformatics team at the PMCC, the RNA data was utilised to extract relevant somatic variants as detailed in 2.14.3. The list of variants was then assessed against the cosmic database of curated genes, in order to ascertain the likelihood of a tumour specific somatic variant. The final list with the variant frequency is presented in Table 4-3.

Table 4-3 Somatic mutations identified from RNA sequencing data

Sample	Gene	Variant Frequency
1	<i>EP300</i> , P925S	44%
	<i>MLH1</i> , 117-2A>T	70%
2	<i>MTOR</i> , P116S	25%
3	<i>PIK3CA</i> , E545K	53%
	<i>SMAD4</i> , I525V	35%
5	<i>TP53</i> , 96+1G>T	100%
6	<i>PTEN</i> C71Y	23%
	<i>PTEN</i> 210-1G>T	50%
7	<i>PIK3CA</i> , E542K	31%
	<i>PIK3CA</i> , M1043I	39%
8	<i>PIK3CA</i> , E545K	54%
	<i>TET2</i> , L34P	61%
11	<i>FBXW7</i> , T536A	22%

In conjunction with the cancer 2015 project, this increased the overall cohort of patients with sequencing data up to 33. This increases the current pool of NGS data on ASCC by a magnitude of 20%.

4.3 Discussion

At the commencement of my PhD, the immune and genomic landscape of ASCC was relatively unexplored. There existed a single publication on the prognostic value of the immune infiltrate²⁰¹ and no publication on the use of NGS in this disease. Consequently, a major focus of my PhD was to attempt to delineate the underpinnings of this disease in an effort to better expose predictive and prognostic markers and possible therapeutic targets. At present, there are in excess of three publications on each of these areas, which work to complement the results of this chapter.

While there has been some discordance in the three publications^{111, 201, 202} on the prognostic role of TILs in ASCC, the data presented within this chapter is in agreeance with two of the three published studies. This is in regard to a favourable treatment response and improved overall survival for those patients who demonstrate a high CD8+ or TIL infiltrate. The differing methodologies used in performing each of the studies and furthermore the TIL population examined, are important factors that need to be acknowledged as limitations when attempting to directly compare results.

The significance of the TIL infiltrate in terms of its predictive power, relates to the ability to improve stratification of patients into those who are likely to achieve a complete response to treatment from those who are likely to require further treatment. The importance of such a predictive biomarker is further magnified by the present difficulties and variability in assessing treatment response. The most recent recommendation is that treatment assessment should continue out to 6 months after the completion of chemoradiotherapy, given tumour regression can be ongoing over this period of time.¹⁰⁵ Having the ability to more accurately stratify patients, would create less anxiety for patients and clinicians alike. Most

importantly, for those patients with a low TIL infiltrate that have not achieved a complete response in the early post-treatment phase, a more rapid decision to proceed to salvage surgery would circumvent the cohort of patients who progress to unresectable disease and consequently palliative treatment.

The limitations with the data not only relate to the retrospective nature of the study, but also the limited sample size and skewed dataset of minimal Stage I/II patients. Furthermore, the findings presented need to be further explored in the setting of a trial in order to validate the limited retrospective studies that have been undertaken. Despite the impressive results of the worldwide consensus validation on the use of the immunoscore by Galon et al in colorectal cancer,²⁴⁵ there has been limited uptake into the clinical arena within Australia to date. The logistics of employing such a marker include the cost and time spent performing the test, which often hinder the uptake into clinical practice.

The genomic work undertaken in this chapter is complementary to that already published. *PIK3CA* mutations were identified as the most frequent genomic aberration, consistent with the published works.^{187, 197-200} The other mutations identified in the Cancer 2015 study and RNA sequencing datasets involved genes that have been previously reported in this disease. The specific mutations for several of the genes have themselves however not been reported, increasing the current pool of knowledge on the genomic landscape of this disease.

The genomic data on the combined cohort of 30 patients supports the strong association of up-regulation of the PI3K pathway in ASCC. This insight provides a druggable target on which to focus further research. Furthermore, with the emerging field of ctDNA as a method for identifying treatment failure and relapse in other cancers, awareness of such frequent genomic events in ASCC

may help identify a common marker that could be used with such platforms for surveillance and assessment of treatment response.

There was a significant limitation on interpretation and analysis of the data generated, given firstly the small number of patients on which sequencing was successfully performed and secondly the limited patients who suffered treatment failure. Nonetheless, the dataset will be added to the COSMIC database and cBioPortal with matching clinical data to increase the overall pool of ASCC patients on which genomic data is available by over 20%. As this cohort size continues to increase, it will facilitate the correlation of mutations with treatment response and outcome, thereby further assisting with the identification of predictive and prognostic genomic markers.

4.4 Conclusions

The identification of the prognostic role of CD8⁺ TILS in ASCC and the exploratory findings of the genomic and immune underpinnings in ASCC adds greatly to the limited body of knowledge in this disease.

Chapter 5: Establishment and Characterisation of a Panel of ASCC Cell Lines

5.1 Introduction

Human tumour cell lines are an incredibly useful resource, enabling the study and assessment of cancer cell growth, differentiation, migration and metastasis. This is both generically as well as specifically to the cancer from which they were derived. Furthermore, they act as a means of assessing resistance mechanisms and sensitivity to therapeutic agents both *in vitro* as well as *in vivo* by way of cell line tumour xenografts. They consequently represent an effective means of investigating tumour response to potential novel therapeutics and once bio-banked, can be utilised by researchers simultaneously across the world.

With clinical trials being a challenge in ASCC secondary to the rarity of the disease, preclinical investigation is key to further research and focus on therapies most likely to demonstrate benefit in the clinical setting. A single publication from 2009 reports on an ASCC cell line derived from a lymph node metastasis.²⁰⁶ However, this line has never been banked and the authors are uncontactable, relegating the line from further study. Consequently, there is a clear need for *in vitro* models to address this hiatus.

5.2 Aims

To establish a panel of human ASCC cell lines that will serve as the first *in vitro* preclinical models upon which to further explore the biology of ASCC and assess existing and new therapeutics.

5.3 Results

5.3.1 Prospective Cohort and Cell Line Success

Patients were recruited prospectively to have biopsies taken from primary, loco-regionally relapsed or metastatic ASCC over a period of 2.5 years. Fifteen patient samples were attained over this period of time, with preclinical models established as outlined in Table 5-1, including the successful establishment of five cell lines.

Primary tumours were biopsied with the tissue passaged through Nod-SCID-IL2R γ ^{null} (NSG) mice prior to cell line development for expansion of the human tissue. Relapsed patient tumours were biopsied, and the tissue subsequently processed for immediate cell line development as well as xenograft and tumouroid establishment where adequate tissue was available.

Three of the cell lines were derived from xenograft tissue, where the human tumour samples had been passaged through NSG mice to expand the volume of tissue available for use. The xenograft success rate from implantation of tissue taken from the 15 human samples was 47% (7/15) over this period of time. The greatest limitation with the success of engraftment, was the amount of primary tissue taken at the time of biopsy and therefore available for implantation into multiple NSG mice. The take rate has also been demonstrated to be higher in other tumour types when it is from a tumour at an advanced stage.²⁴⁶

Without successful passage through mice to expand the volume of tissue, the establishment of cell lines from primary human tumours was 0% (0/6). Consequently, where no xenograft was established for primary human tumours, a reasonable attempt at cell line establishment was not possible. The overall success rate of establishing cell lines from xenograft tumours was 60% (3/5). One

of the xenograft tumours from which a line could not be established, was derived from a relapsed patient, with failure to establish the line directly from adequate human tissue or following xenografting. Primary tumours however were successfully established as another *in vitro* model of this patient's disease.

Of the five relapsed human samples, four had adequate tissue for a direct attempt at establishing a line, with two successes (50%, 2/4). The fifth sample was inadequate in size for successful xenografting, and another failed to engraft despite adequate tissue. The remaining sample derived from a relapsed patient was discussed above.

Table 5-1 Success of establishing pre-clinical models from human ASCC tissue

Patient Sample	Xenograft	Cell Line	1 ^o Tumours
LH462 (P2)	Yes	Yes	N/A
MW753 (P3)	Yes	Yes	N/A
SJ770	No	N/A	N/A
MS512	No	N/A	N/A
KM149 (P1)	Yes	Yes	N/A
GB370	No	N/A	N/A
CS909	No	N/A	N/A
KB019	Yes	No	No
IM528	No	N/A	No
LS654 (LRF1)	Yes	Yes	Yes
DF437	No	N/A	No
<u>MC861 (LRF2)</u>	Yes	Yes	Yes
<u>CJ163</u>	No	No	No
JC301	No	N/A	No
JB168	Yes	No	Yes

N/A – Not Applicable; Bold – Relapsed; () – Suffix Name of Cell Line; HPV -ve underline

5.3.2 Patients and Source of Cell Lines

The patient demographics, clinical and tumour features and treatment and response details of the patients from which the cell lines were derived are outlined in Table 5-2. Patient age ranged from 45 – 58yo, which sits slightly below the expected range of 55-64yo.² There were 3 females and 2 males, consistent with the disease having a female predominance. In terms of risk factors, four of the five patients were active or ex-smokers, with one patient having sexual practices involving anoreceptive intercourse (MSM). No patient was HIV positive or immunosuppressed. All tumours were anal canal in origin. Four were p16 positive on IHC with those four being subtyped for an extensive panel of HPV types and identified to harbour only HPV 16 DNA.

The nomenclature of the lines was with a prefix denoting the organisation and histological type, PMASCC (Peter MacCallum Anal Squamous Cell Carcinoma), and the suffix denoting the treatment state of the tumour, P (Primary, treatment naïve) vs LRF (LocoRegional Failure, post primary treatment). After the first mention of the complete name, they are subsequently referred to by the unique suffix.

All primary tumours were found to display either moderate to poor differentiation with some evidence of focal keratinisation in areas of moderate differentiation on H & E staining. Adjacent to the invasive component of PMASCC-P1 was evidence of HSIL. Only one of the primary lines (PMASCC-P3) displayed evidence of LVI (without PNI), while both relapsed tumours had evidence of LVI and PNI. The stage of the patients at diagnosis ranged from Stage I – IIIC based on the most recent AJCC 8th edition TNM classification (Table 1-2).

Table 5-2 Cell Line Origin: Demographics, Tumour Characteristics, Treatment & Response

PM ASCC	Age / Sex Risk Factors	Location	HPV Type	Differentiation	LVI / PNI	Stage	Origin	Treatment	Response
P1	58 M MSM (HIV -) Ex-smoker	Canal	+ / 16	Moderate HSIL adjacent	Nil	T2N0 (IIA)	Primary	5FU / MMC IMRT - 54Gy / 30#	Complete Alive 3.5y
P2	45 F Non-smoker	Canal	+ / 16	Moderate Frequent mitoses Focal keratinisation	Nil	T2N3 (IIIA)	Primary	5FU / MMC IMRT - 54Gy / 30#	Complete Alive 5y
LRF1	46 M Smoker	Canal	+ / 16	Moderate to Poor Frequent mitoses Focal keratinisation	LVI / PNI	T3N3 (IIIC)	Local Relapse	5FU / MMC IMRT – 54Gy / 30# <i>Palliative Chemo Carboplatin/Paclitaxel</i>	Locoregional + Distant Failure <i>Died 20m</i>
P3	46 F Ex-smoker	Canal	+ / 16	Poor Basaloid, SCCis	LVI	T3N3 (IIIC)	Primary	5FU / MMC IMRT - 54Gy / 30#	Complete Alive 3.5y
LRF2	51 F Ex-smoker	Canal	-	Moderate to Poor Focal keratinisation	LVI ++ / PNI	T1N0 (I)	Local Relapse	Incidental Excision Capecitabine / MMC VMAT – 54Gy / 30# <i>Salvage APR</i>	Locoregional Failure <i>Died 2y</i>

M - Male, F – Female, # - Fractions, *Relapsed treatment & outcome – Italics*; SCCis – Squamous Cell Carcinoma in situ surrounding invasive

There were no significant differences in the patient demographics, tumour characteristics or treatment and response details between those samples from which cell lines were derived and those which failed.

All patients had received definitive CRTx in the form of 5FU, MMC and 54Gy as primary treatment for their localised disease at initial presentation. The three patients from whom the primary samples were derived all achieved a complete response to primary treatment (Figure 5-1), with no relapse at a minimum of 3.5 years of follow up. The two patients from whom the relapsed samples were obtained, both suffered from persistent disease following the completion of primary therapy.

PMASCC-LRF1 initially endured locoregional only disease following the completion of primary treatment, and was considered for salvage resection, however, vascular involvement in the groin prevented this. Within months he subsequently developed metastatic disease to the liver, with both sites of disease failing to respond to palliative chemotherapy (5FU, cisplatin). LRF2 had an incidental T1N0 HPV negative tumour identified after haemorrhoidectomy. Following discussion at the multidisciplinary meeting, full primary treatment with CRTx was recommended and completed. On surveillance post-completion of treatment, abnormal uptake was identified with subsequent biopsy confirming persistence of disease. She subsequently underwent a salvage resection after re-staging excluded distant disease. This achieved an R0 resection with evidence of nodal involvement. She developed unresectable bilateral inguinal node relapse within 6 months of her salvage operation, which greatly affected her functional status and precluded her from further treatment. Both patients with relapsed disease had succumbed to their disease within 2 years of the initial diagnosis of their cancer.

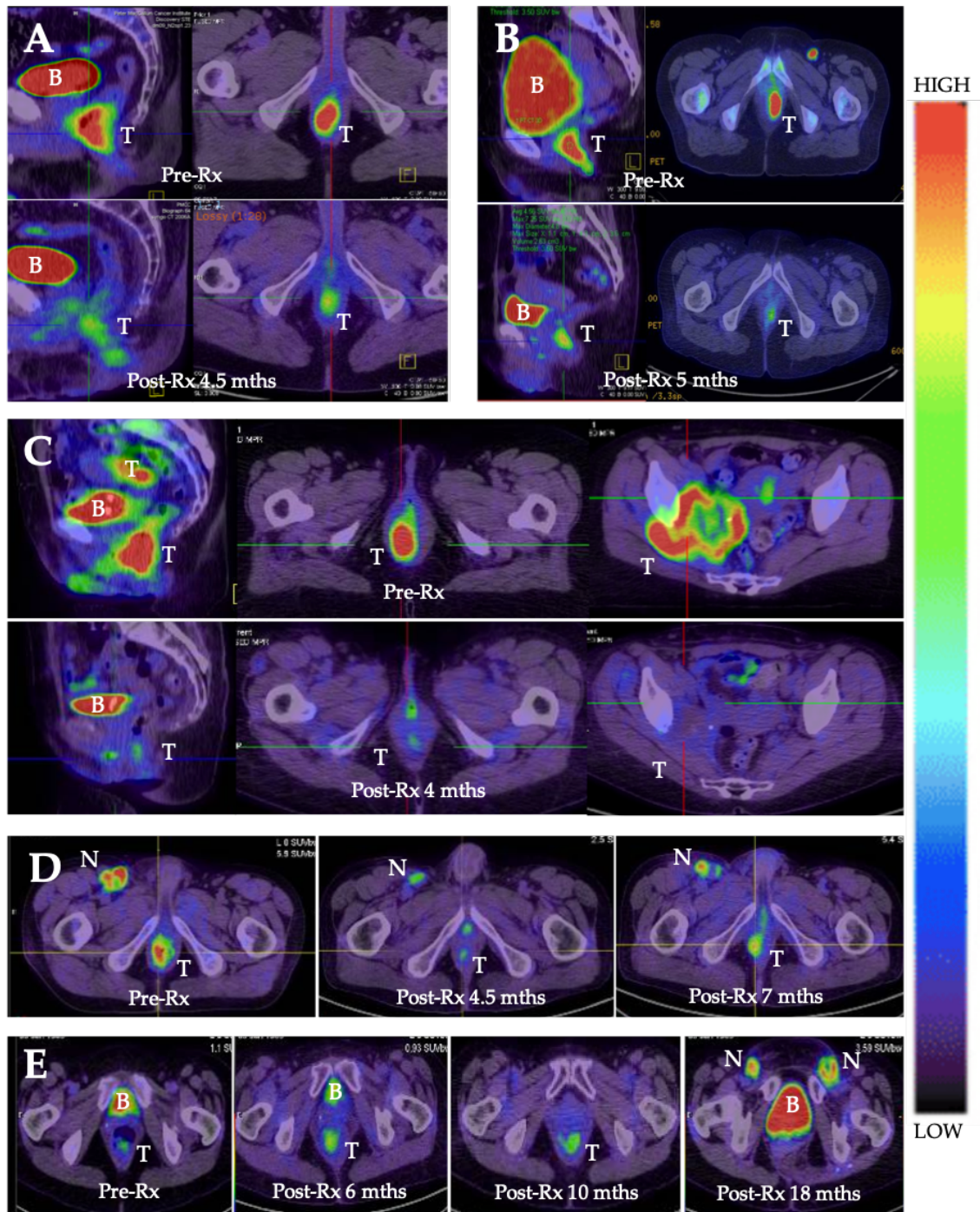


Figure 5-1 18-FDG-PET Imaging pre and post treatment for the five ASCC cell line patients

The images demonstrate the activity of the tumour (T) or involved nodes (N) heralded by the colour spectrum as a marker of high (tumour) or low-intensity (normal tissue) using the scale bar at the right of the image. The bladder (B) activity indicates radio-urine which is normal excretion of the 18-FDG tracer. (A) PMASCC-P1 – Sagittal and axial images demonstrating a near complete metabolic response in the primary tumour on post-treatment (Post-Rx) compared with pre-treatment (pre-Rx) scans, confirmed on biopsy and with ongoing surveillance at 3 years. B) PMASCC-P3 – Sagittal and axial images demonstrating a partial metabolic response on post treatment scans, confirmed to be a complete response with follow-up imaging and surveillance. C) PMASCC-P2 – Primary tumour associated with a large 8cm right nodal mass extending through the sciatic notch, both of which demonstrated a complete metabolic response on post treatment scans. D) PMASCC-LRF1 – Primary and inguinal nodal disease demonstrated an incomplete metabolic response on the initial post treatment scan, followed by disease progression at both sites on the follow up scan, precluding resection due to large vessel involvement E) PMASCC-LRF2 – Incidental primary on haemorrhoidectomy without evidence of residual or nodal disease on the pre-treatment scan. Post treatment scan demonstrated mild anal canal uptake which persisted on follow up at 10 months, was confirmed as persistent disease and resulted in an APR. Post-resection scan demonstrated development of new inguinal nodal disease.

5.3.3 Establishment of Cell Lines

5.3.3.1 Explant Development

All five lines were established via the explant technique of cell line development (Figure 5-2 and Figure 5-3). The three primary ASCC cell lines were established after a minimum of two passages through NSG mice prior to explantation. Their evolution in culture was over a much longer period of time than with the development of the two relapsed ASCC cell lines. The three primary lines were in culture for passages one through five for a minimum of 6 months. In contrast, the relapsed lines were much more rapid in their establishment and evolution into a robustly passaged line.

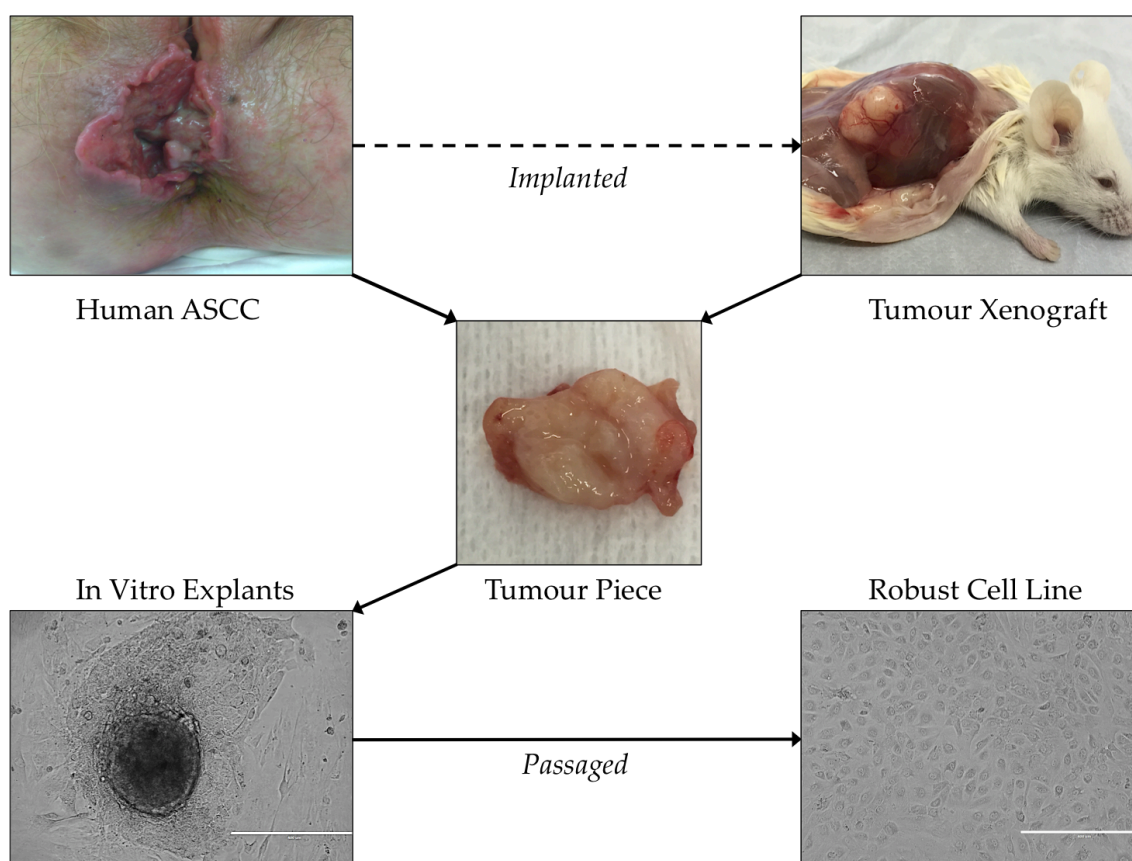


Figure 5-2 Method of Cell Line Establishment

ASCC cell lines were established by a tumour explantation method with serial passaging to develop a robust line. The tumour tissue was processed for explantation either directly from the human biopsies in the case of relapsed disease or following passages through NSG mice in the case of primary tumours. (Objective x10, scale bar 400µm)

5.3.3.2 Cryopreservation

Cryopreservation was undertaken routinely with the newly developed human and mouse ASCC lines out to passage thirty. In the early phases of cell line establishment, the cell number frozen down was dictated by the need to ensure successful passaging of the fragile line, and the size of the tissue culture plate or flask from which they were lifted. Consequently, the cell number was often limited and generally restricted to a single cryovial. Once the lines were robust, adequate cell numbers were frozen down per cryovial to re-populate a T175 flask on resurrection with a 30% loss of viability margin (P1-P3: 5×10^6 , LRF1: 1×10^6 , LRF2: 2×10^6)

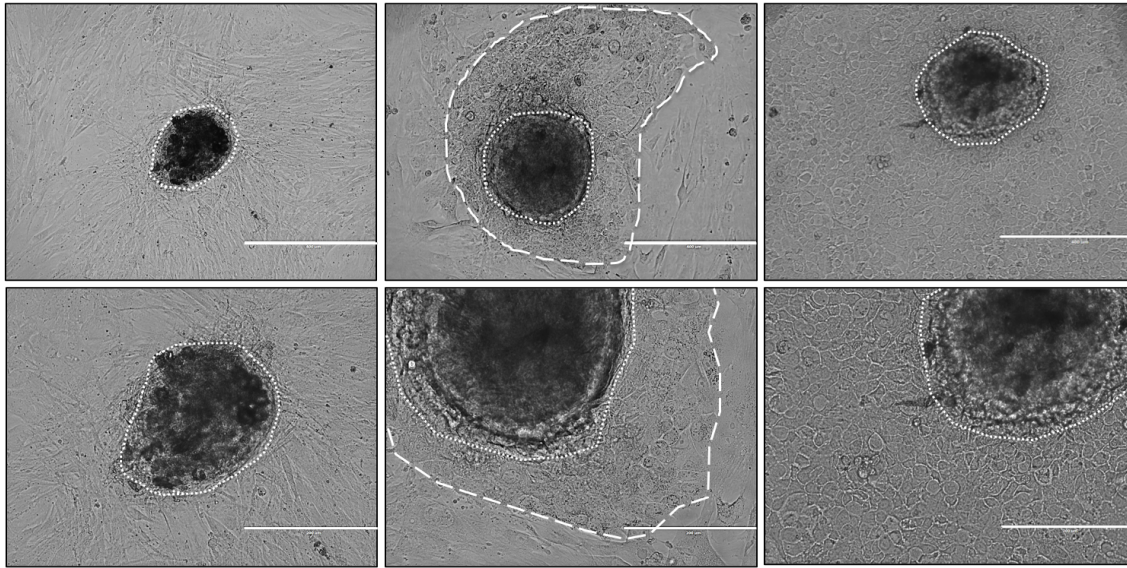


Figure 5-3 Process of Tumour Explantation

Brightfield photomicrographs from left to right demonstrate initial tumour explant (indicated by dotted line) adherence with fibroblast outgrowth, followed by squamous cell expansion (indicated by dashed line) onto the fibroblast bed over a period of 3 weeks (left images – day 7, middle images – day 14, right images – day 21). (upper row – x 10 image, scale bar 400µm, lower row – x 20 image, scale bar 200µm)

5.3.3.3 Fibroblast Elimination

One of the greatest challenges following the establishment of the three primary ASCC lines was the elimination of contaminating fibroblasts. Eradication of mouse fibroblasts was required following explanation for patient derived tumour xenografts harvested from NSG mice. Following the establishment of a robustly passaged cell line, multiple techniques were employed to assist with fibroblast elimination.

5.3.3.4 Differential lift

Media was removed from the flask and the adherent cells washed with PBS -/- for a period of 3 mins. The PBS -/- was then aspirated and an ample amount of TrypLE Express pre-warmed to 37°C was added to the flask ensuring adequate cell coverage. The fibroblast cells subsequently go through a process of

retraction, aggregation and rounding followed by displacement from the flask surface in response to the TrypLE Express at an earlier time point than occurs with the epithelial cells. This effect on the cells was closely monitored over the ensuing minutes after TrypLE Express addition, by observation under an inverted microscope. Gentle taping of the flask assists with the preferential lifting of the fibroblasts while leaving the epithelial cells adhered. Once lifted, the TrypLE Express was aspirated, following which the flask surface was washed three times with PBS +/- and aspirated. This process was repeated at serial time-points over weeks to months, both in isolation when the fibroblasts are dense or outcompeting the epithelial cells, and also preceding each epithelial cell passage.

If the TrypLE Express was found to have too rapid of an effect on the epithelial cells preventing a competitive advantage being offered over the fibroblasts, PBS +/- was used in place of it, necessitating a longer exposure period, often 10-20 minutes before fibroblast displacement was effected.

5.3.3.5 Foetal Calf Serum Reduction

Explant establishment requires 20% FCS which is maintained in the early phases of cell line evolution. Once the epithelial component is established, reduction in the FCS concentration is essential to aid fibroblast eradication. This is undertaken sequentially, with gradual exposure to 15% followed by 10% over a period of 6 weeks to 2 months.

5.3.3.6 L-glutamine Free RPMI-1640

A further essential step in the eradication of mouse fibroblasts involves exposure to L-glutamine free RPMI-1640. The cells are exposed to this media with 10% FCS for a period of 48-72 hours, following which a differential lift is undertaken and standard RPMI-1640 + 10% FCS replaced. This process can be repeated,

multiple times if necessary, however, the effect on the epithelial cells must be closely monitored and the duration, frequency altered depending on the effects.

5.3.3.7 Critical Factors in Cell Line development

Minimising mechanical disruption to the explants in the early phases of plate adherence is critical to subsequent explant outgrowth. Housing the plate in a low traffic incubator is important in this regard. This also mandates a delay in the first media change until day 4. Only a half media change should be undertaken at this stage to further reduce media forces on the fragile attachment of the explant, and this is subsequently undertaken three times a week.

In the early phases of cell line passaging, a ROCK inhibitor is of great importance, preventing anoikis, which is the induction of apoptosis due to the loss of adherence with extracellular matrix or neighbouring cells. This is a major factor in cell attrition and failure of passaging success particularly with squamous lines derived from primary tumours without metastasis. This is based on the low metastatic potential of a cancer where the cells have not lost their susceptibility to anoikis. In line with this, passaging at high densities is an essential step in the early phases of cell line evolution to combat the low plating efficiency of squamous cell lines. While a large number of cells may initially adhere reflecting attachment efficiency, most will rapidly migrate together as small clusters to ensure adequate cell-cell adhesion. Those that fail to migrate or become isolated undergo anoikis. Consequently, the colony forming efficiency of these lines is often low, further justifying the need for higher passage number.

A high FCS concentration is another critical factor in the early phases of cell line establishment and passaging, offering a favourable environment for the growth of the cancer cells. The outgrowth of the epithelial component of the cell line

however can be slow, requiring media changes often for months prior to the initial first passage.

5.3.4 *Mycoplasma Assessment*

Mycoplasma contamination was assessed and confirmed as absent across the panel of established cell lines by PCR, as demonstrated in Figure 5-4.

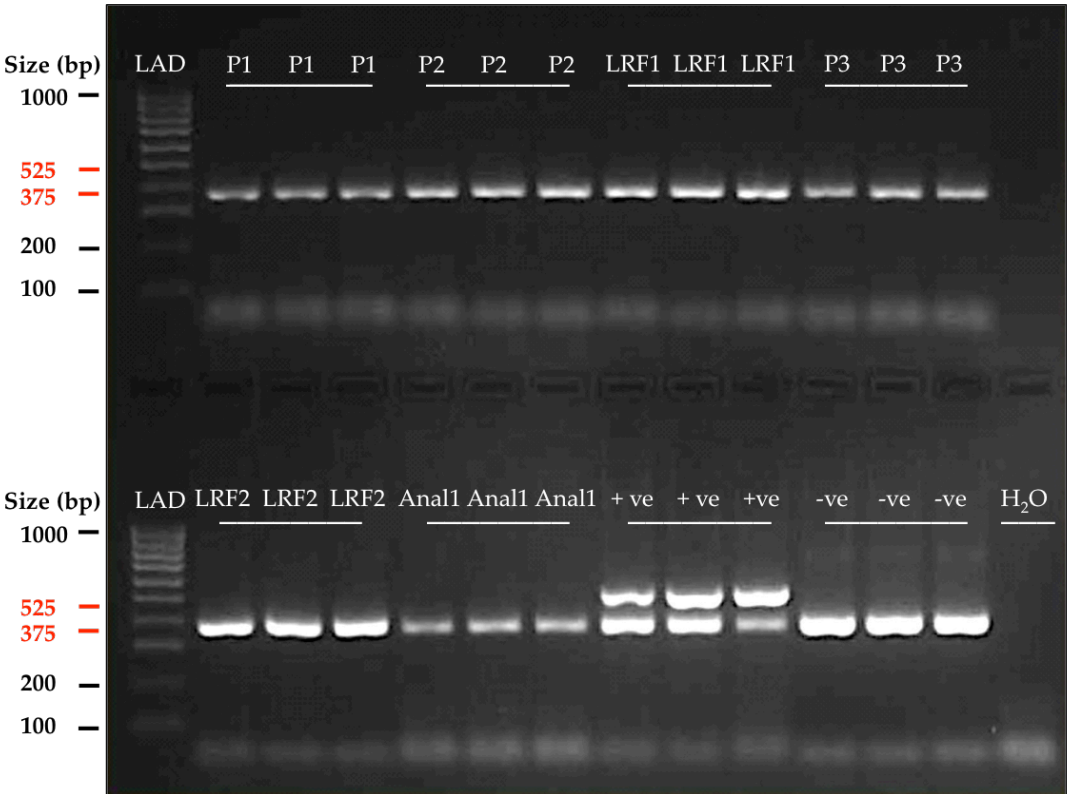


Figure 5-4 Mycoplasma Assessment by PCR Gel Electrophoresis

PCR for mycoplasma (520bp) and cytochrome C (375bp; loading control) run on 1.5% agarose gel with Midori Green utilising gDNA extracted from cell line pellets by utilising the Qiagen DNeasy kit and protocol. (+ve – mycoplasma positive control –ve – mycoplasma negative control; H₂O – DNA negative control; LAD – 100bp ladder)

5.3.5 Short Tandem Repeat Analysis

STR analysis was undertaken on the panel of cell lines and compared with genomic DNA extracted from the parent tumour tissue from which the cell line was derived. Comparison was made across 10 loci, as recommended by ATCC with the STR profiles demonstrated in Table 5-3. All lines demonstrated greater than 80% conservation across the loci assessed, a minimum standard for asserting the line's origin. ASCC line P1 was derived from a male patient but subsequently lost the Y chromosome, which is a not infrequent event reported in over 100 cell lines within the ATCC Cell Biology Collection.²⁴⁷

Table 5-3 STR Analysis of Human ASCC Cell Lines and Primary Tumour Samples

Loci	P1		P2		P3		LRF1		LRF2	
	CL	T	CL	T	CL	T	CL	T	CL	T
D5S818	11	11	10 12	10 12	11	11	11 12	11 12	12 12	12 12
D13S317	8	8	13	7 12	11	11	8 12	8 12	13 13	8 13
D7S820	10 12	10 12	9 12	9 12	8 9	8 9	10	10	9 13	9 13
D16S539	9 13	9 13	11 12	11 12	11 12	11 12	12	12	11 12	11 12
vWA	14 17	14 17	17 18	17 18	15 17	15 17	17 19	17 19	16 17	16 17
TH01	7	7	6 9.3	6 9.3	9 9.3	9 9.3	6	6	6 9.3	6 9.3
TPOX	8 11	8 11	8 11	8 11	8	8	8 9	8 9	8 11	8 11
CSF1PO	11 12	11 12	10 11	10 11	10 12	10 12	11	11	11 11	11 11
Amelogenin	X	X Y	X X	X X	X X	X X	X Y	X Y	X X	X X
D21S11	30.2 32	30.2 32	28 29	28 29	29 30	29 30	30 32.2	30 32.2	30 31.2	30 31.2
Match	94%		89%		100%		100%		95%	

*Red denotes those with a difference between the cell line and primary tumour

5.3.6 Whole Exome Sequencing

Whole exome sequencing (WES) was undertaken on the ASCC cell lines, matched parent tumour samples and matched PBMCs.

The Oncoplot demonstrated in Figure 5-5 demonstrates a selection of the mutated genes in the cohort of five ASCC lines. The majority of the 809 somatic mutations identified in the ASCC lines were missense mutations (715), followed less frequently by nonsense mutations (61), splice site aberrations (14) and frameshift deletions (10) and insertions (3). This was in keeping with the summary of mutation types reported in the COSMIC (Catalogue of Somatic Mutations in Cancer) v90 database for 119 ASCC samples.²¹⁹ A total of 20 genes were identified to be present in more than one cell line, however, no more than two of the five cell lines shared a mutation in the same gene.

When compared to the COSMIC database and publications on the reported genomic aberrations in Anal SCC,^{187, 197, 199, 200} many were identified to be present within the panel of ASCC cell lines. This included mutations in *PIK3CA*, *KMT2C*, *PIK3R1*, *TP53*, *EP300*, *FGFR3*, *TGFBR2*, *FAT1* and *LRP1B*. There were also 18 previously unreported mutations which were identified in two of the cell lines. This included three *MUC* genes, with elevated expression reported in multiple other cancer types, including *MUC4* in colorectal adenocarcinoma and *MUC16* (*CA125*) in ovarian and pancreatic cancer.²⁴⁸ Given the finding of these mutations within two cell lines, the likelihood that they are representative of a true genomic event in ASCC is increased, however the findings should be validated by Sanger sequencing.

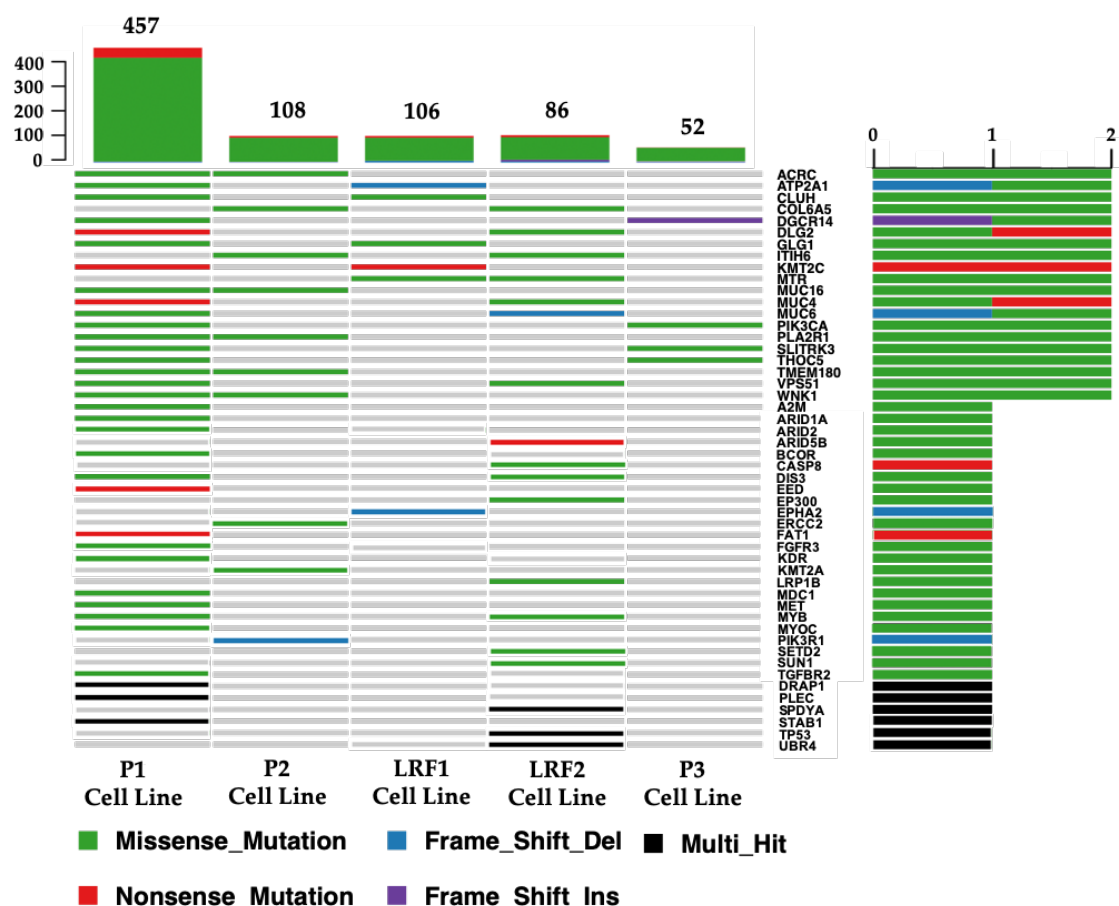


Figure 5-5 Summary of the most frequent and relevant somatic mutations in each of the five ASCC cell lines

The Oncoplot derived from the WES analysis demonstrates a selection of the mutated genes identified in the cohort of five ASCC lines. The total mutations in each cell line are represented in the barplot at the top of the image, with the number of lines harbouring each gene aberration displayed in the right barplot. The 50 genes listed were selected based on: the most frequently mutated genes (total of 20, identified in no more than two of the five cell lines); those mutated in only one cell line and reported previously in ASCC (total of 25); and those genes with multiple hits (5).

The tumour mutational burden of the five anal cancer cell lines is presented in Figure 5-6 compared to the mutational load of a list of other TCGA cohorts. This demonstrates that the panel of lines sit at a similar level to HNSCC, which shares many similarities with ASCC, including being HPV associated with a similar genomic landscape. Four of the cell lines also had a mutational burden similar to the mean somatic burden of 5.7 mutations/Mb published previously for a cohort of ASCCs.¹⁹⁷ The cell line P1 had a much higher mutational load (19.86 Mut/Mb)

than the other four cell lines, however this was well within the 0.8 – 35.5 mut/Mb range reported, with a small proportion of ASCC's having a very high mutational burden.

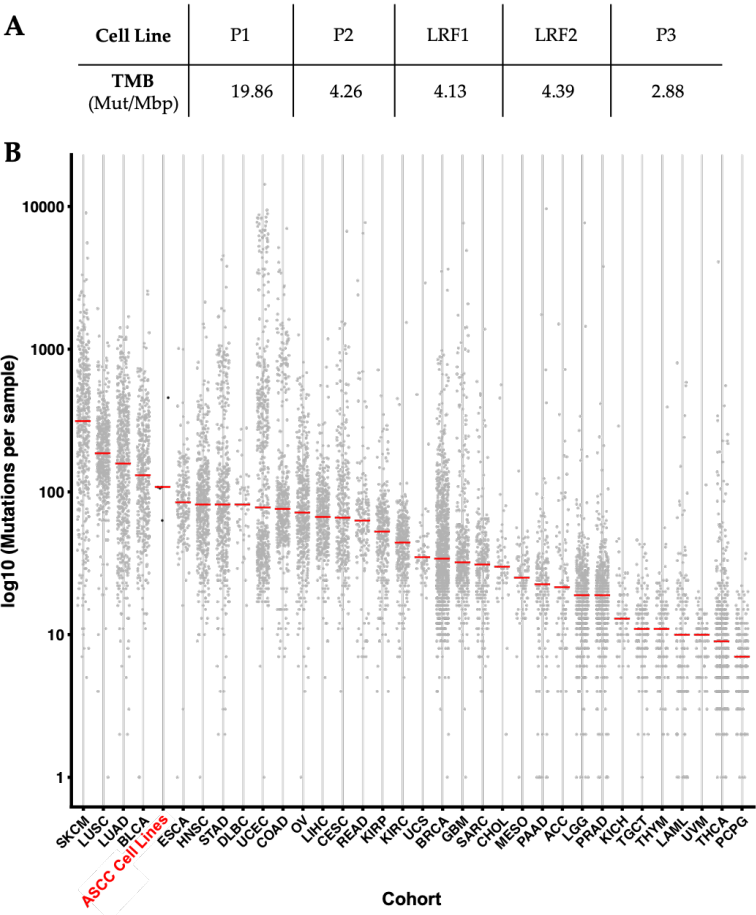


Figure 5-6 Tumour Mutational Burden of ASCC Cell Lines

The Tumour mutational burden derived from the WES analysis for each of the cell lines is represented. (B) The mutational burden of the panel of ASCC cell lines is plotted against the mutational burden of the other TCGA cohorts (<https://www.cancer.gov/tcga>) (Grey dot – Individual patient, Red bar – Median, SKCM – Melanoma, LUSC – Lung SCC, LUAD – Lung Adenocarcinoma, BLCA – Bladder Cancer, ESCA – Oesophageal SCC, HNSCC – Head and Neck SCC, STAD – Stomach Adenocarcinoma , DLBC – Diffuse Large B Cell Lymphoma, UCEC – Uterine Corpus Endometrial Carcinoma, COAD – Colorectal Adenocarcinoma, OV – Ovarian cancer, LIHC – Liver Hepatocellular Carcinoma, CESC – Cervical SCC, READ – Rectal Adenocarcinoma, KIRP – Kidney Renal Papillary Cell Carcinoma, KIRC – Kidney Renal Clear Cell Carcinoma, UCS – Uterine Carcinosarcoma, BRCA – Breast Carcinoma, GBM – Glioblastoma Multiforme, SARC – Sarcoma, CHOL - Cholangiocarcinoma, MESO – Mesothelioma, PAAD – Pancreatic Adenocarcinoma, ACC – AdrenoCortical Carcinoma, LGG – Low Grade Glioma, PRAD – Prostate Adenocarcinoma, KICH – Kidney Chromophobe, TGCT – Testicular Germ Cell /Tumours, THYM – Thymoma, LAML – Acute Myeloid Leukaemia, UVM – Uveal Melanoma, THCA – Thyroid Carcinoma, PCPG – Pheochromocytoma and Paraganglioma)

The somatic copy number analysis (SCNA) for the panel of ASCC cell lines (Figure 5-7 A) revealed gains in 3q and 5p throughout the panel of lines and this has been reported as a frequent event in ASCC.¹⁹⁹ These regions include the genes *PIK3CA*, *TP63*, *SOX2*, *FGF10*, *TERT*, *RICTOR* and *SDHA*. Other focal gains included regions harbouring genes with mutations reported in ASCC including *ARID1A*, *STK3*, *MYC*, *ABL1*, *NOTCH1*, *CCND1*, *FGF3*, *AKT1*, *CDH1*, *JAK3* and *BCL2L1*.^{187, 197-199}

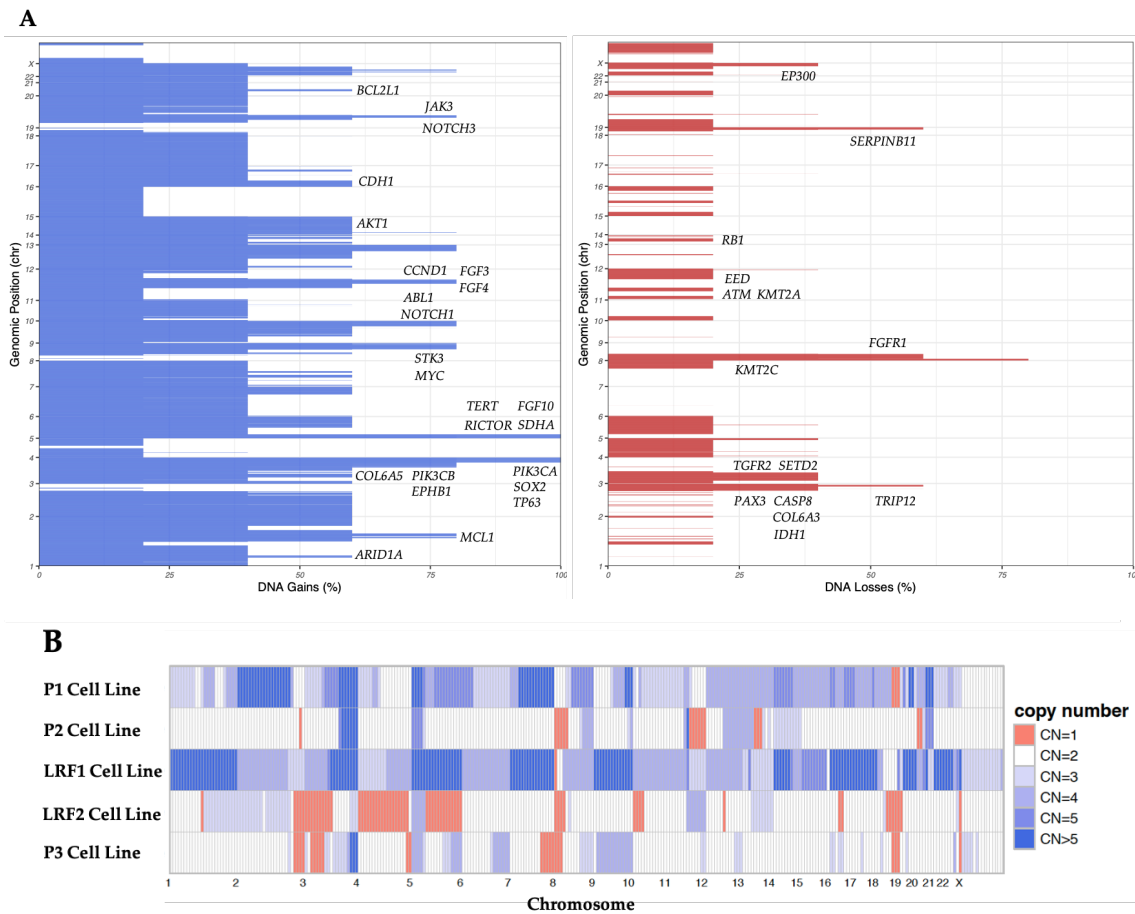


Figure 5-7 Copy number analysis of the panel of ASCC Cell Lines

(A) From the WES analysis, the percentage of the five ASCC cell lines with a copy number gain or loss at each chromosomal region are presented. (B) The cytoband represents the copy number of the gains and losses across the chromosomal regions for each of the five individual ASCC cell lines. (CN = copy number).

