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The Genetic Status of the *PIK3CA* Oncogene and Activity of the PI3K-AKT-mTOR Pathway in Penile Squamous Cell Carcinoma

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MD(Res) Thesis

Submitted in partial fulfilment of the requirements of the Degree of Doctor of Medicine

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STATEMENT OF ORIGINALITY

I, Anthony Adimonye, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated.

Mrs Sakunthala Kudahetti provided help and advice with tissue banking and cutting of fresh frozen tissue specimen. Miss Elzbieta Stankiewicz helped me with qPCR, FISH, Western Blot and IHC methodology. Prof Dan Berney provided assistance by examining all H&E sections and with the help of Dr Giorgia Trevisan scored all IHC TMA samples. Mr Bernard North (Senior Statistician - Wolfson Institute of Preventative Medicine; Queen Mary University of London) assisted me with the univariate and multivariate analyses.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

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I confirm that this thesis has not been previously submitted for the award of a degree by this or any other university.

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Date: 16/10/16

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ABSTRACT

Penile squamous cell carcinoma (PSCC) is rare; hence little is known about its aetiology and pathogenesis. Two challenges exist in the clinical management of PSCC patients. Firstly, finding a non-invasive method to aid the detection of occult lymph node metastasis to improve patient selection for inguinal lymphadenectomy. Secondly, the development of novel treatment strategies for those with advanced PSCC, as current treatment options are limited.

A high prevalence of copy number gain in the chromosome 3q arm has been identified and linked to poor cancer-specific and disease-free survival in PSCC. Within this region lies the *PIK3CA* oncogene, which is mutated/amplified/gained and results in the activation of the PI3K-AKT-mTOR pathway. *PIK3CA* copy number gain has not been fully investigated in PSCC and it has the potential to be a driver gene in penile carcinogenesis and the PI3K-AKT-mTOR pathway presents an opportunity for targeted therapeutics in PSCC.

I demonstrated an increasing frequency of *PIK3CA* copy number gain with evolving PSCC disease state (penile intraepithelial neoplasia (10/58; 17%), primary PSCC (83/199; 42%), advanced primary PSCC (20/26; 77%); $p<0.0001$) with few *PIK3CA* mutations in PSCC (3/51; 6%). *PIK3CA* copy number gain correlated with more aggressive PSCC subtypes ($p=0.0028$), higher tumour grade ($p<0.0001$) and stage ($p=0.0043$) and thus could be used as a marker of high-risk disease. However, it shows no significant association with lymph node metastasis or prognostic value for cancer-specific survival in PSCC.

Overall, I confirmed that the PI3K-AKT-mTOR pathway activity is primarily involved in early penile carcinogenesis and based on these findings the therapeutic targeting of this pathway in those with advanced PSCC disease is unlikely to produce significant clinical benefit. Future studies will need to focus on the identification of new clinically relevant candidate genes and signalling pathways, which offer prognostic value and the potential for targeted therapeutics in this rare cancer.

ABBREVIATIONS

aCGH	Array Comparative Genomic Hybridisation
BAC	Bacterial Artificial Chromosome
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	Base Pair
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CIN	Chromosomal Instability
CSCC	Cervical Squamous Cell Carcinoma
CSS	Cancer-Specific Survival
CT	Computed Tomography
C_T	Cycle Threshold
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
FISH	Fluorescence *In-Situ* Hybridisation
FFPE	Formalin-Fixed Paraffin Embedded
H&E	Haematoxylin and Eosin
HISCC	Human Papilloma Virus Induced Squamous Cell Carcinoma
HNSCC	Head and Neck Squamous Cell Carcinoma
HrHPV	High Risk Human Papilloma Virus
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase

IHC	Immunohistochemistry
IRS1	Insulin Receptor Substrate 1
Kb	Kilobase
LB	Lysogeny Broth
LrHPV	Low Risk Human Papilloma Virus
M-MLVRT(H-)	Moloney Murine Leukaemia Virus Release Transcriptase, RNAse H Minus Point Mutant
MIN	Microsatellite Instability
mTORC1	mTOR/raptor complex
mTORC2	mTOR/rictor complex
NBT	Nitro Blue Tetrazolium
OSCC	Oesophageal Squamous Cell Carcinoma
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PeIN	Penile Intraepithelial Neoplasia
PET	Positron Emission Tomography
PI3K	Phosphatidylinositol 3-Kinase
PI3Kα	Phosphatidylinositol 3-Kinase p110α Protein
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic Subunit Alpha
PIP_2	Phosphatidylinositol (4,5) Bisphosphate or Phosphatidylinositol (3,4) Bisphosphate
PIP_3	Phosphatidylinositol (3,4,5) Trisphosphate
PCR	Polymerase Chain Reaction
pRB	Retinoblastoma