The SCNA also revealed focal losses in 2q and 8p in at least three of the five cell lines. These regions include the genes *TRIP12*, *COL6A3*, *IDH1*, *PAX3* and *FGFR1*.

Other areas of focal loss included regions with mutations reported in ASCC including *TGFR2*, *SETD2*, *KMT2C*, *KMT2A*, *EED*, *ATM*, *RB1*, *EP300* and *SERPINB11*. All of these genes are or have been implicated as tumour suppressor genes, with their loss suggesting a role in tumourigenesis.^{187, 197-199}

The cytoband (Figure 5-7 B) demonstrated significant alterations in the SCNA across the cell lines, with copy number gains being more common than losses, with no line having loss of both copies. Of the cell lines, P1 and LRF1 had the greatest variations predominated by gains, including LRF1 having multiple whole chromosomal gains. This was in comparison to the remaining three lines, which had less frequent alteration, many of which were losses, particularly for LRF2.

There was significant correlation across the panel of cell lines for the *PIK3CA* gene. While both P1 and P3 harboured activating mutations, all five lines also demonstrated evidence of copy number gain across this locus, supporting *PIK3CA* as one of the most frequently altered genes in ASCC. *COL6A5* was also found to be mutated in both P2 and LRF2, with copy number gain in the other three cell lines, however, it's role in carcinogenesis is currently unclear.²⁴⁹

A comparison of the variants between the cell lines and the parent tumour from which they were derived is displayed in Figure 5-8. This demonstrated that three of the cell lines (P2, P3, LRF2) have a significant number of shared variants with the parent tumour. However, both P1 and LRF1 have almost no shared variants with their parent tumour, with both parent tumours having a low number of variants compared with the respective cell lines. This was further demonstrated in a comparison of the copy number analysis between the cell lines and parent tumours in Figure 5-9. This revealed that for P1 and LRF1, the parent tumour tissue had almost no copy number variation. Furthermore, the cellular fraction

of identified copy number alteration in all five parent tumours was very low, consistent with low tumour purity. This fits with the limited parent tumour tissue available for sequencing, particularly for P1 and LRF1, with the majority of the tissue likely representing normal adjacent tissue or stroma.

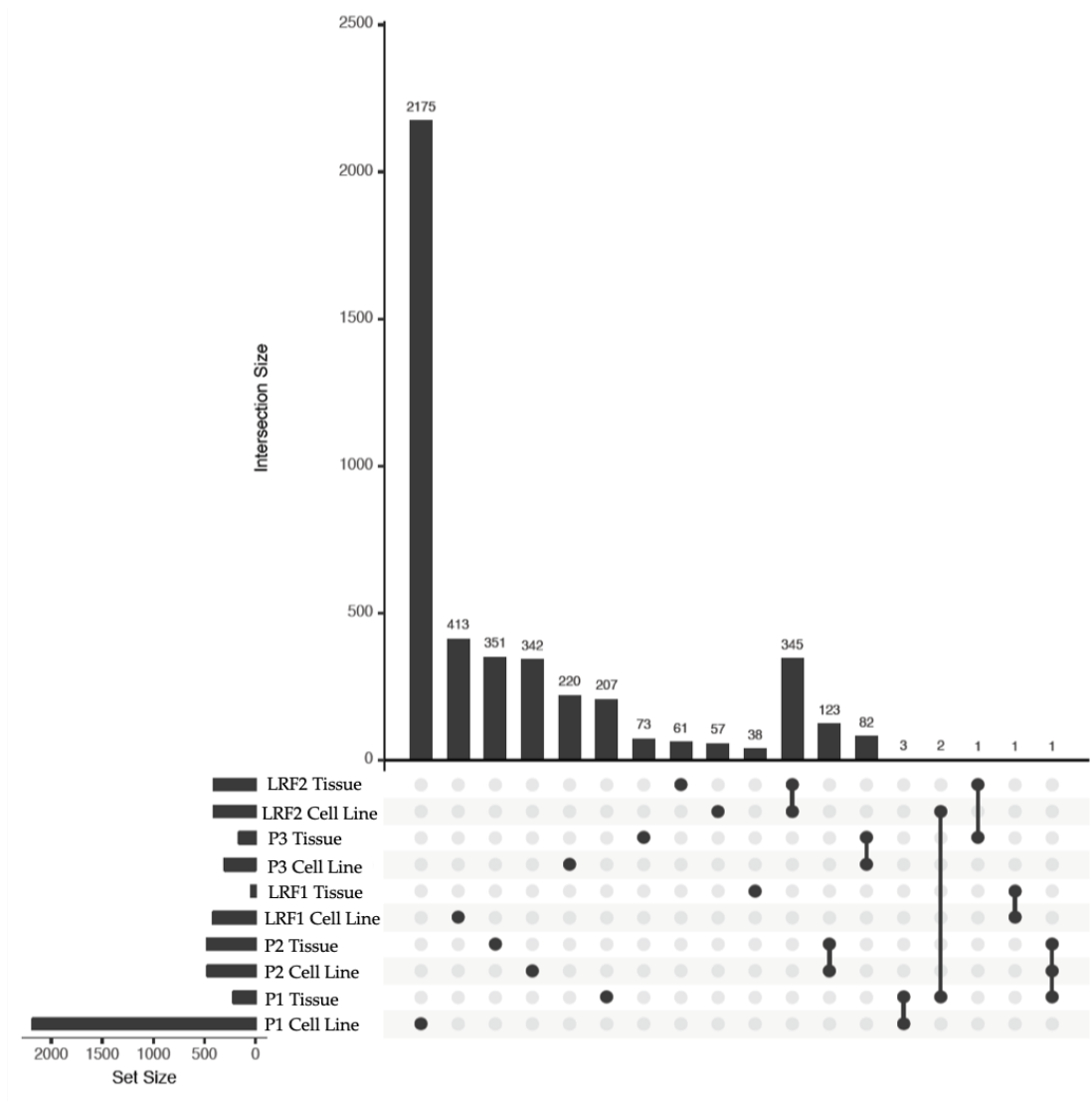


Figure 5-8 UpSetR Plot of unique and shared mutations across the panel of ASCC cell lines and parent tumour samples

UpSetR plot of somatic mutations across the panel of cell lines and parent tumour samples. The set size refers to the total number of somatic mutations for each sample. The intersection size refers to the number of shared somatic mutations between the samples identified by the linked dots or unique to the sample alone (single dot). There was substantial overlap in somatic mutations between cell lines and their parental tumours for P2, P3, and LRF2, but not for P1 and LRF1 due to the low purity of the parent tumour in both samples. There was also minimal overlap between the non-matched samples.

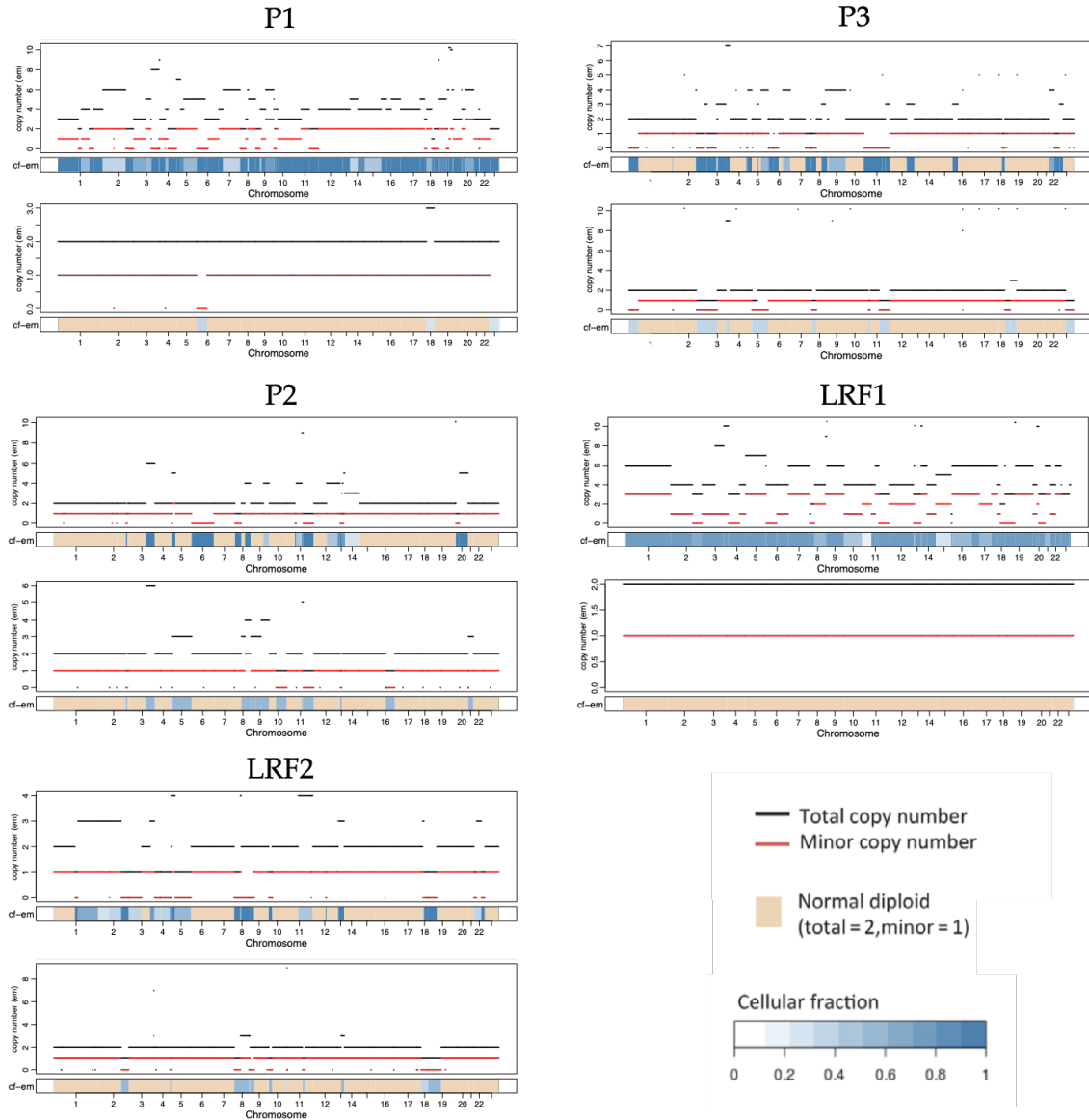


Figure 5-9 Copy number analysis for ASCC cell lines and matched tumour

The images display the copy number variation for each sample, with the minor copy number indicating the number of copies of the least frequent allele, and the total copy number the sum of the major and minor allele counts. The cellular fraction harbouring the copy number variation is displayed in the bottom bar (tan – normal (diploid), shades of blue – estimated fraction; cf – cellular fraction, em – estimated mean). The top image for each line represents the cell line SCNA and the bottom image the matched parental tumour SCNA. This demonstrates that the cell lines had a greater variation in the SCNA than the matched parent tumour. For P2, P3 and LRF2, there was significant overlap in the SCNA between the cell line and the parent tumour. However, for P1 and LRF1, the parent tumour had almost no SCNV in contrast to their matched cell lines. The cellular fraction of the identified SCNVs for all parent tumours was also much less than the matched cell lines, indicating a low tumour purity.

The individual SCNA (Figure 5-9) also demonstrated that the cell lines had a greater variation in copy number than the matched parent tumour. However, for P2, P3 and LRF2, there was significant overlap in the SCNA between the cell line and the parent tumour. In conjunction with the finding of shared SNVs with the parent tumour, this further validates the lines as an accurate representation of the parent tumour from which they were derived.

Mutational trinucleotide signatures are characteristic combinations of mutation types arising from specific mutagenesis processes. The mutational trinucleotide signatures were examined across the five cell lines and are represented in Figure 5-10. Based on the classification by Alexandrov et al²¹⁷, all five cell lines were identified to have more than one mutational trinucleotide signature. Signature 1 was identified in all five cell lines, consistent with the literature, where 100% of assessed ASCC samples demonstrated this signature.¹⁹⁹ It was found to be the dominant signature in four of the five lines (P2, P3, LRF1, LRF2). It is the result of an endogenous mutational process by spontaneous deamination of 5-methylcytosine and is associated with age.²¹⁷

Signature 2 was identified in all four HPV positive lines but not in LRF2, the viral negative line. This signature has been attributed to activity of the AID/APOBEC cytidine deaminases, for which viral infection has been proposed as the underlying factor.²⁵⁰ It has also recently been identified to link *PIK3CA* helical domain mutations to HPV driven tumourigenesis.²⁵¹ Signature 13 has also been attributed to activity of the AID/APOBEC family, and is commonly found with signature 2, as identified in both P1 and P2 cell lines. Signature 4 is associated with smoking, and was identified only in LRF1 cell line, which was derived from the only current smoker of the five patients from which the panel of lines was developed.²¹⁷

Signature 10 was also identified in both P1 and P2 cell lines. It has been linked with the generation of huge numbers of mutations in a small subset of samples, termed ultra-hypermutators.²¹⁷ This is in fitting with the mutational load of P1 being far above that of the other four ASCC lines (Figure 5-6). A somatic or germline mutation in *POLE* or one of the genes involved in DNA mismatch repair which are frequently identified in this group of ultra-hypermutators was not identified in P1.²¹⁷ However, pathogenic missense somatic mutations in *ARID1A* and *PALB2*, which are involved in homologous recombination deficiency were identified and may partly explain its high mutational rate.²⁵²

Signature 7 was identified in P1 and LRF2 and has been associated with exposure to ultraviolet light. This has been reported in other ASCC patient samples.¹⁹⁹ Signature 24 was identified in P3, LRF1 and 2, but has not been reported in ASCC previously. However, it has been identified in liver cancers and appears to be related to exposure to aflatoxins.²¹⁷ Signature 3, 15 and 20 have also not been identified previously in ASCC.

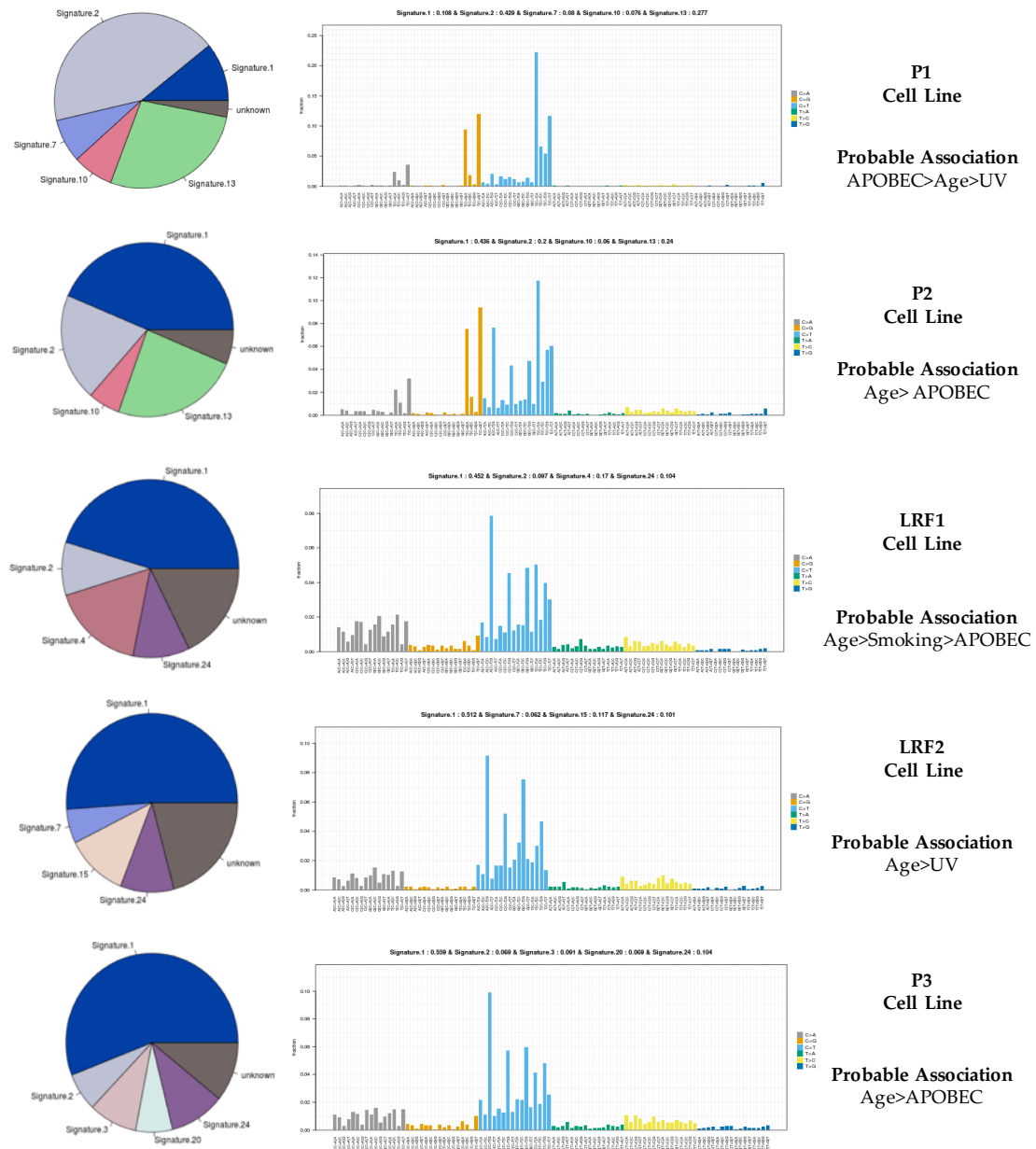


Figure 5-10 Mutational trinucleotide signatures across the panel of ASCC cell lines

The mutational trinucleotide signature for the panel of five ASCC cell lines was determined from the WES analysis. The pie chart demonstrates the relative predominance of mutational signatures present in each cell line based on the fraction of each mutation type demonstrated in the bar graph. This revealed that all lines had more than one signature, with signature 1 (age) shared across all lines and signature 2 (APOBEC) being identified in the four viral positive lines (P1-3, LRF1).

5.3.7 Morphological Features

In vitro culture of the panel of ASCC lines exhibited a well-attached monolayer with a distinct pavement-like epithelial appearance characteristic of squamous cancers (Figure 5-11). They also demonstrated irregular size and shape with a high nuclear to cytoplasmic ratio with prominent nucleoli, consistent with malignant change (Inset, Figure 5-11). Lines P1-P3 require a higher seeding density for successful passaging and demonstrate evidence of significant anoikis represented by the multiple small round apoptotic single cells compared with the two relapsed tumour derived lines LRF1 & 2.

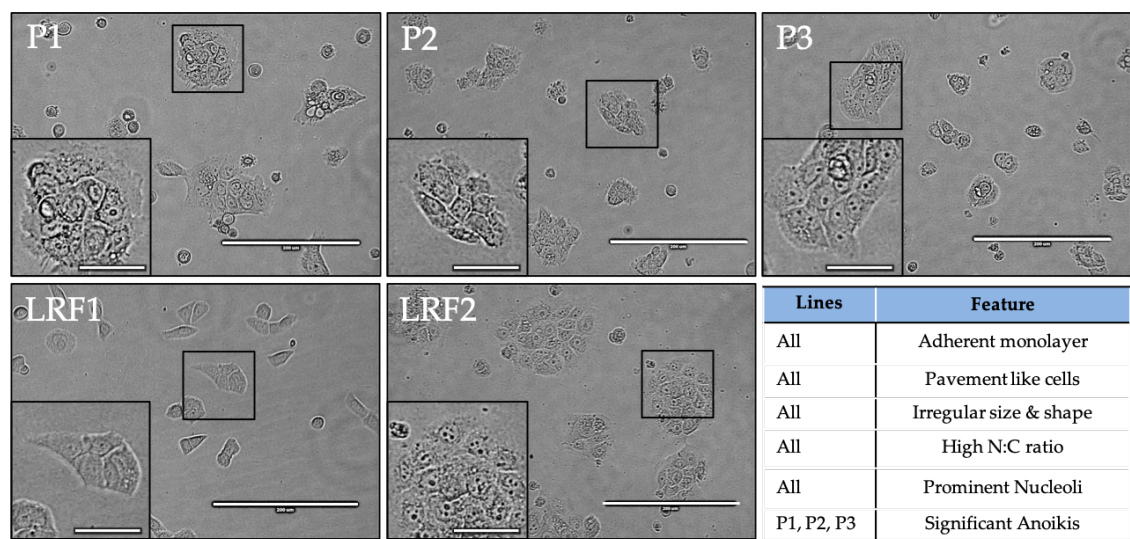


Figure 5-11 Cell Line Morphology

Brightfield photomicrographs of the five human ASCC cell lines. Cells were seeded in a 96-well plate (P1-P3 20,000 cells / well; LRF1 – 5,000 cells / well; LRF2 – 10,000 cells / well) and images taken at 48 hours. The three primary lines demonstrate a higher proportion of apoptotic cells (single cells) compared with the two relapsed lines, consistent with the higher seeding efficiency of LRF1 & 2. (x20 Objective, scale bar 200µm; inset - 50µm).

5.3.8 Immunohistochemical Markers

Assessment of CK 5/6 and p63 demonstrated that all five lines had almost uniform expression of these two markers (Figure 5-12). CK5/6 revealed diffuse

cytoplasmic staining and peri-nuclear enhancement, with p63 localised to the nucleus. This is consistent with the lines having expression of the classic squamous markers and confirms the near purity without fibroblast contamination.

The marker of cell proliferation, Ki67, was found to be very highly expressed across all lines with appropriate staining localised to the nucleus (Figure 5-13). This is consistent with the lines being in the exponential phase of growth, with most cells undergoing cell division. Assessment of p16 expression demonstrated strong nuclear and cytoplasmic staining across all lines except LRF2, which had a complete absence of staining. This is abnormal and may indicate methylation of the promoter of both CDKN2A alleles, given the absence of CDKN2A mutations.

5.3.9 HPV Assessment

HPV genotyping was undertaken utilising six different assays (refer 2.3.5). Five assessed for HPV 16, 18 and 12 other high-risk genotypes while the Linear Array® HPV Genotyping assay assessed for 37 genotypes in total. LRF2 was found to be HPV negative consistent with the absence of p16 staining. The other four cell lines were found to be positive for only HPV subtype 16 (Figure 5-13A). A study published in 2015 identified that HPV16 was identified in 89% (134/151) of all assessed ASCC specimens and 93% (134/144) of those with HPV positivity, with mono-infection accounting for 75% (108/144) of samples.

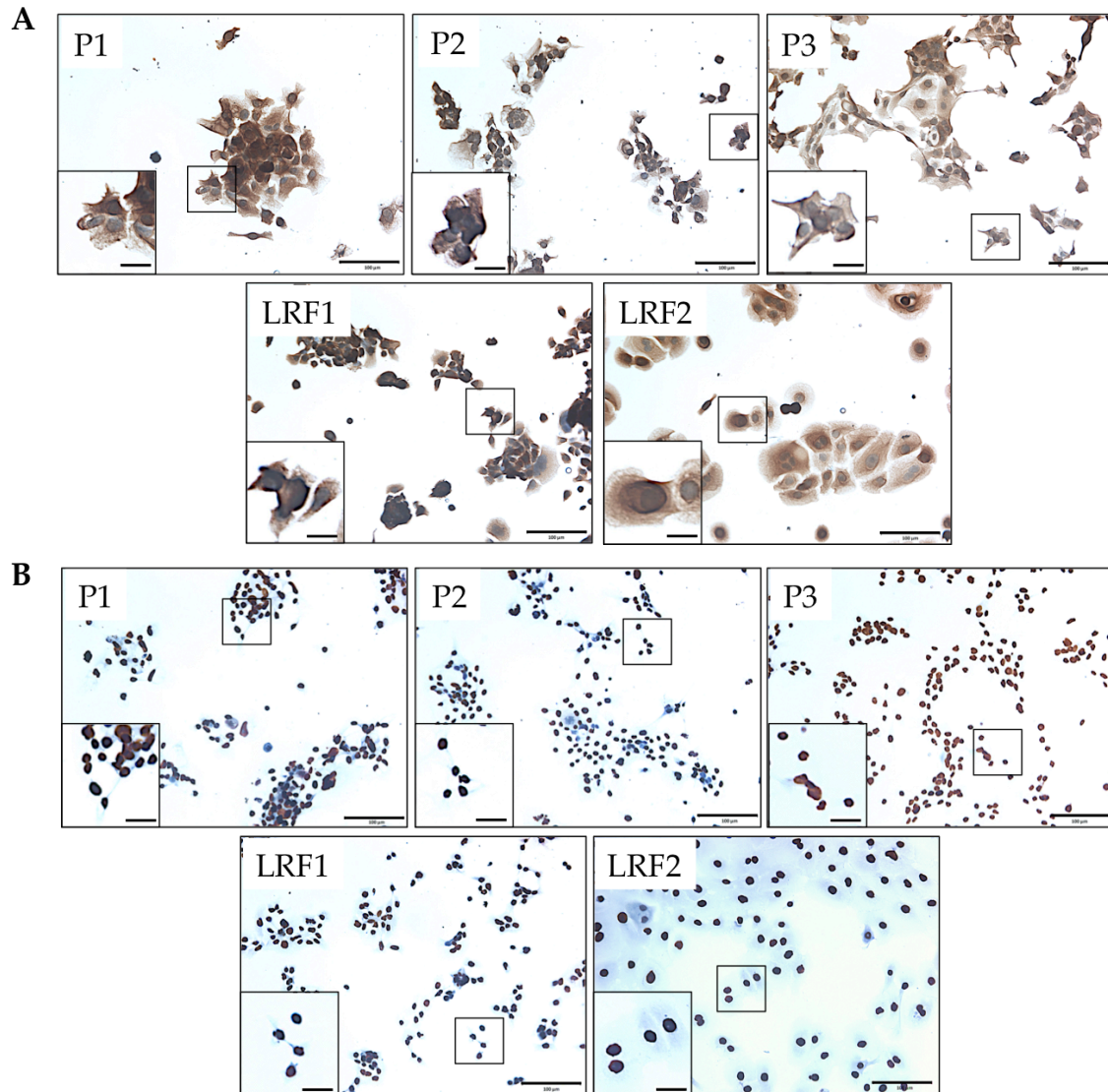


Figure 5-12 CK5/6 & p63 Immunocytochemistry

Cells were seeded in a 96 well plate at the following densities (P1-P3 20,000 cells / well; LRF1 – 5,000 cells / well; LRF2 – 10,000 cells / well). At 48 hours, they were fixed with 4%PFA and permeabilised with Triton X before undergoing ICC staining for CK5/6 (A) and p63 (B). Main scale bar is 100µm with inset scale bar 25µm. The CK5/6 ICC demonstrates appropriate cytoplasmic and peri-nuclear staining, with p63 localised to the nucleus alone, consistent with cells being of squamous origin.

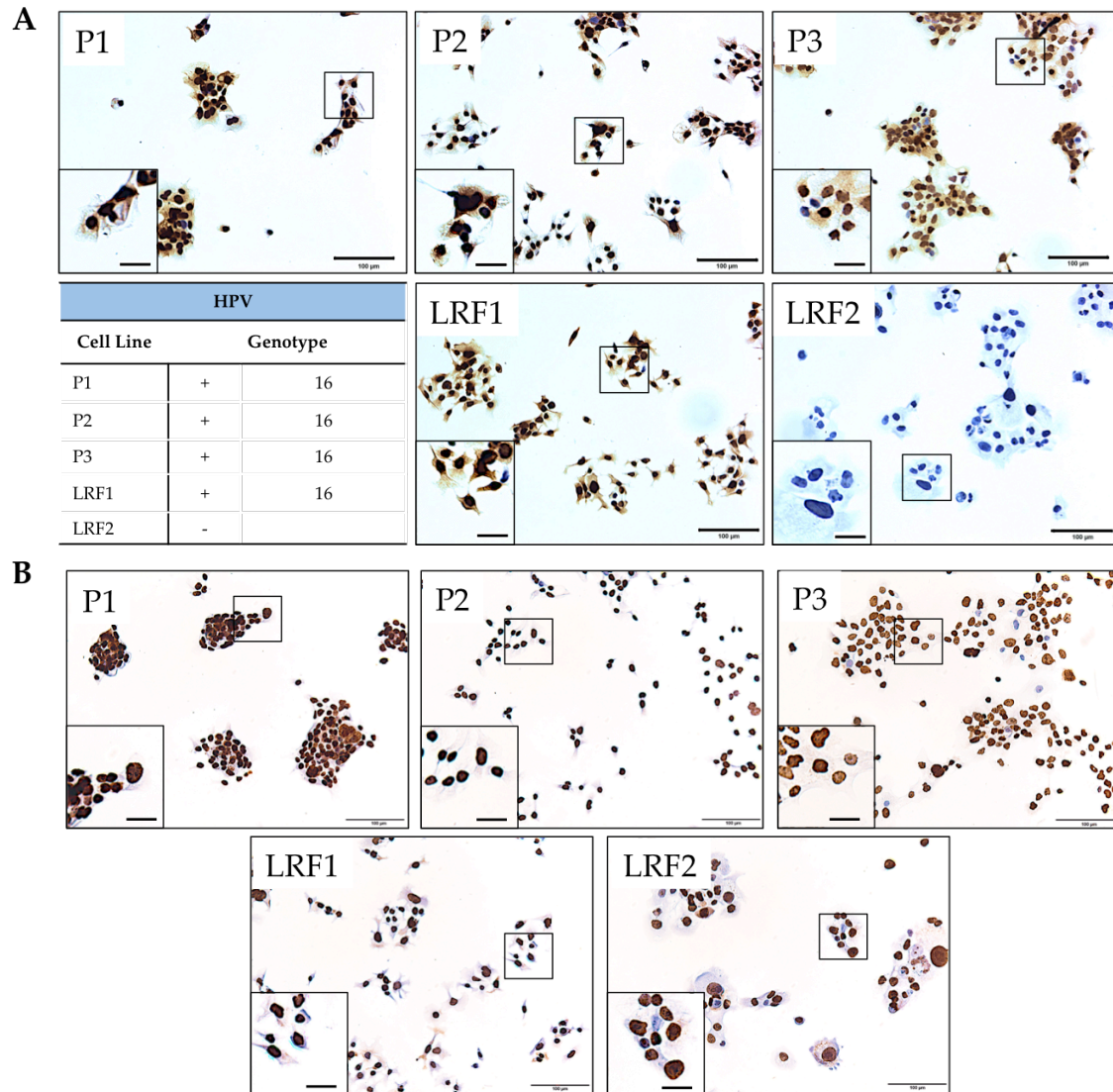


Figure 5-13 p16 & Ki67 Immunocytochemistry & HPV Genotyping

Cells were seeded in a 96 well plate at the following densities (P1-P3 20,000 cells / well; LRF1 – 5,000 cells / well; LRF2 – 10,000 cells / well). At 48 hours, they were fixed with 4% PFA and permeabilised with Triton X before undergoing ICC staining for p16 (A) and Ki67 (B). Main scale bar is 100µm with inset scale bar 25µm. The p16 staining is present across both cytoplasm and nucleus in lines P1, P2, P3 and LRF1, with LRF2 being clearly negative. HPV genotyping was undertaken on a series of platforms utilising PCR based assays for over 100 subtypes. This identified HPV 16 as the only subtype present in P1, P2, P3 and LRF1. It confirmed LRF2 as HPV negative. Ki67 was present in >90% of cells across all lines, consistent with the lines being in the exponential phase of growth.

5.3.10 Cell Line Tumouroids

To further explore the characteristics of the ASCC cell lines and perform specific assays, cells were seeded in the extracellular matrix Matrigel to form tumour spheroids. All cell line tumouroids were grown in the same media as the counterpart cell lines, RPMI-1640 + 10% FCS.

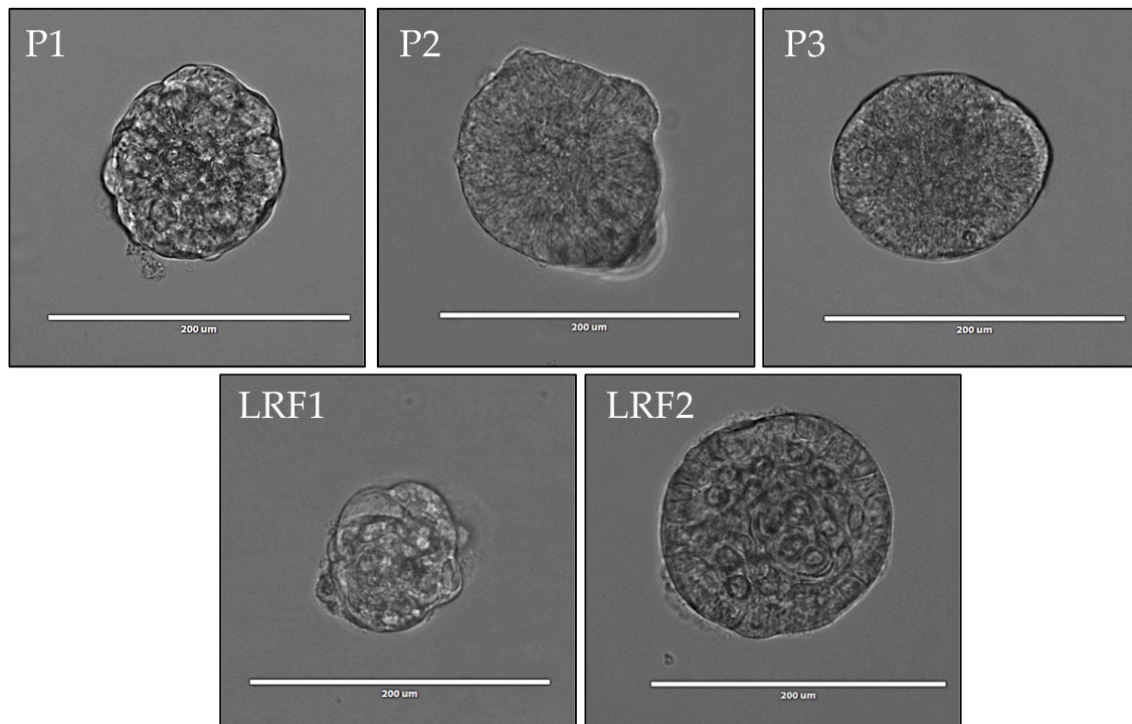


Figure 5-14 Microscopy Image of Cell Line Tumouroids

Cell lines were seeded at 1000 cells per 50μl droplet of Matrigel into a 24-well plate and allowed to grow in the presence of RPMI + 10% FCS. Phase contrast microscopy at 14 days post-seeding demonstrated a non-budding spheroid morphology and well-defined external pseudo-capsule across all cell line tumouroids. (Objective x20, scale bar 200μm)

The morphology of the lines was assessed initially with phase contrast microscopy, demonstrating a predominantly spheroid form without budding across the lines, and evidence of an outer pseudo-capsule encompassing the tumouroids as demonstrated in Figure 5-14. This was further delineated with scanning electron microscopy (ScEM, Figure 5-15) revealing closely interconnected flat pavement-like squamous cells and the appearance of an outer pseudo-capsule particularly with the larger tumouroids. The HPV negative line

LRF2 had larger cells and a more irregular surface compared with the viral associated tumouroids. The larger cell size of LRF2 was similar to that identified in the brightfield photomicrographs (Figure 5-11).

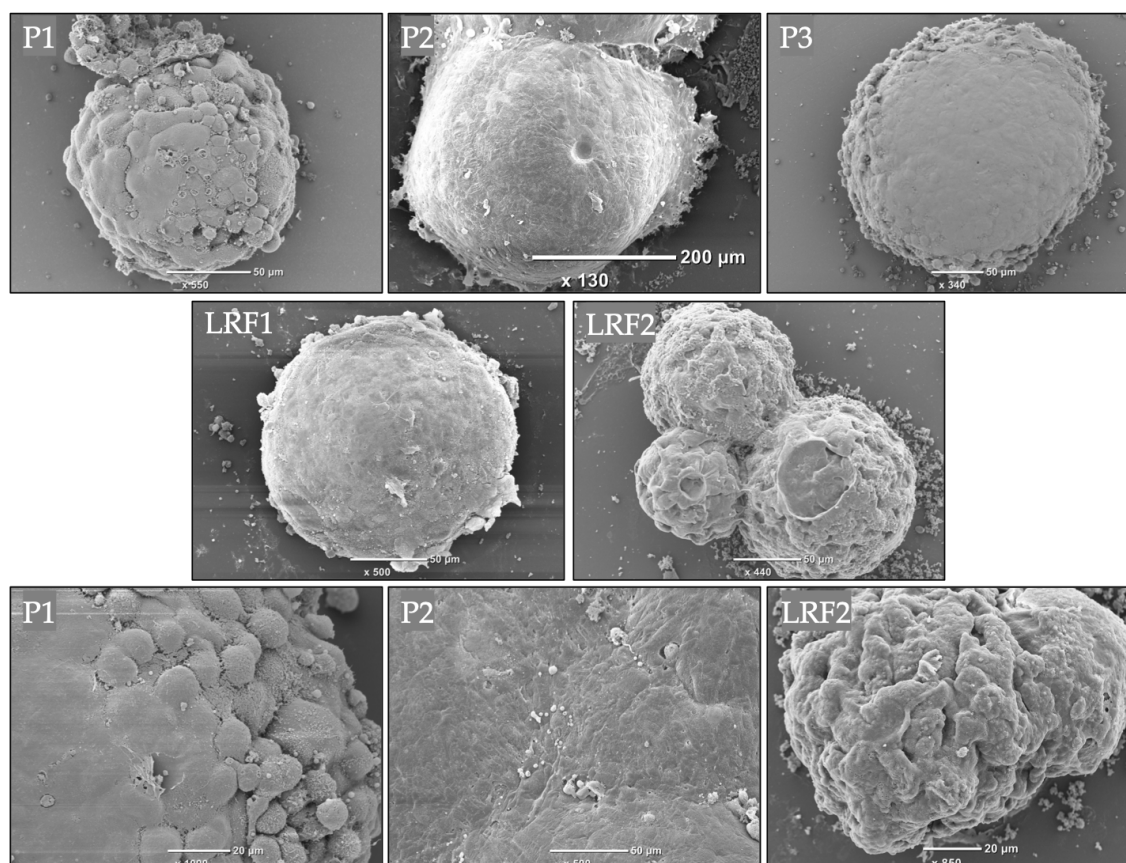


Figure 5-15 Surface Architecture of Cell Line Tumouroids by ScEM

Cell lines were seeded in a 24-well plate (1000 cells in 50µl Matrigel) and allowed to grow for 14 days in RPMI + 10% FCS. The cell line tumouroids were then retrieved, fixed and processed for ScEM. All tumouroids demonstrate a spheroid shape with closely interconnected flat pavement-like squamous cells as demonstrated at high magnification of P1 (bottom row). Larger tumouroids (P2/3 & LRF1) develop the appearance of an outer pseudo-capsule demonstrated at higher magnification of P2 (bottom row). The HPV negative line LRF2 had evidence of larger cells and a more irregular surface demonstrated at higher magnification in the bottom row. (Scale bar 50µm top & middle rows - except image P2 - 200µm; 20µm bottom row except P2 - 50µm)

The tumouroids were also fixed and blocked to allow assessment histologically and with immunohistochemical markers. The H & E images demonstrated significant recapitulation of the degree of cellular differentiation and the

architecture of the parent tumour (Figure 5-16 compared with Figure 5-22). It also again demonstrated the much larger cell size of LRF2 compared with the virally driven ASCC tumouroids.

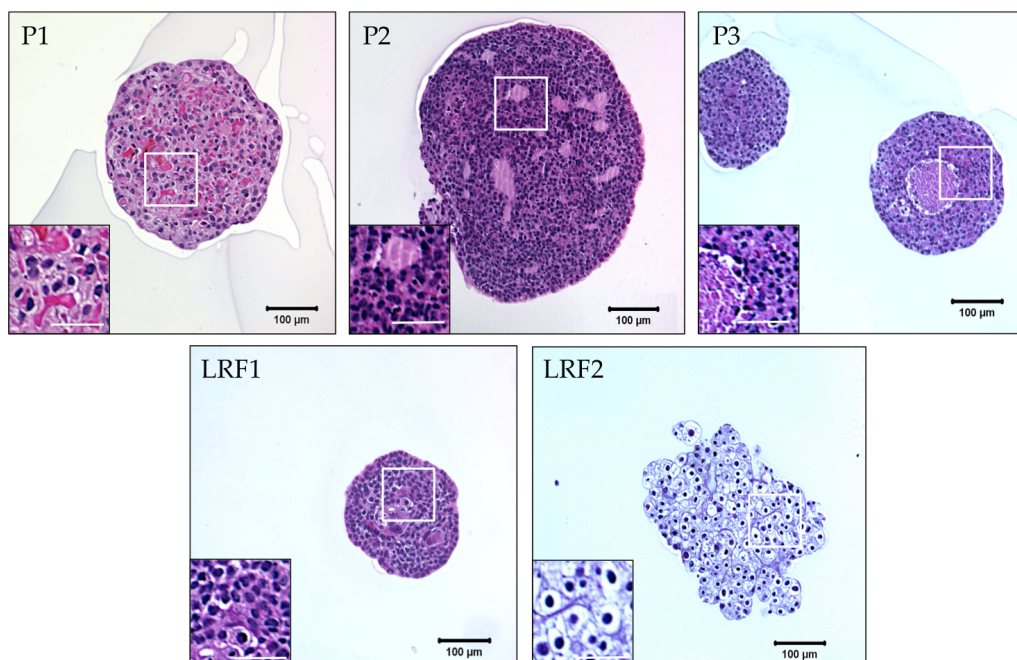


Figure 5-16 Cell Line Tumouroid Histology on H & E Staining

Cell lines were seeded in a 24-well plate (1000 cells in 50μl Matrigel) and allowed to grow for 14 days in RPMI + 10% FCS. The cell line tumouroids were then retrieved, fixed with 4% PFA, embedded in histogel and sectioned for H & E staining. (Scale bar 100μm). The cellular differentiation and architecture of the tumouroids closely recapitulated that of the parent tumour from which they were derived (compare with Figure 5-22).

The profile of immunohistochemical markers was also comparable with the primary lines, demonstrating appropriate and uniform CK5/6 and p63 expression (Figure 5-17). This further validates the purity of the five cell lines without fibroblastic contamination. The co-stain of p16 and Ki-67 also demonstrated the appropriate findings of a high Ki67 across all lines with p16 positivity in all but LRF2.

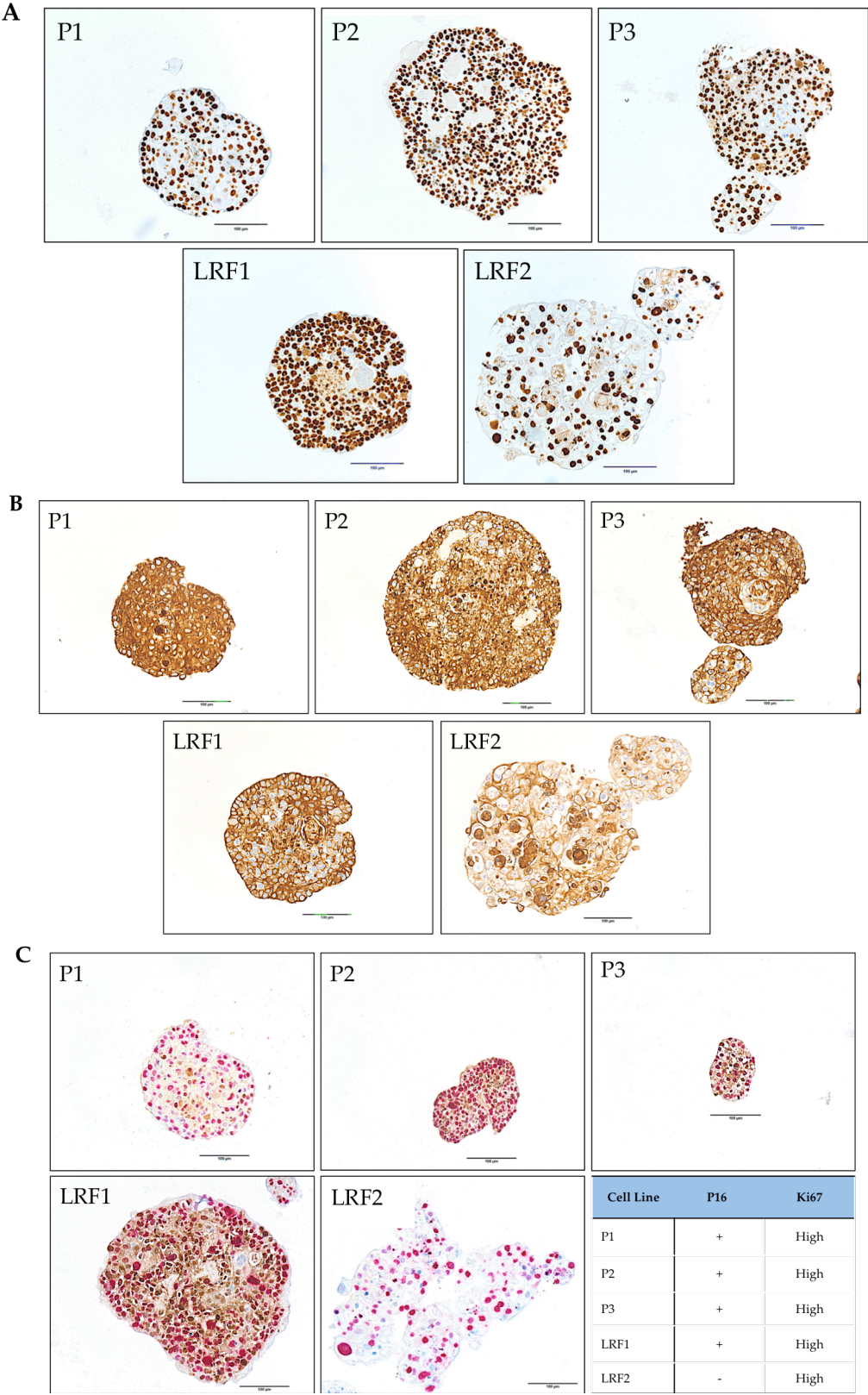


Figure 5-17 Cell Line Tumouroid Immunohistochemical Markers

Cell lines were seeded in a 24-well plate (1000 cells in 50µl Matrigel) and allowed to grow for 14 days in RPMI + 10% FCS. The cell line tumouroids were then retrieved, fixed with 4% PFA, embedded in histogel and sectioned for (A) p63, (B) CK5/6 single stains and (C) Ki67 (red chromagen) / p16 (brown chromagen) dual stain immunohistochemistry. (Scale bar 100µm). Appropriate cytoplasmic CK5/6 staining and nuclear p63 staining is consistent with the tumouroids being squamous in origin. Nuclear Ki67 staining was present at a high level in all cell line tumouroids, with p16 staining in all but LRF2, consistent with it being HPV negative.

5.3.11 Parental Tumour IHC

The parental tumours from which the cell lines were derived also underwent IHC to assess the p16 staining for comparison with the cell lines and to assess the p53 IHC staining pattern in light of the HPV findings. This demonstrated that the four viral positive tumours were p16 positive in comparison to the viral negative tumour LRF2, which had normal p16 expression. The p16 expression was heterogeneous in the primary tumours from which P1 and P3 were developed, in contrast to the cell lines, which demonstrate uniform expression of p16. The p53 staining appeared normal in the four p16 positive lines and was highly over-expressed in the HPV negative line.

5.3.12 Seeding (Plating) Efficiency

The plating efficiency of each cell line was determined by seeding cells into a six well plate at three different cell densities (P1 – 2 / 4 / 8 x 10³; P2 – 10 / 30 / 60 x 10³; P3 – 1 / 2 / 4 x 10³; LRF1/LRF2 – 0.2 / 0.5 / 1 x 10³) to determine the optimal density for passaging. This was reliant on the attachment efficiency, ability to migrate into clusters and subsequent colony forming efficiency of the cells. The optimal seeding efficiency of the lines is represented in Table 5-4.

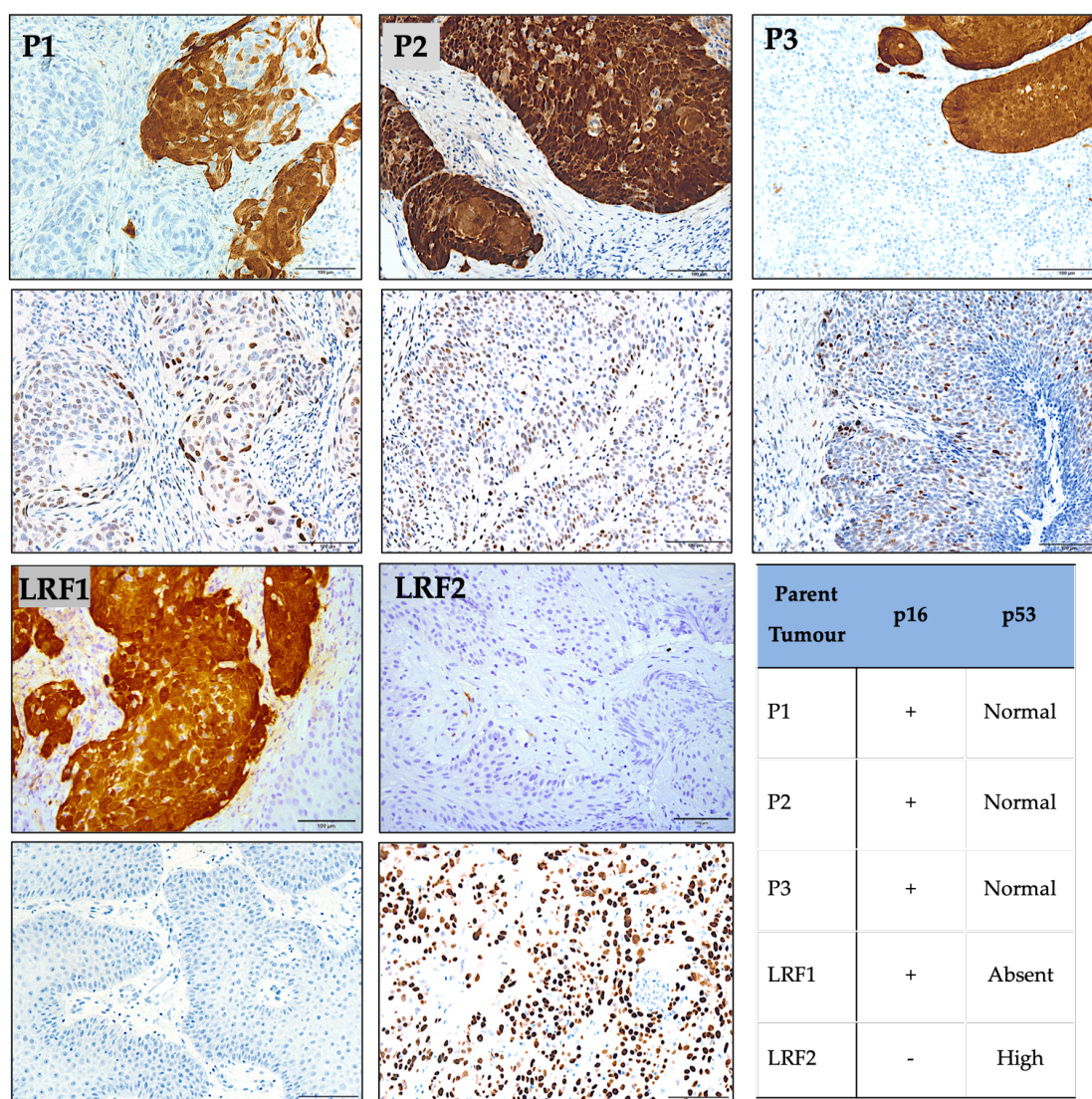


Figure 5-18 Parent Tumour Expression of p16 and p53

The parental tumours from which the cell lines were established underwent IHC staining for p16 and p53. The top images of each of the parental tumours demonstrates the p16 IHC results with p53 demonstrated in the image below (scale bar 100µm). The results are summarised in the table, demonstrating normal or absent p53 staining in the four p16 positive tumours, with heavy p53 staining in the p16 negative tumour. Interestingly, the p16 staining was found to be heterogeneous in the P1 and P3 parental tumours, while the derived cell lines demonstrate uniform expression.

5.3.13 Proliferative Capacity and Doubling Time

The proliferative capacity of the lines was assessed over a period of 150 hours using the xCELLigence platform as outlined in section 2.7. The seeding density to reach an appropriate level of exponential growth was optimised before

undertaking the assay in triplicate, on two separate occasions. The resultant growth curves are demonstrated in Figure 5-19, where the cell index is a surrogate measure of proliferation over time. The initial 12 - 24 hours of the curve relates to the settling, adherence, flattening and migration of the squamous cells prior to undergoing proliferation. Consequently, all of these events are associated with greater electrode contact and therefore increased resistance, leading to a non-growth phase related increase in the cell index. Following this period, the cells begin to proliferate at variable rates dependent on their doubling time and represented by the exponential aspect of the curve. The final plateau relates to either cell confluence or exhaustion of the media.

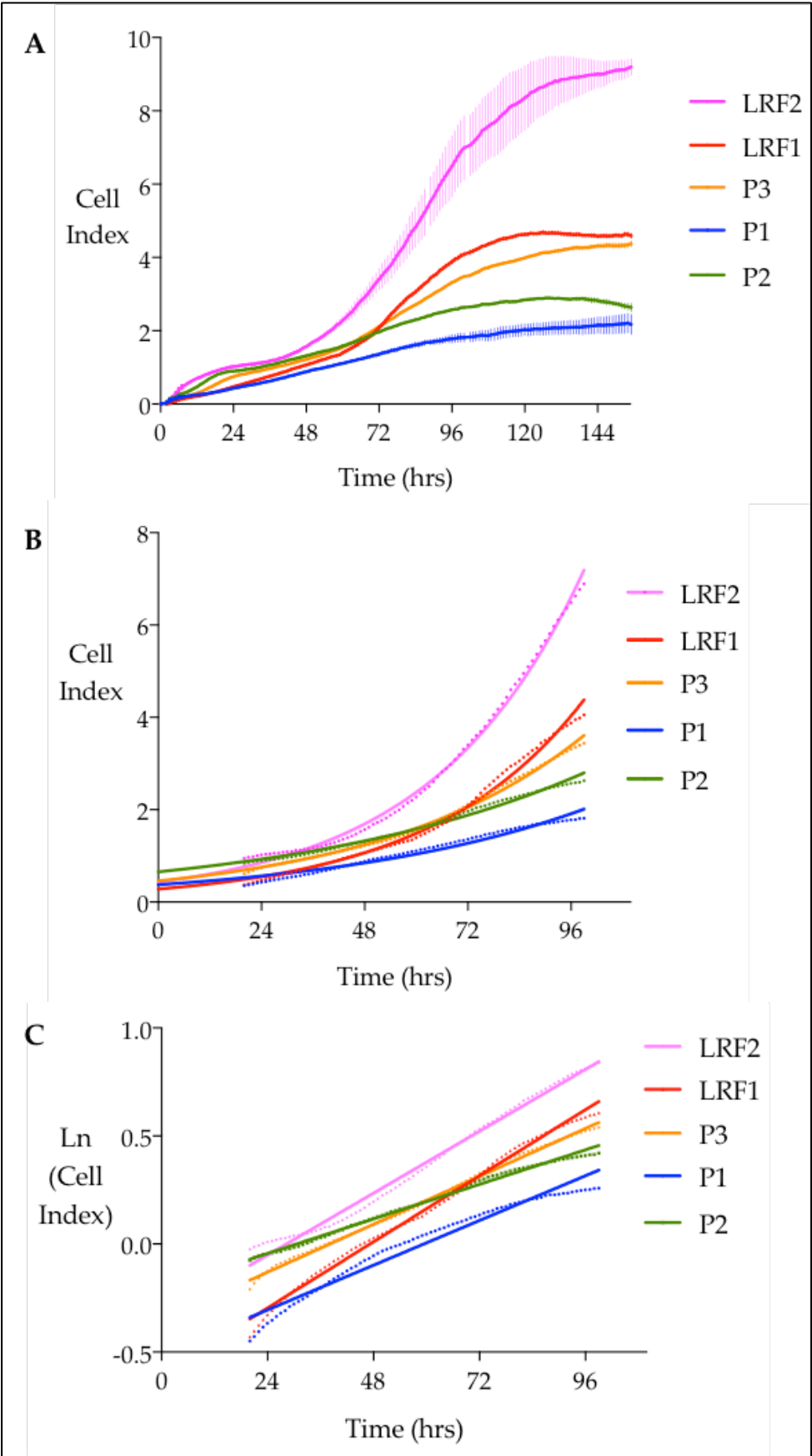


Figure 5-19 Proliferation Assay

A) Cells were seeded in triplicate in an xCELLigence DTPA 16 well E-plate with RPMI-1640 + 10% FCS and the real time cell index recorded over a period of 150 hours (Mean + SEM depicted across each time point, SEM very small for P2, P3 & LRF1); B) The exponential growth equation (solid lines) was applied to the exponential growth phase of the real-time curves (24 - 96 hours – dotted lines), demonstrating close alignment with the real time data. C) Natural log transformation (dotted lines) followed by linear regression analysis (solid lines) demonstrated close alignment of the resultant lines, confirming the consistency in the doubling time of each cell line during this period of growth.

To assess the doubling time of each of the lines, the exponential aspect of the real-time curve was selected (24 - 96 hours). This was to minimise the effect of cell size and degree of cell adhesion and spreading (initial 24 hours) on the results. The exponential growth equation [$Y = Y_0 \cdot \exp(k \cdot x)$] was then applied in Graphpad prism. Y_0 is the value when (x) is zero, k is the rate constant (inverse minutes) and Tau is the time constant (reciprocal of k). The resultant curve is co-represented in Figure 5-19 B (solid line) and closely aligned with the real time cell index data (dotted line). The doubling time is calculated as $[\ln(2)/k]$ for each line and is presented in Table 5-4.

Table 5-4 Cell Line Passaging Characteristics

Line	Doubling Time (hrs)	Seeding Efficiency (%)
P1	41	6
P2	47	1
P3	33	9
LRF1	25	33
LRF2	24	24

The constant doubling time was also validated by performing a log (natural) transformation of the exponential phase growth data followed by linear

regression [$Y = k \cdot x + Y_0$] with the resultant lines represented in Figure 5-19 C. This demonstrated a predominantly linear relationship with a varied gradient dependent on the doubling time for each line. The results demonstrated that the two relapsed lines both had a higher seeding efficiency (section 5.3.12) and shorter doubling time compared with the primary ASCC lines.

5.3.14 Migration Potential

The migration capacity of each cell line was assessed using the xCELLigence platform as detailed in section 2.8. The assay was undertaken in triplicate on two separate occasions with a representation demonstrated in Figure 5-20 A. In line with the protocol by ACEA, the first three hours during which the experimental conditions are being established and the cells seeded are excluded from the migration analysis as demonstrated in Figure 5-20 B. Linear regression was then performed to assess the gradient of the cell index for each cell line as a surrogate marker of the degree of migration. The resultant lines demonstrate close alignment with the real time data, and the inverse of the slope is used as the marker of migration to compare between lines as reported in the table in Figure 5-20 C. The lower the inverse slope, the greater the motility of the line.

From the migration data, both relapsed cell lines demonstrated the greatest cell motility, a key factor required for the development of lymphatic or haematogenous metastasis. Of the three primary complete response lines, P3 demonstrated evidence of a high level of cell migration not dissimilar to the relapsed lines. Increased motility has been previously identified to relate to helical domain *PIK3CA* mutations, a known genomic aberration possessed by P3 (Figure 5-5), which may go some way towards explaining its substantially increased migration compared with the other primary lines.²⁵³

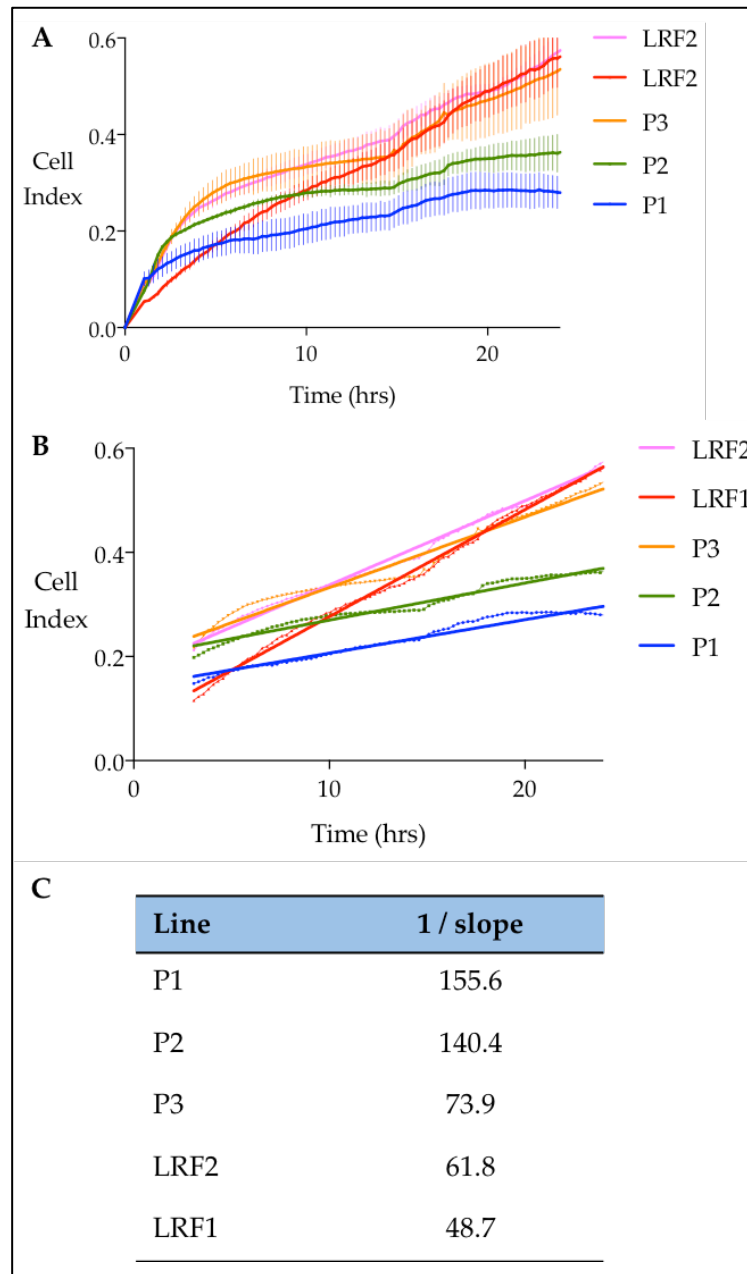


Figure 5-20 Migration Assay for Human ASCC cell lines

A) Cells were seeded in triplicate in an xCELLigence DTPA 16 well E-plate with RPMI-1640 + 10% FCS and the real time cell index recorded over a period of 24 hours (Mean + SEM depicted for each time point); B) The first 3 hours of the assay during experimental setup is removed as stipulated in the migration protocol (dotted line). Modelling with linear regression (solid line) demonstrates close alignment with the real time data. C) The 1/slope of the linear regression performed in B was calculated as a surrogate of the migration potential of each cell line, with the lower the number the higher the migratory potential.

5.3.15 *In Vivo* Tumourigenicity

Tumourigenicity was assessed in both female NSG and BALB/c Nude mice, which respectively represent the most immunocompromised and least immunodeficient mice for xenografting. All five lines were found to be tumourigenic in both mouse strains, however the rates of tumour formation were variable in Nude mice, ranging from 3/5 to 5/5 after subcutaneous injection of 5×10^6 cells (Figure 5-21).

The mean rate of growth of the cell line xenografts for P1, LRF1 & 2 was more rapid in the NSG than Nude mice as demonstrated in Figure 5-21. On the contrary, for the Nude mice injected with P2 and P3 that developed tumours, the mean growth rate was similar to that in the NSG mice. Of the mice that developed tumours, growth was to a minimum 500mm³ by Day 70. The two most rapid growing lines in both mice strains were P1, followed by LRF1, with the other 3 lines demonstrating slower *in vivo* growth.

Analysis of the morphology and histology of the CL xenografts with H & E staining demonstrated close recapitulation of the parent tumour and primary xenografts with regard to cellular differentiation and tissue architecture (Figure 5-22). Anti-human mitochondrial stains demonstrated evidence of the mouse tumours being human in origin.

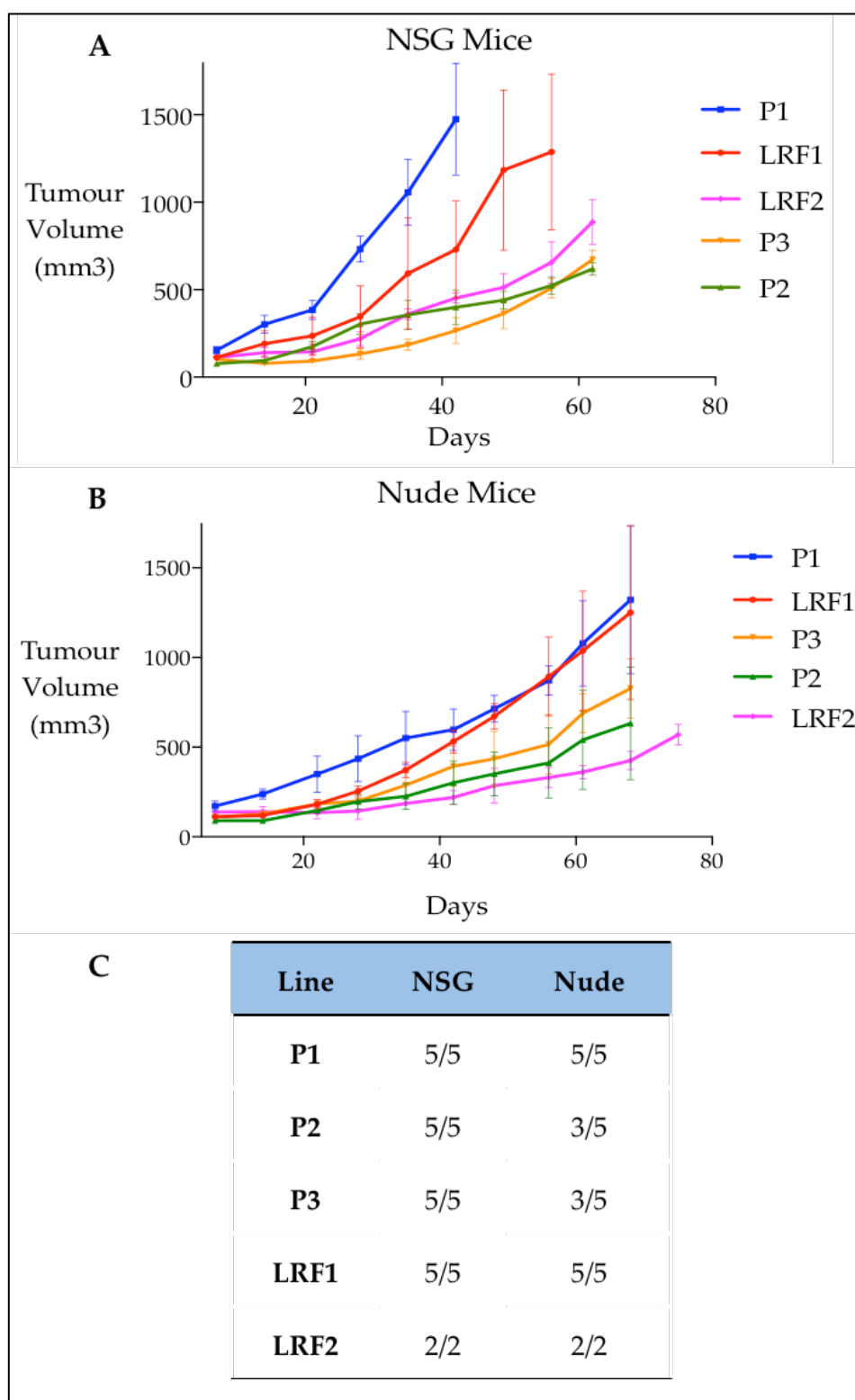


Figure 5-21 Cell Line Tumourigenicity

The tumourigenic potential of the five human ASCC lines was assessed in both NSG and Nude mice following subcutaneous injections of 5×10^6 cells in 100 μ l of 1:1 Matrigel:PBS. The tumours were measured with the growth demonstrated (Mean $\pm$ SEM) of those mice with tumours for each cell line A) & B). The proportion of mice developing established tumours is displayed in C).

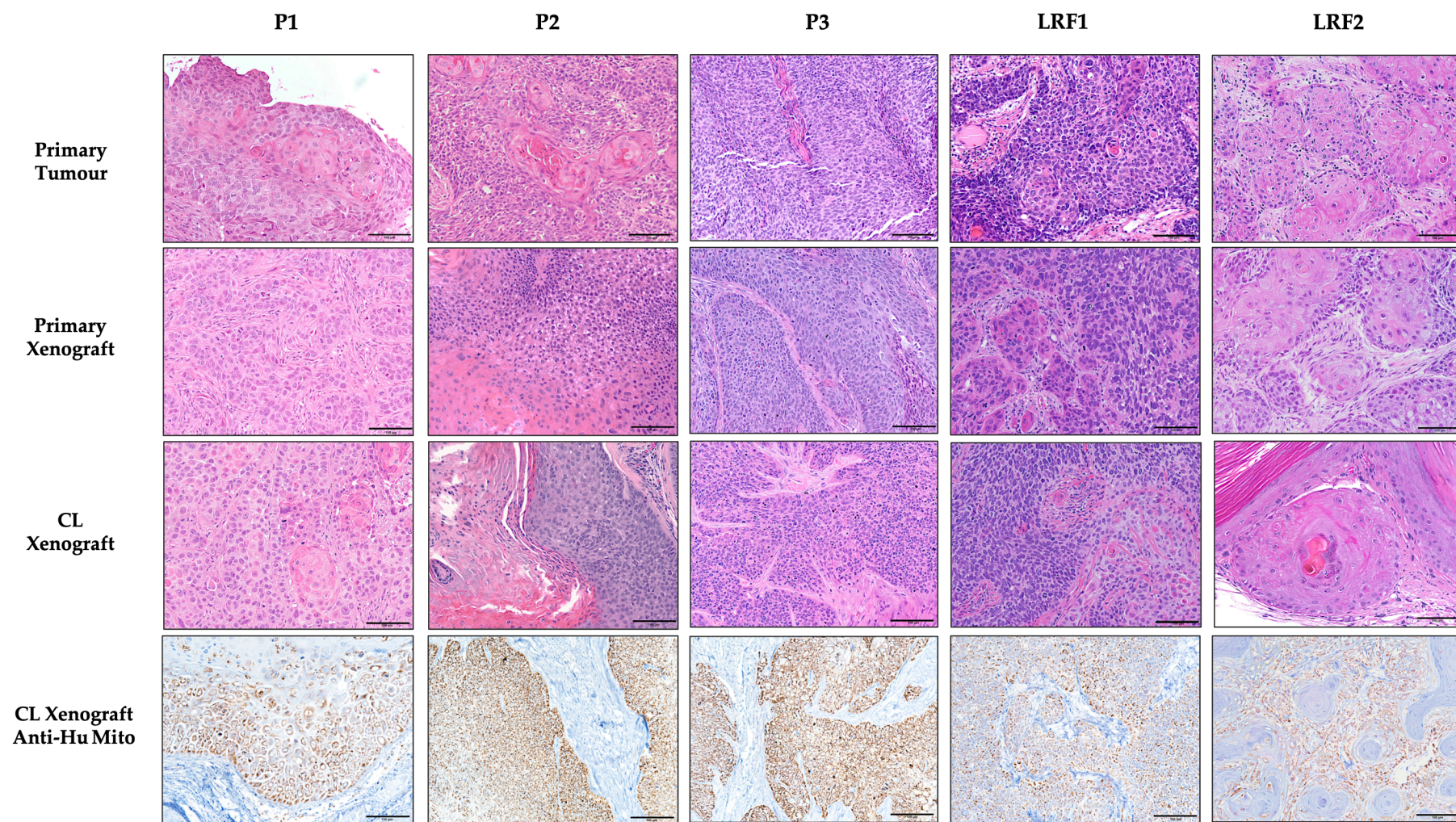


Figure 5-22 Histological architecture & anti-human IHC of the five primary human ASCC tumours and derived tumour models

H & E and IHC images following formalin fixation of tumour tissue with paraffin embedding, sectioning and staining (Scale bar 100µm). The H & E images (Rows 1-3) demonstrate the preserved cellular and architectural features between the five primary human ASCC tumours (1st row) from which primary (2nd row) and cell line (3rd row) xenografts were subsequently derived. Immunohistochemistry with anti-human mitochondrial antibody (4th row) demonstrating brown chromagen staining of the human epithelial tumour cells, with absent staining of the mouse stroma, validating the mouse CL tumours as human in origin. CL – Cell Line; Anti-Hu Mito – Anti-human mitochondrial.

5.3.16 IFN- γ modulation of MHC I and PDL1 Expression and Growth Effects

The gating strategy used for the evaluation of MHC I and PDL1 expression is outlined in Figure 5-23. The expression of PDL-1 and MHC-I at baseline and following exposure to IFN- γ is displayed in Figure 5-24 A & B. Compared to isotype, at baseline there was variable PDL1 expression, which is better visualised by IHC on the CL tumouroids in Figure 5-25. This revealed that the PDL1 expression appeared to be patchy. On exposure to IFN- γ , there was significant upregulation of PDL1 expression in all lines except for P1, which had minimal expression at baseline or with stimulation. This may be secondary to copy number loss at the *JAK2* locus which was identified on the CNA for P1 (Figure 5-9), which is implicated in *JAK/STAT* pathway control of both MHC1 and PDL1 membrane expression.²⁵⁴

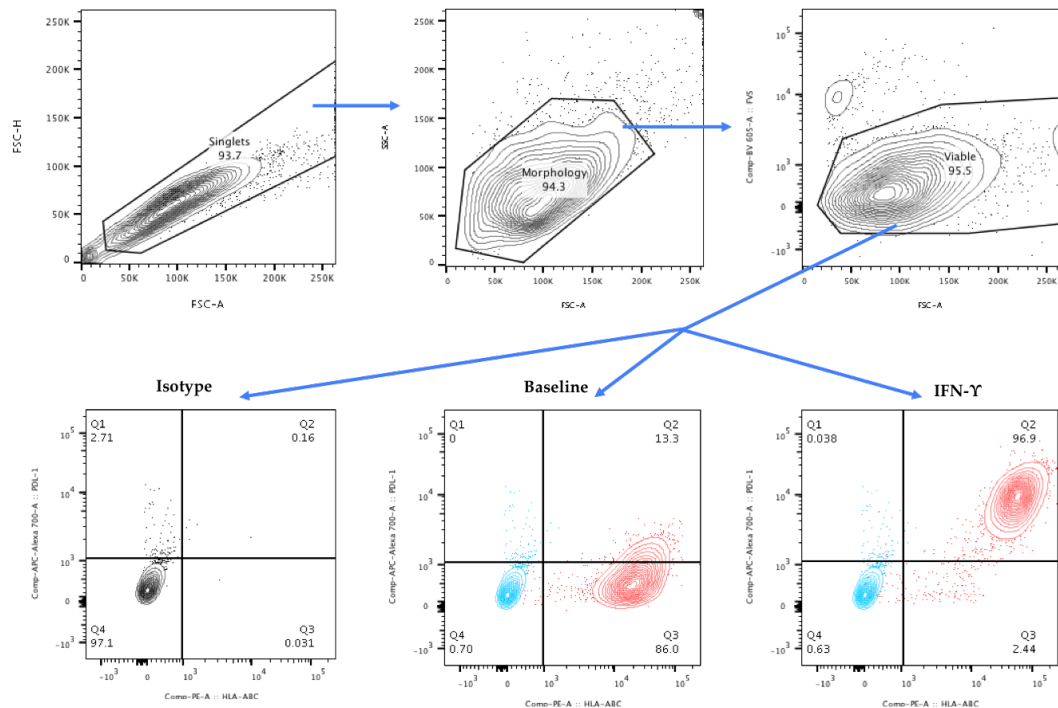


Figure 5-23 Flow Cytometry gating for assessing the expression of MHC-I and PDL1 at baseline and following IFN- γ stimulation

Following stimulation with IFN- γ , control and test cells were stained and subsequently analysed with flow cytometry for expression of MHC 1 and PDL1. They were gated sequentially on the following populations – singlets, morphology and viability before assessing expression of both MHC-I and PDL1. An isotype control was used to determine the cut-off for expression of both markers, which was assessed at both baseline (control) and following IFN- γ (100ng/ml) stimulation (Blue – Isotype, Red – Test).

Similar findings were identified in assessing the parent tumour specimens stained for anti-PDL1 with IHC. The parent tumour of P1 had only stromal evidence and no tumoural expression of PDL1, with a plausible explanation offered above. P3 had more widespread expression of PDL1 with greatest intensity at the invasive margin, suggesting a low-level constitutive expression with an inducible component at the interface with the stromal microenvironment. P2, LRF1 & 2 appeared to have a patchy distribution of PDL1, consistent with an inducible expression.

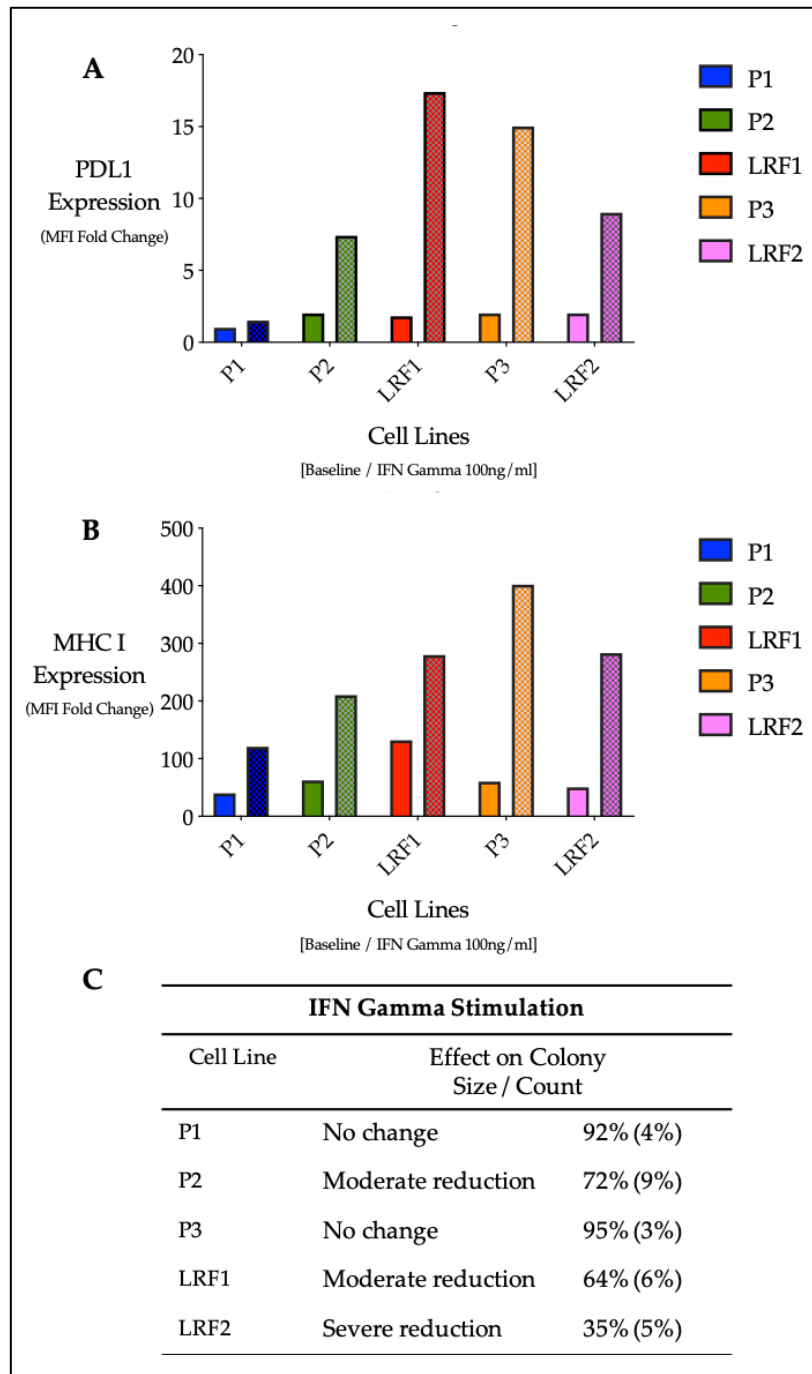


Figure 5-24 Modulation of PDL1 and MHC I Expression and Direct Cytotoxic Effects of IFN- γ

The effect of IFN- γ on the expression of PDL1 and MHC-I and its direct cytotoxic effect across the panel of human ASCC cell lines. Expression of PDL1 and MHC-I assessed by flow cytometry at baseline (plain bar) compared with up-regulated expression (hashed bar) in the presence of IFN- γ after 48 hours exposure is presented in A) and B) respectively. Results are expressed as the log-fold change of the MFI in relation to the respective isotype control. C) Direct cytotoxic effect of IFN- γ represented by changes in colony size (qualitative) and count (mean percentage of control $\pm$ SEM) in comparison to control.

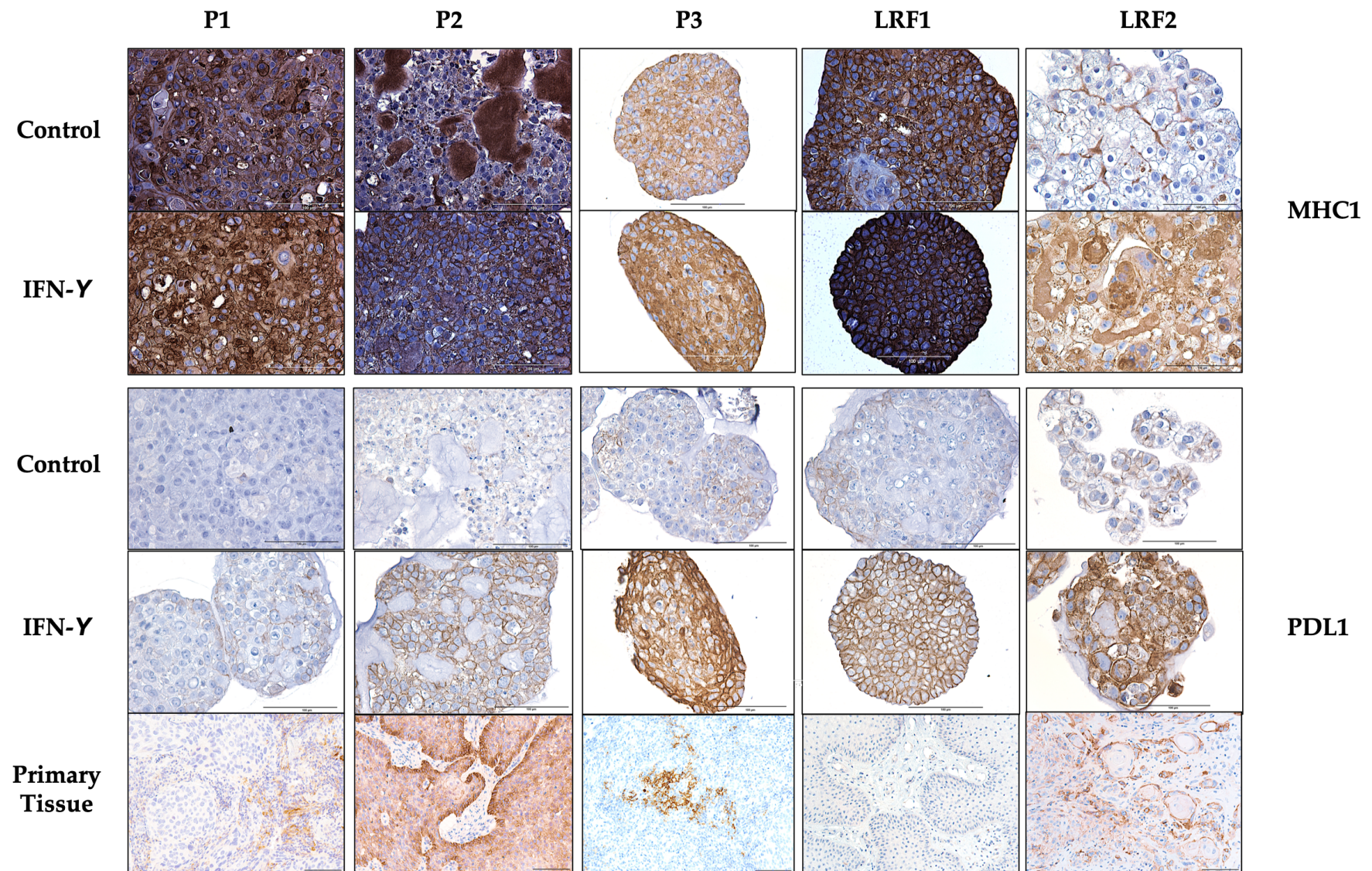


Figure 5-25 IFN-gamma effect on CL Tumouroid MHC I & PDL1 Expression, compared to parental tumour PDL1 expression

The panel of human ASCC cell lines were passaged as tumouroids and subsequently exposed to IFN- γ for 48 hours. They were subsequently fixed, embedded, sectioned and stained by IHC for MHC I and PDL1 expression at baseline (control) and following 48-hours exposure to IFN- γ (haematoxylin counterstain). Bottom row demonstrates the parent human ASCC tumour tissue PDL1 staining for comparison (Scale bar 100 μ m).

While MHC-I down-regulation can be utilised by some cancers as a means of immune evasion, this does not appear to be the case in this cohort of five tumours. MHC-I expression at baseline was substantial across all cell lines. In the presence of IFN- γ , there was significant up-regulation of expression, as demonstrated in both the FACS and IHC results. Consequently, the MHC-I expression would have allowed appropriate presentation of tumour specific antigens and therefore engagement of the adaptive arm of the immune system if it were appropriately primed. Given the poor outcomes of both LRF 1 & 2, other tumour and host immune and molecular factors may have been at play in the treatment resistance of both of these tumours.²⁵⁵

IFN- γ also appeared to have a direct effect on the viability and growth of several of the cell lines, a known effect of IFN- γ .²⁵⁶ This effect can be of significant importance *in vivo* and particularly in the setting of radiotherapy, with IFN- γ being one of the key arbiters in inducing the anti-tumour effects of radiation.²⁵⁷ Both P1 & P2 demonstrated no significant changes in colony number or growth in the presence of IFN- γ , however, P3 & LRF1 demonstrated a moderate reduction and LRF2 a severe reduction in both the number and size of colonies. Given the poor outcomes of the patients from which LRF1 & 2 were derived, this suggests a poor Th1 immune response may have been a significant contributor to the failure of treatment in both these individuals.

5.3.17 *In Vitro* Cytotoxicity and Targeted Therapy Assays

The sensitivity of the panel of ASCC cell lines to the two chemotherapeutic drugs that are used in the primary treatment of patients with ASCC was assessed. Both relapsed patients from whom LRF1 and LRF2 were derived had been exposed to both drugs prior to the establishment of the cell lines. The three primary lines were all derived from treatment naïve patients.

The 5FU dose-response curves and representative GI₅₀ for each cell line are presented in Figure 5-26 A. This revealed that both relapsed lines demonstrated substantial resistance to 5FU. Amongst the three primary lines, there was a similar profile of sensitivity. P1 had the highest GI₅₀ of the 3 primary lines, however LRF2 and LRF1 had a GI₅₀ that was 16 and 7 times higher respectively than P1. The maximum plasma concentration for 5FU has been documented as 60-200ug/ml, which is far above the GI₅₀ of even LRF2, however, the plasma concentration does not necessarily reflect the concentration at the site of the tumour.

Head and neck SCC and cervical SCC have many similarities to ASCC, in terms of the genomic profile of the disease, the presence of HPV positive and negative subsets, and the treatment involving chemoradiotherapy with the option of surgery.²⁵⁸ Consequently, the use of published head and neck or cervical SCC cell line data is useful as a comparator in the absence of other ASCC lines.