PSCC	Penile Squamous Cell Carcinoma
PTEN	Phosphatase and Tensin Homolog
PVDF	Polyvinvlidene Difluoride
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RQ	Relative Quantification
RTK	Receptor Tyrosine Kinase
SCC	Squamous Cell Carcinoma
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SGK	Serum and Glucocorticoid-Inducible Kinase
SHIP	Src homology 2-containing inositol 5-phosphatase
SPF10	Short PCR Fragment Primers
SSC	Saline-Sodium Citrate
SSCT	4xSSC and 0.5% Tween 20
SSCTM	5% Marvel Milk/4xSSC and 0.5% Tween 20
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline/0.1% Tween 20
TIL	Tumour Infiltrating Lymphocyte
TIP	Taxane, Ifosfamide and Cisplatin
TMA	Tissue Microarray
TNM	Staging System: T-primary tumour, N-number of involved lymph nodes, M-metastasis

TPF	Taxane (Docetaxel), Cisplatin and 5-Fluouracil
TTR	Time To Recurrence
VPS34	Vacuolar Protein Sorting 34
WB	Western Blot
WT	Wild-Type

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CHAPTER ONE

INTRODUCTION

1.1 Cancer

1.1.1 Definition, Epidemiology and the Hallmarks of Cancer

Cancer is the nomenclature given to a collection of diseases, which have uncontrolled cellular growth and can invade and metastasize to distant sites of the body. It is a major cause of morbidity and mortality in Europe with 3.45 million and 1.75 million new cases and deaths from cancer, respectively, each year (1, 2). In the UK, there are 352,197 and 161,823, new cases and death from cancer a year and these figures are expected to increase due to an ageing and ever growing population (3).

The evolution of cancer is a complex and multi-step process, which involves the successive accumulation of genetic alterations such as mutations that confer a selective growth advantage that drives the transformation of normal cells into malignant cancers (4). Despite the existence of various types of cancers, it is widely accepted that for normal cells to evolve progressively into a neoplastic state, they must acquire some or all of a succession of specific biological hallmark capabilities which include: (i) sustaining proliferative signals, (ii) resisting cellular apoptosis, (iii) enabling replicative immortality, (iv) resistance to growth inhibitory signals, (v) immune system evasion, (vi) induction of angiogenesis, (vii) tissue invasion and metastasis, (viii) reprogramming of cellular energy metabolism, (ix) tumour-promoting inflammation and (x) genomic instability and mutations (5, 6). (Figure 1.1)

Figure 1.1 Cancer Hallmarks. Adapted from Hanahan *et al.* (5)

The molecular bases of each of these essential biological hallmarks are common to most if not all malignancies and each capability represents a potential target for mechanistic-based cancer therapeutics.

1.1.2 Genomic Instability in Cancer

Genomic instability can be described as the increased rate of spontaneous genetic alterations that impairs normal cellular biological mechanisms that repair, replicate and ensure the accurate segregation of the genome during cell division. Thus promoting the acquisition of further DNA alterations, clonal evolution and tumour heterogeneity (4, 7, 8). Genomic instability, which is an almost universal feature of cancer cells, occurs at two distinct levels: nucleotide (microsatellite instability) and chromosomal (chromosomal instability) (9).

Microsatellites, also known as short tandem repeats, are simple repeated short motifs usually of one to six base pairs long, which are scattered throughout the genome and

are particularly prone to DNA replication errors (10, 11). Microsatellite instability (MIN) manifests from defects in the DNA mismatch repair genes (such as the MLH1, MSH2, PMS2 genes among others), which causes random deletions, insertions and expansion of microsatellites and leads to a hypermutable phenotype (9, 11). This facilitates cellular malignant transformation via accumulation of mutations leading to activation and inactivation of key oncogenes and tumour suppressor genes, respectively, which have fundamental cellular functions (10, 12). MIN is a characteristic feature of a number of cancers, such as gastric and colorectal cancer, the latter of which, is where it was first described and has been extensively studied (13-15).

Among the inherent types of genomic instability, chromosomal instability (CIN) is the most common form. CIN is characterised by the high rate of gains and/or loss of whole chromosomes or chromosomal segments and hence structural and numerical chromosomal alterations and an abnormal karyotype (16, 17). Numerical CIN results from chromosome segregation defects that are caused by mechanisms such as multipolar spindles and centrosome amplification for example (18). Whilst structural CIN results from chromosome breakage and rearrangement due to defects in cell cycle checkpoints and the loss of telomeres integrity among other mechanisms (17, 19). These alterations lead to aneuploidy, translocations, amplifications, insertions, inversions and deletions and thus karyotypic instability and the increased probability of cellular dedifferentiation, malignancy and survival in stressful microenvironments. The human papillomavirus (HPV) is well known to interfere with these aforementioned processes, causing CIN and tumour formation in infected epithelial cells (4, 18).

It is still unclear whether genomic instability is just an incidental by-product of the microevolution of the tumour or whether it is the primary driving force of tumourigenesis occurring early, late or throughout this on going process. However, it has been proposed that the different cancers follow alternative pathways of initiation and hence the role of genomic instability in carcinogenesis may vary depending on the tumour type (17, 20).

1.2 Penile Cancer

1.2.1 Epidemiology

Penile cancer is a rare disease in Europe and North America, representing approximately 0.5% of all male malignancies (21). The incidence of PSCC in Europe varies geographically ranging from 0.45 – 1.7 per 100,000 population (22), with 600 new cases diagnosed each year in the UK (23). This malignancy occurs predominantly in elderly men, with an increasing incidence with age, with the highest rate being between 50 and 70 years (24).

The clinical course of penile cancer is interesting with two distinct groups of patients seen. The first is a large patient cohort with a high cure rate (80%) and excellent long-term survival as their disease is organ-confined with no or minimal regional lymph node involvement (24, 25). The second is a smaller patient cohort with highly aggressive disease with significant propensity to metastasise and poor prognosis (25, 26). Management of the latter group with aggressive and advanced tumours still remains difficult as most are chemo/radio-resistant with limited treatment options available (27).

One of the main challenges in penile cancer remains patient selection for radical inguinal lymphadenectomy, which can cause significant morbidity, in those with clinically node negative disease as around 25% will have occult metastasis. Standard clinico-pathological parameters are well established and the mainstay in guiding current patient management and prognosis. The most important of these remains the presence and extent of lymph node metastases, which is a key determinant of long-term patient survival in penile cancer (26, 27).

Several molecular markers such as squamous cell carcinoma antigen and Ki-67 have been evaluated, in hope of finding a potentially useful adjunct to conventional pathological variables. Unfortunately, the clinical application of these markers remains limited (28-30). Nonetheless, novel molecular markers are required not only for prognostication and the accurate determination of lymph node status but also to serve as chemotherapeutic targets and aid in tailoring therapeutic treatments to individual patients (30).

1.2.2 Risk Factors and Pathology

Several risk factors have been identified which are thought to contribute to the development of penile cancer. One of the most important and extensively studied is infection with HPV. Around 33% of all penile cancer cases are associated with HPV (31), similar to vulvar (32) and head and neck cancers (33, 34). Other risk factors include phimosis, chronic inflammation, poor penile hygiene, penile trauma and smoking (22, 35).

Penile tumours originate most commonly from the epithelium of the penile glans, inner prepuce (foreskin), and coronal sulcus and less commonly on the penile shaft (36). They may arise from the malignant transformation of precursor lesions - penile intraepithelial neoplasia (PeIN), the rate at which this occurs is not well defined, but is thought to arise in approximately 30% of cases, if left untreated (37, 38) (Figure 1.2).

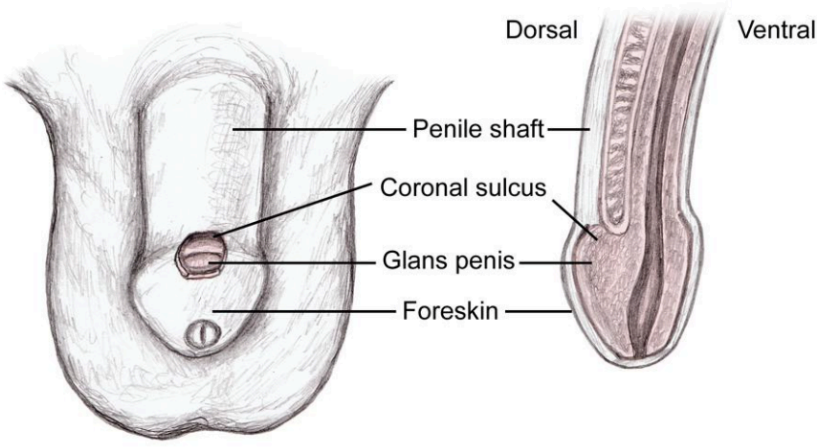

Figure 1.2 Penile Anatomy. Adapted from Medscape (39)

Squamous cell carcinoma accounts for around 95% of all malignant disease of the penis (40). The remaining 5% comprise of various rare tumour entities from clear cell and basal cell carcinomas to melanomas and sarcomas among others (41). Despite penile squamous cell carcinoma (PSCC) being the most common penile neoplasm, various distinct histological subtypes (including those with a mixed histological background) exist each with histotype specific associations with human papilloma virus and clinical aggressiveness (41-45). (Table 1.1)

Table 1.1 PSCC Histotypes with Frequency, HPV Association, Metastatic Rate and Prognosis

PSCC Subtype	Frequency (%)	HPV Positivity (%)	Metastatic Rate (%)	Prognosis
Usual	48-65	24-59	28-39	Intermediate
Basaloid	4-10	70-100	50-100	Poor
Warty	7-10	22-78	17-18	Good
Verrucous	3-8	0-23	Nil	Excellent
Papillary	5-15	8-15	12	Good

Data from the following references: (34, 43, 45)

1.2.3 Diagnoses and Staging

To ensure the appropriate treatment selection for each patient careful diagnosis and staging of the malignancy is paramount. Clinical examination is important as it may identify the primary lesion, which may present as a localised mass, ulcer or inflammatory lesion (27). To accurately diagnose penile cancer, biopsy of the lesion is required for histopathological confirmation.

Determination of lymph node involvement in penile cancer is a crucial step for staging, treatment and prognosis, which is carried out by careful physical examination of each groin (46). In patients with non-palpable inguinal nodes with intermediate and high-risk disease, invasive nodal staging with bilateral dynamic sentinel lymph node biopsy is recommended. Whilst those with low risk disease are put under a surveillance program (24, 47-49). In those patients with confirmed lymph node metastasis, radiological staging for distant metastasis and systemic disease is required which is carried out with abdomino-pelvic computed tomography (CT) or positron emission tomography/CT (PET/CT) scan, chest radiography and a bone scan if indicated (50, 51).