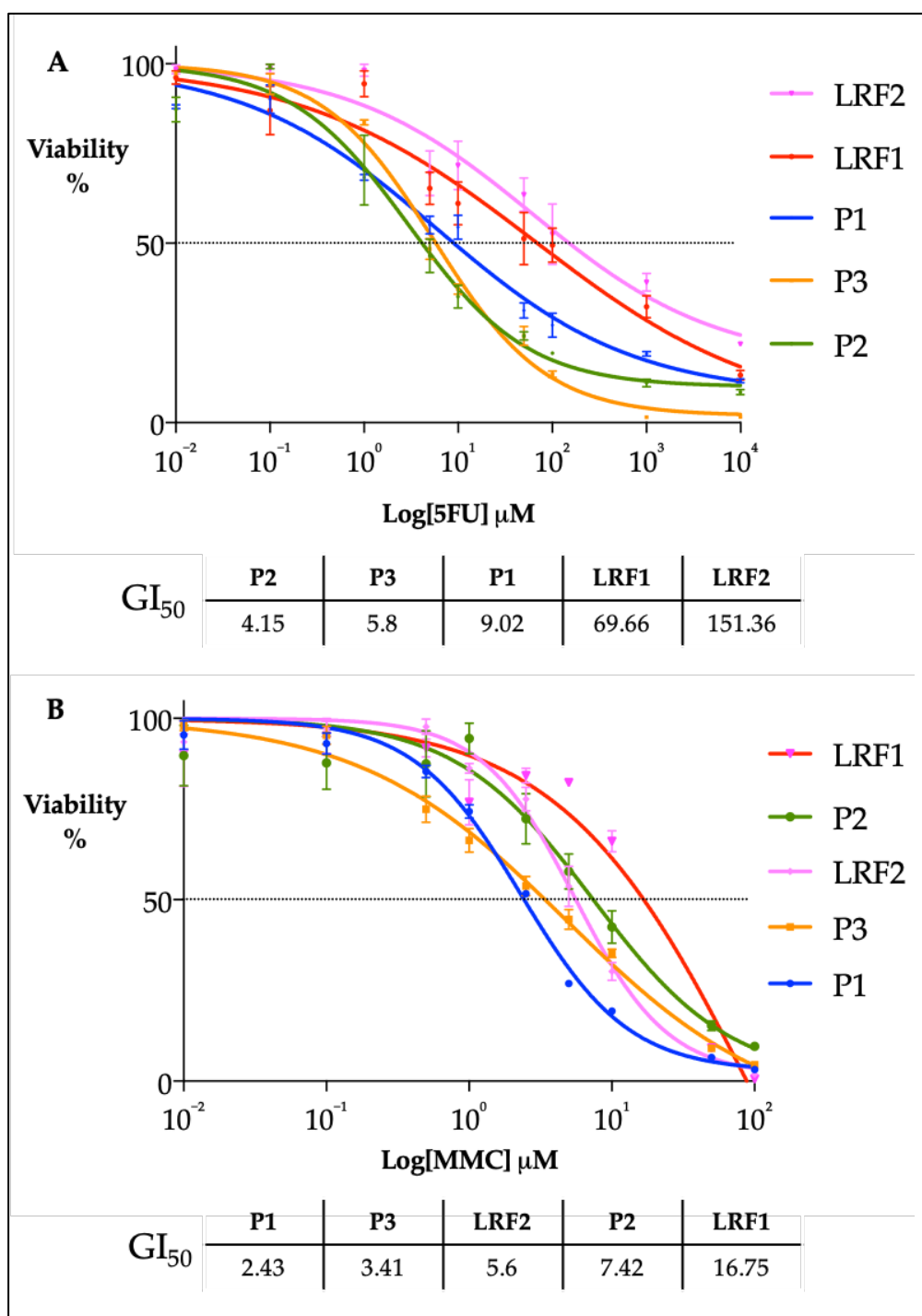


Figure 5-26 In Vitro 5FU and MMC Cytotoxic Assays

Dose-response curves for single agent 5-FU (A) and MMC (B) treatment of the panel of ASCC cell lines. Cells were seeded at densities of 1×10^4 cells/well (P1, P2, P3) or 5×10^3 cells / well (LRF 1 and 2) and treated with a range of concentrations of both drugs for 96 hours, and the viable cells quantified by AlamarBlue® Assay. Both demonstrate dose-dependent inhibition of cell proliferation and viability. The data represented are the mean $\pm$ SEM for three experiments. The GI_{50} are presented in the table below. The two lines developed from relapsed patients demonstrated the greatest resistance to 5FU, while all lines and particularly LRF1 demonstrated resistance to MMC.

In comparison to a published series of head and neck SCC lines, the spectrum of sensitivity by comparing dose-response curves is similar, however IC_{50} has been utilised instead of GI_{50} for comparison²⁵⁹. GI_{50} is the absolute IC_{50} , which results in 50% cell death. The IC_{50} however measures the concentration which gives 50% of the maximal response. Consequently, for a resistant line, where near 100% cell death is not reached, the IC_{50} is not measuring the same endpoint. As long as at least 50% cell death is achieved, the GI_{50} can be calculated and acts of a measure of the same endpoint across the cell lines. This requirement was met by the panel of ASCC lines, and consequently the GI_{50} represents the concentration of 5-FU leading to 50% of cell death.

The MMC does response curves and GI_{50} values are represented in Figure 5-26 B. Again, LRF1 demonstrated a much higher GI_{50} than the primary lines, while LRF2 had a GI_{50} within the range of the primary lines. However, in comparison to a panel of head and neck SCC cell lines, the entire panel of human ASCC lines demonstrated substantially increased GI_{50} values.²⁶⁰ This may have related to the activity of the mitomycin C, or alternatively the conditions under which the assay was undertaken.

Two molecular targeted therapies were also assessed based on the genomic landscape of ASCC. Given the frequency of *PIK3CA* mutations and PI3K aberrations in ASCC, assessment of sensitivity across the panel of cell lines to the *PIK3CA* p110 α specific inhibitor BYL719 (Alpelisib) was undertaken with results and the GI_{50} data listed in Figure 5-27.

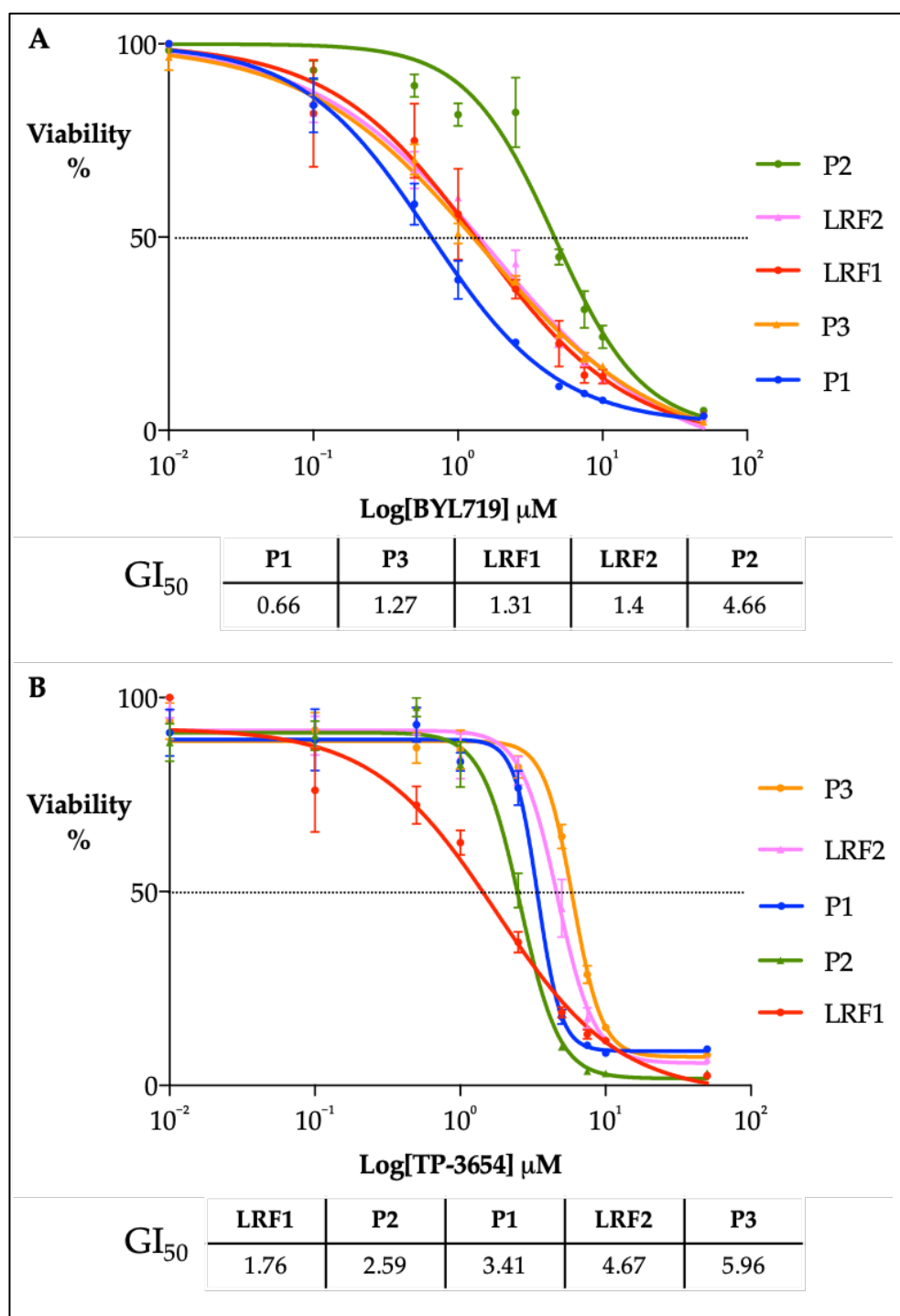


Figure 5-27 *In Vitro* BYL719 and TP-3654 Molecular Targeted Therapy Assays

Dose-response curves for single agent BYL719 (A) and TP-3654 (B) treatment of the panel of ASCC cell lines. Cells were plated at densities of 1×10^4 cells/well (P1, P2, P3) or 5×10^3 cells / well (LRF 1 and 2) and treated with a range of concentrations of both drugs for 96 hours, and the viable cells quantified by AlamarBlue® Assay. Both demonstrate dose-dependent inhibition of cell proliferation and viability. The data represented are the mean $\pm$ SEM for 3 experiments. The GI_{50} are presented in the table below. P2 demonstrated significant resistance to BYL719, with LRF2 and P3 demonstrating resistance to TP-3654.

This revealed that four of the five cell lines demonstrated a similar GI₅₀ of 0.66 – 1.4, which is in the sensitive to intermediate range based on previously published head and neck SCC lines and *PIK3CA* mutant breast cancer cell lines.^{261, 262} Of the four sensitive lines, P3 has a *PIK3CA* mutation, and P1 and LRF1 had copy number gains at the *PIK3CA* locus (Figure 5-9), providing an explanation for sensitivity to this drug. The viral negative line LRF2 however had no evidence of upregulation or mutation of *PIK3CA* to explain its intermediate sensitivity. The primary line P2, demonstrated a 3-fold higher GI₅₀ than the next highest GI₅₀ (LRF2) of the human ASCC lines, falling in the range of resistance based on previously published results.²⁶¹ This was despite having evidence of copy number gain at the *PIK3CA* locus on WES (Figure 5-9). An explanation for this resistance is offered by the findings of copy number gain of *FGF3* and *FGF10* (Figure 5-9), both of which have been defined as resistance mechanisms to BYL719 previously in breast cancer.²⁶³

The last molecular targeted therapy to be assessed for efficacy was TP-3654, a PIM1 inhibitor with some PI3K inhibitor activity.²⁶⁴ Previous RNA sequencing in ASCC samples had identified PIM1 as over-expressed in patients with locally advanced disease.²⁶⁵ However, WES data on the panel of ASCC lines failed to identify a mutation or copy number variation at this locus. When the drug sensitivity results are compared to published data relating to urological cell lines, LRF1 and P2 fall within the sensitive range, however, the remaining three lines appear to fall into the category of being resistant based on their GI₅₀ values.²⁶⁴ An explanation for these findings may be further identified with RNA sequencing.

5.3.18 Radiotherapy SF2 and Dose-Response Curve

The sensitivity of the panel of human ASCC cell lines to radiotherapy was assessed by determining the surviving fraction across 2, 4, 6, 8 and 10 Gy of

radiotherapy. The seeding density was optimised as outlined in Table 5-5. The lines were grown for variable times, LRF2 for 9 days, LRF1 and P3 for 14 days and P1 and P2 for 18 days, until they had reached the appropriate colony size. The surviving fraction of 2 Gy (SF2) and the dose response curves derived from the linear quadratic equation [$SF(D)=e^{-\alpha \cdot D - \beta \cdot D^2}$] are displayed in Figure 5-28 A and B.

Table 5-5 Seeding density for 6-well plate radiotherapy clonogenic assay

RTx Dose (Gy)	Seeding Density (x 10 ³)				
	P1	P2	P3	LRF1	LRF2
0	4	30	2	0.5	0.5
2	4	60	2	0.5	0.5
4	8	60	4	1	1
6	16	120	8	8	2
8	32	240	16	16	4
10	32	240	16	32	4

The linear quadratic model describes the surviving fraction (SF) of clonogenic or stem cells as a function of radiation dose (D).²⁶⁶ The α term describes the linear component of the curve while the β term describes the quadratic part of the curve. Consequently, the cell death resulting from the α component increases linearly with dose compared with the cell death from the β component, which increases in proportion to the square of the dose. The α and β therefore represent the intrinsic radiosensitivity of the cells, with a higher α and β indicating increased sensitivity to radiation. The ratio of both parameters α/β is a measure of the fractionation sensitivity, with a higher ratio indicating less sensitivity to the effect of fractionation. While there are several models to describe the radiobiological response, the linear quadratic has been best validated by both experimental and clinical data.

These data demonstrate the HPV negative line LRF2 to be the most resistant to radiotherapy in terms of SF2 and across increasing doses. This is consistent with the theory that non-virally driven ASCC tumours are more radio-resistant, explained by the high incidence of mutant p53 mutations in this cohort.¹⁶⁶ This is in keeping with the sequencing data on LRF2 which has confirmed a truncating and separate missense gain of function TP53 mutation. LRF1, derived from a treatment resistant tumour, was however found to have a reasonable sensitivity on assessment of the SF2 and exquisite sensitivity at increasing doses, with a consequent very low α/β ratio. While this patient eventually succumbed to their disease, had this knowledge been to hand at the time of their treatment, an evolving technique such as stereotactic ablative radiotherapy (SABR) may have demonstrated clinical utility where no other option was available.

The curves and α/β ratios had some similarity across the three primary HPV positive tumours, however, assessment of the SF2 for these three lines demonstrated some differences in radio-sensitivity. The line P3 was found to have an SF2 of 0.56, lying not far below that of LRF2 (0.58). Consequently, while intrinsic tumour radio-resistance may be a factor contributing to the clinical outcome of the patient, treatment response or failure is also likely contributed to by other non-tumour factors including the stroma and micro-environment.

Performing a comparison between the clinical α/β ratios of human tumours and cell lines is difficult, with a wide range of values when treating a particular human cancer type across individuals. This has been well exemplified in a recent publication, demonstrating in a systematic review of the literature that there is a very large amount of heterogeneity in the reported values within and between studies even when they are stratified by tumour site and tumour histology.²⁶⁶ When the SF2 of the four human ASCC lines is compared to published data on cervical SCC lines, the range of SF2 values is similar for the HPV + lines. With

the only cervical HPV- SCC line having an SF2 of 0.47, which lies below that of LRF2 (0.58). When compared to HNSCC lines, the four HPV+ HNSCC lines assessed had a lower SF2 (0.18-0.27) than the ASCC HPV+ lines (0.24-0.56).²⁶⁷ The HPV- HNSCC lines assessed had a wide range of SF2 values (0.33 – 0.80) with LRF2 sitting midway within this range.²⁶⁸ The HPV –ve HNSCC lines clearly demonstrated a higher median SF2 than the +ve lines assessed, in keeping with the trend identified in the ASCC data.

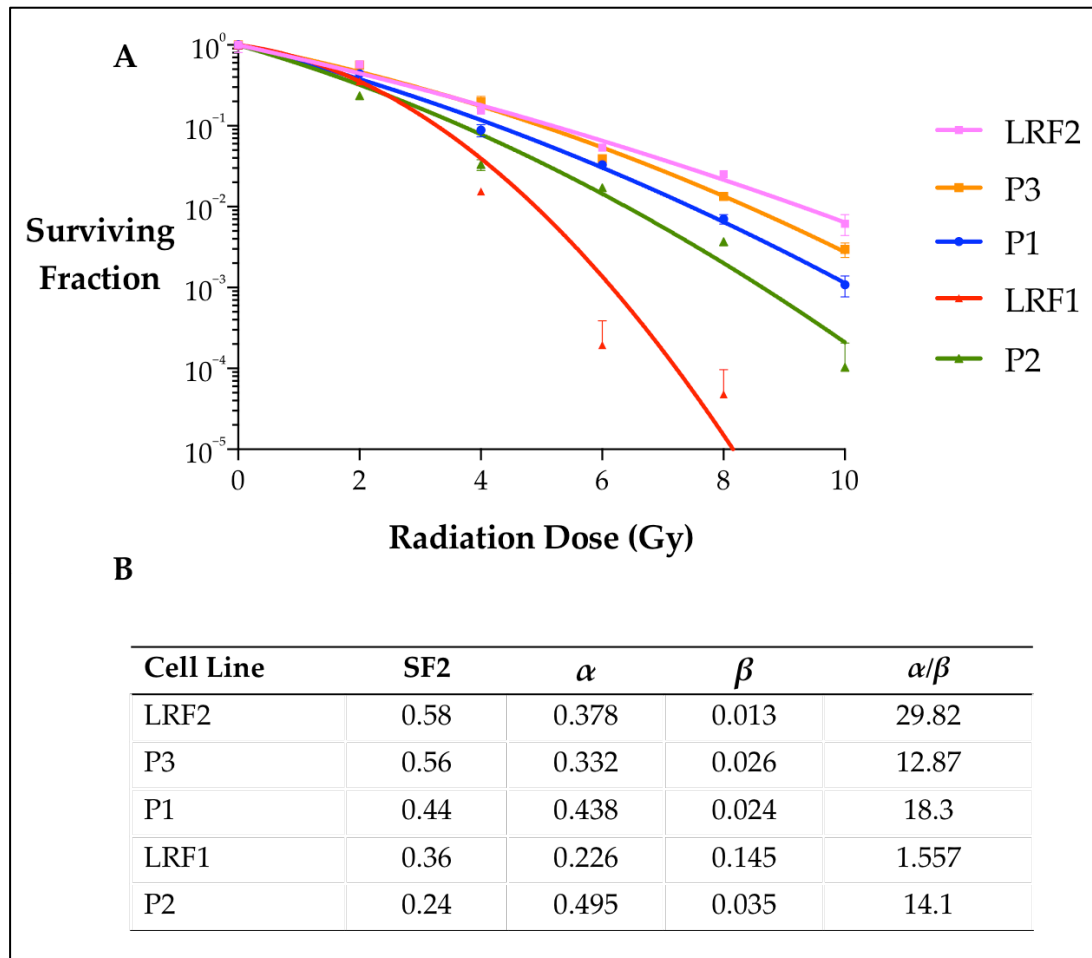


Figure 5-28 Radiotherapy SF2 and Dose-Response Curve for Human ASCC Lines

Radiation survival curves demonstrating the survival fraction (log10) following irradiation with 2, 4, 6, 8 and 10 Gy for the panel of 5 human ASCC cell lines. Data points are mean +/- SEM for 3 experiments. Data points are fitted with the linear quadratic equation A). The table (B) displays the SF2 for each line and the α and β values and α/β ratio for each line to better characterise the radiobiology of the lines and facilitate comparison with published data.

5.3.19 TIL Expansion

The expansion of TILs from the parent tumours of three of the cell lines was possible. All three of these TIL populations were utilised to perform the co-culture cytotoxic assay (section 2.2.5). However, one patient had adequate TILs remaining after the assay that could be maintained in culture. This TIL population was then subsequently phenotyped with flow cytometry for a limited panel of immune markers to compare with the TILs defined from phenotyping the original tumour. These results are demonstrated in Figure 5-29 and reveal a significant change in the proportion of immune cells. Of greatest significance, the large number of CD45+CD3-CD56- cells dramatically diminished in culture and were replaced by CD45+CD3+ cells. There was also perturbation in the CD4:CD8 ratio, which was pushed higher by increasing the CD4+ cells and reducing the CD8+ cells.

An explanation for this finding is the preferential effect of expanding T cells in the presence of IL2 and particularly CD4+ cells. It also highlighted the importance of undertaking the co-culture cytotoxic assay as early after TIL expansion as possible.

5.3.20 CL Tumouroid and TILs Co-Culture Cytotoxic Assay

The establishment of CL tumouroids and the expansion of matched tumour infiltrating lymphocytes allowed further exploration of the immune systems role in tumour control in a subset of the human ASCC cell line panel.

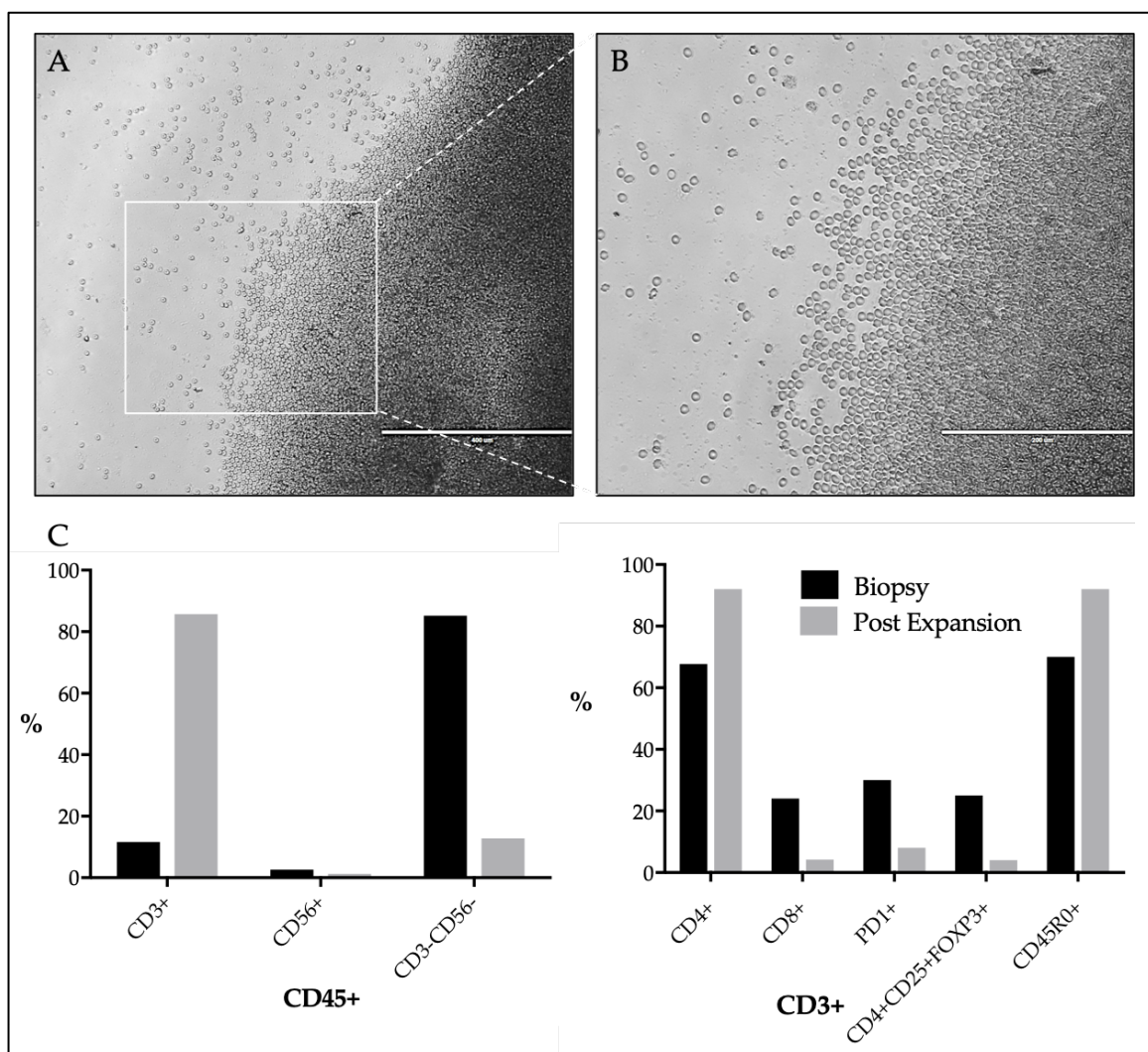


Figure 5-29 TIL expansion and alteration in the phenotype after 3 weeks in culture – LRF1

Primary tumour explants are plated in a 24-well plate with RPMI-1640 + 10% HS + IL2. A) and B) demonstrate the expansion of TILs with IL-2 from a tumour explant with phase contrast photomicrographs at x10 objective (scale bar 400µm) and x20 objective (scale bar 200µm) respectively. C) TILs extracted from the primary tumour and those expanded over three weeks in culture were fixed and stained for a panel of immune markers and subsequently analysed by flow cytometry. This demonstrated an alteration in TIL subpopulations (expressed as a percentage of the parent population, CD45+ in left image and CD3+ in right image) from the initial tumour sample compared with expansion at 3 weeks. This includes the outgrowth of CD3+ cells with the loss of CD45+ DN cells. The majority of the CD3+ cells expanded were CD4+ with a subsequent reduction in the CD8+ population.

The process for undertaking the assay is defined in 2.2.5 which we have recently published²⁶⁹ and a schematic of the process is demonstrated in Figure 2-2. The

co-culture assay was undertaken on 3 of the 5 ASCC cell lines. The remaining two could not be performed, because both patients had no remaining primary tissue from which to extract TILs.

The results of the three assays are demonstrated in Figure 5-30. Increasing the TIL to tumouroid cell number ratio from 1:1 to 5:1 to 10:1 did not have any additional influence on the tumouroid killing out to 24 hours as demonstrated in panel A. To maintain consistency, a TIL : tumouroid cell ratio of 10:1 was utilised for the other two experiments. TILs expanded from the two relapsed samples (cryopreserved) matched to the lines LRF1 and LRF2 both demonstrated minimal cytotoxic effect when compared to the TILs expanded from the primary complete response patient (cryopreserved tissue) from which P1 was derived. These data are demonstrated in Figure 5-30 C. Fluorescent phase contrast images are demonstrated in Figure 5-31 to depict the dramatic difference in TIL induced cytotoxicity between the P1 and LRF1 lines with matched TILs.

For the relapsed line LRF2, given the poor killing and the known baseline and inducible expression of PDL1, an anti-PD1 antibody (Keytruda, hAnti-PD1, Merck) was utilised as a separate experimental condition to determine whether the TIL cytotoxic function could be increased. The results demonstrated in Figure 5-30 B revealed that there was no change in killing compared with the TILs + tumouroids alone condition. Despite adequate TILs to perform the co-culture assay, an inadequate number of immune cells were extracted from the initial human sample designated to FACS, which precluded the ability to assess the PD1 status of the TILs. This would have been useful information, as a lack of PD1 expression may explain why the TILs had no response to the anti-PD1 antibody.

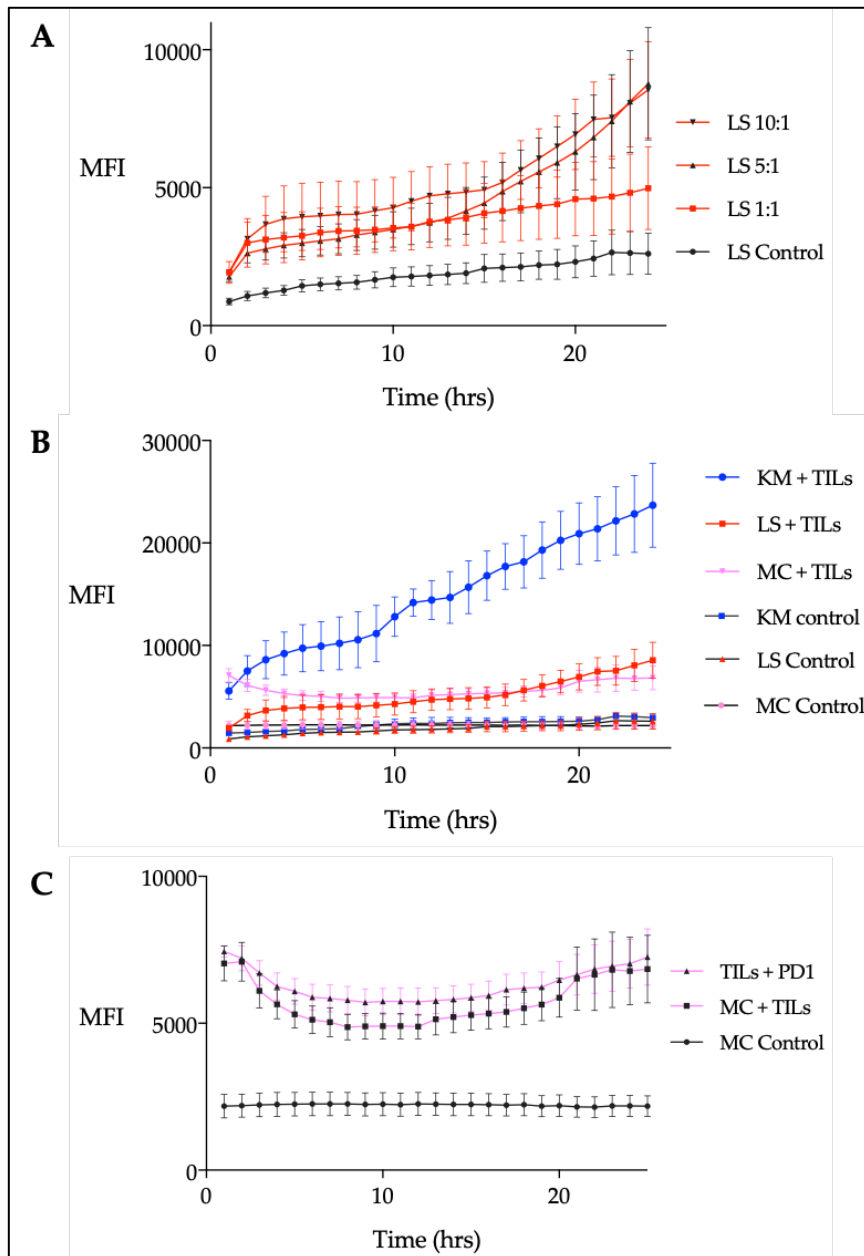


Figure 5-30 CL Tumouroid – TIL Co-Culture Cytotoxic Assay

TILs were expanded from cryopreserved parent tumour biopsies in the presence of IL-2 and then co-cultured with matched CL tumouroids in the presence of PI over 48 hours, to allow the Mean Fluorescence Intensity (MFI) of individual tumouroids to be measured as a surrogate marker of tumouroid death (Mean $\pm$ SEM from 10 tumouroids from a representative experiment) A) TIL to tumouroid cell ratios of 1:1, 5:1 and 10:1 were assessed to determine the effect on cytotoxicity. There was no significant difference between these conditions out to 24 hours. B) An anti-PD1 Ab was assessed for efficacy in the TIL:Tumouroid co-culture assay (10:1 ratio) of LRF2. There was minimal killing both in the absence and presence of an anti-PD1 Ab. C) The three CL tumouroids with matched TILs are plotted on the same axes, demonstrating the significantly greater cytotoxicity in P1's assay compared with the two relapsed patients' assays. (MFI $\pm$ SEM is represented across 8 tumouroids per condition)

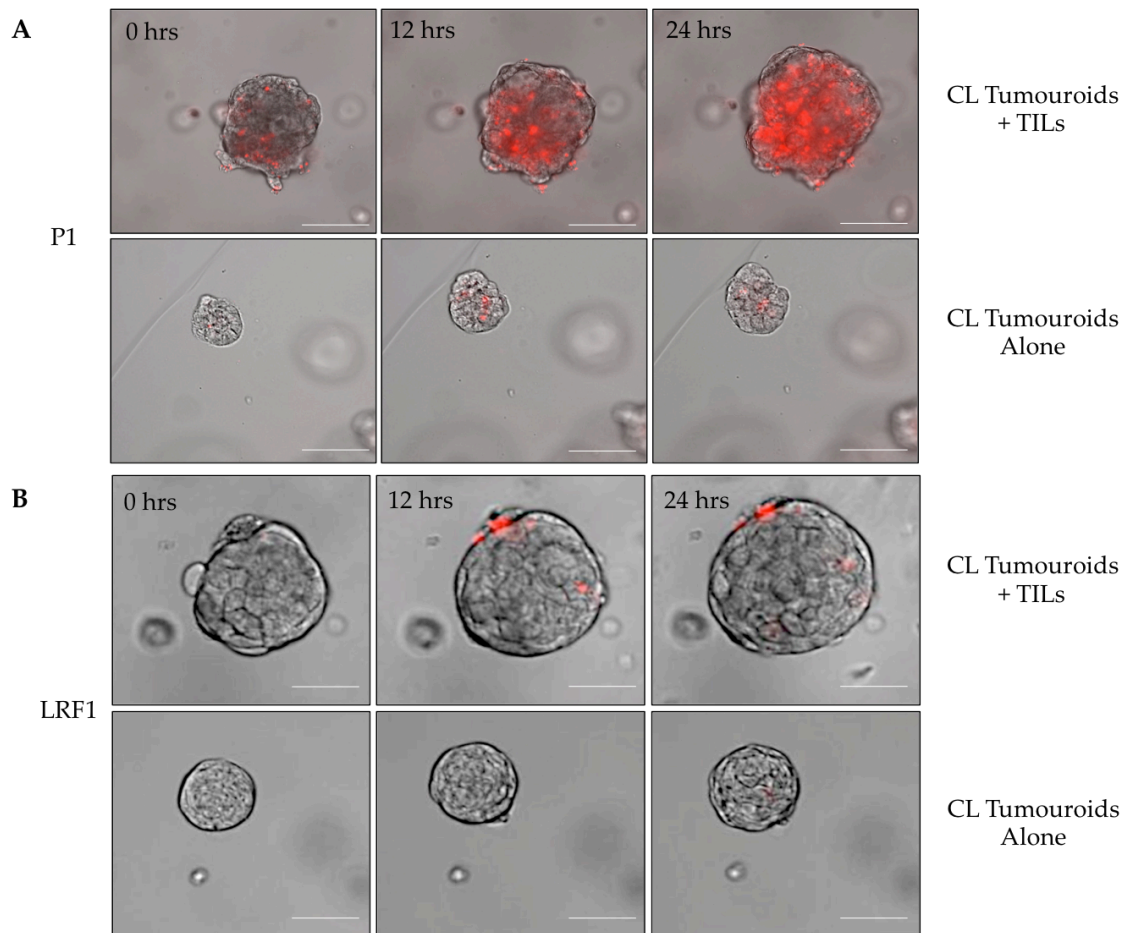


Figure 5-31 TIL-Tumouroid Cytotoxic Assay Fluorescent Phase Contrast Images

*The assay as outlined in **Figure 5-30** with images taken at set time-points The PI in the co-culture media is used to indicate cell death by the red flare. CL Tumouroids co-cultured with matched TILs from the parent tumour demonstrating dramatic killing over 24 hours for P1 (A) compared with the limited killing above the level identified in the negative control (CL Tumouroids alone) for LRF1 (B) (scale bar 100µm).*

5.4 Discussion

This chapter outlines the establishment, validation and characterisation of the world's first panel of human ASCC cell lines. As a new resource, they begin to address the hiatus in the absence of preclinical models for this disease. Consequently, they will likely form a useful platform upon which further investigation into the biology of ASCC can be undertaken, and novel therapeutics assessed.

The panel of cell lines are representative of the spectrum of both disease stage and treatment response, with two lines importantly established from treatment resistant relapsed tumours. Of greatest significance, one of the relapsed lines represents viral negative disease with mutant TP53. This line represents an *in vitro* model of the most resistant form of the disease that will become the predominant type encountered in future decades, once HPV vaccination has taken effect.

The establishment of the squamous cell lines presented a significant challenge, particularly with biopsies taken from primary pre-treatment tumours. This was partly due to the limited size of the tissue obtained but was also a reflection of the intrinsic characteristics of the cells, which were susceptible to anoikis and, consequently, failure in establishing a line. This may also relate to the high incidence of HPV positivity amongst ASCC samples. Despite more than 70% of HNSCC being HPV related, there are only 17 HPV + cell lines available compared to over fifty HPV negative lines.²⁷⁰ Furthermore, in cervical cancer, only 8 HPV positive lines are available and in penile, all reported cell lines are HPV negative.²⁷⁰ This suggests that HPV + tumours are a more challenging group from which to establish cell lines. Consequently, the establishment of 5 cell lines from

9 samples with adequate tissue (56%), four of which are HPV+, represents a reasonable success rate.

An explantation technique was utilised, in an attempt to reduce the risk of anoikis and maintain stromal cell support during the early phases of evolution. This is in contrast to the more classical method of tumour digestion with plating out of the resultant cell suspension. Numerous other factors were modified and optimised in order to streamline the process and improve the success rate, with the technique having now been applied successfully to 6 other cancer types (oesophageal, colorectal, penile, merkel, adenoid-cystic and carcinoma of unknown primary).

Characterisation of the panel of human ASCC cell lines included performing WES, once all lines had been fully established and validated. This allowed for an in-depth assessment of the SNVs, CNV, mutational load and trinucleotide signature of the lines. This revealed that many of the SNV's were shared with those reported in the literature, with several new SNV's identified in two of the anal SCC lines, increasing the likelihood of these aberrations being a true reflection of a genomic event in human ASCC.

The SCNA also revealed the cell lines to share genomic losses and gains similar to those previously reported in ASCC. The mutational load of the lines was also similar to that reported in the literature, with the exception of P1, which was found to lie at the high end of the spectrum but within the reported range. The trinucleotide signatures were also found to be consistent with a previous report, dominated by an association with age (signature 1) and the HPV+ lines demonstrating the APOBEC signature, proposed to be secondary to viral infection.

Matched parent tumour of the cell lines was also sequenced as a comparator for the cell lines. Unfortunately, the remaining primary tumour tissue at the time of sequencing was limited both in size and in terms of the squamous carcinoma component, with most having predominantly stroma and surrounding normal tissue remaining. Consequently, the results of the SNV's and CNA demonstrate that the parent tissue, particularly for P1 and LRF1 contain very few aberrations and minimal CNVs, consistent with it being of low purity.

Further characterisation of the panel of human ASCC cell lines, demonstrated the appropriate features of squamous cancers both *in vitro* and *in vivo*, including cytological morphology, cytokeratin expression and histological architecture. They were confirmed free of contamination from mycoplasma, and STR analysis demonstrated appropriate conservation across the 10 assessed loci with each line's respective parental tumour. Thorough HPV genotyping revealed four lines to be positive, harbouring HPV 16 alone, which has been identified as the most frequent finding in ASCC tumours.¹⁵⁴ Furthermore, it confirmed the absence of all HPV sub-types in the TP53 mutant line LRF2.

Further characterisation of the panel revealed that the relapsed lines had a quicker doubling time, greater seeding efficiency, proliferative rate and migration potential. They also demonstrated greater resistance to the chemotherapeutic drug 5FU. LRF1 had more pronounced resistance to MMC, with the profile of resistance of LRF2 similar to the primary lines. When assessed against the *PIK3CA* targeted agent BYL719, three lines with mutation or copy number gain of *PIK3CA* demonstrated sensitivity, while resistance of P2 was identified despite a *PIK3CA* copy number gain, which may be secondary to copy number gain of FGF3 and FGF10.²⁶³

By assessing the sensitivity to radiotherapy across the panel, it was revealed that the HPV negative line had greater resistance than the viral positive lines. Furthermore, the finding of a very low α/β ratio for the line LRF1, offers some insight into the fact that relapsed tumours may remain radiosensitive dependent on the dose per fraction. This may suggest that a novel therapy such as stereotactic ablative radiotherapy (SABR) could have provided a role in disease control for this patient in preference to palliation, had this information been to hand.

The expression of MHC1 and PDL1 was also assessed at baseline and following exposure to IFN- γ , revealing heterogeneity at baseline and with regard to response. This provides insight into the immune evasion mechanisms employed by ASCC tumours, and the direct cytotoxic effects of a Th1 tumour response. The host immune system's role in controlling ASCC was also further explored by way of the cytotoxic co-culture assay. This revealed an element of utility in predicting treatment response in the three lines assessed, with strong anti-tumour activity by TILs expanded from the primary complete response tumour compared with TILs from the two relapsed samples against their respective matched tumour cell line. The cohort of three patients alone however is too small to draw any significant conclusions, but provides a proof of principle that the anti-tumour activity of TILs can be assessed *in vitro*.

The panel of cell lines also demonstrated tumourigenicity in mice, increasing their application for the assessment of novel therapeutics across both *in vitro* and *in vivo* settings. While only *in vitro* assessment has currently been undertaken, this has provided some insight into understanding the biology of this disease and factors that contribute to treatment response. Variable sensitivity has been demonstrated to both chemotherapeutic and molecular targeted therapies across the panel of lines. This combined with the wide range of radio-sensitivity, goes

some way to teasing out the intrinsic response of individual tumours to the different treatment components that they are subjected to. Furthermore, by being able to partly explore the immune response to a subset of these tumours, another facet of tumour control and treatment response has been assessed, building a greater understanding of the multiple factors that contribute to the clinical responses in this disease.

Future areas of interest include performing an invasion assay, to further our understanding of the biology of the individual cell lines and assess for an association with the presence of lymph node metastasis. Undertaking an *in vitro* assay combining both 5FU, MMC and radiotherapy together would also better recapitulate the clinical scenario. Furthermore, undertaking CL xenograft studies to assess the tumour responsiveness *in vivo* to chemoradiotherapy will provide an ideal accompaniment to the *in vitro* data. By combining these experiments with further genomic studies, including RNA sequencing, a greater understanding will be gained of the multiple factors at play in the response of ASCC to treatment.

5.5 Conclusions

The world's first panel of human ASCC cell lines has been established, validated and characterised. They have been utilised to provide insight into the biology of both the HPV driven and viral negative forms of the disease, as well as furthering our understanding of the complex interplay of factors that determine treatment response and outcome. They now provide a new and incredibly useful preclinical resource through which we can further develop our understanding of this rare disease and assess new therapeutic options.

Chapter 6: Establishment and Characterisation of a Mouse Model of Anal SCC

6.1 Introduction

Preclinical investigation is key to further our understanding of the pathogenesis of ASCC and assess new potential therapies. The development of an appropriate murine model of this disease will create a platform to address both of these objectives. There are two anal cancer mouse models that currently exist in the published literature.^{208, 210} The greatest limitations with both of these models surround, firstly, the relevance of the genomic alterations initiating and driving carcinogenesis and, secondly, the lack of targeting of the anal canal as the origin for the cancers.

The first described murine model was based on a transgenic mouse with the HPV 16 E6/7 oncogenes linked to a K14 Cre on a FVB/N background.²⁰⁸ However, this line does not lead to the spontaneous development of anal cancer, but requires topical treatment with DMBA to induce tumours. Ras mutations are the predominant target of DMBA, but an uncommon (<2%) finding in ASCC, limiting the relevance of this model. The second model was based on a combined deletion of *tgfb β 1* and *PTEN* linked to a tamoxifen inducible K14-Cre on a mixed background.²¹⁰ The delivery of oral tamoxifen purportedly induces ASCC, however, close scrutiny of the images from this manuscript depict what appears to be gluteal skin cancers with a normal appearing anal canal Figure 6-1.

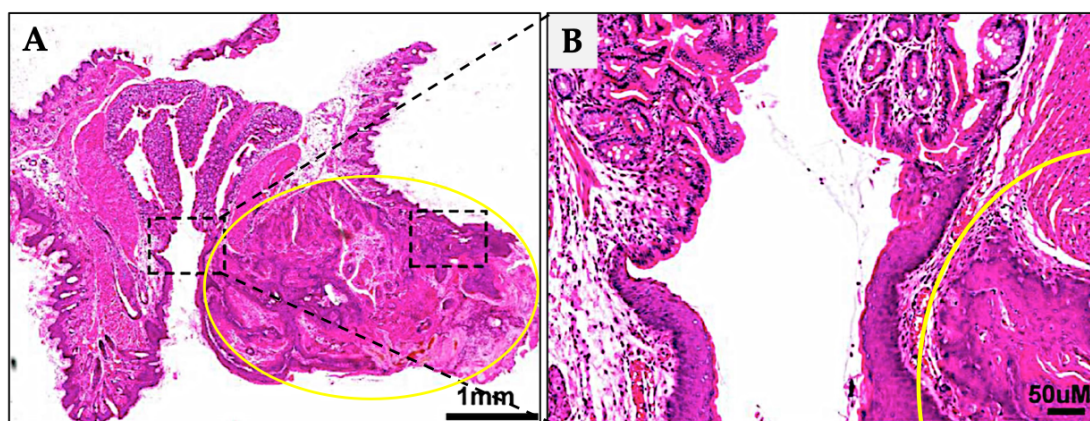


Figure 6-1 Transgenic mouse with cutaneous SCC and a normal anal canal

*H & E Images of a mouse tumour and anal canal adapted from the published manuscript of Sun et al (creative commons attribution (CC BY) license).²¹⁰ The tumours arise in a transgenic mouse with deletion of *tgfr1* and *PTEN* linked to a tamoxifen inducible K14-Cre after exposure to tamoxifen. A) demonstrates tumour formation (highlighted by yellow circle) arising from adjacent gluteal skin with the magnified image in B) revealing normal mucosa in the anal canal (yellow demonstrating tumour invading from distant gluteal skin)*

From the recently published genomic studies of ASCC,^{187, 197, 198} *PIK3CA* has been identified as the most frequently mutated gene in anal cancer and that amplification of this gene and aberrations in the PI3K pathway are a frequent occurrence in over 60% of ASCC tumours.¹⁸⁷ The PI3K aberrations are not associated with disease stage and as such are likely early events in oncogenesis. Furthermore, both Chung et al.¹⁸⁷ and Mouw et al.¹⁹⁷ identified multiple ASCC samples to harbour both a *PIK3CA* activating mutation with inactivation of *PTEN*, providing a rationale for utilising both of these genomic alterations in the backbone of my transgenic mouse model.