Staging of penile cancer is essential for the determination of patient treatment and prognosis and is linked to patient survival and disease progression (52). Penile

cancer can be staged pathologically and/or clinically (24). Both staging methods follow the TNM classification, were the extent of the primary tumour (T category) and nodal involvement (N category) is taken into account, as is the presence or absence of distant metastasis (M category). Tumour grade, which is based on the degree of cellular differentiation, is also a key part of the pathological TNM classification due to its prognostic relevance (53, 54). Table 1.2 and 1.3 - both adapted from (24).

Table 1.2 Penile Cancer TNM Clinical Staging Classification

Penile Cancer TNM Clinical Staging		
T - Primary Tumour	*TX*	Primary tumour cannot be assessed
	T0	No evidence of primary tumour
	Tis	Carcinoma in-situ
	Ta	Non-invasive carcinoma
	T1	Tumour invades sub-epithelial connective tissue
	T2	Tumour invades corpus spongiosum and/or corpora cavernosa
	T3	Tumour invades urethra
	T4	Tumour invades other adjacent structures
N - Regional Nodes	*NX*	Regional lymph nodes cannot be assessed
	N0	No palpable or visibly enlarged inguinal node
	N1	Palpable mobile unilateral inguinal node
	N2	Palpable mobile multiple unilateral or bilateral inguinal nodes
	N3	Fixed inguinal nodal mass or pelvic node(s)
M- Distant Metastasis	*M0*	No distant metastasis
	M1	Distant metastasis

Table 1.3 Penile Cancer TNM Histopathological Staging Classification

Penile Cancer TNM Histopathological Staging	
T - Primary Tumour	*pTX* Primary tumour cannot be assessed
	pT0 No evidence of primary tumour
	pTis Carcinoma in-situ
	pTa Non-invasive carcinoma
	pT1 Tumour invades sub-epithelial connective tissue
	pT2 Tumour invades corpus spongiosum and/or corpora cavernosa
	pT3 Tumour invades urethra
	pT4 Tumour invades other adjacent structures
N - Regional Nodes	*pNX* Regional lymph nodes cannot be assessed
	pN0 No regional lymph node metastasis
	pN1 Intranodal metastasis in a single inguinal node
	pN2 Metastasis in multiple unilateral or bilateral inguinal nodes
	pN3 Metastasis in pelvic nodes(s) or extranodal extension of any regional lymph node
M- Distant Metastasis	*pM0* No distant metastasis
	pM1 Distant metastasis
G - Tumour Grade	*GX* Grade of differentiation cannot be assessed
	G1 Well differentiated
	G2 Moderately differentiated
	G3 Poorly differentiated
	G4 Undifferentiated

1.2.4 Clinical Management

The choice of definitive treatment of the primary tumour depends on the grade and stage of the primary lesion, local clinical practice and the physical wellbeing of the patient and their wishes. However, the fundamental goal remains to achieve complete tumour eradication with the implementation of penile preservation treatment whenever possible (24, 46).

Surgical intervention remains the most common method for treatment with penile sparing surgeries such as glans resurfacing and glansectomy with reconstruction preferred due to good oncological outcomes and cosmetic results (55, 56). Treatment with topical chemotherapy such as 5-flurouracil, laser ablation and radiotherapy still remain alternatives in certain penile cases for example in those with early stage non-invasive disease, small tumours or in those unfit for surgical intervention (37, 57-59).

The management of the regional lymph nodes is fundamental in penile cancer and treatment of confirmed inguinal lymph node metastasis is with radical inguinal lymphadenectomy (60). Pelvic lymphadenectomy is indicated in only those with pelvic lymph nodes seen on radiological imaging, evidence of extra-capsular nodal metastasis or when more than two inguinal lymph nodes are positive, due to the high risk of pelvic lymph node metastasis between 23 - 56% (61-63).

Radiotherapy is only used for palliation in those with advanced disease and may improve loco-regional control in patients with extensive metastases and/or extra-nodal spread (24). Chemotherapy for penile cancer is used for the treatment and palliation of advanced or metastatic disease, as well as in the neo-adjuvant and adjuvant setting for locally invasive or fixed inguinal lymph nodes. But due to the rarity of this tumour, there is a paucity of prospective randomised clinical trial data resulting in multiple small heterogeneous studies being published, making evidence-based recommendations in current guidelines difficult (64).

Current first-line chemotherapy for penile cancer consists of a taxane, cisplatin, and 5-fluouracil or ifosfamide (TPF or TIP) as these regimes demonstrated good objective tumoural response (65-68), but significant toxicity has been noted with TPF treatment (69, 70).

However, penile cancer is inherently radiotherapy and chemotherapy resistant. There are limited second-line chemotherapeutic options for the systemic treatment of penile cancer, once first-line therapy eventually fails (64). A few targeted therapeutic agents have been used as second-line treatment for patients with refractory disease with modest results. These include anti-epidermal growth factor receptor (EGFR) monoclonal antibodies such as cetuximab and panitumumab (71-73), and receptor tyrosine kinase (RTK) inhibitors such as sorafinib and sunitinib (74). These targeted therapeutics bind to the extracellular domain of the EGFR and multiple RTKs, respectively. Consequently, they block the receptor-dependent activation of downstream signal transduction, hence providing multiple anti-tumour effects from inhibition of angiogenesis to cell cycle arrest and induction of apoptosis (75, 76).

1.3 Molecular Pathogenesis of Penile Cancer

The molecular mechanisms underlying penile carcinogenesis are still largely unknown and seldom studied. This is largely due to the rarity of the disease and hence the small number of cases and limited tissue material available for molecular and translational research.

However, the establishment of supra-regional penile cancer networks with centralisation of penile care services in the UK and organisation of international and national research collaborations will undoubtedly improve patient management and our understanding of this disease (77, 78). A greater understanding of the molecular mechanisms of penile carcinogenesis will lead to identification of biomarkers and novel treatment strategies in penile cancer.

Our fundamental understanding of the underlying aetiology of penile cancer is in recognition of its heterogeneity and two distinct molecular pathways of carcinogenesis: the HPV and non-HPV mediated pathways (79).

1.3.1 HPV Dependent Penile Carcinogenesis

HPVs are non-enveloped small circular double-stranded DNA viruses. There are more than 100 known variants of HPV of which around 40 are known to infect the anogenital mucosa (80). These mucosal HPVs are classified in to high- and low-risk groups, which reflect the malignant potential of the lesion they cause to progress into invasive cancer such as cervical, anal, vulvar or penile cancer (81, 82). (Table 1.4)

Table 1.4 Oncogenic Potential of HPV Subtypes

Classification	HPV Subtypes
High Risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Likely Oncogenic	26, 53, 66, 68, 73, 82
Low Risk	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81

Adapted from Munoz *et al.* (81)

High-risk HPVs (hrHPV) are associated with around 33% of all penile cancer cases, with HPV 16 the most prevalent subtype (34). Low-risk HPV (lrHPV) such as HPV 6 and 11, are more commonly associated with benign lesions such as condylomata acuminate (41) and respiratory papillomas (83). These lrHPV variants are not thought to be actively involved in carcinogenesis, as it has been shown that their oncoproteins do not cause significant dysregulation of RB and p53 tumour suppressor pathway, leading to minimal $p16^{INK4A}$ upregulation (84). However, these lrHPV variants are commonly found in PSCC with HPV 6 the second most common HPV subtype found in this tumour (3.7%) (31).

HPV virions are thought to gain access to and infect the basal cells of the epithelial mucosa via micro-abrasions and specific receptors such as heparan sulphate proteoglycans and α_6 integrins (85). Persistent epithelial HPV infection and integration of HPV DNA into the host cell genome, leads to a transformed malignant phenotype (Figure 1.3) (82). This results in genomic instability and overexpression of high-risk viral oncoproteins E6 and E7 that exert a dysregulating effect on cell cycle control that is key to the maintenance of the cancer phenotype (80, 86).

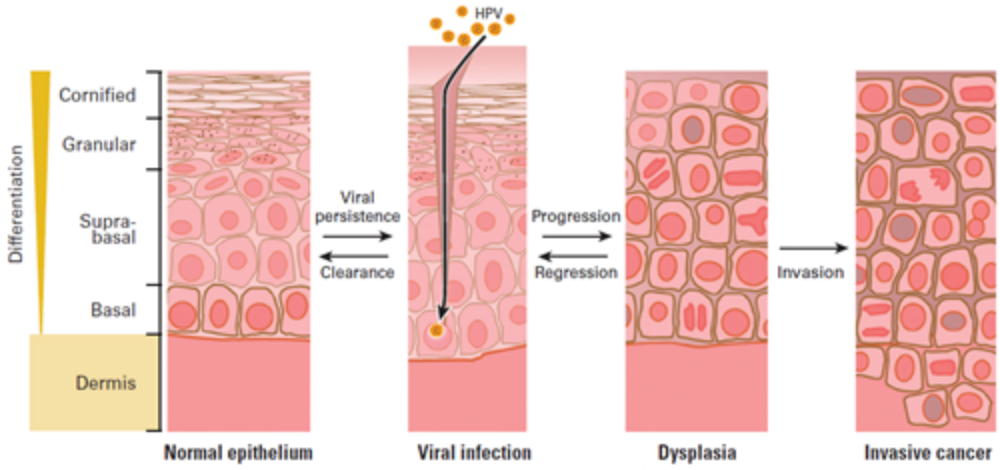

Figure 1.3 HPV Infection of the Basal Cells of the Epithelial Mucosa. HPV virions gain access to and infect the basal cells of the epithelial mucosa via micro-abrasions and specific receptors. Persistent infection with hrHPV can lead to dysplasia (PeIN) then to invasive squamous cell carcinoma, which is poorly differentiated. Progression is rare and a slow process, with many lesions regressing spontaneously. Adapted from Hellner *et al.* (82).