Given the establishment of immunotherapy as the fourth pillar in the treatment of cancer, the creation of an immunocompetent mouse model on which targeted and immune therapies could be assessed was a primary focus. Given the great volume of data and the utility of C57Bl/6 for immune studies, this was the chosen background for my mouse model.

6.2 Aims

To develop a representative mouse model of ASCC that can be used as a platform to further investigate the biology of ASCC and assess new therapeutic modalities.

6.3 Results

6.3.1 Transgenic Mice

Two genetic alterations were chosen as the backbone for the transgenic mouse to increase the potential of this model to develop invasive carcinoma based on the results of previous cancer mouse models.²⁷¹ *Pik3ca* was selected given it is the most frequent genetic alteration identified in ASCC,^{187, 197, 198} and *pten* loss was chosen given PI3K alterations resulting in overactivity of this pathway have been identified in over 60% of ASCC's. Furthermore, the finding of both mutations has been identified and reported in multiple ASCCs.¹⁸⁷

C57Bl/6 mice with a Cre-recombinase (Cre) - conditional knock-in of the *Pik3ca*^{H1047R} mutation and deletion of *PTEN* were crossed with C57Bl/6 mice expressing a tamoxifen-inducible Cre under the control of the ubiquitin C promoter (UBC-Cre^{ERT2}). This cross was performed in order to allow the genomic alterations to be inducible only on exposure to Tamoxifen. This reduced the complicating risk of developing tumours in other mouse organs.

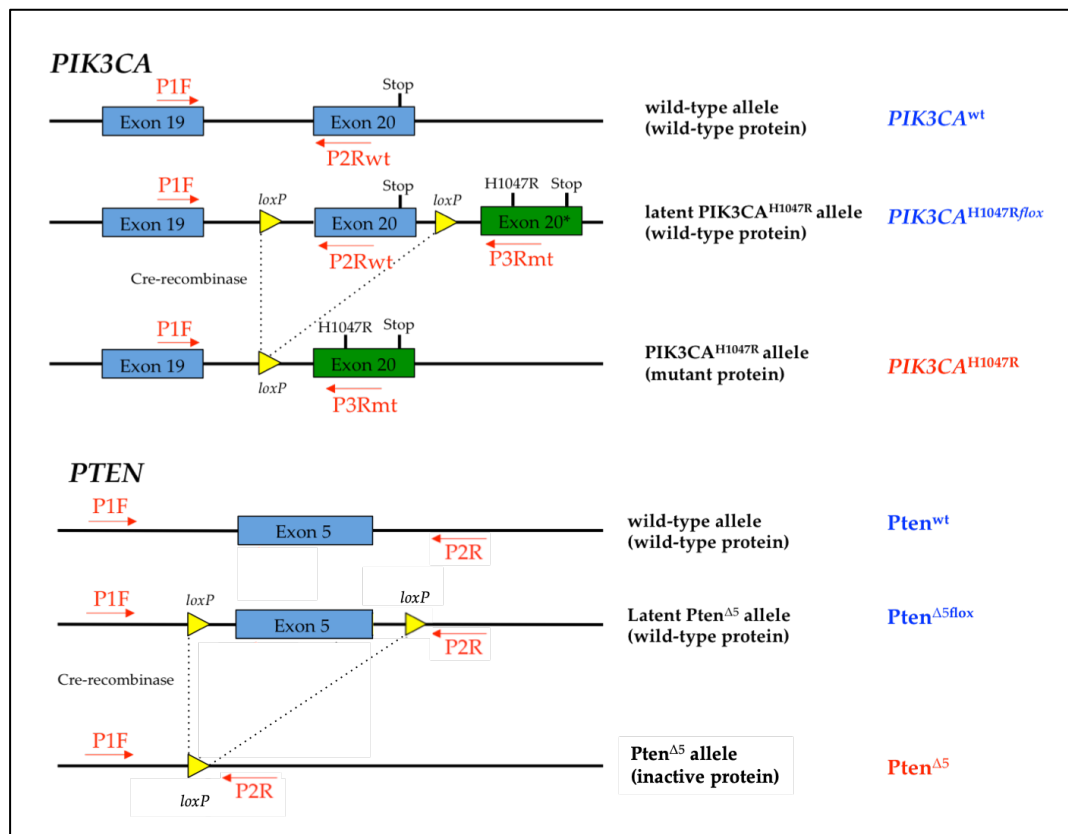


Figure 6-2 Inducible *Pik3ca*^{H1047R} and *Pten*^{flox/flox} knock-in mouse

Schematic demonstrating the location of the LoxP sites in the *pik3ca* and *pten* transgenes utilised for the anal cancer murine model. The primers (red) are for identifying transgene induction and are demonstrated at their sites of annealing (sequences listed in **Table 2-1**). The *pik3ca* primers are on cDNA (demonstrated overlying the corresponding exon regions). *Pten* primers are on genomic DNA. (P1F – Primer 1 forward; P2R – Primer 2 Reverse; P2Rwt – Primer 2 reverse wildtype, P3Rmt – Primer 3 reverse mutant)

Pik3ca^{H1047R} mice have been generated by inserting firstly *LoxP* sites (excision between *LoxP* sites mediated by Cre recombinase) on either side of the final exon (exon 20, which contains the stop codon) of the *pik3ca* (p110 α) gene and then adding a duplicate version of exon 20 harbouring the H1074R mutation (hotspot activating oncogene mutation) to one allele of the germline DNA.²¹³ *Pten* mice possess *loxP* sites flanking exon 5 of both alleles of the targeted gene (knockout of both *pten* tumour suppressor genes)²²⁶. When used in conjunction with the UBC-Cre^{ERT2} recombinase - expressing mouse strain and after treatment with tamoxifen, this generates *Pik3ca*^{H1047R} mutations and double knock-out of *pten* in exposed cells.

6.3.2 Validation of the Technique

Oral tamoxifen is absorbed and transported to the liver where it is metabolised to the active form 4-hydroxy tamoxifen (4-OHT). Given the presence of a UBC-Cre in the chosen transgenic mouse, systemic delivery would lead to widespread induction of the genomic aberrations in all mouse cells. Consequently, the active metabolite 4-OHT was utilised in order to target induction of the genomic aberrations in the mouse anal canal.

In order to avoid the possibility of inducing transgenic mutations in the adjacent rectal mucosa and anal margin skin, the 4-OHT was applied topically to the anal canal with a purpose modified viral culture swab (Figure 6-3 A, B). The viral swab was altered to have a shortened cotton tip length of 2mm beyond the shaft of the swab and sub-millimetre bristle length (radial projection). The mice had limited shaving of their perineum immediately adjacent to the anal margin skin in order to improve access without inadvertent exposure to the surrounding perineal and gluteal skin. The tail of the mouse was grasped between the left index finger and thumb and curled cephalad over the mouse to facilitate exposure of the area. The anal canal was then intubated with a P200 pipette tip two to three times to make the anal canal patulous and pout, facilitating entry of the modified culture swab and preventing accidental exposure to the adjacent perianal skin (Figure 6-3 C & D). The swab was then dipped into the 4OHT solution prior to anal intubation, with appropriate targeting of the anal canal without contamination of the adjacent anatomical regions.

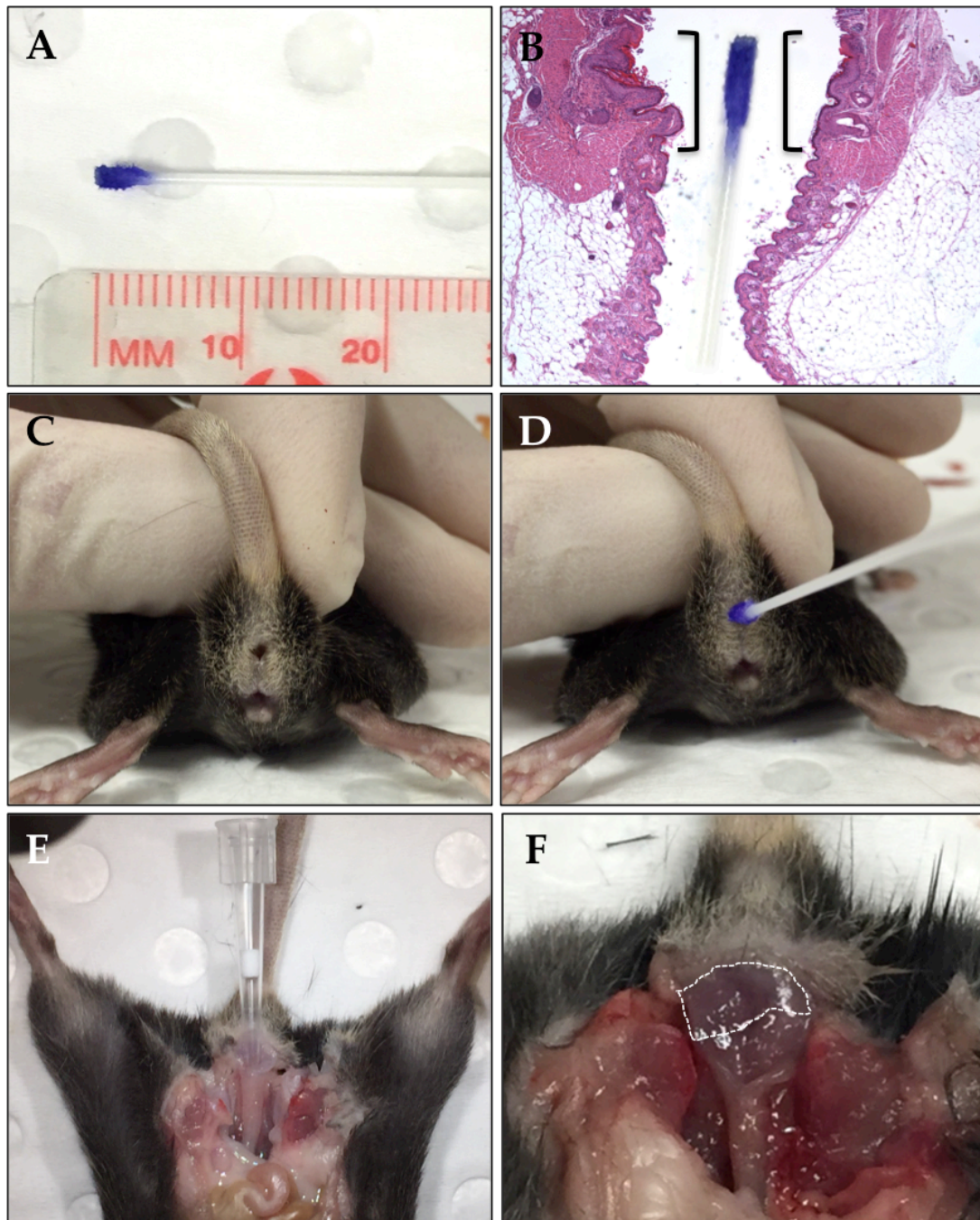


Figure 6-3 Technique for Targeted Topical Application to the Anal Canal

A) Purpose modified viral culture swab after dipping in methylene blue; B) H & E slide of mouse anal canal demonstrating the theoretical targeting with a culture swab overlaid on the image; C) Anal canal dilatation with grasping technique and following P200 pipette tip intubation; D) Technique for anal canal topical treatment in an anaesthetised mouse (methylene blue validation); E) Culled mouse after transection of anterior pelvis and with P200 pipette tip splinting anal canal to facilitate dissection; F) Culled mouse with anal canal longitudinally splayed open to display accurate blue staining of the anal canal mucosa (represented by the dotted line).

Two separate validation techniques were utilised to assess the accuracy of this technique in targeting the anal canal alone. The first validation technique involved the use of methylene blue in place of 4-OHT to stain the targeted area in anaesthetised wtC57Bl/6 mice utilising the modified viral swab. Mice were subsequently culled with the anal canal opened longitudinally in a sagittal plane for macroscopic inspection. This demonstrated that the anal canal was well targeted with minimal staining of the adjacent rectum and skin (Figure 6-3 E & F).

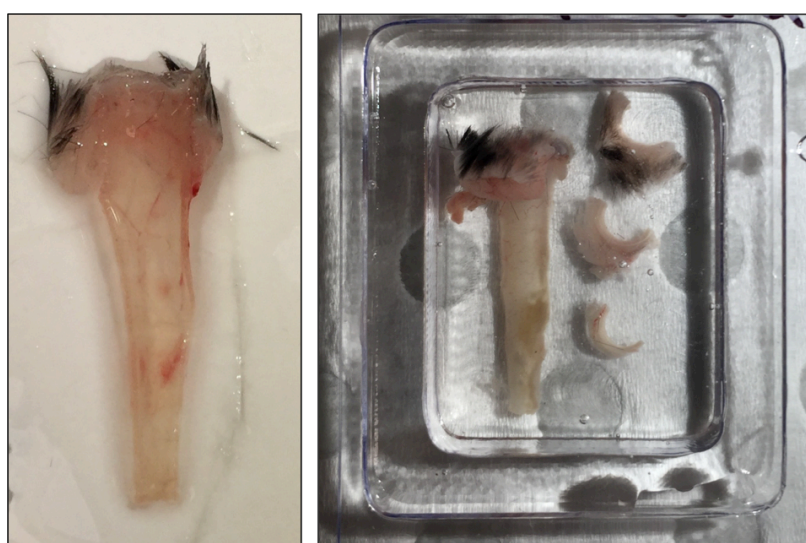


Figure 6-4 Specimen fixation, dissection and orientation for OCT embedding

*K5Cre^{ERT2}.ROSA26^{lacZ} C57Bl/6 mice had targeted application of 4-OHT to the anal canal for 3 consecutive days followed by culling 48 hours later. Fixation of the harvested anal canal and rectum was undertaken, and the specimen was then bisected in a longitudinal plane (left image) with one of the two bisected segments further sectioned serially in an axial plane before all were oriented in a cryomould and embedded in OCT medium (right image) for subsequent sectioning and Xgal staining for β -galactosidase activity (*lacZ* reporter).*

The second validation technique involved the use of K5Cre^{ERT2}.ROSA26^{lacZ} C57Bl/6 mice. These mice have a *LacZ* transgene (coding for β -galactosidase) linked to a keratin specific Cre, allowing localisation of transgene (*LacZ*) activation in the epithelium by performing X-gal staining (chromagenic substrate for β -galactosidase) on the fixed tissues of interest.²²⁸ The same technique of

targeting the anal canal employed in performing the methylene blue staining was utilised. Targeted 4-OHT application to the anal canal of anaesthetised mice for 3 consecutive days, followed by culling 48 hours post the last application. The anal canal was resected en-bloc with rectum and surrounding skin utilising a P200 pipette tip to splint open the anal canal to provide scaffolding for the dissection (Figure 6-4). The specimen was then fixed, sectioned and stained with the LacZ protocol (refer section 2.12) to confirm the area of exposure and Cre activation (Figure 6-5).

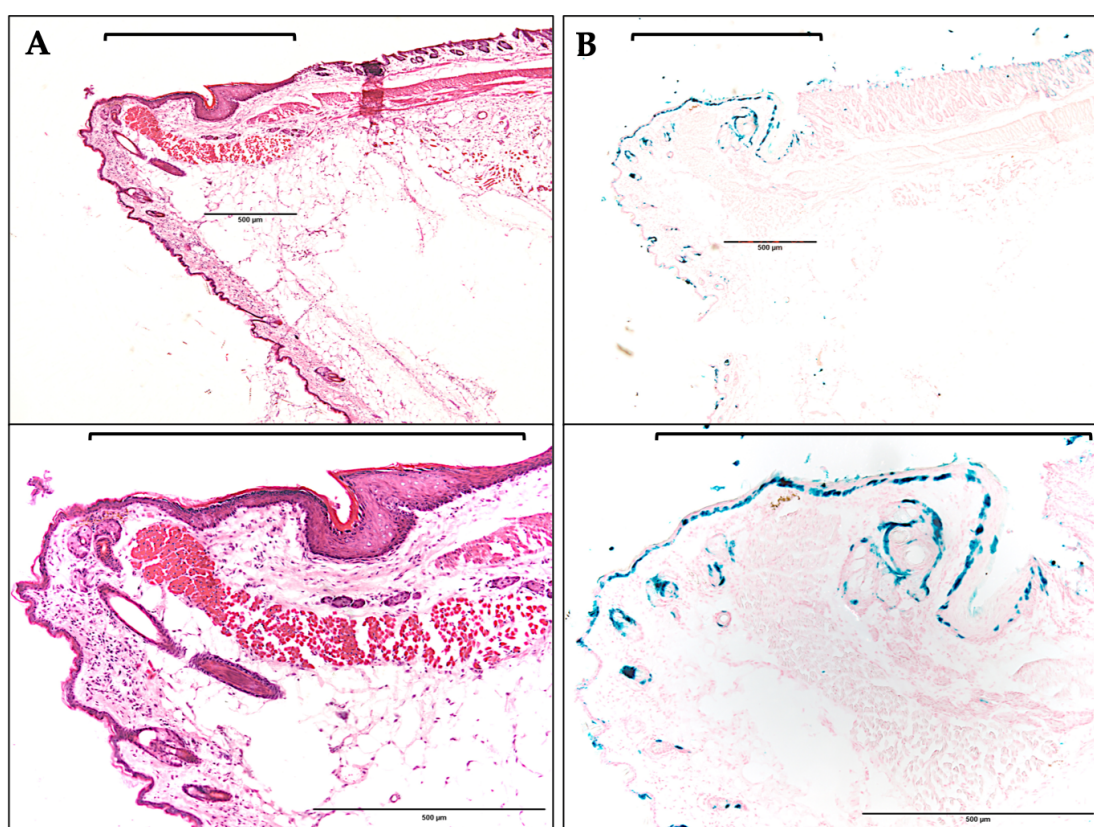


Figure 6-5 X-Gal staining to validate the appropriate targeting of the Anal Canal epithelium

Serial H & E and X-gal stained images of the anal canal of a K5Cre^{ERT2}.ROSA26^{lacZ} C57Bl/6 mouse after targeted application of 4-OHT. The anal canal was fixed and embedded in OCT media and cryosectioned with subsequent H & E (A) and X-gal (B) staining. The tamoxifen inducible LacZ transgene encodes for β -galactosidase, with X-gal being the chromagenic substrate of β -galactosidase. The blue (chromagenic) staining in the X-gal images demonstrated targeting of the anal canal with minimal exposure of the adjacent rectal and perianal / gluteal epithelium (upper images x4 objective and lower images x10 objective, scale bar 500 μ m, anal canal indicated by black calipers)

6.3.3 Treatment Cohort

Twenty-three UBC-Cre.*pik3ca*^{H1047R}.*pten*^{fl/fl} C57Bl/6 mice were treated as a cohort utilising the validated technique with 2µg / µl of 4-OHT for three consecutive daily applications. The total dose applied per dose was less than 1µl. Mice were then observed and weighed twice weekly, with all mice developing evidence of anal canal tumours within a period of 3 weeks. While a significant component of the tumour growth was exophytic out of the anal canal, the tumours reached a size where they induced partial bowel obstruction necessitating the mouse to be culled as early as three weeks after the last dose of topical 4-OHT.

The anal tumours harvested from these mice were subsequently resected, rinsed in RPMI tumour wash and dissected into segments that were processed for 1) fixation and blocking and 2) passaging and engrafted to the back of both immunocompromised NSG and immunocompetent wtC57Bl/6 mice. Fixation and blocking, and passaging was undertaken as previously described (2.4.1 and 2.15.4). Engraftment was successful with tumour growth occurring within weeks of implantation, with the resultant tumours being fixed, blocked and re-passaged.

Macroscopically, the tumours began to become dark in colouration and frequently blood filled in stark contrast to the original anal tumour and all other human ASCC specimens and xenograft tumours. When H & E slides were assessed, evidence of squamous carcinoma was evident, however, there was increasing outgrowth of dysplastic stroma with each passage. This became a major issue, whereby at passage three, more than 90% of the tumour was stromal with limited squamous carcinoma identified (Figure 6-7). This was consistent with the outgrowth of a sarcoma secondary to transgene activation in the stromal compartment beneath the anal epithelium following 4-OHT treatment, with subsequent transformation and outgrowth on serial passaging.

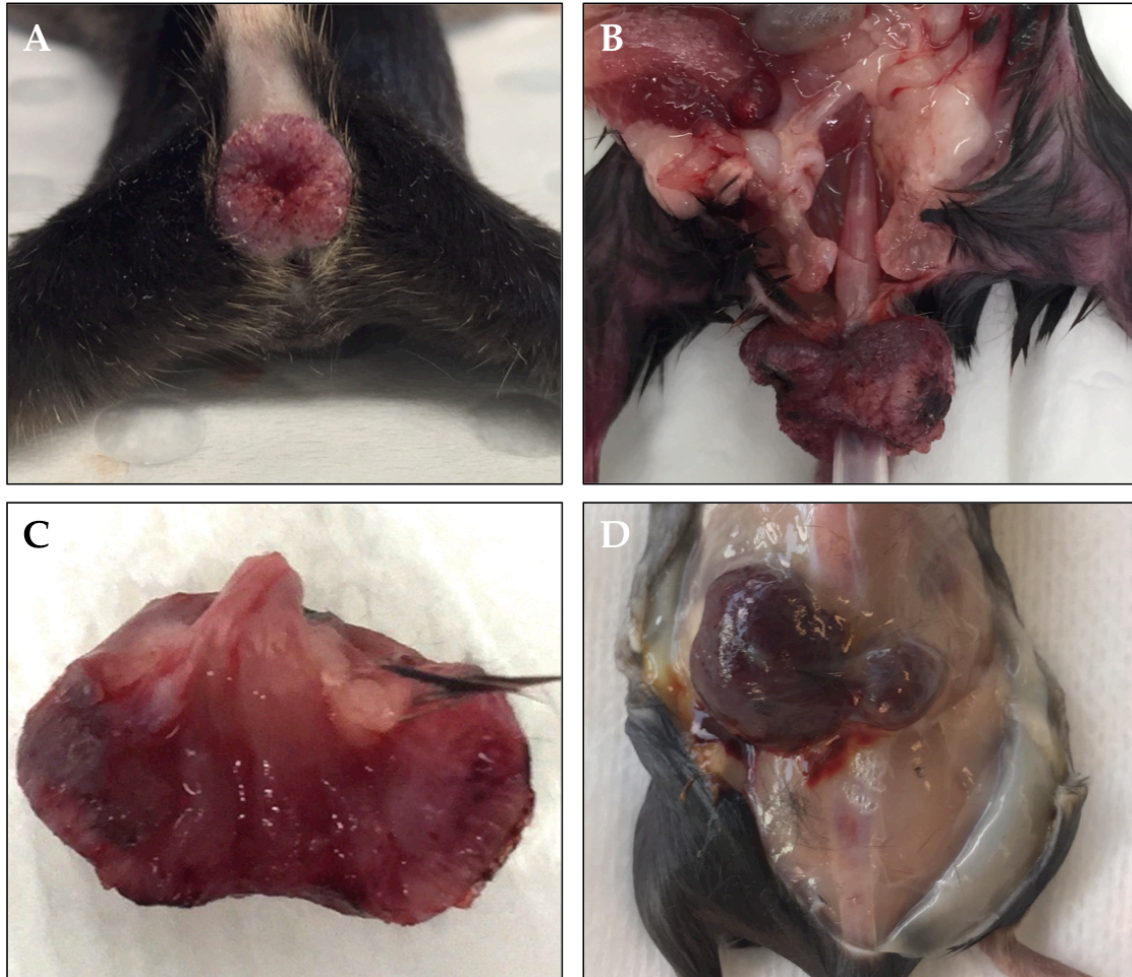


Figure 6-6 Development of anal canal tumours following targeted 4-OHT application in UBC-Cre.pik3ca^{H1047R}.pten^{fl/fl} C57Bl/6 mice

Transgenic mice were exposed to 4-OHT on three consecutive days with a novel technique to target the anal canal of the UBC-Cre.pik3ca^{H1047R}.pten^{fl/fl} C57Bl/6 mice. All mice developed anal canal tumours within 3 weeks of exposure as demonstrated in (A). Following necropsy dissection with splitting of the anterior pelvis (B) demonstrated a normal rectum proximal to the anal canal tumour. This is seen more clearly following longitudinal transection of the tumour (C). On passaging of the anal tumour to the back of wtC57Bl/6 mice, the tumour was found to macroscopically change to a darker colouration secondary to it being predominantly blood-filled (D) compared to the original anal canal tumour (C).

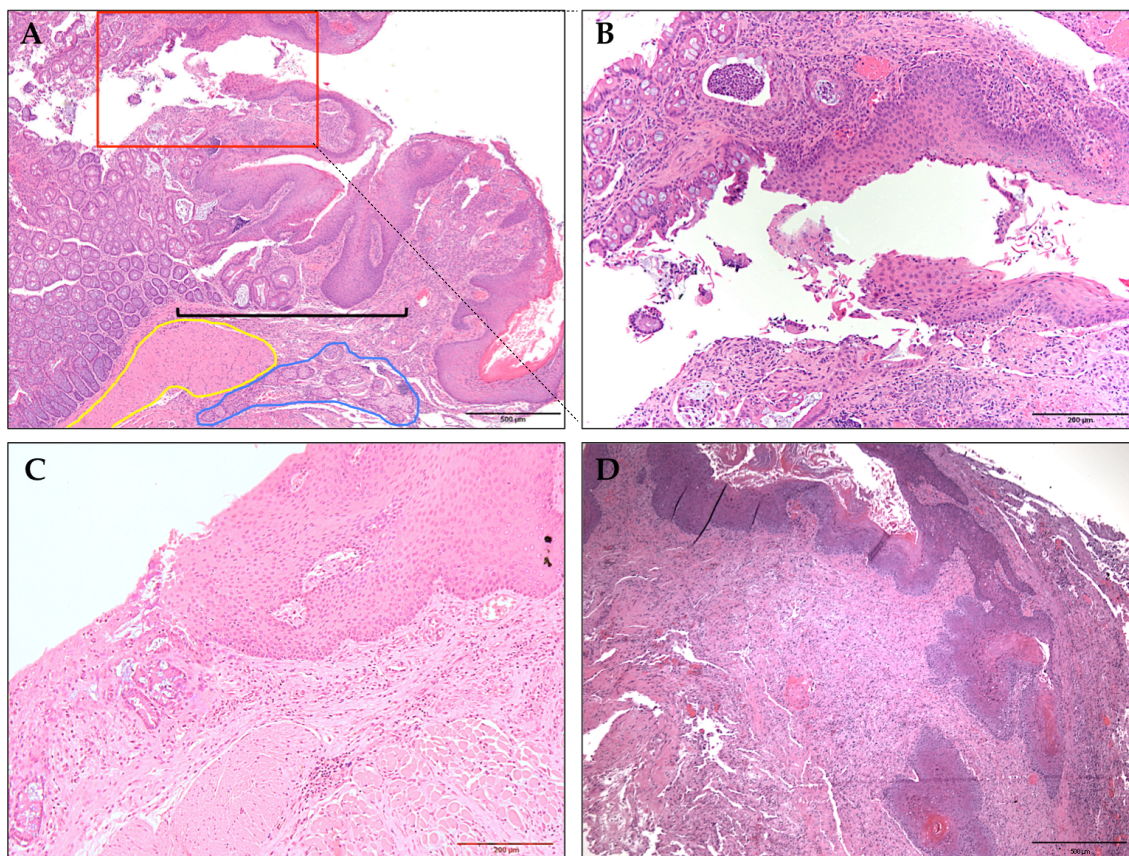


Figure 6-7 Anal canal tumourigenesis in UBC-Cre.pik3ca^{H1047R}.pten^{fl/fl} C57Bl/6 mice followed transgene induction with stromal overgrowth on passaging

UBC-Cre.pik3ca^{H1047R}.pten^{fl/fl} C57Bl/6 mice had targeted application of 4-OHT to the anal canal. Tumourigenesis occurred within weeks and the tumour was resected for formalin fixation, sectioning and H & E staining. Shown (A) is a representative image of the anal canal tumour (yellow line - anal sphincter; blue line - perianal glands specific to mouse anatomy; black calipers indicate the anal canal; x4 objective, scale bar 500µm) B) Focused view of the anal canal area (x10 objective, scale bar 200µm) as outlined by the red box in A). C) Transition from rectal mucosa to dysplastic squamous epithelium in an anal canal tumour (x10 objective, 200µm). D) Tumour outgrowth following passaging of an anal canal tumour to a wtC57Bl/6 mouse, with subsequent resection, fixation, embedding and staining, demonstrating significant stromal outgrowth over the squamous component, suggesting transformation of the stroma (x4 objective, 500µm).

6.3.4 Syngeneic Cell Line Establishment

An alternative methodology was developed to combat the outgrowth of transformed stromal cells. This involved the establishment of an ASCC cell line from the induced mouse tumour. Ten days after induction of the anal canal epithelium, the C57Bl/6 transgenic mouse was culled and the anal canal, rectum

and surrounding anal margin skin were dissected free en bloc. This required the anterior bony pelvis to be split open with the use of strong dissecting scissors and a P200 pipette tip to splint open the anal canal and rectum, facilitating its identification and precise dissection. Once dissected free, the P200 pipette tip was left *in situ* to provide a firm platform onto which the anal canal and rectum was opened along its longitudinal axis. The splayed open anal canal was then immersed into a solution of 6 units / ml warmed Dispase within a sterile 10cm Petri dish for a period of 30 mins to assist with the dissociation of the epithelium (rectal, anal canal tumour and skin) from the underlying tissue. The specimen within the petri dish was then examined under a dissecting microscope, following which fine tooth forceps were utilised to facilitate the teasing apart of the epithelium and accurate removal of only the anal canal tumour for subsequent cell line development.

The methodology for the establishment of the Anal1 cell line was similar to that detailed in section 2.2.2. However, rather than solid tumour explants, the dispase and microdissection technique resulted in small epithelial clusters of the anal canal that were subsequently plated out and treated in a similar fashion to that described in section 2.2.2. After adherence to the plastic, slow outgrowth of an epithelial line ensued without a significant stromal component. This eliminated the risk of transformed stromal cells outcompeting the epithelial component *in vitro*.

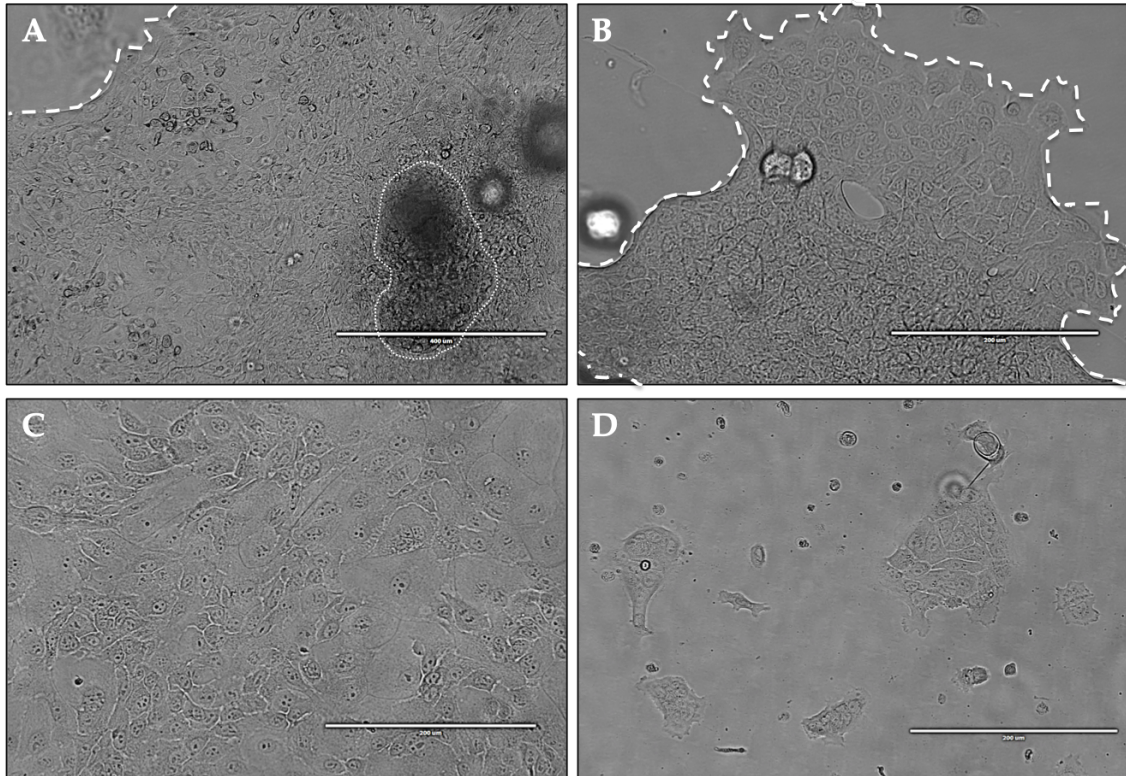


Figure 6-8 Evolution of Anal1 cell line from initial explant through to robust cell line

Brightfield photomicrographs of an epithelial explant derived from the anal canal of a 4-OHT treated (10 days prior) UBC-Cre.pik3ca^{H1047R}.pten^{fl/fl} C57Bl/6 mouse. (A) Outgrowth of squamous cells is seen from the explant without fibroblasts at D14 post plating (dotted line indicating explant; dashed line indicating epithelial outgrowth margin, x10 objective, scale bar 400µm); B) Expanding squamous outgrowth from an explant at D14 with classic cobblestone like interlinked cells (x20 objective, scale bar 200µm). Squamous cells of C) the evolving Anal1 cell line, 72 hours following passage number 2 (high seeding density, 100% of lifted cells passaged from 12-well to 6-well plate) and D) the established Anal1 cell line 24 hours following passage 10 (seeded at 1×10^6 to T175; scale bar 200µm, x20 objective).

6.3.5 Human HPV 16 E6/7 Transduction

With the knowledge that over 90% of ASCC is associated with HPV, and predominantly subtype 16, a decision was made to transduce the Anal1 cell line with the two HPV 16 oncogenes, to better recapitulate the genomic underpinnings and biology of human HPV positive ASCC.

6.3.5.1 Transduction Efficiency

Once established, the Anal1 line (Passage 10) was virally transduced with the HPV 16 E6/7 oncogenes. The transduction efficiency of the Anal1 line was first determined using a GFP reporter lentiviral construct. This was undertaken to perform a limited assessment of the Multiplicity of Infection (MOI) that would generate an adequate number of transduced cells. Evaluation was undertaken for a MOI of 0.5, 1 and 2 only, as a high efficiency was not required for the E6/7 transduction given puromycin would select out the pool of transduced cells. The results of the assessment are presented in Figure 6-9, with a MOI of 1 selected for the HPV 16 E6/7 transduction.

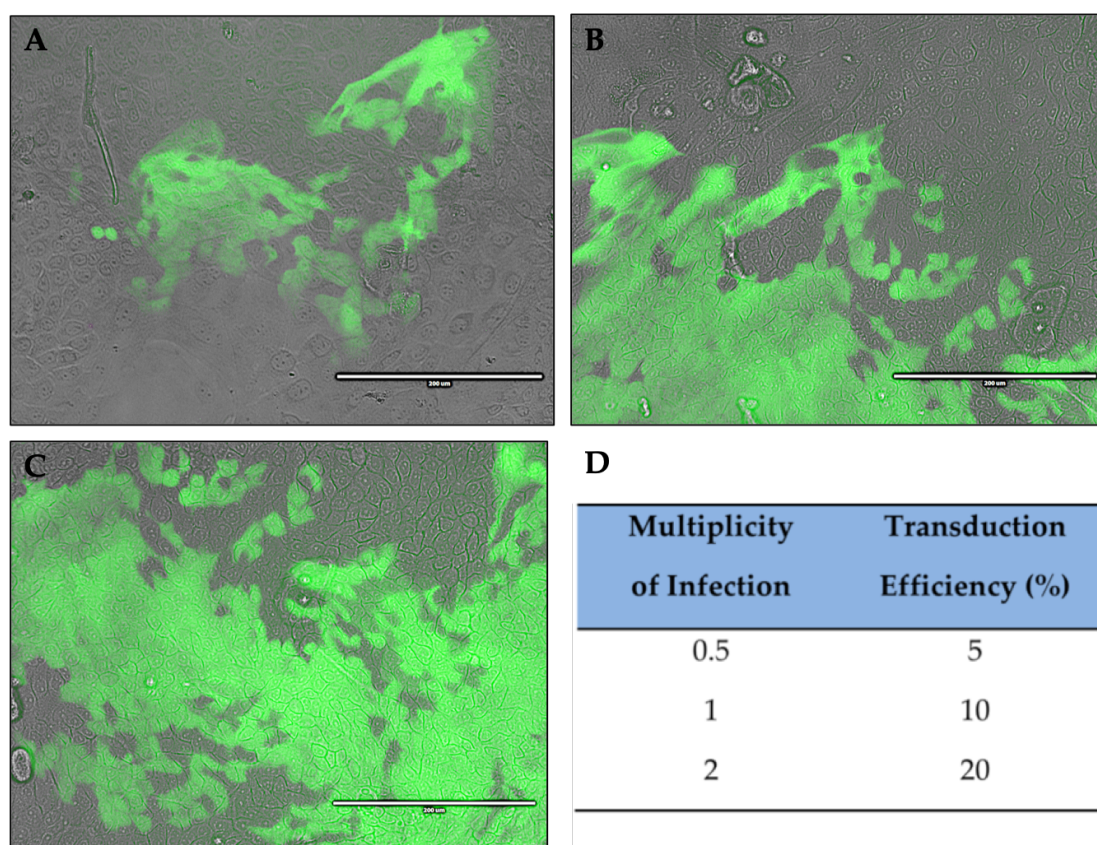


Figure 6-9 Transduction efficiency across three Multiplicity of Infection ratios.

GFP-control lentiviral transduction of Anal1 cell line across MOI of 0.5, 1 and 2. Fluorescence capable phase contrast photomicrographs with x20 objective demonstrating clusters of GFP positive cells at a MOI of 0.5 (A) 1 (B) and 2 (C) (scale bar 200 μ m); (D) Table summarising the quantified efficiency per MOI

6.3.5.2 Puromycin Selection

The lentiviral construct contained a puromycin resistance gene that when stably incorporated and expressed, allows for the selection of transfected cells. A puromycin dose viability curve was undertaken against non-transduced Ana11, in order to determine the lowest puromycin dose at which all cells were killed. This dose was found to be 2µg/ml for Ana11 (Figure 6-11). This dose was then utilised to select out only those clones transduced with stable incorporation and expression of the oncogenes.

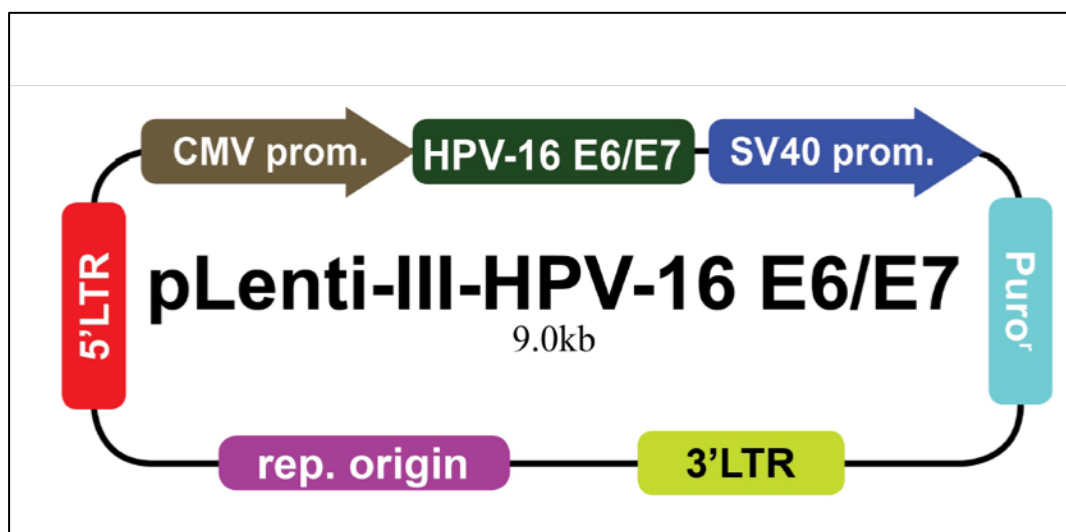


Figure 6-10 HPV 16 E6/7 Lentiviral Construct

Lentiviral construct of 9kb length, containing the HPV 16 E6/7 oncogenes with a puromycin selection gene to obtain the population of stably incorporated and expressing transduced cells.

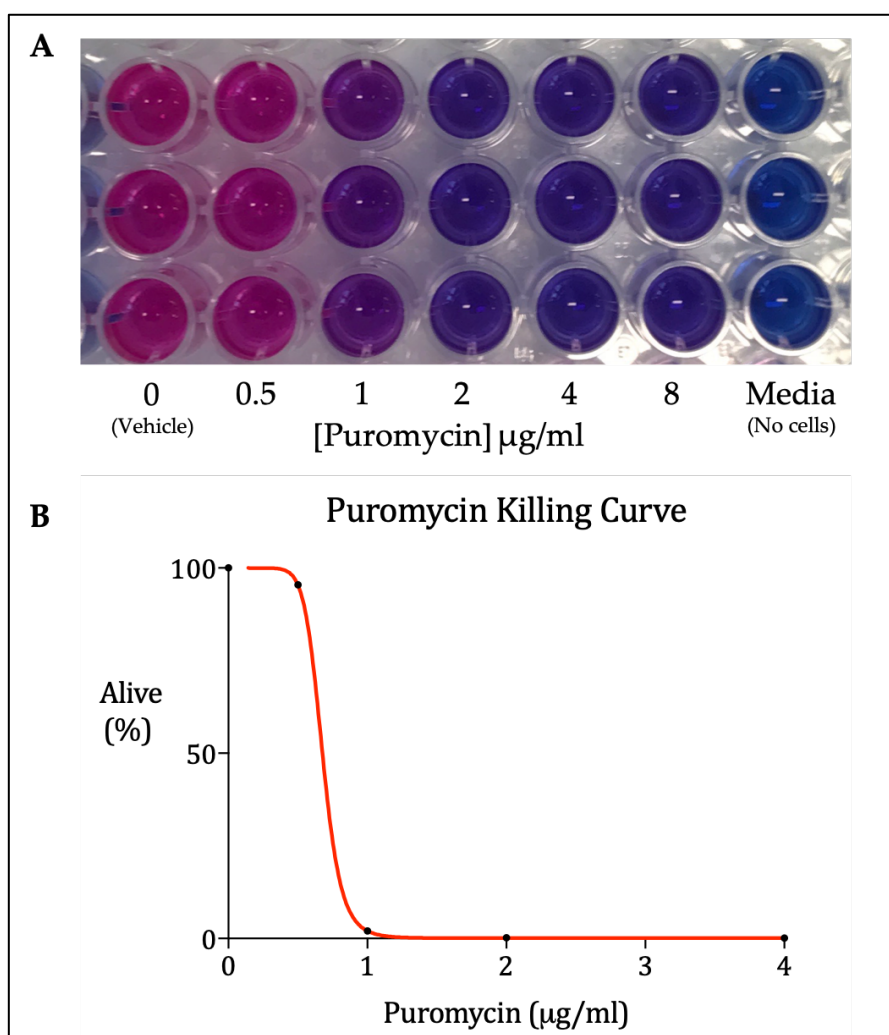


Figure 6-11 Puromycin Killing Curve for Anal1

A) AlamarBlue[®] assay used to determine the cell viability of non-transduced Anal1 after exposure to the differing concentrations of Puromycin for 72 hours (Pink - high viability, purple - low viability); B) Puromycin killing curve calculated from the above results of the six puromycin concentrations against the parental non-transduced line Anal1.

6.3.6 Validation

6.3.6.1 HPV 16 E6/7 Oncogene

PCR was used to confirm the successful transduction of HPV 16 E6/7 into Anal1 E6/7 cell line. Genomic DNA was extracted from a snap frozen cell pellet of Anal1, Anal1 E6/7, P1, P2, P3, LRF1 and LRF2 as outlined in section 2.3.1, with the yield and quality assessed using the nanodrop (Table 6-1). The gDNA was used in a PCR for identifying the HPV 16 E6/7 oncogenes using the technique

and primers described in section 2.3.9. This confirmed the absence of the E6/7 oncogenes in the parental line Anal1 and the HPV-ve human ASCC cell line LRF2 (negative control). It demonstrated the presence of the transgenes in the Anal1 E6/7 line and the four human HPV 16+ ASCC cell lines (P1, P2, P3 and LRF1 - positive controls).