The major biological functions of the high-risk viral oncoproteins E6 and E7, lie in their inactivation of the tumour suppressors proteins, p53 and retinoblastoma (pRB), respectively. High-risk HPV E7 oncoprotein associates with and inactivates pRB via binding and disruption of the pRB-E2F complex and/or inducing proteasome-mediated degradation of pRB. This releases the transcription factor E2F to activate genes involved in DNA synthesis and results in uncontrolled cell cycle progression (87). This process leads to the overexpression of $p16^{INK4A}$, due to the disruption of the negative feedback loop between $p16^{INK4KA}$ and pRB (34, 88). This allows $p16^{INK4KA}$ overexpression to be used as a surrogate marker of transcriptionally active HPV infection in penile caner, which has been shown by multiple groups (89-91).

HrHPV E6 oncoprotein targets the p53 tumour suppressor protein for proteasome-mediated degradation by forming a tripartite complex with p53 and the cellular ubiquitin ligase E6-associated protein. This leads to prevention of DNA repair, growth arrest and apoptosis and predisposes the infected cell to accumulation of secondary genetic events such as mutations that eventually lead to cancer (92). (Figure 1.4)

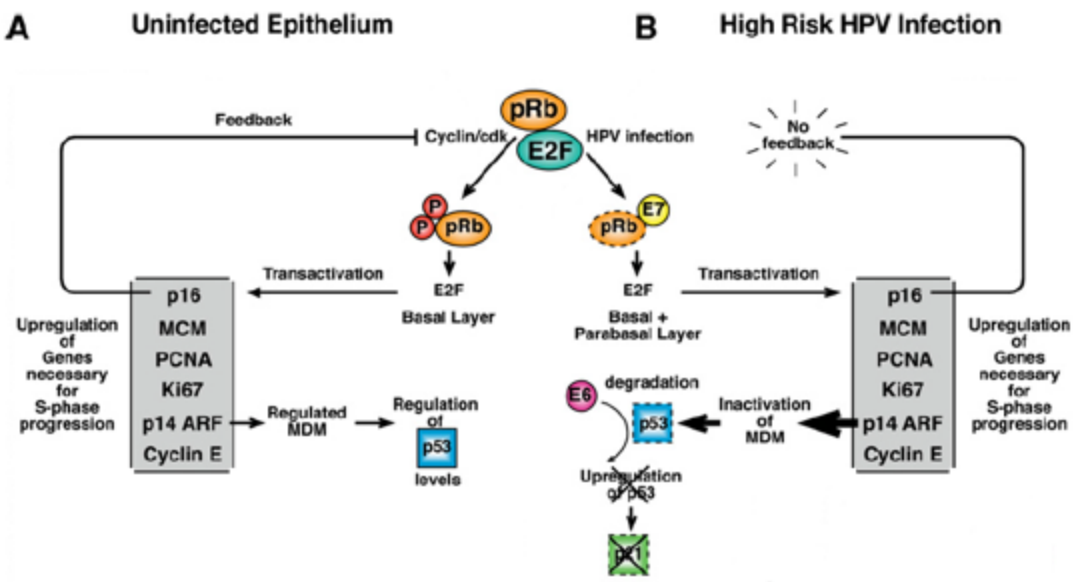

Figure 1.4 Cell-Cycle Dysregulation caused by HrHPV Infection. (A) In normal uninfected epithelium, growth factors activate cyclinD/Cdk4/6 complexes, which lead to pRB phosphorylation and release of the transcription factor E2F, which drives the expression of proteins, needed for S-phase progression. $p16^{INK4A}$ regulates the levels of active cyclinD/Cdk, by binding Cdk4 and thus preventing the phosphorylation of pRB leading to cell cycle arrest. This provides a negative feedback mechanism that regulates the levels of cyclin E and PCNA. $p14^{ARF}$ regulates MDM ubiquitin ligase, which in turn regulates p53 levels **(B)** HPV oncoprotein E7 binds and degrades pRB and facilitates E2F mediated DNA transcription. Although, $p16^{INK4A}$ levels rise, normal feedback is by-passed due to cyclin/Cdk independent cell-cycle progression. The rise in $p16^{INK4A}$ leads to a concomitant rise in $p14^{ARF}$, which leads to inhibition of MDM function, and increase in p53. This is countered by the viral oncoprotein E6 that associates with E6-associated protein ubiquitin ligase and stimulates the degradation of p53 preventing growth arrest and/or apoptosis. Adapted from Doobar *et al.* (93)

Previous studies have also noted other important carcinogenic functions of the oncoproteins E6 and E7. One example includes the HPV E6 oncoprotein mediated activation of telomerase via c-myc induced hTERT expression (80) (94) and degradation of the NFX1-91, a repressor for the hTERT promoter (95) that leads to cellular immortalisation. Both E6 and E7 oncoproteins can induce genomic instability by mechanisms such as aberrant centrosome synthesis and subversion of mitotic checkpoints causing a higher incidence of mutations and structural chromosome abnormalities leading to chromosomal rearrangements (96).

1.3.2 HPV Independent Penile Carcinogenesis

The genetic mechanisms behind HPV negative penile carcinogenesis are less well understood. Two studies analysed the genomic changes in penile cancer by array comparative genomic hybridisation (aCGH) methods. La-Touche *et al.* (97) performed the largest aCGH thus far on 64 PSCC cases. They reported recurrent gains in chromosomes 1p13.3-q44 (88%), 3p12.3-q29 (86%), 5p15.33-p11 (67%) and 8p12-q24.3 (84%) and losses in chromosomes 2q33- q37.3 (86%) and 11q12.2-q25 (81%). Similar patterns of genetic aberrations were found between HPV positive and negative PSCC cases, which they suggested provide evidence that similar molecular pathways are targeted by HPV positive and negative PSCC and both may utilise a final common pathogenetic pathway.

Busso-Lopes *et al.* (98) in 46 PSCC cases discovered, significantly lower cancer-specific and disease-free survival in cases with losses of 3p21.1-p14.3 ($p=0.0006$ and $p=0.023$, respectively) and gains of 3q25.31-q29 ($p=0.017$ and $p=0.042$, respectively). These regions may harbour potential prognostic biomarkers and future therapeutic targets in penile cancer. They also identified 19 specific genomic alterations which were more common in HPV positive PSCC cases and nine of which are breakpoints previously described as associated with HPV integration sites in cervical carcinoma (e.g. loss of 5q31.1 and gain of 19q13.32) (99). Thus supporting the hypothesis of two distinct PSCC aetiologies: one dependent and the other independent of HPV infection.

More recently, Feber *et al.* (100) carried out the most comprehensive analysis to date of the somatic mutational landscape of PSCC via whole exome sequencing. They found few recurrent somatic mutations in 27 PSCC cases with only 137 (17%) recurrent events out of 810 mutated genes identified, with the most common in *TP53* (19%) and *CSN1* (17%). Interestingly, despite not finding any significant association between mutational burden and HPV status, on stratification for HPV viral load, they found high viral load tumours, which they defined as >1 HPV copy/cell via qPCR, had a significantly ($p<0.05$) lower mutational load when compared to HPV negative tumours.

Chronic inflammation is a known risk factor for penile cancer and a key mediator of inflammation, cyclooxygenase-2, is strongly expressed in PeIN, invasive PSCC and

lymph node metastasis, but not in normal tissue (101, 102). The overexpression of cyclooxygenase-2 leads to overproduction of prostaglandins and thromboxans, which play a pivotal role in proliferation, invasion and angiogenesis (103).

Dysregulation of major tumour suppressor pathways, $p16^{INK4A}$/cyclin D/RB and $p14^{ARF}$/MDM2/p53 pathways has been found to occur also by HPV independent mechanisms promoting penile carcinogenesis. The *p16*^{INK4A} gene often inactivated by loss of heterozygosity and silencing via DNA methylation with loss of $p16^{INK4A}$ immunoexpression has been significantly associated with lymph node metastasis and disease recurrence (104, 105). In addition, overexpression of *BMI-1* polycomb gene, which results in down-regulation of *p16*^{INK4A} and *p14*^{ARF}, has been found in penile cancer (88). (Figure 1.5)

Allelic loss and mutations of *TP53*, *p14*^{ARF} mutation and methylation, and overexpression of MDM2, which is a negative regulator of TP53, have also been shown in penile cancer (104, 106, 107) (Figure 1.5). Mutation of *TP53* can also lead to increased expression of an inactive p53 mutant protein (90%) or absent p53 protein expression (10%) (40). Multiple studies have investigated p53 immunoexpression in penile cancer with varying detection rates from 41.5% to 89% cited (34). In addition, there is recognition in the literature of an association between p53 immunoexpression and poor prognosis in penile cancer (108, 109).