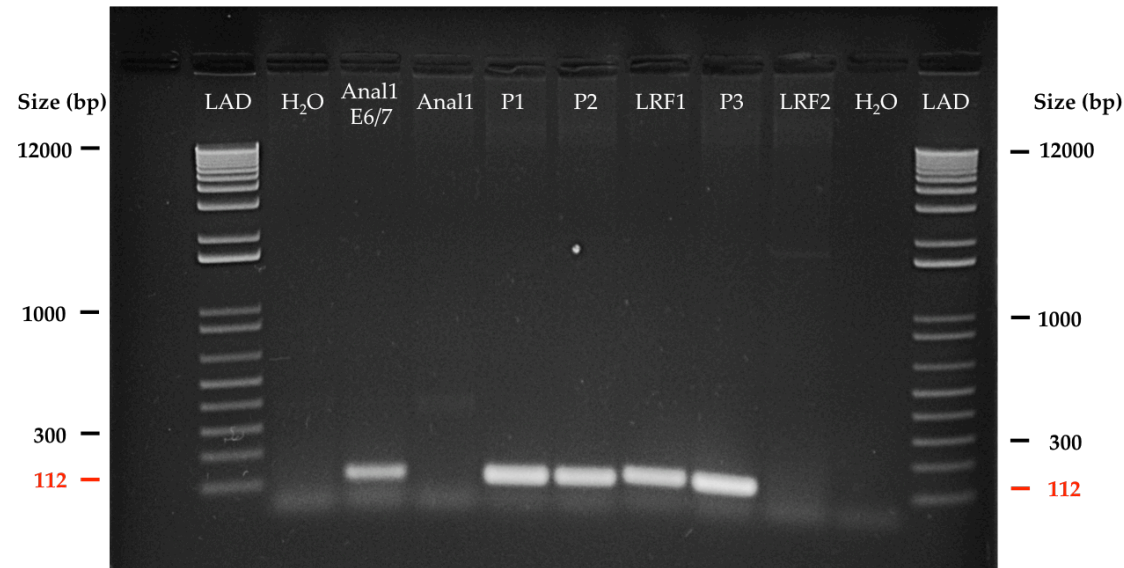


Figure 6-12 HPV 16 E6/7 Oncogene PCR

gDNA was extracted from cell pellets (2×10^6) of Anal1, Anal1 E6/7 and the five human ASCC cell lines and used in a PCR to confirm the presence of HPV 16 E6/7. The products were run on a 1.5% agarose gel with Midori Green. The HPV E6/7 gene product is 112bp, with the human HPV+ve (P1-3, LRF1) and -ve (LRF2) cell lines acting as positive and negative controls respectively (H₂O control – No gDNA ; LAD – 100bp ladder).

6.3.6.2 *Pik3ca* Mutation

PCR was utilised to confirm appropriate expression of the *Pik3ca*^{H1047R} mutant on cDNA synthesised from RNA taken from a cell line pellet. RNA was extracted from snap frozen cell pellets (2×10^6 cells) of parental (Anal1) and transduced cell lines (Anal1 E6/7) using the technique described in section 2.3.2. The RNA yield and quality were assessed with Nanodrop (Table 6-1).

Table 6-1 Nucleic Acid Extraction Yield and Quality

Sample		Concentration (ng/μl)	260/280	260/230
RNA	<i>Anal1</i>	1535.6	2.15	2.20
	<i>Anal1 E6/7</i>	557.7	2.12	2.22
	<i>P3</i>	601.2	2.17	2.28
<i>cDNA</i>	<i>Anal1</i>	833.4	1.83	1.30
	<i>Anal1 E6/7</i>	1689.3	1.80	1.40
	<i>P3</i>	2095.4	1.45	1.75
<i>gDNA</i>	<i>Anal1</i>	298.4	2.11	2.15
	<i>Anal1 E6/7</i>	260.5	2.12	2.09
	<i>P1 Primary</i>	23.6	1.90	0.85
	<i>P1 CL</i>	81.2	1.81	1.37
	<i>P1 PBMCs</i>	855	1.92	2.44
	<i>P2 Primary</i>	17.1	1.90	0.73
	<i>P2 CL</i>	52.9	1.94	2.47
	<i>P2 PBMCs</i>	208.3	1.94	2.46
	<i>P3 Primary</i>	75.9	1.97	1.31
	<i>P3 CL</i>	74.4	1.81	1.48
	<i>P3 PBMCs</i>	180.4	1.94	2.49
	<i>LRF1 Primary</i>	174.6	1.99	1.12
	<i>LRF1 CL</i>	69.7	1.95	2.25
	<i>LRF1 PBMCs</i>	66.3	1.89	2.27
	<i>LRF2 Primary</i>	116.9	1.90	1.38
	<i>LRF2 CL</i>	132.9	1.88	1.85
	<i>LRF2 PBMCs</i>	111.6	1.91	2.74

cDNA was synthesised using reverse transcription described in section 2.3.3. The cDNA was assessed for resultant quantity and quality (Table 6-1). The cDNA was used in a PCR for identifying wildtype or mutant *pik3ca* using the technique and primers described in section 2.3.7.1. This demonstrated that both *pik3ca* wildtype and mutant alleles were present in both the mouse parental and transduced lines but absent in the human ASCC line PMASCC-P3 (primers specific for mouse) when compared to controls (Figure 6-14).

Exon 19 Wildtype + Exon 20 Wildtype

```
CTGTTTCATATAGATTTTGGGCACCTTTTGGATCACAAGAAGAAAAAATTGGCTATAAGCGGGA
ACGTGTGCCATTTGTGTTGACACAGGATTTCTTGATTGTGATTAGTAAGGGAGCA CAAGAGTACAC
CAAGACCAGAGAGTTTGAGAGGTTTCAGGAGATGTGTTACAAGGCTTACCTAGCAATTCGGCA
GCATGCCAATCTCTTCATCAACCTTTTTTCAATGATGCTTGGCTCTGGAATGCCAGAACTACA
ATCTTTTGATGACATTGCATATATCCGAAAGACTCTAGCCTTGGACAAAACCTGAGCAAGAAG
CTTTGGAATATTTACAAAGCAAATGAATGATGC ACATCATGGTGGATGGACGACA AAAATG
GATTGGATCTTCCACACCATCAAGCAGCATGCTTTGAACTGAGATGGGAGCTGGGACTGCCGAG
CTGGCTCCCGGCTTCTCCACTGCATGGCAGTGAGTGGCAGCAGGCAGGCAGTGGCATGGGATGGC
ACAGTCAGGAACAACATTAGGACTTGAGCAAGAACATAAACAA
```

Exon 19 Wildtype + 20 Mutant

```
CTGTTTCATATAGATTTTGGGCACCTTTTGGATCACAAGAAGAAAAAATTGGCTATAAGCGGGA
ACGTGTGCCATTTGTGTTGACACAGGATTTCTTGATTGTGATTAGTAAGGGAGCA CAAGAGTACAC
CAAGACCAGAGAGTTTGAGAGGTTTCAGGAGATGTGTTACAAGGCTTACCTAGCAATTCGGCA
GCATGCCAATCTCTTCATCAACCTTTTTTCAATGATGTTGGGATCCGGCATGCCTGAGTTGCA
ATCTTTTGATGACATTGCATATATCCGAAAGACTCTAGCCTTGGACAAAACCTGAGCAAGAAG
CTTTGGAATATTTACAAAGCAAATGAACGACGC TAGGCACGGAGGGTGGACGACA AAAAT
GGATTGGATCTTCCACACCATCAAGCAGCATGCTTTGAACTGAGATGGGAGCTGGGACTGCCA
GCTGGCTCCCGGCTTCTCCACTGCATGGCAGTGAGTGGCAGCAGGCAGGCAGTGGCATGGGATGG
CACAGTCAGGAACAACATTAGGACTTGAGCAAGAACATAAACAA
```

Figure 6-13 *Pik3ca* wildtype and allele cDNA sequences

Pik3ca wildtype and mutant allele cDNA gene sequences. Blue - exon 19; black – exon 20 with underline indicating base substitution of A > G, H1047R mutation; red – part of 3'-untranscribed region; blue highlighting – forward primer; green highlighting – reverse wildtype primer; yellow highlighting – reverse mutant highlighting.

A control without reverse transcriptase was not used to exclude the amplification of contaminating gDNA, however the product size is consistent with amplification of the appropriate cDNA and not gDNA. The mutant and wildtype cDNA primers have been previously confirmed as specific by assessment against homozygous wildtype and mutant controls.²¹³

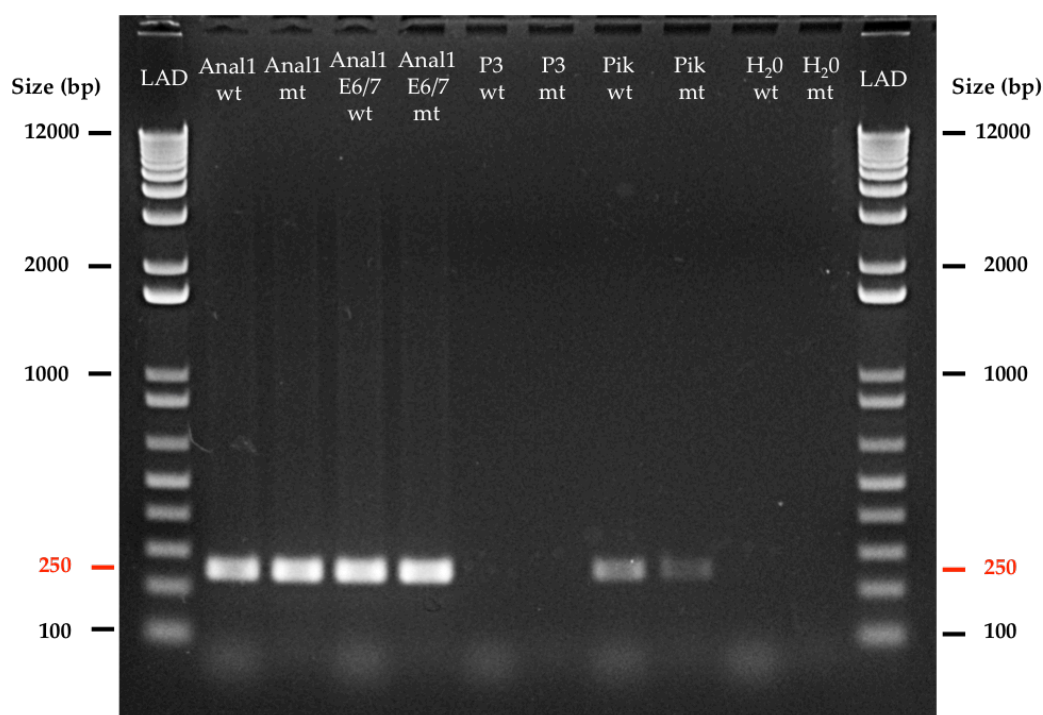


Figure 6-14 *Pik3ca* PCR

Pik3ca PCR run on 1.5% agarose gel with Midori Green utilising cDNA synthesised from RNA extracted from mouse anal cancer cell lines Anal1 and Anal1 E6/7 and the human ASCC line P3. The bands for wildtype and mutant *pik3ca* are both 250bp, requiring wt and mutant specific primers and appropriate controls (*pik* = control mouse tissue expressing both wt and mutant *pik3ca* alleles). (wt = wt specific primer; mut = mutant specific primer; H₂O control – No cDNA; LAD = 100bp ladder)

6.3.6.3 *Pten* knockout

PCR was undertaken to confirm the bi-allelic loss of exon 5 in the *pten* gene. Genomic DNA was extracted from a snap frozen pellet of both Anal1 and Anal1 E6/7 as outlined in section 2.3.1, and the yield and quality assessed using the nanodrop (Table 6-1). The PCR demonstrated that the *Pten* was wildtype across both alleles, despite the cell line having evidence of induction of the *Pik3ca* mutation (Figure 6-14). This was unexpected, as the *Cre recombinase* theoretically acts at all *loxP* sites within tamoxifen exposed cells, leading to mutant *pik3ca* and biallelic *pten* knockout cells. In order to assess whether the genotyping was incorrect, the genomic DNA was genotyped with appropriate primers (Table 2-5). This confirmed that both *Pten* transgenes with *loxP* sites were present

(Figure 6-15). However, the *Cre recombinase* had only induced the *Pik3ca* mutation and not the double knockout of *pten*.

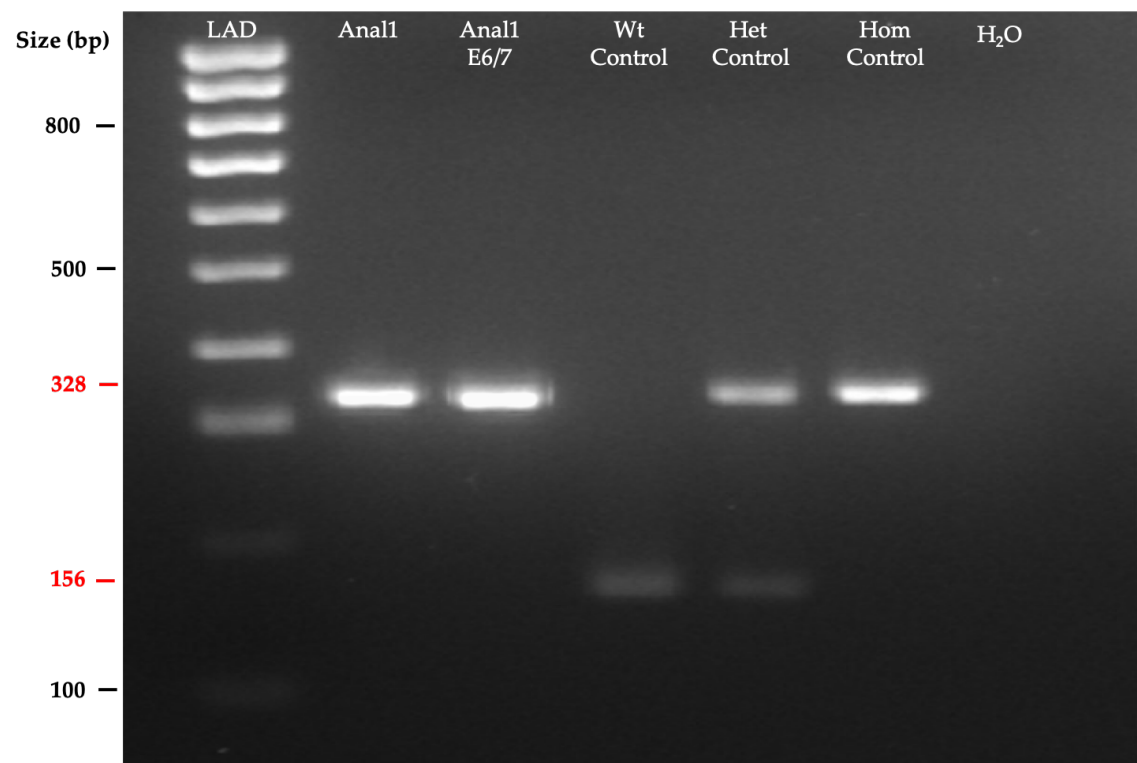


Figure 6-15 *Pten* genotyping PCR

*Genomic DNA was extracted from cell line pellets (2 x 10⁶) of Anal1 and Anal1 E6/7 cell lines and a PCR undertaken to identify the presence of the *pten*^{wt} gene (156 bp gene product) and *pten*^{Δ5} transgene (loss of exon 5, 328 bp gene product) alleles on genotyping with primers described in Table 2-5. A) *Pten* genotyping demonstrating homozygous *pten* transgenes in both Anal1 and Anal1 E6/7. (Het – Heterozygous, Hom – Homozygous; H₂O – water control)*

This finding was further explored, by performing an allele specific PCR on the original mouse tumour from which the Anal1 line was derived (Figure 6-17). This revealed that the majority of the tumour had evidence of *pten* knockout, however there was a light band revealing that some cells had retained *pten*^{wt/wt}. With the establishment of the Anal1 line from culturing this tumour *in vitro*, it is possible that the *pik3ca*^{H1047R} and *pten*^{wt/wt} cells had a growth advantage over those with the double knockout of *pten*.

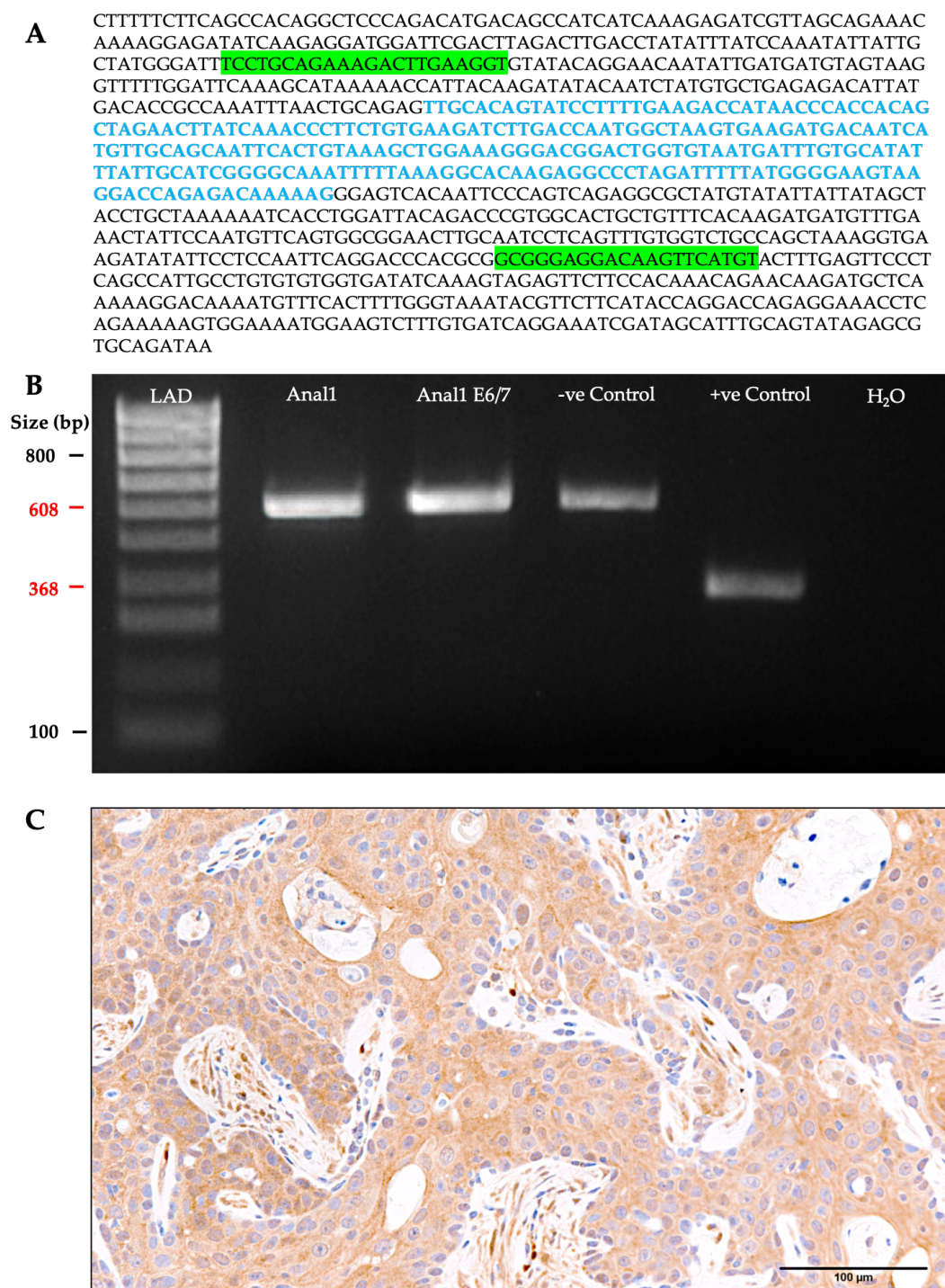


Figure 6-16 *Pten* gene sequence, allele specific PCR and tumour IHC

A) *Pten* gene sequence with exon 5 in blue. Green highlights the forward and reverse allele specific primers in the flanking sequences around exon 5. B) gDNA was extracted from Anal1 and Anal1 E6/7 cell lines and an allele specific PCR performed, demonstrating homozygous wildtype *pten* (608 bp gene product) in Anal1 and Anal1 E6/7 without loss of exon 5 (368 bp gene product). C) *Pten* IHC performed on an Anal1 E6/7 syngeneic tumour demonstrating normal retained staining (scale bar 100μm; Het – Heterozygous, Hom – Homozygous; H₂O – water control)

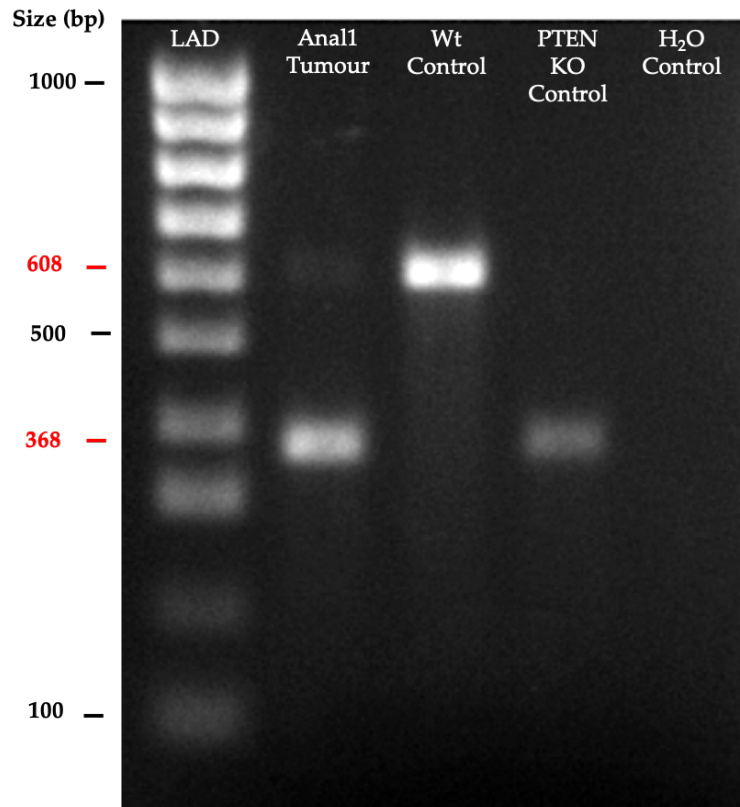


Figure 6-17 Pten allele specific PCR on original mouse tumour

Genomic DNA was extracted from cryopreserved tumour tissue taken from the mouse tumour of which the cell line Anal1 was developed. The tumour was digested and processed to extract DNA in accordance with section 2.3.1. An allele specific PCR was then undertaken as in Figure 6-16 B. This demonstrated a strong band at 368bp consistent with pten knockout, with a light band at 608 bp consistent with pten wt. (KO – knockout; LAD – 100bp ladder; Wt – wildtype; H₂O – Water control).

6.3.6.4 Up-regulation of the PI3K Pathway

PI3K pathway activity is increased secondary to *PIK3CA* or other genomic aberrations in over 60% of human ASCC's.¹⁸⁷ In order to confirm induction of the *pik3ca*^{H1047R} hotspot mutation with consequent upregulation of the PI3K pathway, the downstream marker pS6 was assessed by IHC (section 2.4) on Anal1 cell line syngraft tumours (Figure 6-18). This confirmed that there was increased expression of pS6 in the cytoplasm of Anal1 syngeneic tumour cells compared with normal squamous mucosa of a wtC57Bl/6 mouse.

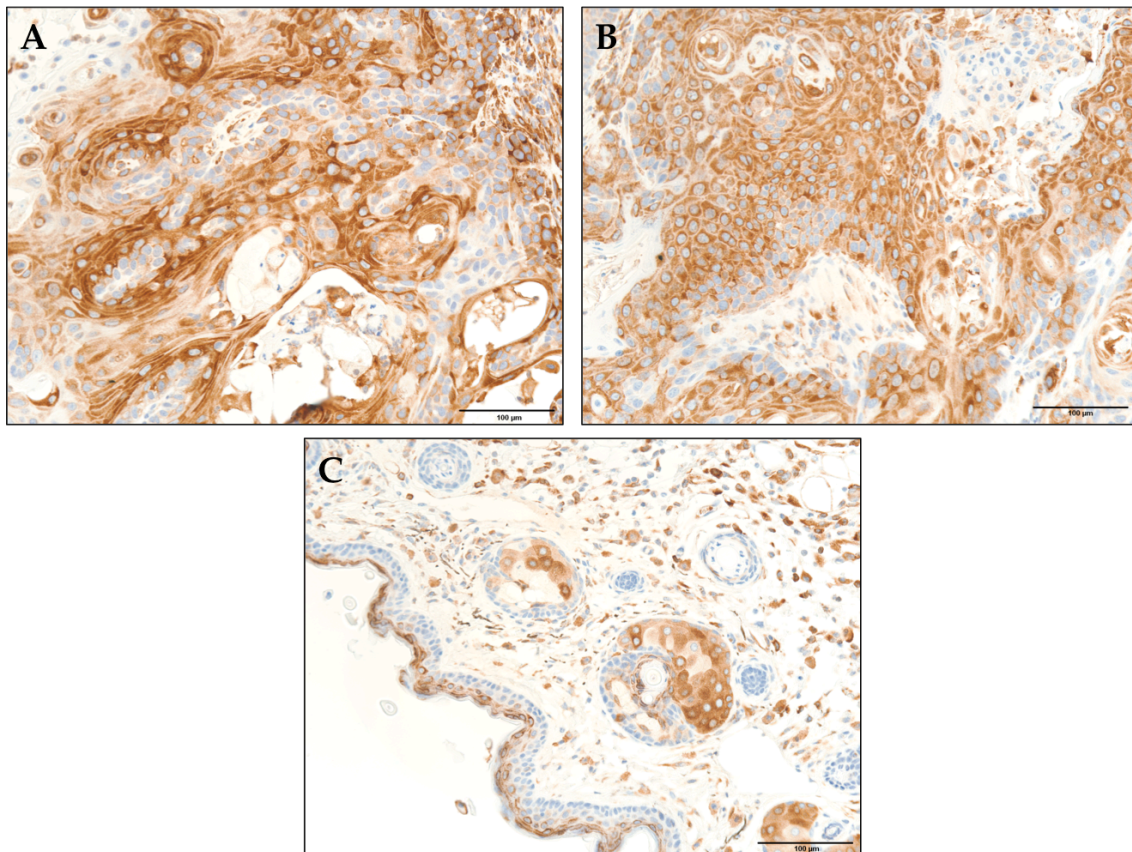


Figure 6-18 pS6 IHC demonstrating increased expression across the Anal1 syngeneic tumour

Anal1 cell line syngeneic tumours were resected, formalin fixed, embedded and sectioned with subsequent pS6 IHC staining. A) and B) demonstrate two separate areas of an Anal1 syngeneic tumour with increased expression of pS6 compared with normal squamous tissue from a wtC57Bl/6 control mouse (C) (Scale bar 100µm)

6.3.6.5 *Mycoplasma*

Both the parental and transduced cell lines were confirmed free of Mycoplasma on PCR, in the same manner as the human ASCC lines with methodology (refer 2.3.6) and results (Figure 5-4) demonstrated in the respective chapters.

6.3.7 Morphology, Architecture and Cytokeratin Expression

Anal1 and Anal1 E6/7 were confirmed to have appropriate morphology growing as a 2-D culture *in vitro* with classic squamous cobblestone like cells as demonstrated with Anal1 in Figure 6-19 A & B. The cell line was also grown as

cell line-derived tumouroids, with H & E staining demonstrating appropriate morphology consistent with a squamous carcinoma (Figure 6-20). Expression of both CK5/6 and p63 was confirmed in both the cell line and cell line tumouroids, confirming the expression of cytokeratin markers consistent with a squamous carcinoma (Figure 6-19 & Figure 6-20).

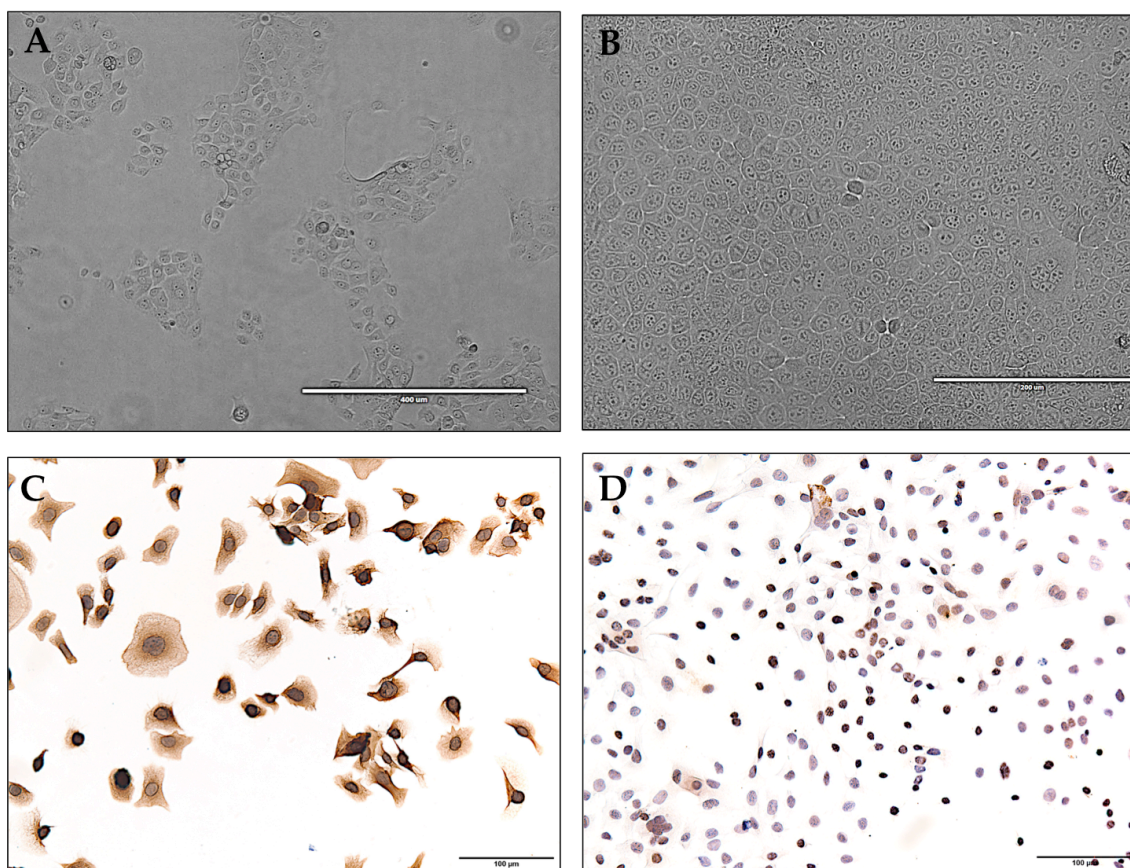


Figure 6-19 Morphology and Cytokeratin Expression of Anal1 cell line

Anal1 cells were seeded in an 8-well chamber slide. Brightfield photomicrographs demonstrating the typical adherent and flat cobblestone like cells and colonies typical of a squamous carcinoma line after 24 hours A) (x10 objective, scale bar 400μm) and 72 hours of culturing B) (x20 objective, scale bar 200μm). For IHC, the cells were fixed and stained following incubation overnight for cytokeratins CK5/6 and p63. The CK5/6 staining is cytoplasmic with peri-nuclear enhancement (C) and the p63 is nuclear specific (D), confirming the line as squamous in origin.

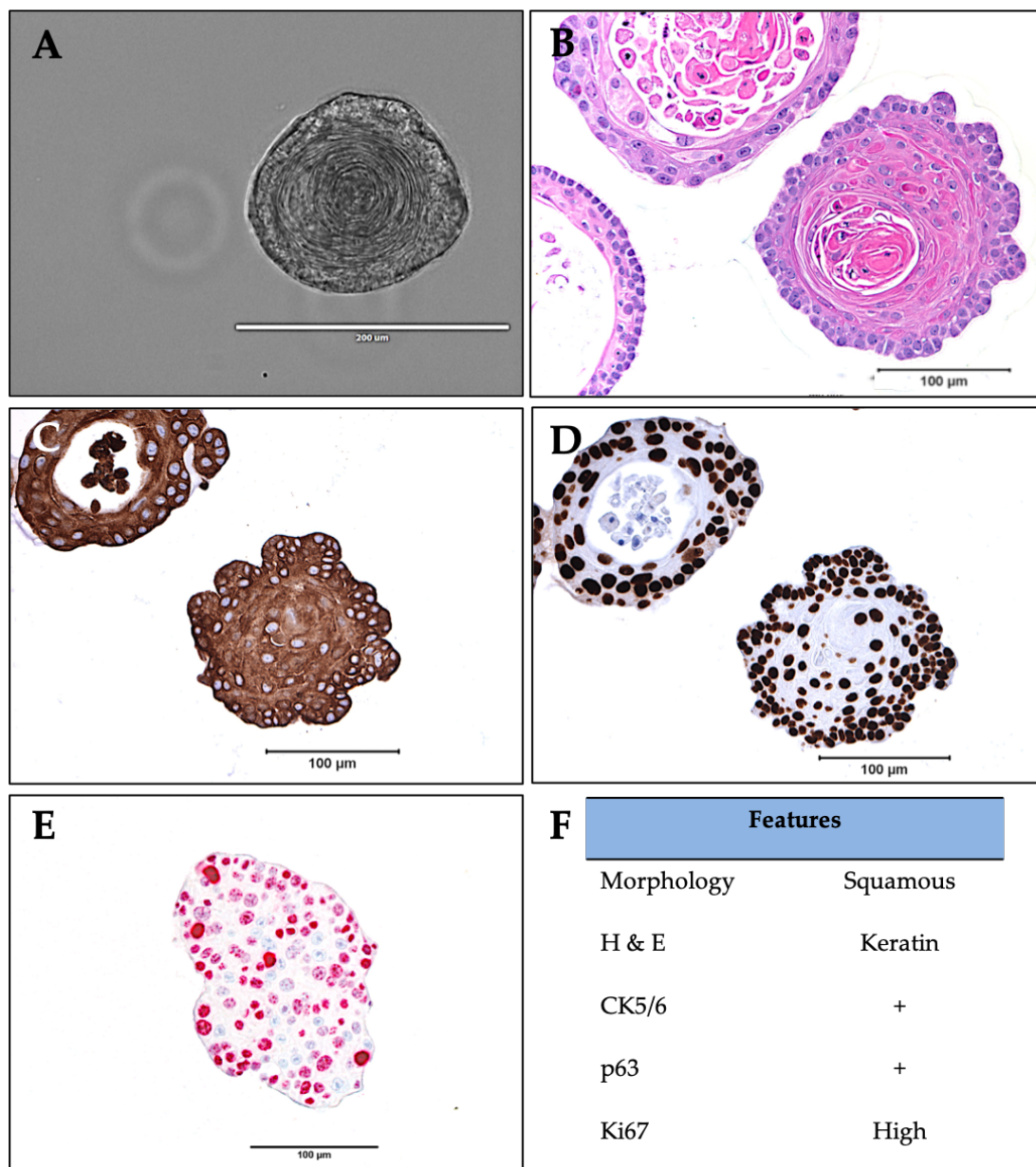


Figure 6-20 Morphology and IHC profile of Anal1 Tumouroids

Anal1 cells were seeded in matrigel and allowed to grow for 14 days. A) Inverted microscope image of Anal1 CL tumouroid at D14 post seeding (scale bar 200µm). Retrieval, fixation, histogel embedding and sectioning for staining was then performed. B) H & E staining of Anal1 CL tumouroids demonstrating squamous architecture with some keratin centrally. C) CK5/6 IHC staining of the cytoplasm and D) Nuclear specific p63 staining. E) High Ki67 nuclear staining (red chromagen). F) Table summarises the features consistent with a squamous cell carcinoma line.

6.3.8 Seeding (Plating) Efficiency

The plating efficiency of each cell line in 2D culture was determined by seeding cells into a six well plate at three different cell densities (1×10^2 , 2×10^2 , 5×10^2 cells) to determine the optimal density for passaging. This was reliant on the attachment efficiency, ability to migrate into clusters and subsequent colony forming efficiency of the cells. The resultant seeding efficiency (colonies (>50 cells) established / cells seeded) and that of the panel of human ASCC cell lines is displayed in Table 6-2. The seeding efficiency was found to be higher for both mouse lines when compared to the human ASCC lines.

Table 6-2 Cell Line Passaging Characteristics

Line	Doubling Time (hrs)	Seeding Efficiency (%)
P1	41	6
P2	47	1
P3	33	9
LRF1	25	33
LRF2	24	24
Anal1	17	72
Anal1 E6/7	16	44

6.3.9 Proliferative Capacity and Doubling Time

The proliferative potential of the cell lines was determined by utilising the xCELLigence platform as outlined in section 2.7. The seeding density to reach an appropriate level of exponential growth was optimised before undertaking the assay in triplicate, on two separate occasions. The results are represented in Figure 6-21 A, where the cell index is a surrogate measure of proliferation over time. The events related to each aspect of the curve have been defined in 5.3.13.

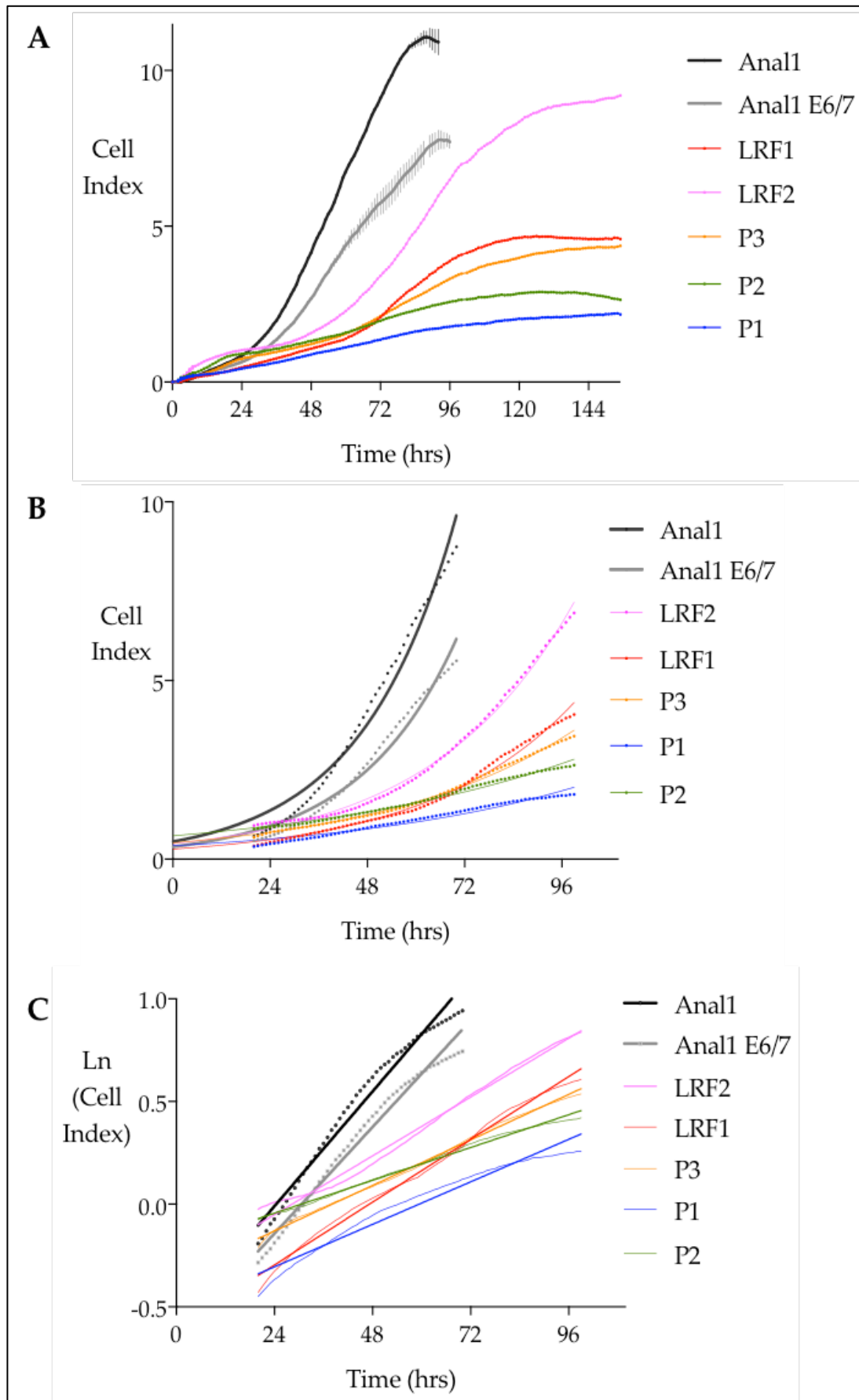


Figure 6-21 Proliferation of ASCC cell lines

A) Cells were seeded in an xCELLigence RTPA DP 16-well E-plate in RPMI-1640 + 10% FCS and the real time cell index recorded over a period of 150 hours; Data represents the mean of the cell index +/- SEM (n=3, human lines – mean alone) for the replicates of a representative experiment B) Period of exponential growth for each line was selected and the exponential growth equation (based on a constant doubling time) applied with the resultant curves (solid line) demonstrating close alignment with the real time data (dotted line); C) The cell index was natural log transformed (dotted lines) and linear regression undertaken with the resultant lines demonstrated (solid lines). This revealed the linearity of the population doubling of each line demonstrated by the close correlation between dotted and solid lines.

Assessment of these data identified that the mouse ASCC lines had a higher proliferative capacity than the human ASCC lines. To assess the doubling time of each of the lines, the exponential aspect of each curve was selected and the exponential growth equation applied in Graphpad Prism as further explained in section 5.3.13. The resultant curve (solid line) is co-represented in Figure 6-21 B and closely aligned with the real time cell index data (dotted line). The doubling time was calculated for each line by performing a natural $\log(2)/K$ of the non-linear regression analysis and is presented in Table 6-2. The doubling time was shorter for both mouse lines compared to the panel of human ASCC lines. Anal1 was found to have more rapid growth kinetics *in vitro* when seeded at the same cell number as Anal1 E6/7. This appears to be secondary to the higher seeding efficiency, with a similar proliferative potential and doubling time for both lines. The constant doubling time was also validated by performing a log (natural) transformation of the exponential phase growth data (dotted line) followed by linear regression (solid line) with the resultant lines represented in Figure 6-21 C.

6.3.10 Migration Potential

The migration capacity of each cell line was assessed using the xCELLigence platform as detailed in section 2.8. The assay was undertaken in triplicate on two

separate occasions with a representation demonstrated in Figure 6-226.3.10. Both mouse cell lines demonstrated similar migration characteristics, confirmed on linear regression with 1/slope values (maintain scientific convention and simplify comparison) closely aligned. Compared to the human ASCC lines, both mouse lines demonstrated a considerably increased level of migration.

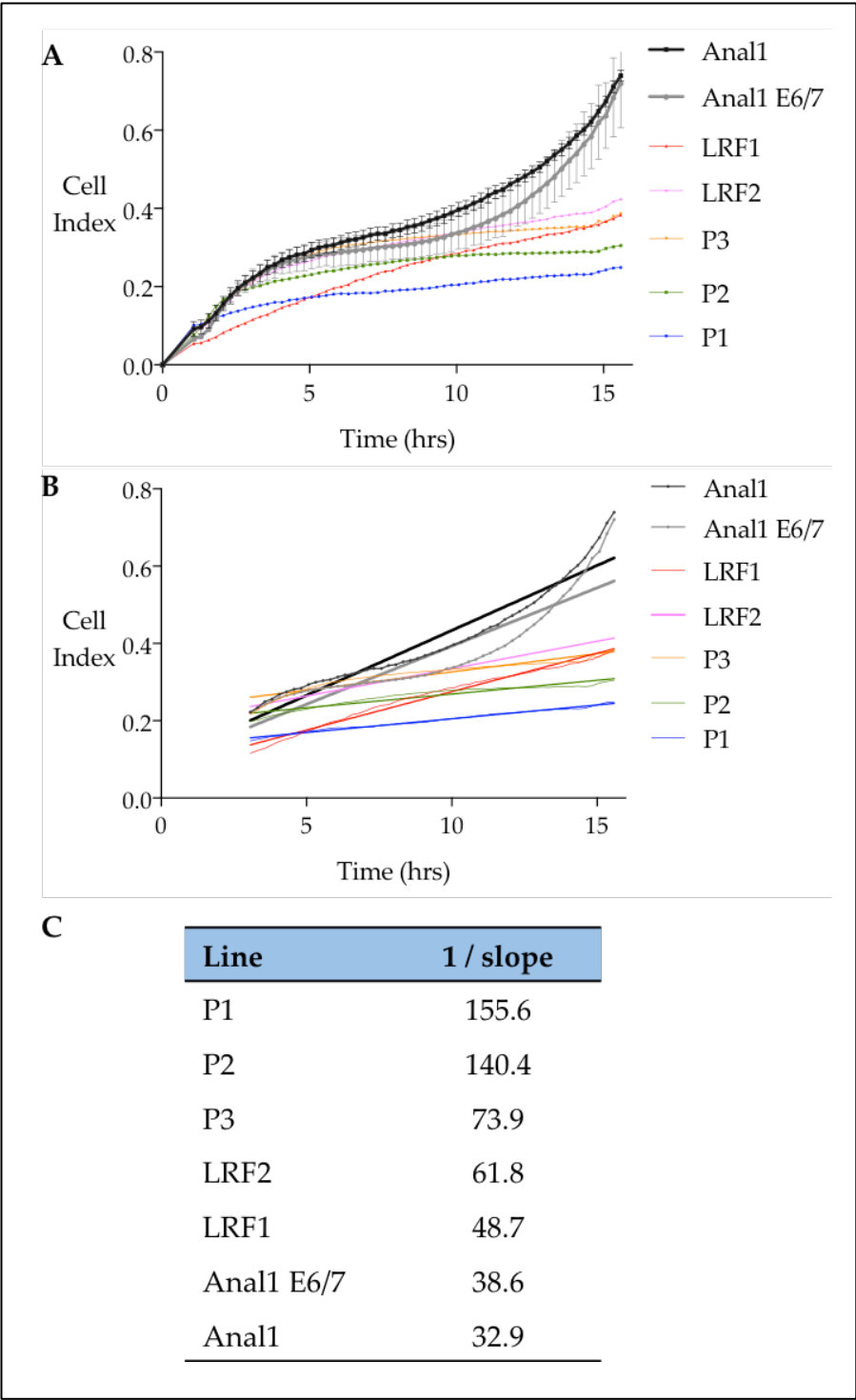


Figure 6-22 Migration Assay of ASCC lines

A) Cells were seeded in an xCELLigence RTCA DP 16 well E-plate in RPMI-1640 + 10% FCS and the real time cell index recorded over a period of 24 hours; Data represents the mean of the cell index +/- SEM (n=3, human lines – mean alone) for the replicates of a representative experiment. B) The period of migration following the initial 4.5 hours of cell settling and adherence revealed a consistent level of migration for each cell line (thin solid lines). This was quantified with linear regression with the resultant curves demonstrating similar alignment with the real time data (thick solid lines). C) From the linear regression performed in B, the inverse of the slope was calculated as a surrogate of the migration potential of each cell line (higher the value, lower the migration).

6.3.11 *In Vivo* Tumourigenicity

A cell number titration was undertaken with Anal1 E6/7 to determine the tumourigenicity in syngeneic wildtype C57Bl/6 mice and the effect on the rate of growth as displayed in Figure 6-23. Injections of $1, 2$ and 5×10^6 were all tumourigenic in C57Bl/6 mice, however only 2 of 3 mice developed an established tumour in the 1×10^6 group compared with 3 from 3 for the higher cell number injections. The growth was similar between the three groups and for future experiments, injections were standardised with 5×10^6 cells.

The tumourigenicity of the Anal1 and Anal1 E6/7 lines were also compared and assessed in immunocompromised NSG and immunocompetent syngeneic wtC57Bl/6 mice. The lines were tumourigenic in both mice with more rapid growth kinetics of Anal1 (Mann-Whitney U Test, $p=0.03$) in the immunocompromised NSG mice (Figure 6-23 B & C). The growth kinetics of Anal1 E6/7 did not differ between the two mouse strains ($p=0.49$). A non-statistically significant trend was seen with the Anal1 E6/7 line growing more rapidly than Anal1 in the wtC57Bl/6 mice and the reverse trend in NSG mice. This may have been due to the limited numbers of mice (3 in each group) that were assessed for each strain.

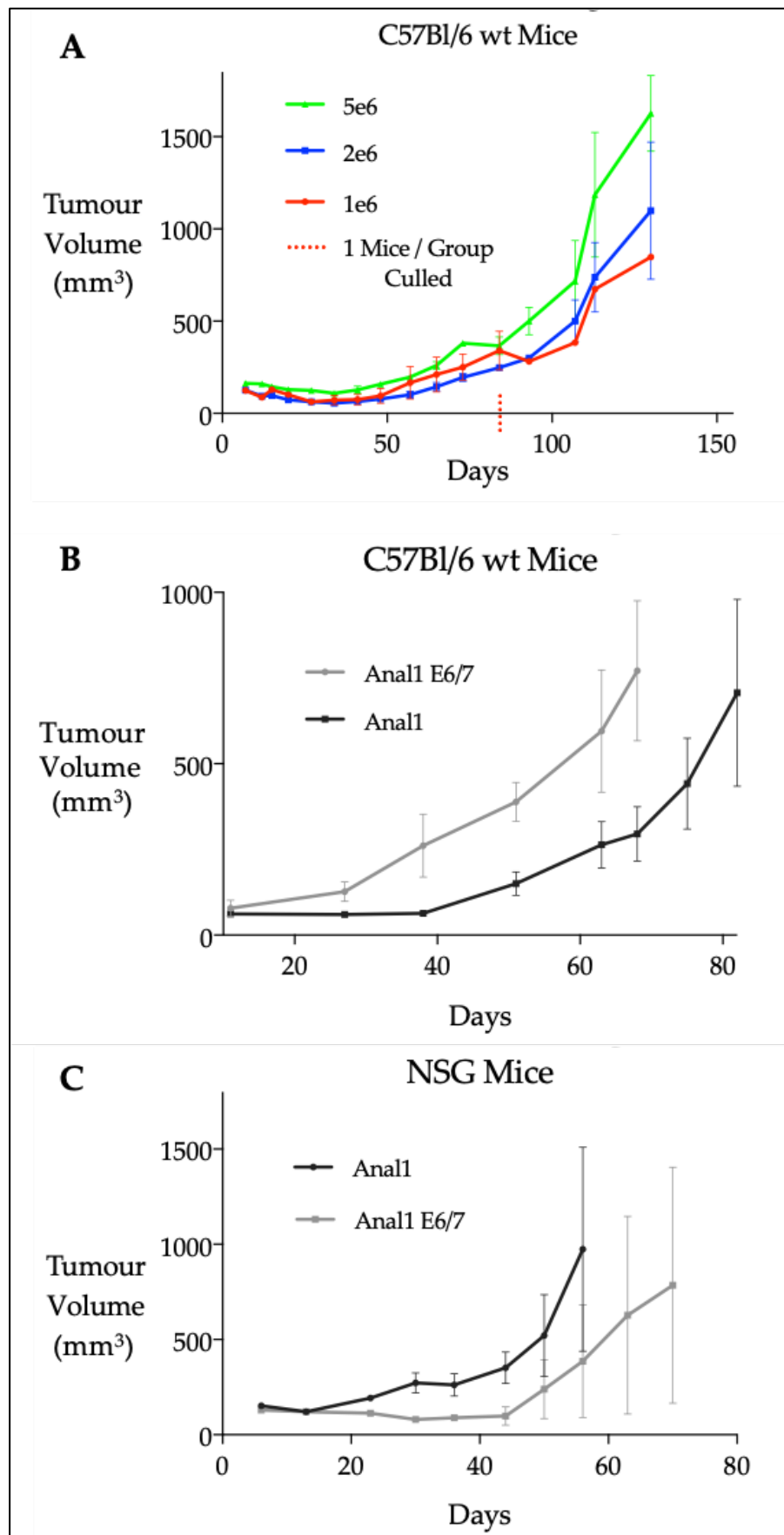


Figure 6-23 Cell Number Titration of Anal1 E6/7 and Tumourigenicity of Anal1 and Anal1 E6/7 between NSG and C57Bl/6^{wt} mice

A) Anal1 E6/7 cells were lifted and suspended in a 1:1 Matrigel:PBS mixture and injected at 1, 2 and 5 x 10⁶ cells per mouse to the right leg in a subcutaneous position. Mice were then monitored, and tumour growth recorded as displayed. One mouse from each group was culled at day 84 and processed for immune phenotyping by flow cytometry. Anal1 and Anal1 E6/7 were injected subcutaneously at 5 x 10⁶ to the right leg of 3 wtC57Bl/6 mice (C) and 3 NSG mice (D) with the subsequent tumour growth recorded as displayed.

6.3.12 Orthotopic Model

To better recapitulate ASCC, an experimental orthotopic model was assessed in 3 mice of both NSG and C57Bl/6 background. This involved the use of a 30-gauge needle to inject 2 x 10⁶ cells in 50µl of PBS into the subepithelial region of the anal canal. All mice developed tumours that rapidly reached ethical limits within 6 weeks. The growth kinetics appeared more rapid than those of the subcutaneous injections, however, monitoring the size of the tumours was problematic, given the significant proportion within the anal canal and surrounding pelvic soft tissues. A fixed specimen is demonstrated in Figure 6-24, with the anal tumour taken en-bloc with the rectum and surrounding skin to demonstrate the anatomy. The specimen was then blocked and sections stained with H & E and CK5/6 and p63 (Figure 6-24). The H & E sections demonstrated significant infiltration into the surrounding tissues including the anal sphincters, a common finding in both locally advanced and relapsed human ASCC. The cytokeratin expression was found to be consistent with a squamous carcinoma.

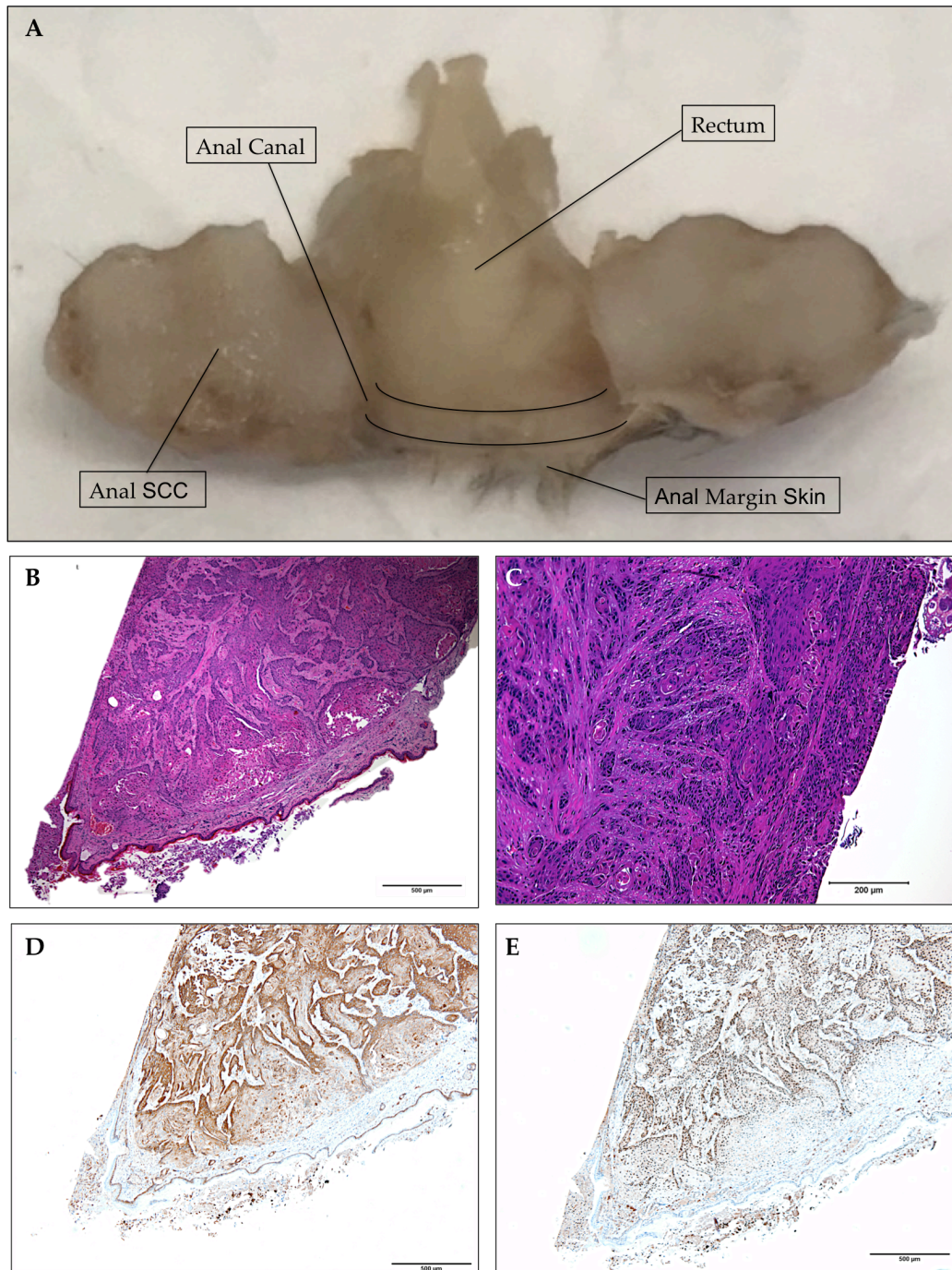


Figure 6-24 Exploration of an Orthotopic model

Orthotopic injection of 2×10^6 cells of Anal1 E6/7 into the anal canal with the subsequent tumour resected, formalin fixed and bisected to display the anatomy as demonstrated in A). The tumour was then paraffin embedded and processed for histology. H & E photomicrographs demonstrate extensive infiltration into the surrounding perianal and pelvic tissues including the anal sphincter (B) (x4 objective, scale bar 500µm) and (C) (x10 objective, scale bar 200µm). Cytokeratins CK5/6 (D) and p63 (E) appropriately stain and confirm the tumour component as squamous (x4 objective, scale bar 500µm).

6.3.13 Establishment of a Metastatic Subclone – Anal1 E6/7 Met

When performing necropsies on the NSG mice with Anal1 E6/7 intra-anal syngrafts that had reached ethical limits, pulmonary metastases were noted macroscopically in all 3 of 3 mice (Figure 6-25 A). Following 10% NBF fixation and blocking, H & E and IHC staining subsequently confirmed the lesions to be metastatic SCC (Figure 6-25 B-D) consistent with disease from the Anal1 E6/7 line.

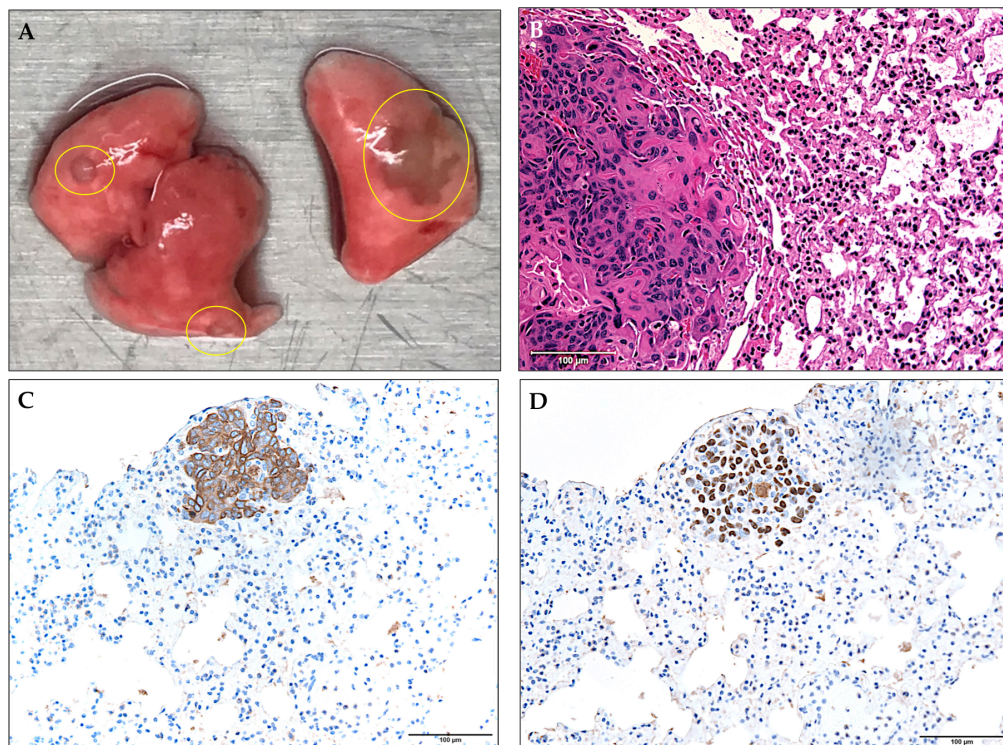


Figure 6-25 Establishment of a Metastatic subclone – Anal1 E6/7 Met

Following intra-anal injection to NSG mice, and the primary anal tumour reaching ethical limits, the mouse was culled with the identification of bilateral pulmonary metastases. Macroscopic view of NSG bilateral pulmonary metastases highlighted with yellow circles (A). Following fixation and blocking, a H & E slide (B) demonstrates evidence of a squamous metastasis surrounded by normal pulmonary parenchyma. Cytokeratin expression of CK5/6 (C) and p63 (D) confirms the lesion as squamous in origin (x20 objective, 100µm) .

Tissue taken at the time of necropsy was utilised to establish a metastatic subclone cell line of Anal1 E6/7 via the described explant technique (section 2.2.2). The morphology of this adherent line was assessed with brightfield microscopy. This demonstrated smaller colonies and numerous spindle and mesenchymal-like cells with lamellipodia when compared with the parental lines (Figure 6-26 A). This suggested a proportion of cells with a more mesenchymal than epithelial phenotype. When Anal1 E6/7 Met line was assessed as CL tumouroids, several also demonstrated a budding morphology not seen in the two parental lines (Figure 6-26 B). This morphology however could be reproduced in all three lines when cultured in colorectal organoid media.²⁷²

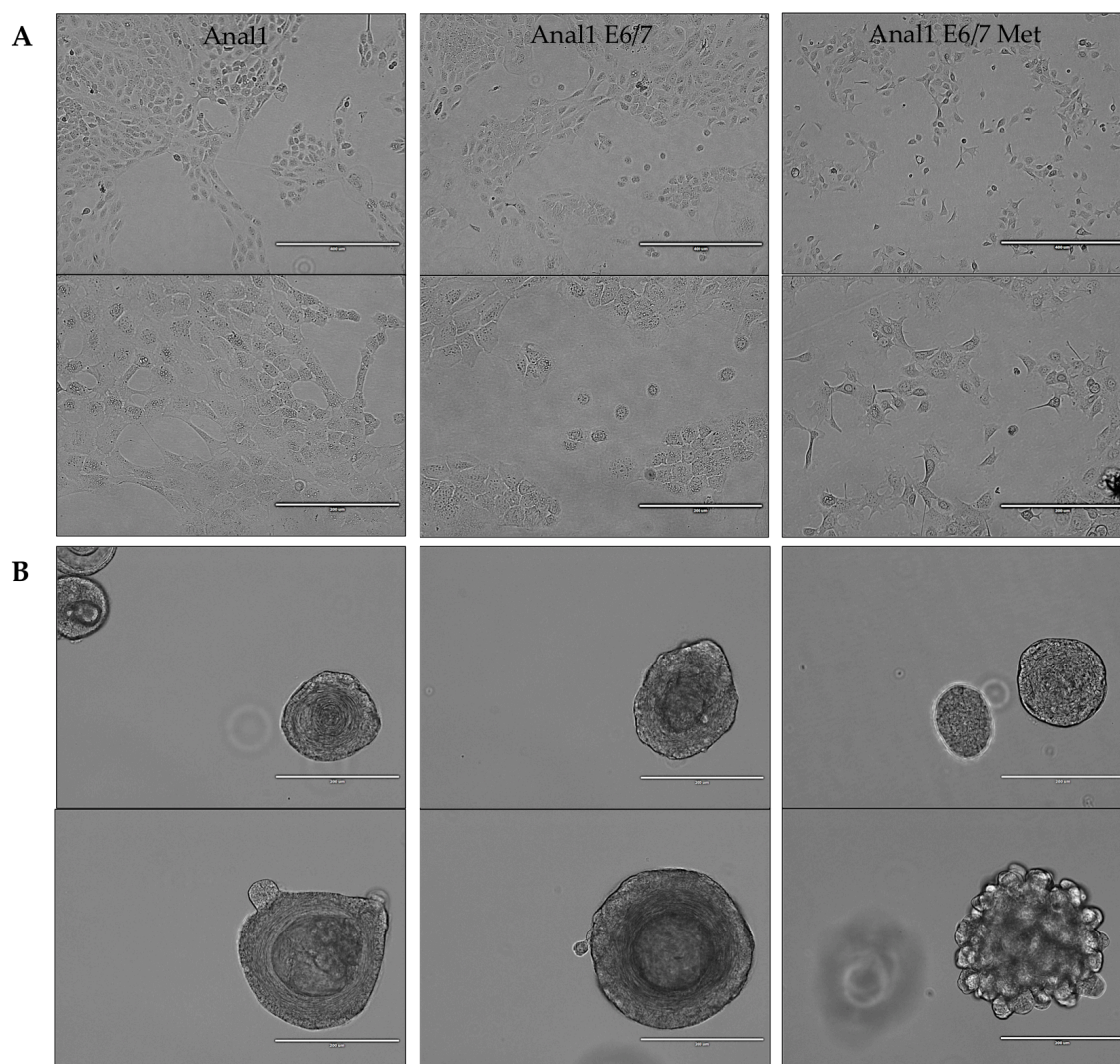


Figure 6-26 Morphology of Anal1 and derived sub-lines Anal1 E6/7 and Anal1 E6/7 Met

The cell lines Anal1, Anal1 E6/7 and Anal1 E6/7 Met were seeded into (A) a 96-well plate (5×10^2 cells) and imaged at 48 hours and (B) into Matrigel in a 24-well plate at 100 cells / 50 μ l and allowed to grow for 14 days at which point they were imaged under an inverted microscope with the x10 and x20 objectives. Anal1 and Anal1 E6/7 demonstrate a similar morphology as an adherent line with large colonies, while Anal1 E6/7 Met demonstrates evidence of a higher number of spindle and mesenchymal-like cells with lamellipodia scattered between smaller colonies (A). When assessed as CL tumouroids (B), Anal1 and Anal1 E6/7 demonstrate a consistent spheroid shape with an outer rim of cells not dissimilar to that identified in the human ASCC CL tumouroids. However, while the Anal1 E6/7 Met line had some smaller spheroid tumouroids (top image of B), several demonstrated a budding morphology (bottom image of B). This budding could be reproduced when any three of the CL tumouroids were placed in colorectal organoid media.²⁷² (scale bar 400 μ m top row of A, remainder 200 μ m)

6.3.14 *In Vitro* Chemotherapeutic and Targeted Therapy Assays

The two mouse ASCC cell lines, Anal1 and Anal1 E6/7 were assessed for sensitivity to both 5FU and MMC, the two drugs used in the primary treatment of ASCC patients and a comparison made with the sensitivity of the panel of human ASCC cell lines.

The 5FU dose-response curves and representative GI₅₀ for each cell line are presented in Figure 6-27 A. This revealed that both murine lines had a very similar sensitivity to 5FU and were found be more sensitive than the panel of human lines. The GI₅₀ of both mouse lines were almost 4 times lower than the most sensitive human line, P2.

The MMC does-response curves and GI₅₀ values for the mouse and human cell lines are represented in Figure 6-27 B. Both anal lines again demonstrated significant similarity in their sensitivity to MMC and had GI₅₀'s that fell within the range of the least resistant human anal SCC lines.

The sensitivity of Anal 1 and Anl1 E6/7 to the PIK3CA p110 α specific inhibitor BYL719 (Alpelisib) was undertaken with results and the GI₅₀ data listed in Figure 6-28. This revealed that Anal1 demonstrated substantial sensitivity in a similar range to the most sensitive human anal SCC cell line, P1. Anal1 E6/7 however demonstrated resistance with a GI₅₀ of 2.19 μ M, which falls within the range of resistance based on published results of head and neck and breast cancer cell lines.^{261, 262} A reason for this resistance may be revealed with sequencing.

The last molecular targeted therapy to be assessed for efficacy was TP-3654, a PIM1 inhibitor with some PI3K inhibitor activity. When compared to published

data relating to urological cell lines, both Anal1 and Anal1 E6/7 fall within the sensitive range based on their GI₅₀ values (Figure 6-28).²⁶⁴

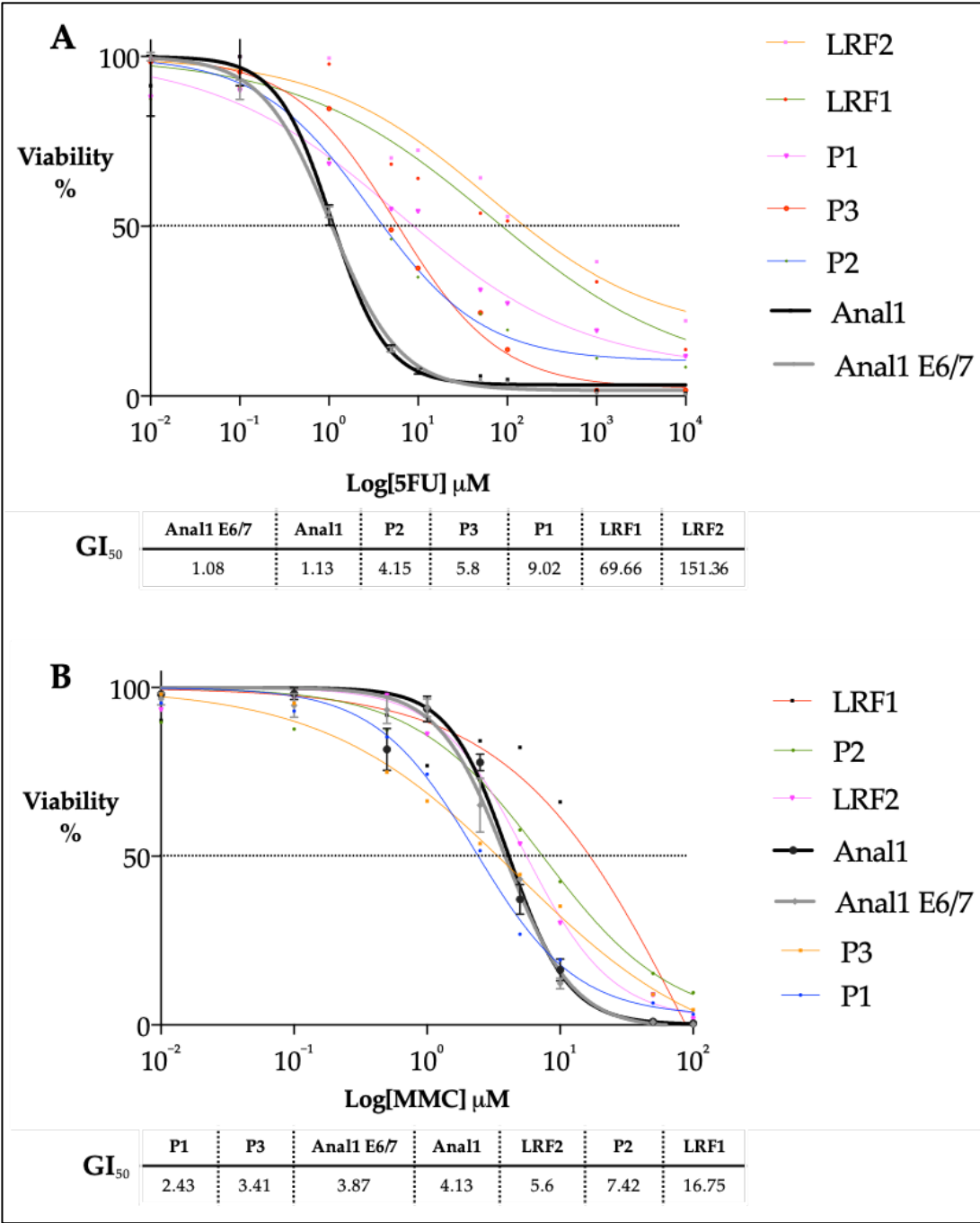


Figure 6-27 5FU and MMC *in vitro* Cytotoxic Assays

Dose-response curves for single agent 5-FU (A) and MMC (B) treatment of Anal1 and Anal1 E6/7 compared with the human ASCC cell lines. Anal1 and Anal1 E6/7 were seeded at 5×10^2 cells / well and treated with a range of concentrations of both drugs for 96 hours, and the viable cells quantified by AlamarBlue[®] Assay. Both demonstrate dose-dependent inhibition of cell proliferation and viability. The data represented are the mean $\pm$ SEM for three experiments. The GI₅₀ are presented in the tables. Both mouse lines

demonstrated similar sensitivity to both agents. When compared to the human lines, the mouse lines were considerably more sensitive to 5FU (A) but demonstrated a similar profile of response to MMC as the more sensitive human ASCC lines (B).

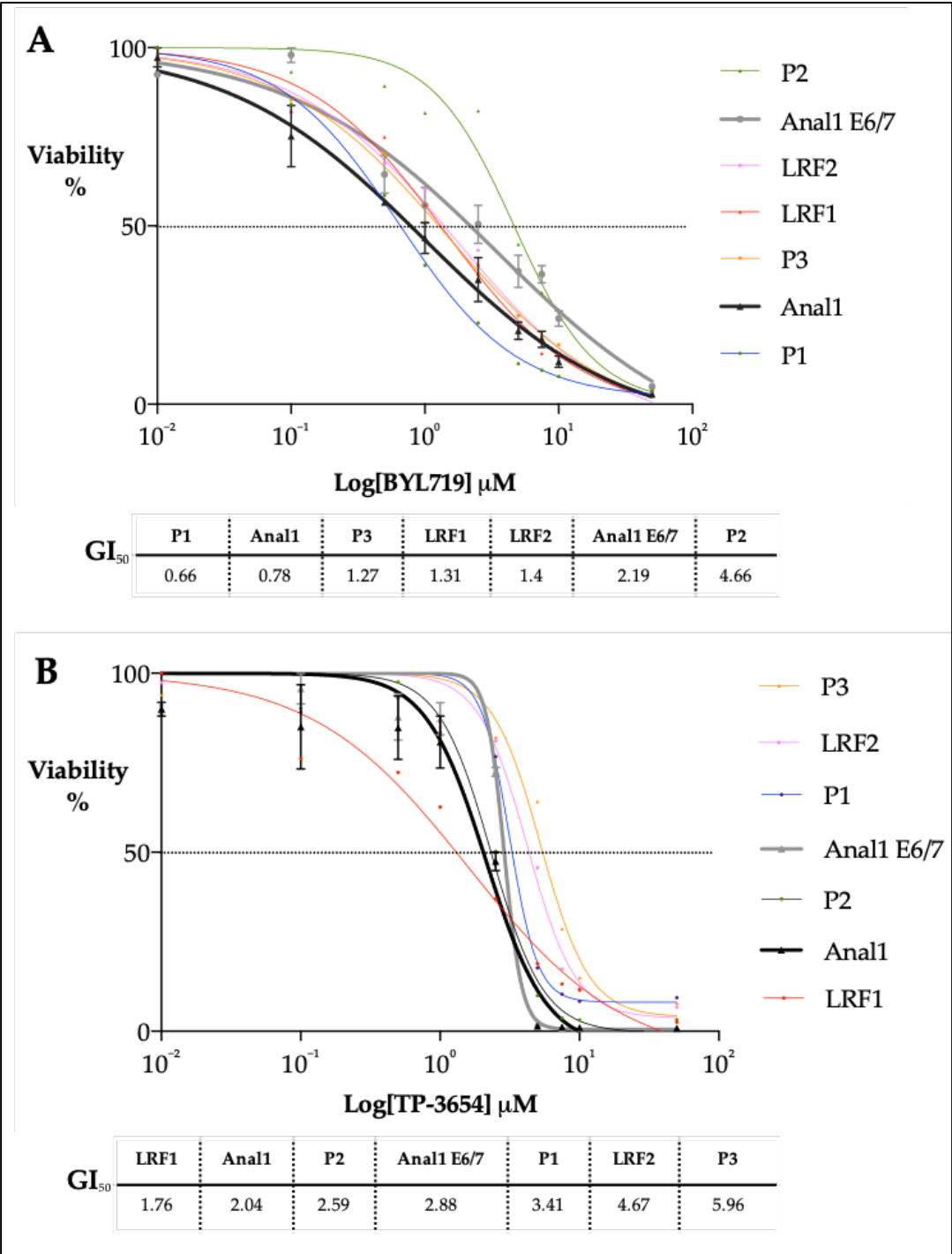


Figure 6-28 *In vitro* TP-3654 and BYL719 Molecular Targeted Therapy Assays

Dose-response curves for single agent BYL719 (A) and TP-3654 (B) treatment of Anal1 and Anal1 E6/7 compared with the human ASCC cell lines. Anal1 and Anal1 E6/7 were seeded at 5×10^2 cells / well and treated with a range of concentrations of both drugs for 96 hours, and the viable cells quantified by AlamarBlue[®] Assay. Both demonstrate dose-

dependent inhibition of cell proliferation and viability. The data represented are the mean +/- SEM for three experiments. Anal1 E6/7 demonstrated increased resistance to BYL719 compared to the parental line Anal1 (A). Significant sensitivity to TP-3654 was demonstrated in a similar fashion by both lines (B).

6.3.15 *In Vitro* Radiotherapy SF2 and Dose-Response Curve

The sensitivity of Anal1 and Anal1 E6/7 cell lines to radiotherapy was assessed by determining the surviving fraction across 2, 4, 6, and 8 Gy. The seeding density was optimised for the radiotherapy clonogenic assay with the final density for each dose outlined in Table 6-3. Anal1 and Anal1 E6/7 were grown for 4 days before reaching the appropriate colony size. The surviving fraction of 2 Gy (SF2) and the dose-response curves derived from the linear quadratic equation [$SF(D)=e^{-\alpha \cdot D - \beta \cdot D^2}$] are displayed and compared against the results of the panel of human anal SCC lines in Figure 6-29 A and B. An explanation of this equation and the respective parameters is detailed in section 5.3.18.

Table 6-3 Seeding density for radiotherapy clonogenic assay

RTx Dose (Gy)	Seeding Density (x 10 ³)	
	Anal1	Anal1 E6/7
0	0.2	0.2
2	0.2	0.2
4	0.4	0.4
6	0.4	0.8
8	0.8	1.6

These data demonstrate that both mouse cell lines compared to the panel of human ASCC lines, are very resistant to radiation in terms of SF2 and with increasing doses. Anal1 E6/7 was more resistant than Anal1 with regard to SF2, however, Anal1 E6/7 demonstrated increasing sensitivity at higher dose fractions. Anal1 on the other hand had an incredibly high α/β ratio, indicating minimal sensitivity to fractionation.

When the mouse lines are compared to published data on cervical SCC and HNSCC cell lines, the two mouse anal cell lines had a similar SF2 to the most resistant HPV-ve HNSCC lines (SF2 range 0.3 – 0.80).²⁶⁸

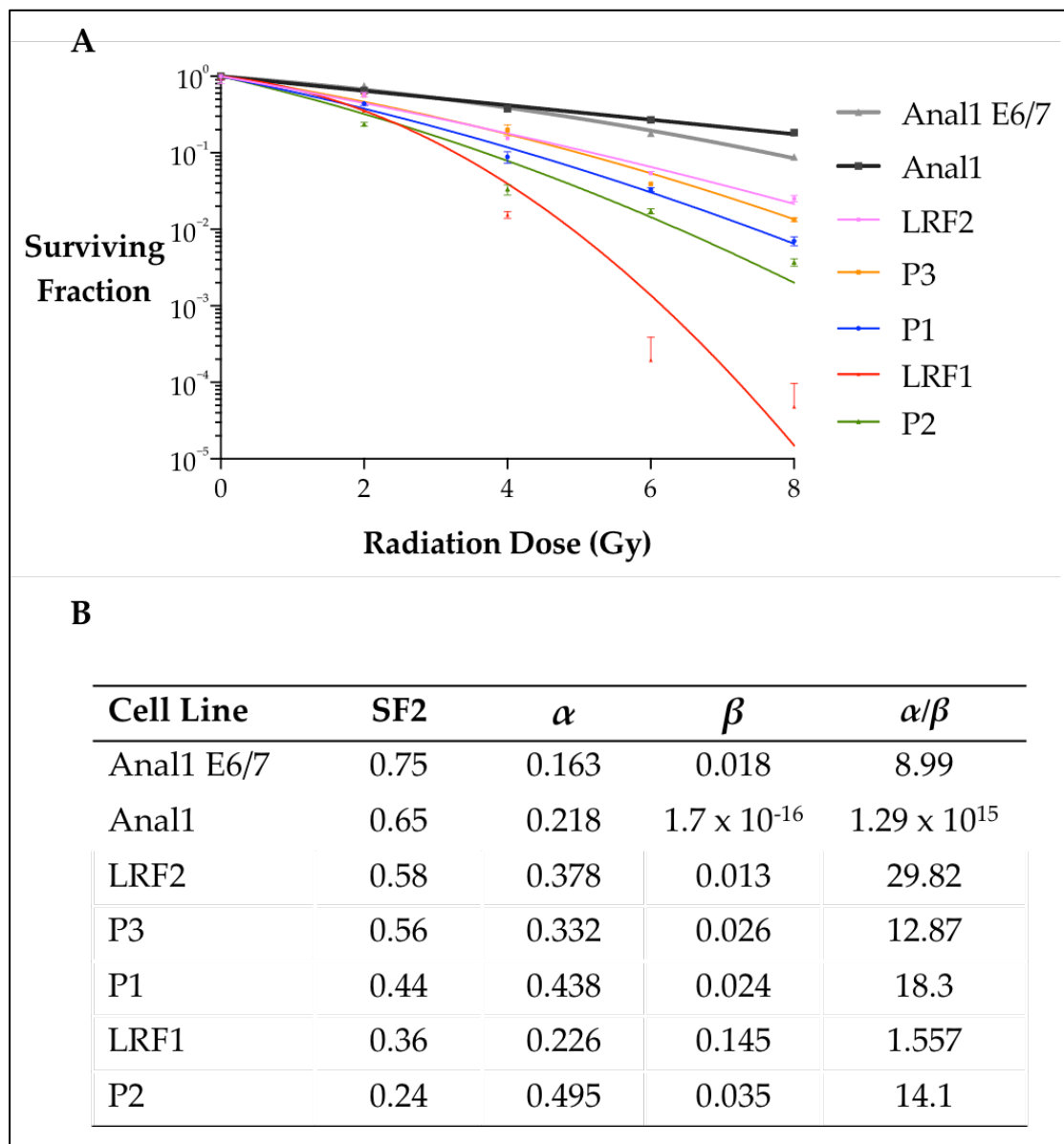


Figure 6-29 Radiotherapy SF2 and Dose-Response Curve for Mouse and Human ASCC Lines

Radiation survival curves demonstrating the survival fraction (log10) following irradiation with 2, 4, 6, 8 and 10 Gy for the two mouse ASCC lines compared to the panel of 5 human ASCC cell lines. Data points are mean $\pm$ SEM for 3 replicates. (A) Data points are fitted with the linear quadratic equation. The table (B) displays the SF2 and the α and β values and α/β ratio for each line to better characterise the radiobiology of the lines and facilitate comparison with published data. Both mouse lines demonstrated greater resistance to radiotherapy both in terms of SF2 and at increasing doses.

6.3.16 Exploring the Immune Phenotype

6.3.16.1 Immunohistochemistry

The immune infiltrate of Anal1 E6/7 subcutaneous and intra-anal tumours was explored initially by chromogenic IHC. The CD3+ marker was used to assess the T-cell infiltrate (

Figure 6-30). This revealed that there was significant heterogeneity within tumours, with areas of dense infiltrate and others of minimal to no infiltrate. The majority of the CD3+ cells were also identified within the peri-tumoural stroma rather than being intra-tumoural in location. This is consistent with the findings in human ASCC, where the majority of immune cells are located with the stroma. The greatest density of CD3+ cells was identified in those tumours in an orthotopic position. The reasoning for this could be secondary to an improved vascular supply, given the vascularity of the anal canal is substantially greater than in the subcutaneous plane. It could also relate to a greater density of immune cells residing within the gastrointestinal tract than within the subcutaneous tissue plane, given the higher antigenic exposure of the gastrointestinal tract than the subcutaneous fat plane.

PDL1 expression was also assessed in the Anal1 E6/7 subcutaneous syngrafts, demonstrating significant baseline expression on the tumoural cells and heavy expression on specific cells within the peri-tumoural stroma (Figure 6-31).

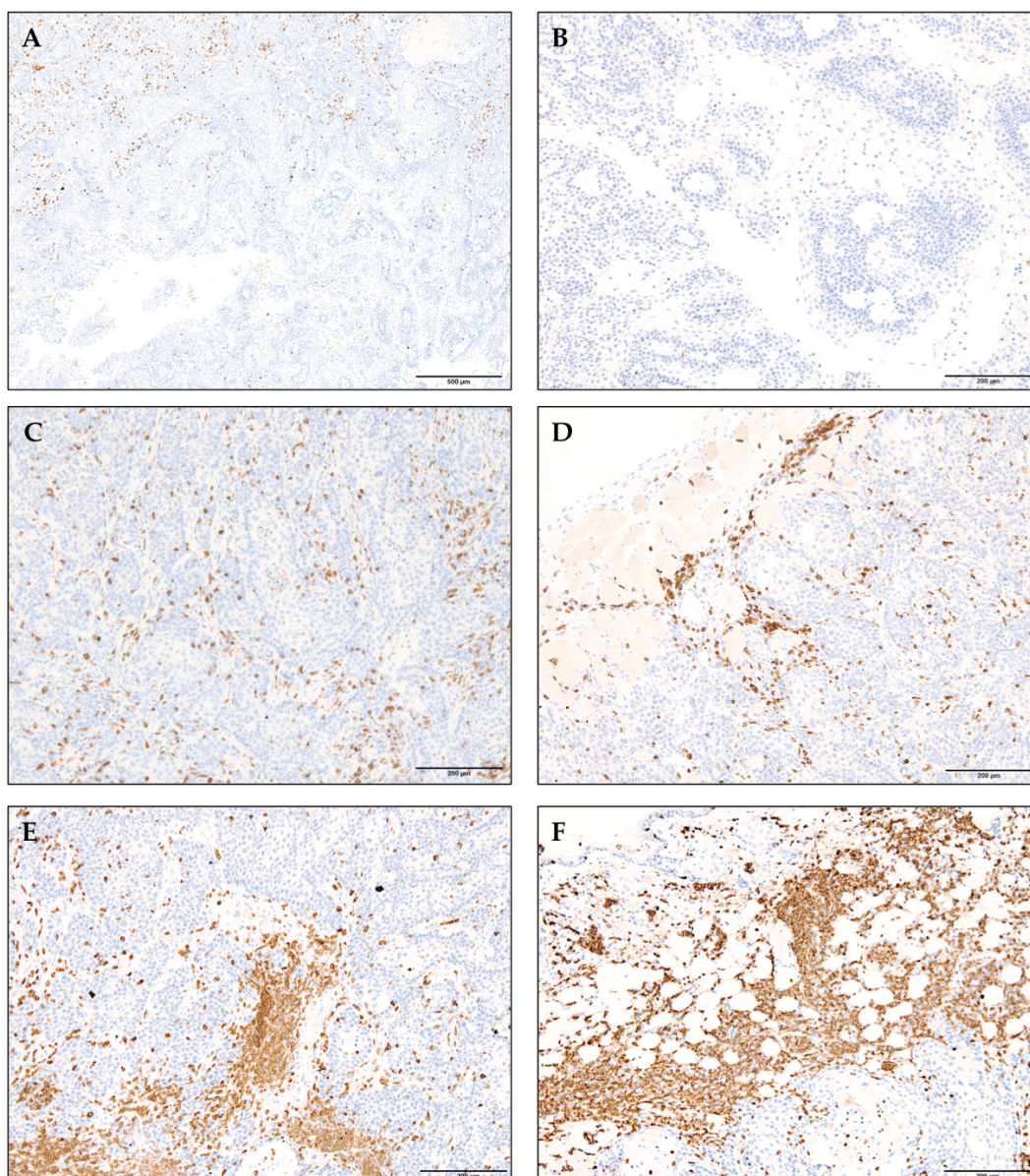


Figure 6-30 CD3+ TIL in Anal1 E6/7 Syngraft tumours

Anal1 E6/7 CL syngrafts (subcutaneous or intra-anal) harvested from wtC57Bl/6 mice, fixed, blocked and sectioned for CD3+ IHC staining. (A) Demonstrates the significant heterogeneity of the CD3+ infiltrate identified in syngraft tumours (x4 objective, scale bar 500µm). An area of moderate infiltration (C) contrasted with a region of desert appearance (B). Where present, the majority of the CD3+ TIL infiltrate is located in the peri-tumoural stroma rather than intra-tumoural, consistent with that identified in human ASCC. D) Demonstrates the highest density of the CD3+ cells at the invasive tumour margin (muscle invasion). E) & F) Demonstrate the higher CD3+ TIL infiltrate identified in orthotopic Anal1 E6/7 syngraft tumours, again concentrated within the stroma and at the invasive margin (x10 objective, scale bars 200µm).

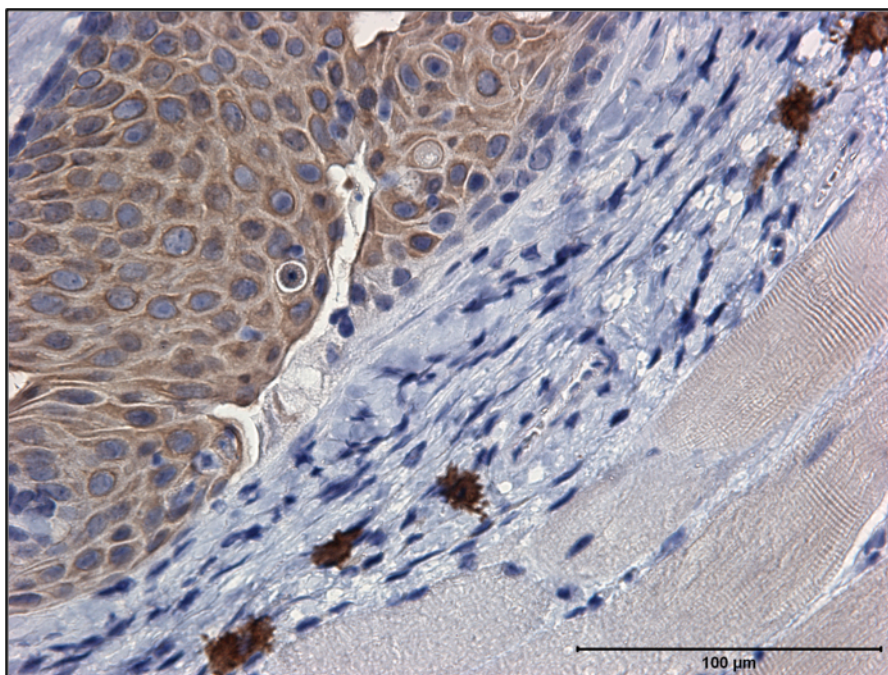


Figure 6-31 Anal1 E6/7 Syngraft demonstrating PDL1 Expression

Anal1 E6/7 subcutaneous CL Syngraft, harvested, fixed blocked and sectioned with chromogenic IHC staining for PDL1 expression. This revealed evidence of baseline tumoural PDL1 expression and heavy PDL1 expression on cells within the peri-tumoural stroma.

6.3.16.2 Flow Cytometry

Analysis of the phenotype of the immune infiltrate by flow cytometry in the Anal1 E6/7 tumours was undertaken for subcutaneous syngrafts alone. This was performed with an extensive panel of markers to delineate both the adaptive and innate arms of the immune system. The gating strategy for the immune markers is demonstrated in Figure 6-32 and Figure 6-33. The placement of the isotype gates for accurate assessment of marker expression is demonstrated for a select number of markers in Figure 6-34.

The results of the TIL phenotype of the Anal1 E6/7 syngrafts are demonstrated in Figure 6-35, representing the average of results from four mouse tumours. This revealed a median of 64% of cells as CD45+ with a median of 9% of this group

being CD3+, both similar to the percentages identified in human ASCC tumours (Figure 4-8). The proportion of CD3+ cells being CD4+ (median 51%) and CD8+ (median 42%) in the mouse tumours was also similar to human ASCC, with an increased expression of PD1 identified (median 30%). The proportion of CD4+ cells being FOXP3+ was found to be a median of 10% in the mouse tumours compared with 21% in human tumours. The majority of the CD3+ cells were also identified to be of a memory phenotype (CD44^{hi}CD6L^{Lo}, median 78%) again consistent with the findings in human ASCC (CD45R0+ cells, median 82%).

Of interest, the Anal1 E6/7 tumours also demonstrated a high proportion of CD45+CD3- cells (median 74%), similar to human ASCC tumours (median 79%). These cells were further defined in the mouse tumours by gating for lineage negative cells (TCR-, CD56-, CD19-) and assessing for MHCII and CD11b expression. This identified that the majority of these cells were MHCII- and CD11b+ (median 81%), consistent with a myeloid derived suppressor cell population, and similar to the HLA DR-CD11b+CD33- population (median 87%) in the human ASCC tumours. The PDL1 expression in this subgroup was found to be particularly high (median 81%), and likely accounts for the heavy PDL1 expression that was identified on discrete stromal cells on chromogenic IHC for PDL1 (Figure 6-31). In the human ASCC tumours, PDL1 expression on this subset only reached a median of 9%.

The MHCII-CD11b+ MDSC population was further assessed for expression of Ly6G and Ly6C, demonstrating that the majority of cells were Ly6G⁺⁺Ly6C⁺ or Ly6G-Ly6C-. Tumour expression of PDL1 was also assessed (Figure 6-35 B) and found to be at a median of 39%. MHCI expression was also assessed and found to be at a median of 32%.

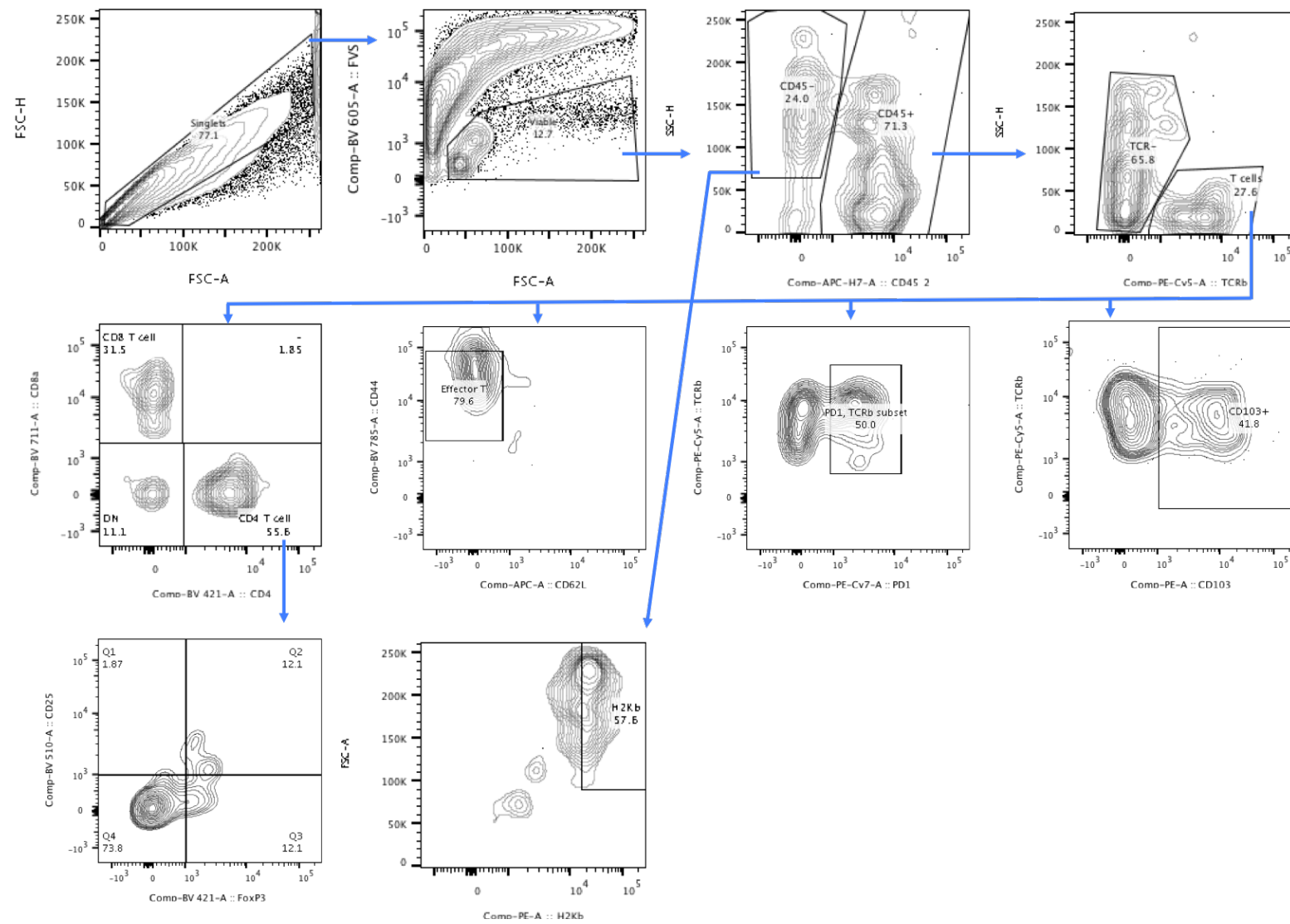


Figure 6-32 Anal1 E6/7 Syngraft TIL Phenotype Gating Strategy – Adaptive Immunity

Anal1 E6/7 subcutaneous syngraft tumours were harvested, processed to single cells, stained and analysed by flow cytometry. The gating strategy for determining the expression of adaptive immune cell markers is demonstrated. This involved gating sequentially for singlets, viability and CD45+ cells with subsequent assessment of TCR expression, CD4+, CD8+, CD44, CD62L, PD1, CD103, CD25 and FOXP3. H2Kb (MHCI) was also assessed on the CD45- cell population (non-immune

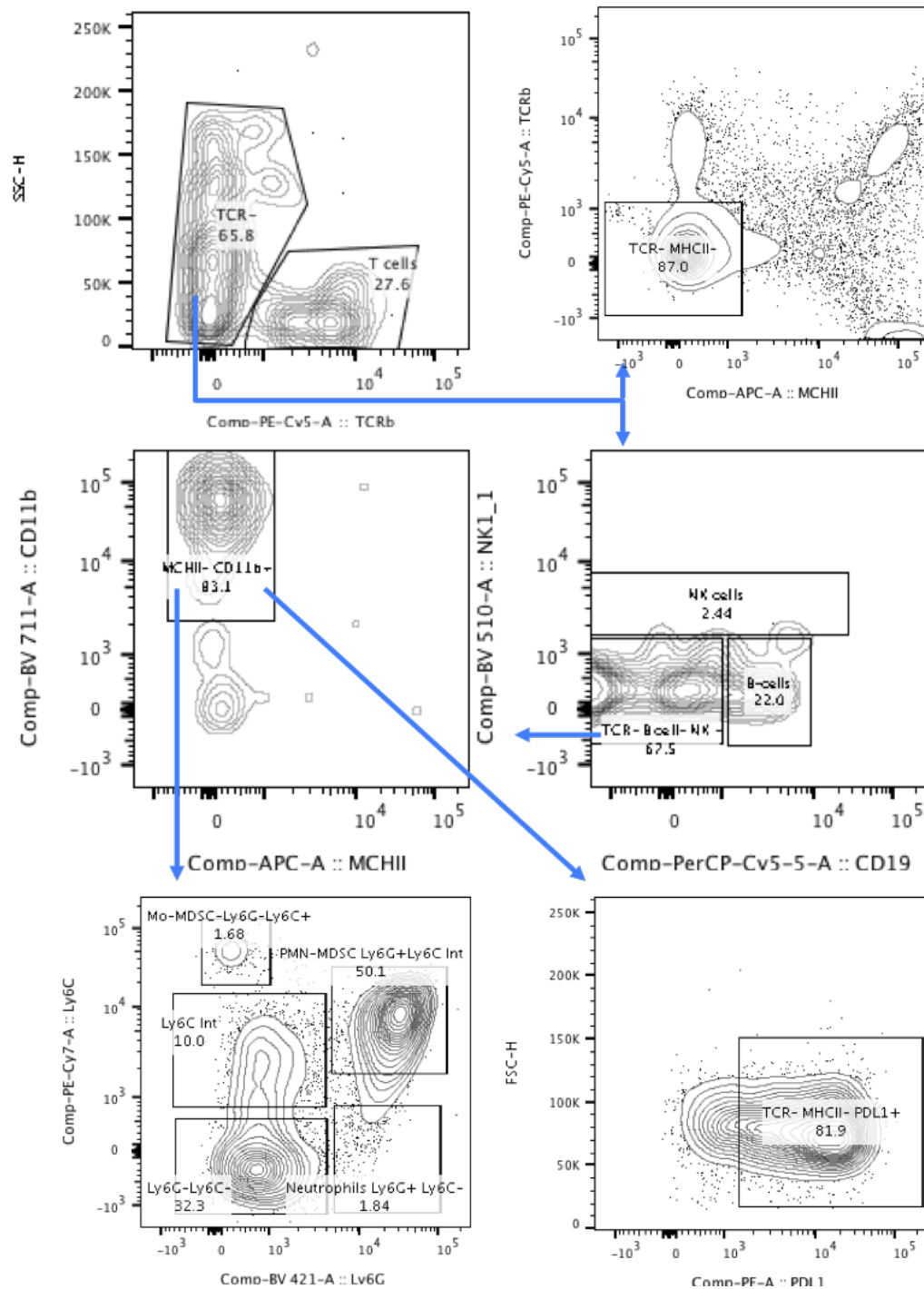


Figure 6-33 Anal1 E6/7 Syngraft TIL Phenotype Gating Strategy – Innate Immunity

Anal1 E6/7 subcutaneous syngraft tumours were harvested, processed to single cells, stained and analysed by flow cytometry. The gating strategy for determining the expression of innate immune cell markers is demonstrated. This involved gating sequentially for singlets, viability, CD45+, lineage negative (TCR-ve, CD19 -ve, NK1.1 -ve) cells, MHCII -ve cells and CD11b-ve cells. The myeloid cell sub-populations were then assessed by examining Ly6C and Ly6G expression and determining the proportion of myeloid cells that were PDL1+.

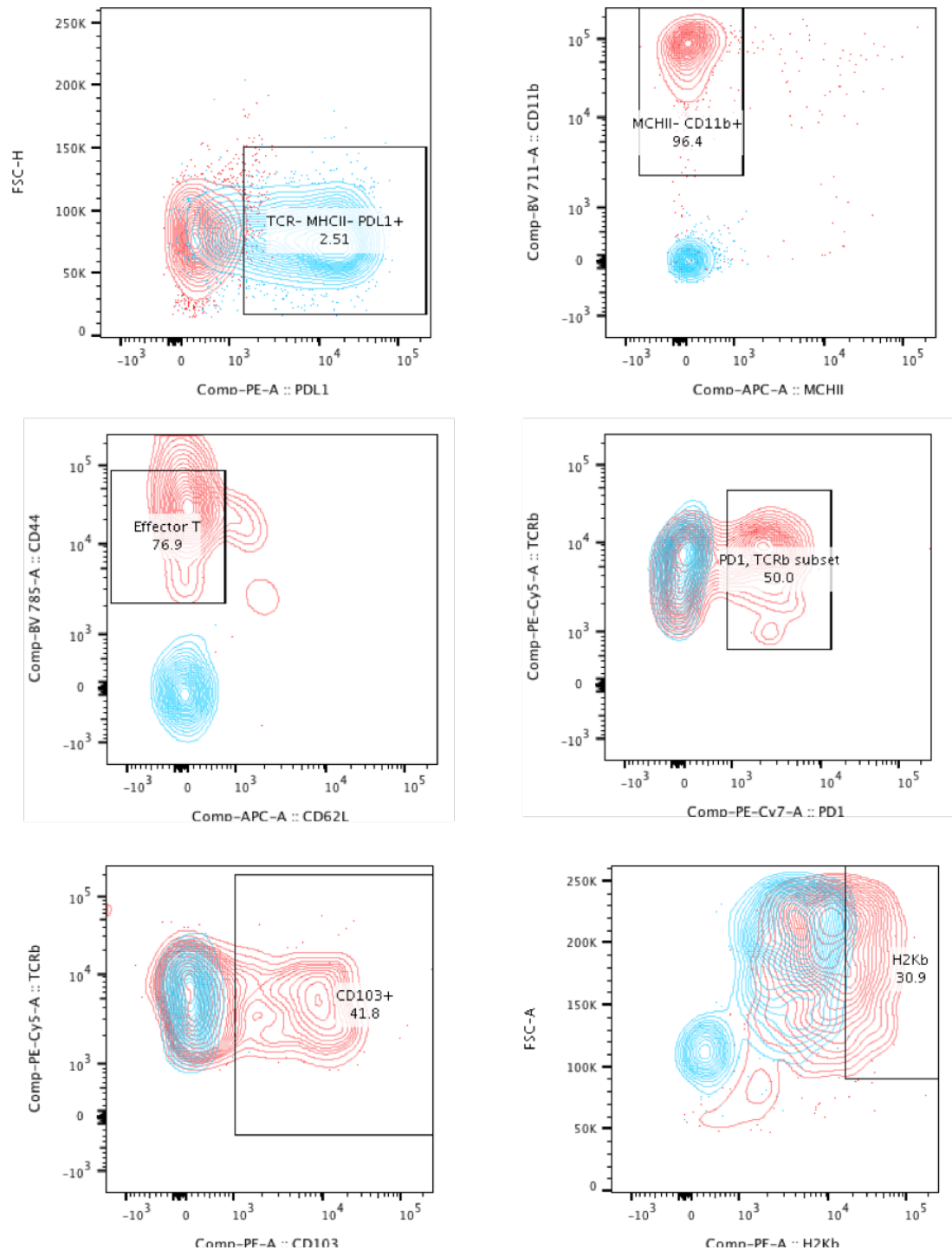


Figure 6-34 Isotype Gating for accurate determination of Mouse Immune Marker Expression

Anal1 E6/7 subcutaneous syngraft tumours were harvested, processed to single cells, stained and analysed for flow cytometry. Isotype controls (blue) were utilised in the staining protocol to determine appropriate gating to ensure accurate assessment of the marker expression (red). Isotype gating for PDL1, MHCII, CD11b, CD44, CD62L, PD1, CD103 and H2Kb are demonstrated.

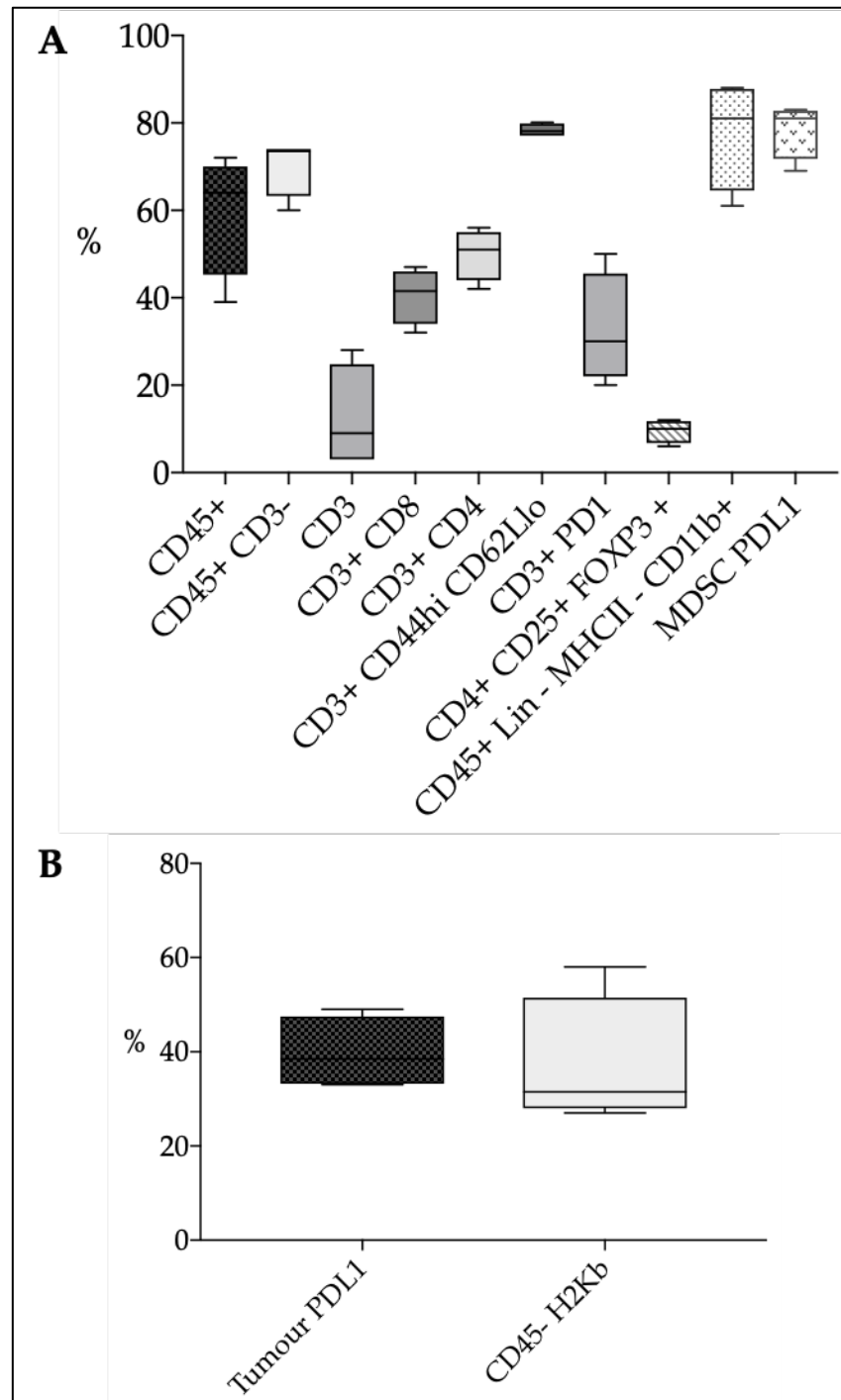


Figure 6-35 Phenotype of the Immune infiltrate in Anal1 E6/7 Syngrafts by Flow Cytometry

Anal1 E6/7 subcutaneous syngraft tumours were processed for flow cytometry to delineate the immune phenotype. (A) Immune subtypes identified in four wtC57Bl/6 mice with subcutaneous CL syngraft tumours. This demonstrated a substantial proportion of CD45+ cells with many similarities shared with the immune phenotype of human ASCC (Figure 4-8). (B) Expression of MHC-I and PDL1 on Anal1 E6/7 tumour cells.

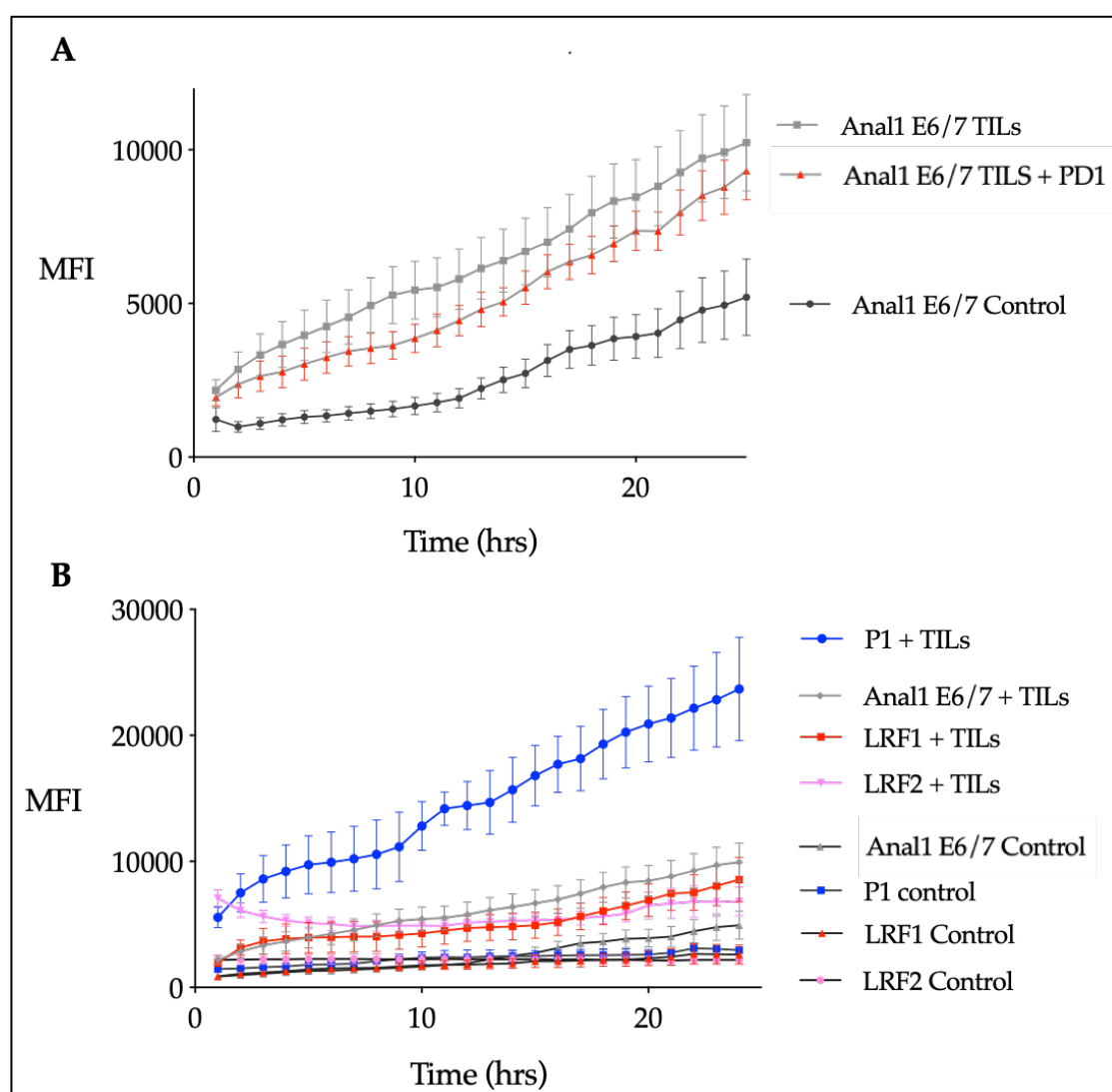


Figure 6-36 Anal1 E6/7 CL Tumouroid – TIL Co-Culture Cytotoxic Assay

TILs were expanded from Anal1 E6/7 syngrafts with IL-2 and co-cultured with Anal1 E6/7 CL tumouroids in the presence of PI, with the cytotoxicity assessed at hourly intervals by measuring the MFI of the CL tumouroids as a surrogate. (A) The cytotoxicity of matched TILs against Anal1 E6/7 CL tumouroids (10:1 ratio) was assessed with and without an anti-PD1 (RMPI-14) antibody and compared to CL tumouroids alone (negative control). This demonstrated minimal killing above the control with no improvement following the addition of an anti-PD1 antibody. (B) When plotted against the three human ASCC lines, the Anal1 E6/7 TILs appear to have a similar cytotoxicity to the two relapsed human patients' assays and well below that of the assay from the patient who achieved a complete response (P1). (MFI +/- SEM is represented across in single experiment with 8 tumouroids per condition)

6.3.17 TIL - Cell Line Organoid Co-Culture Cytotoxic Assay

By expanding tumour infiltrating lymphocytes from an Anal1 E6/7 syngeneic subcutaneous tumour and culturing them with Anal1 E6/7 CL tumouroids, exploration of the immune systems role in tumour control was further assessed.

The process for undertaking the assay is defined in section 2.2.5 which we have recently published²⁶⁹ and a schematic of the process is demonstrated in Figure 2-2. The co-culture assay was undertaken with the Anal1 E6/7 cell line.

The results of the assays are demonstrated in Figure 6-36 A. The TIL : Tumouroid cell ratio utilised was 10:1 to maintain consistency with the human cytotoxic assay. TILs from the syngeneic Anal1 E6/7 tumour demonstrated evidence of cytotoxic activity against matched Anal1 E6/7 CL tumouroids when compared to the negative control. The addition of an anti-PD1 antibody (RMPI-14) did not improve the cytotoxic activity of the TILs. This is despite the expression of PD1 on up to 50% of CD3+ cells. When compared to the human anal ASCC cell lines, the level of killing was similar to that of LRF1 and LRF2 lines and significantly below that seen with the complete response CL P1.

6.4 Discussion

This chapter has outlined the establishment of a novel mouse ASCC model that recapitulates the genomic landscape of human ASCC.

While two mouse models of ASCC have been reported, there are significant limitations, including a lack of clarity over the origin of the tumours and the relevance of employing a carcinogen targeting *ras*, given its infrequency in

human ASCC. The novel model described here addresses these inadequacies, by confirming the targeting of the anal canal region for activation of the transgene and utilising the most frequent genomic aberration in human ASCC, *pik3ca* as the backbone for the model.

One of the greatest limitations and barriers with progress of the mouse model related to the use of UBC-Cre to drive the knock in of the *pik3ca* mutation. DMSO was utilised as the vehicle for 4-OHT, allowing deeper penetration beyond the basal layer of the anal canal epithelium. Consequently, in the setting of a UBC-Cre mouse, cells within the stromal compartment lying beneath the anal canal may have been exposed, allowing transgene activation, with subsequent transformation and outgrowth as a sarcoma with serial passaging.

Ideally, a keratin specific Cre might have been utilised in preference. Keratin expression is limited to the epithelium from where it is theorised squamous cancers arise, compared with Ubiquitin C expression which is present in all cells. Consequently, a keratin specific Cre would target the appropriate epithelial cells, and ameliorate the problems encountered with stromal transformation from transgene induction in other tissues and supporting cells. This would have facilitated the development of a pure anal canal squamous carcinoma reproducibly in the transgenic mouse without malignant stromal contamination.

However, this was not logistically possible given the timing of a physical move of our research lab, animal house and hospital campus to a new location in Melbourne. As part of the move, all mouse lines had to be cryopreserved and re-derived, a process which absorbed 8 months in total. This left inadequate time to perform appropriate back-crossing of mice to obtain a keratin-specific Cre transgenic mouse model on a pure background that would be feasible within the time-line of the PhD. The establishment of a robust cell line Anal1 derived from

the *pik3ca* mutant mouse however has allowed further manipulation including a transduction with HPV 16 E6/7 to better recapitulate the genomic and molecular landscape of human ASCC. Had time permitted, the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology, to knock-out wildtype TP53 and knock-in a mutant TP53 gene to Anal1, would create a model that closely mirrors the genomic landscape of HPV-ve human ASCC.

A pooled transduction technique was utilised with the HPV lentivirus in preference to a single clone transduction, based on the proposed scenario of HPV transformation in human epithelia. Certain tissues, predominantly perineal, penile, cervical and oropharyngeal are at increased risk for infection and stable incorporation of the HPV genome. Of the pool of cells that are infected with the virus, only a reducing proportion will sequentially develop dysplasia and carcinoma. Consequently, a pooled transduction technique would most closely recapitulate this phenomenon, with the possibility of incorporation at multiple sites within the host genome, with certain integrations theoretically having a greater propensity to induce carcinogenesis. Theoretically, the outgrowth of the transduced clones would then subsequently compete against each other to eventually reach a single clone or group of clones in equilibrium with subsequent passaging.

Validation of the genomic backbone of the Anal1 line confirmed the presence of mutant *pik3ca*^{H1047R}, however, it also revealed the presence of both *pten*^{wt} alleles. This was despite the parent tumour from which Anal1 was derived having loss of both *pten* alleles in the majority of the tumour. This lack of fidelity in inducing both genomic aberrations in an appropriately exposed cell has been well documented.²⁷³ With the subsequent culturing of the cells derived from this tumour *in vitro*, the small proportion of *pik3ca*^{H1047R}.*pten*^{wt/wt} cells may have had an inherent growth advantage over the *pik3ca*^{H1047R}.*pten*^{fl/fl} cells. This also provides

evidence that mutant *pik3ca* alone is sufficient to induce tumourigenesis in anal SCC given the tumourigenic potential of this line in subsequent assays.

Further validation was undertaken to demonstrate upregulation of the *PI3K* pathway by the *pik3ca*^{H1047R} mutation as evidenced by increased expression of the downstream product pS6 on IHC. An HPV16 E6/7 specific PCR was also undertaken in addition to puromycin selection in order to confirm stable transduction of the Anal1 E6/7 line. This confirmed the presence of the HPV16 oncogenes in the Anal1 E6/7 line and the four human HPV positive lines. Both mouse syngeneic lines were subsequently proven to demonstrate the characteristics of human squamous anal cancers, including appropriate cytologic features and cytokeratin expression and the ability to grow as tumouroids with appropriate histological architecture.

Both mouse lines demonstrated a more rapid doubling time and higher seeding efficiency than the human ASCC lines, but within the range reported for the NCI-60 cell lines.²⁷⁴ They also demonstrated a significantly increased migration potential compared with the human lines. Both demonstrated robust *in vivo* tumourigenicity, including the ability to grow within an orthotopic position. The Anal1 E6/7 line also demonstrated consistent metastatic potential in immunosuppressed mice, with a metastatic subclone, Anal 1 E6/7 Met established from a metastasis.

Individual assays were performed *in vitro* across chemotherapeutic agents, molecular therapies and radiotherapy, allowing comparison with the response of the panel of human ASCC lines. This demonstrated both lines to have increased sensitivity to 5FU with a similar profile of resistance to MMC. When assessed for sensitivity to the *PIK3CA* inhibitor BYL719, the parental line was found to be sensitive, with a similar GI₅₀ to the human anal SCC lines with mutant

or over-expressed *PIK3CA*. Anal1 E6/7 however demonstrated a level of resistance at the lower end of the range based on the GI₅₀ values, an explanation for which may be identified with sequencing. Both lines were also found to demonstrate pronounced radio-resistance, an important feature in both the parental and transduced models being representative of treatment resistance.

Moving forward, exploration of the responsiveness to combination chemoradiotherapy or chemo-molecular therapy would be more insightful and relevant to the clinical scenario of patient treatment. Furthermore, to complement the *in vitro* work, the next step is to assess the responsiveness of the syngeneic grafts to combined chemoradiotherapy *in vivo*. This would then clarify whether tumour control was adequately provided by this treatment regimen for this model. It also provides an opportunity to explore molecular targeted therapies and immunotherapy in an immunocompetent setting, either alone or in combination with standard treatment. This was not feasible within the time constraints of the PhD.

Utilisation of the syngeneic line in an orthotopic position would be the ideal situation to better recapitulate the human disease. However, monitoring and measurement of the tumours in this location is problematic. Consequently, small animal PET or alternatively transduction of the line with a fluorescent tag may offer another means of regular monitoring of the tumour size and treatment response. Furthermore, the tumour and immune microenvironment are likely to be more representative in an orthotopic than heterotopic model of the actual situation in human disease, and therefore of greater importance when exploring the effectiveness of immunotherapy in this disease.

The initial exploration of the immune system in the mouse model was found to be very closely aligned with that identified in the human ASCC niche. This

included the highest density of immune cells being identified in the peri-tumoural stroma, with a substantial proportion of CD3+ and CD8+ T cells, most having a memory phenotype. It also revealed evidence of PDL1 tumour expression, as well as PDL1 on peri-tumoural stromal cells. There was a large proportion of MDSCs in keeping with our findings in human ASCC, however with a much higher expression of PDL1 expression. These findings validate the utility of this model for further exploring the immune profile of this disease and assessing the utility of immunotherapy. With emerging knowledge on the negative prognostic impact of MDSCs and their role in anti-tumour immunity²⁷⁵, it also provides a platform for further exploration of this subset.

Utilising our immune cytotoxic co-culture assay also provided some insight into the functional capacity of the immune cells *in vitro*. Their cytotoxic ability was limited and approximated that identified in the two human relapsed anal SCC assays. Furthermore, the addition of an anti-PD1 antibody failed to recover further cytotoxic function, despite a high PD1 expression on the CD3+ cells. This may have been secondary to the PD1 being a marker of exhaustion rather than activation. It provides some insights into the host response to this tumour, and may be useful information to understanding future experiments involving *in vivo* assessment of chemoradiotherapy in this syngeneic line.

6.5 Conclusions

A genetically and anatomically relevant and robust syngeneic mouse model of ASCC has been established, validated and characterised. With many features closely aligned with human ASCC, it now acts as an incredibly useful platform for further exploring the molecular biology and host immune response in this

disease. Most importantly, it will allow further exploration and assessment of new therapies in an immunocompetent setting.

Chapter 7: General Discussion and Conclusion

7.1 Background

Anal squamous cell carcinoma is a rare disease that continues to increase in incidence despite widening awareness about its relationship with HPV and immunosuppression. Most patients with ASCC achieve a complete response to primary treatment with chemoradiotherapy. However, this treatment regimen has not changed in over four decades, and consequently the 5-year overall survival rate has remained static at 65%. For patients who endure treatment failure, second line treatment generally consists of salvage surgery if the disease is locoregional and deemed resectable. For those with unresectable or metastatic disease, options are palliative with few lines of therapy available. The lack of consensus and guidelines for management of this subset of patients, highlights the lack of robust scientific or clinical evidence for management decisions.

Much of the stagnation in the development of new therapies in ASCC has been secondary to a lack of preclinical investigation. The genomic underpinnings of human ASCC was undefined at the commencement of my PhD, limiting the understanding of the biology of this disease and preventing the identification of common drivers that can be therapeutically targeted. Furthermore, the immune profile was also unexplored, a factor that has gained increasing significance with the recent dawn of immunotherapy and its establishment as the fourth pillar of cancer treatment. Compounding this situation, has been the paucity of preclinical models on which to trial novel therapeutics.

This thesis has explored the role of salvage surgery in relapsed ASCC and the factors that predict treatment response and a survival outcome. It has profiled the genomic and immune landscape of this disease and established both *in vitro* and *in vivo* pre-clinical models, facilitating a deeper understanding of the biology of this disease and enabling investigation of novel therapies.

7.2 Salvage Surgery in ASCC

Surgery for ASCC was historically first-line treatment until the advent of chemoradiotherapy in the 1970's. Following the results of RCTs confirming the efficacy of CRTx, surgery was resigned to a salvage role, acting as a second line of treatment for those with resectable locoregional failure.

Chapter 3 examined the experience of salvage surgery for locoregional relapse at Peter MacCallum Cancer Centre over a period of 32 years. It identified that locoregional failure was the most frequent pattern of failure, affecting 72% of all relapsed patients. The sole independent predictor for the development of locoregional failure was identified as the T-stage, and of those with locoregional failure, 65% underwent a salvage operation. This offered a 5-year OS rate of 51% with the sole independent predictor of local disease recurrence after salvage surgery being the resection status. A significant proportion of patients within this cohort underwent an exenterative procedure given involvement of other pelvic organs with the recurrence. This highlights the importance of pre-operative staging and patient selection before embarking on salvage surgery, with a positive margin at the time of salvage surgery condemning patients to relapse and a poor outcome.

7.2.1 Future Directions

ASCC is a rare disease, with the management of patients with locoregional relapse being an even less common event. Given the significant proportion of patients with locoregional failure who require exenterative rather than routine salvage surgery, it is increasingly important to audit the volume and outcomes of surgery on this patient cohort. In many countries around the globe, such patients are referred to centralised centres, in order to maximise experience,

improve decision-making and outcomes, and facilitate the maintenance and expansion of a bio-bank and data repository from which useful research can occur.^{276, 277}

With the recent delivery and uptake of the HPV vaccine in many western countries, in the coming decades there will be a reduction in the virally driven cases of Anal SCC. This will leave a predominance of HPV-ve Anal SCC, which are intrinsically more treatment resistant. With this in mind, chemoradiation as first line treatment may need to be re-assessed. With such disparate response rates between the HPV +ve and HPV -ve cohorts, an argument that they should be treated as different diseases in their own right can be made. With ongoing research, this may involve the use of novel agents alone or in combination with chemoradiotherapy for the HPV -ve cohort, in order to improve patient outcomes.

Currently, in the absence of therapeutic alternatives, the employment of surgery early in this HPV -ve cohort, particularly in patients who fail to demonstrate response on re-staging may be an appropriate consideration. This would be in an effort to seize the narrow window of opportunity of performing a successful salvage operation with clear margins in those patients whose disease would otherwise rapidly progress to an unresectable state.

7.3 Immune and Genomic Profile of ASCC

While the TNM system is useful for the prognostication of patients with ASCC, the immune infiltrate has also been demonstrated to be of significant prognostic utility in other cancers, particularly colorectal cancer. This was explored in Chapter 4, by assessing the predictive and prognostic power of the CD8+ immune infiltrate in human ASCC. This revealed that a high pre-treatment CD8+ immune

infiltrate predicted for a complete response to primary chemoradiotherapy. When compared to the TNM system and its ability to stratify for prognosis, the CD8+ immune infiltrate was found to be a better discriminator on statistical grounds within the test cohort. The more significant findings lay with the ability of the CD8+ infiltrate to sub-stratify those patients with locally advanced disease (stage III), signalling which patients are likely to achieve a complete response and good outcome from those that may require further treatment given the increased risk of a poor outcome.

Undertaking an external validation of this quantitation technique of the CD8+ immune infiltrate in ASCC is the next step in determining the validity of the findings. The assessment of the immune infiltrate in a cohort of relapsed patients post chemoradiotherapy, was limited by the small number of patients on which data was available. While a low CD8+ infiltrate post chemoradiotherapy may predict for a poor survival outcome, statistical significance was not met in this study. Furthermore, there was a significant skew towards most patients having a low infiltrate, which combined with the small sample size, further impeded interpretation.

Further exploring the immune landscape of ASCC via flow cytometry, revealed a substantial proportion of CD3+ T cells, however, it also identified a large proportion of MDSC's. With significant evolving research into the protumourigenic effect of this immune subset, they deserve particular attention in focusing further research, as they may offer insight and explanation into those patients suffering treatment failure.

The genomic profiling undertaken in Chapter 4 has added to the very limited body of work currently published in this disease. The most common genomic aberration involved the *PIK3CA* gene, with the *PI3K* pathway upregulated in

over 60% of ASCC samples. These data support the need to further assess the role of PI3K inhibitors in combination with other treatments in this disease, particularly in patients with relapse.

The identification of p53 mutations as the predominant aberration in the majority of HPV -ve patients, also suggests a need to rethink the primary treatment in this cohort. With p53 mutant tumours being inherently resistant to radiotherapy, early consideration of salvage surgery and / or the exploration of novel therapies are needed for this otherwise treatment resistant cohort.

7.3.1 Future Directions

While significant inroads have been made in beginning to define the genomic and immune profile of ASCC, there is a need to focus future research on confirming the role of predicted genomic drivers and the importance of the immune subsets in Anal SCC. This will assist with the identification of appropriate markers for treatment response and therapeutic targets.

7.4 Human ASCC Cell Lines

A lack of preclinical models has significantly hampered the exploration of the underlying biology of anal SCC and the investigation of new therapeutic options. In view of this, the establishment, validation and characterisation of the world's first panel of human ASCC cell lines is a great step forward. As a new resource, they begin to address the hiatus in the absence of preclinical models for this disease, forming an incredibly useful platform.

The five cell lines have been established from anal SCC patients who collectively are representative across the spectrum of both disease stage and treatment

response. This includes two critical lines that have been derived from patients with treatment resistant relapsed tumours. Of greatest significance, one of these two relapsed lines was derived from a patient with viral negative disease and harbours a mutant TP53. This line represents an *in vitro* model of the most resistant form of the disease that will become the predominant type encountered in future decades, once HPV vaccination has taken effect.

An extensive characterisation of the lines included WES, which revealed the lines to be representative of the genomic landscape of ASCC both in terms of SNVs, CNV, mutational load and trinucleotide signatures. The *in vitro* characterisation of the cell lines has further provided insight into understanding the biology of this disease and factors that contribute to treatment response. Assessing the sensitivity of the panel to chemotherapeutic agents, molecular targeted therapies and radiation revealed significant variability. This aids our understanding of the intrinsic response of individual tumours to the different components of treatment that patients are subject to. Furthermore, exploring the immune response to a subset of the cell lines has also begun to unravel another facet of tumour control and treatment response, deepening our understanding of the factors that contribute to clinical responses. With the developed lines also demonstrating robust tumourigenicity in mice, there is now the ability to further our understanding of these factors and assess novel therapeutics in an *in vivo* setting.

7.4.1 Future Directions

The panel of established anal SCC cell lines will now facilitate the expansion of knowledge in this rare disease. Further assays, particularly combination chemoradiotherapy both *in vitro* and *in vivo*, in conjunction with further genomic studies including RNA sequencing, will build upon our knowledge of the biology of the individual cell lines and improve our understanding of the factors

at play in the response of ASCC to treatment. By banking the panel with a commercial cell line repository, they will be within easy reach of researchers from around the globe to achieve this aim of further exploring the biology of this disease and broaden the attempt in identifying new therapies.

7.5 Syngeneic Model of Mouse ASCC

While two mouse models of ASCC have been described, they are overshadowed by significant limitations. Consequently, the establishment of a robust syngeneic mouse model, derived from the anatomical anal canal with the most frequent genomic aberration in human ASCC as the backbone, has created a highly relevant and novel preclinical model. The establishment of the syngeneic cell line also facilitated further manipulation including transduction with the HPV 16 E6/7 transgenes, to better recapitulate the genomic and molecular landscape of HPV +ve human ASCC. With the advent of CRISPR technology, the ability to knock-in a mutant TP53 to the parental line, will also allow the development of a more relevant HPV-ve ASCC model.

The two syngeneic models have been validated and characterised, including *in vitro* assessment in a similar fashion to the panel of human anal SCC cell lines. This has facilitated the comparison of the parental and transduced syngeneic lines to the human lines. Both mouse lines demonstrated a similar but higher proliferation rate and migration potential than their human cell line counterparts. Sensitivity to chemotherapeutic agents, molecular therapies and radiotherapy, was also explored. This revealed sensitivity to 5FU above the level identified with the human panel of cell lines, with a similar profile of response to MMC. While the parental line demonstrated sensitivity to the *PIK3CA* inhibitor BYL719, the transduced line revealed low range resistance despite the presence of a hotspot

PIK3CA driver mutation, an explanation for which may be revealed with sequencing. Both lines also exhibited pronounced radio-resistance above that identified in the human cell lines, an important factor in this model being representative of treatment resistance.

The two syngeneic lines were found to be tumourigenic *in vivo*, including within the native location of the anal canal. To complement the *in vitro* work, the assessment of response of the syngeneic grafts to combined chemoradiotherapy *in vivo*, would clarify whether tumour control was adequately provided by standard treatment in this model. It also provides the opportunity to explore molecular targeted therapies and immunotherapy in an immunocompetent setting, either alone or in combination with standard treatment.

Utilisation of the syngeneic line in an orthotopic position would be the ideal situation to better recapitulate the human disease, particularly with regard to exploring the role of the local immune system in controlling the disease. However, monitoring and measurement of the tumours in this location is problematic. Consequently, small animal PET or alternatively transduction of the line with a fluorescent tag may offer another means of regular monitoring of the tumour size and treatment response. Furthermore, the tumour and immune microenvironment of the human disease are likely to be more representative in an orthotopic than heterotopic model, and therefore of greater importance when exploring the effectiveness of immunotherapy in this disease. This has been partly demonstrated by the close similarity of the immune profile between the mouse model and that of human anal cancer.

7.5.1 Future Directions

This novel syngeneic model now provides a reproducible immunocompetent platform that will facilitate the exploration of chemo-, molecular targeted-, immuno- and radio-therapy, alone or in combination. This will provide insight and the opportunity to unravel the biology underlying treatment response with a closer recapitulation of the clinical scenario of patient treatment.

7.6 Conclusions

While ASCC remains a largely treatable disease, many patients with disease relapse are currently faced with a dismal prognosis. By beginning to explore and define the genomic and immune landscape of ASCC, our understanding of this disease has begun to deepen and many new areas requiring further exploration have been revealed. The establishment of both *in vitro* and *in vivo* preclinical models has now provided platforms upon which to further unravel and validate these exploratory findings. They have also provided the next step in attempting to identify and assess new therapeutics options, particularly for patients with disease relapse.

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