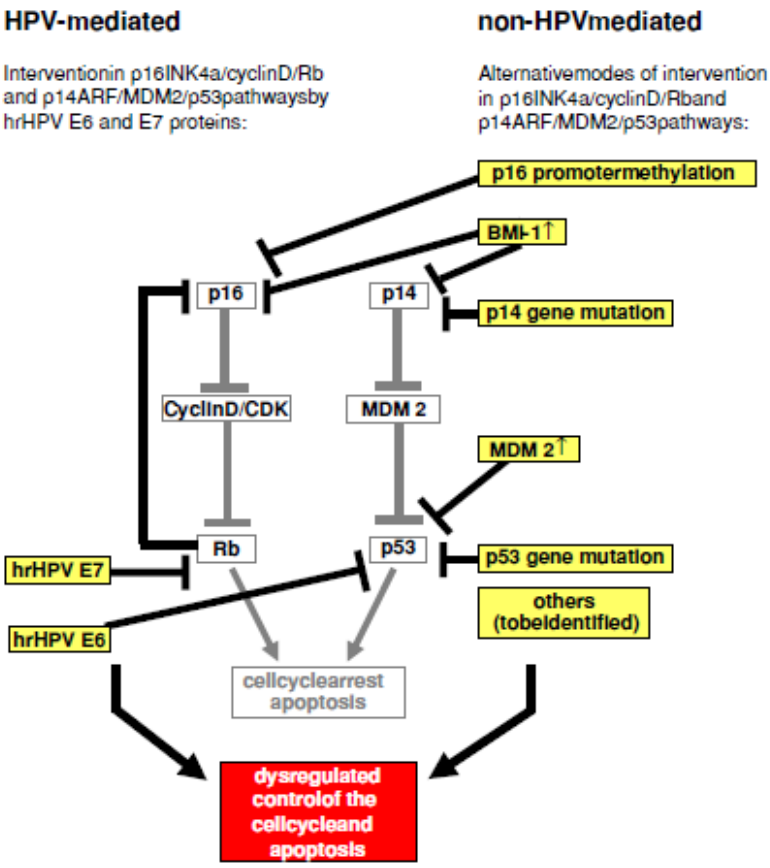

Figure 1.5 HPV- and Non-HPV mediated Dysregulation of Major Tumour Suppressor Pathways, RB and p53. Both the HPV dependent and HPV independent pathways lead to dysregulated cell cycle control and evasion of apoptosis through disruption of the $p16^{INK4A}$/cyclin D/RB and $p14^{ARF}$/MDM2/p53 pathways. HrHPV mediated dysregulation of these pathways is a result of the actions of its hrHPV oncoproteins E7 and E6. The E7 oncoprotein binds and degrades RB thus facilitating cell cycle progression and the E6 oncoprotein stimulates the degradation of p53 preventing DNA repair growth arrest and/or apoptosis. Non-HPV mediated disruption of these two key pathways include p16 promoter methylation which causes transcriptional silencing of the p16 tumour suppressor gene, p14 and p53 mutations and also overexpression of BMI-1 which is an oncogene that represses the tumour suppressor genes, p16 and p14. Adapted from Bleeker *et al.* (41)

Ki-67 is a non-histone nuclear matrix protein that is expressed in all-active phases of the cell cycle except resting cells. Ki-67 immunoexpression is associated with aggressive disease behaviour in penile cancer, however is not found to have any prognostic value for cancer-specific survival and overall survival (110). Other factors likely involved in penile carcinogenesis include e-cadherin and matrix metalloprotease-9, which have been associated with lymph node metastasis and disease recurrence in PSCC, respectively (109, 111). (Table 1.5)

Table 1.5 Common Somatic Genetic Alterations in Penile Cancer

Genetic Change	Gene/ Chromosome	Frequency	References
Point Mutations	*TP53*	13 - 40%	(100, 104, 112, 113)
	PIK3CA	9 - 29%	(113-115)
	CSN1	17%	(100)
	HRAS	6 - 19%	(113-116)
	KRAS	1 - 9%	(114, 115, 117)
DNA Methylation	*FHIT*	88 - 92%	(107, 118)
	TSP-1	46%	(119)
	RUNX3	42 - 44%	(107, 118)
	$p16^{INK4A}$	17 - 42%	(88, 104, 107, 119)
	RASSF1-A	12 - 42%	(107, 118, 119)
Loss of Heterozygosity	$p16^{INK4A}$	62%	(104)
	TP53	42%	(104)
Copy Number Gain/Amplification	1p13.3 - q44	88%	(97)
	3q13.3 - q29	42 - 86%	(97, 98)
	8q21.2 - q24.3	42 - 84%	(97, 98, 120, 121)
Copy Number Loss	2q33 - q37.3	86%	(97)
	3p24.3 - q11.1	34 - 83%	(97, 98)
	11q12.2 - q25	81%	(97)

1.4 Phosphatidylinositol 3-Kinase (PI3K) Family

PI3K are a family of lipid kinases that are characterised by their capability to phosphorylate inositol phospholipids, thus activating signalling pathways that regulate key cellular processes (122, 123). They are divided into three classes according to their structural characteristics, substrate specificity and biochemical function (124-127).

Briefly, class I PI3Ks are divided in to the subclasses, IA and IB based on their mode of regulation. Class IA PI3Ks are heterodimers made up of a single p110 catalytic subunit, of which they are three different isoforms (encoded by the genes PIK3CA - p110α, PIK3CB - p110β and PIK3CD - p110δ) and a single p85 regulatory subunit. There are five regulatory p85 subunit isoforms, which include p85α (and its splice variants p55α and p50α, encoded by PIK3R1), p85β (encoded by PIK3R2) and p55γ (encoded by PIK3R3). Class IB PI3K is a heterodimer composed of a catalytic subunit p110γ (encoded by PIK3CG) coupled with the regulatory subunits p101 (encoded by PIK3R5) or p87 (encoded by PIK3R6). The catalytic subunits, p110α and p110β are expressed ubiquitously, whereas, p110δ and p110γ is mainly expressed in leukocytes. The Class I PI3Ks phosphorylate phosphatidylinositol (4,5) bisphosphate (PIP_2) into phosphatidylinositol (3,4,5) trisphosphate (PIP_3), which leads to various diverse functions from cellular survival and proliferation to motility (124, 125).

Less is known about the class II PI3Ks, which consist of a single catalytic subunit and are thought to preferentially phosphorylate phosphatidylinositol (PI) into phosphatidylinositol (3) phosphate (PIP). There are three class II PI3Ks isoforms that include PI3KC2α, PI3KC2β and PI3KC2γ and are thought to have cellular functions, which include glucose metabolism, cellular survival and migration (124, 128).

The ubiquitously expressed, single class III PI3K, is a heterodimer of the catalytic subunit VPS34 and the regulatory subunit VPS15 which phosphorylates PI into PIP and is found to have a crucial role in intracellular trafficking and autophagy (125). (Figure 1.6)

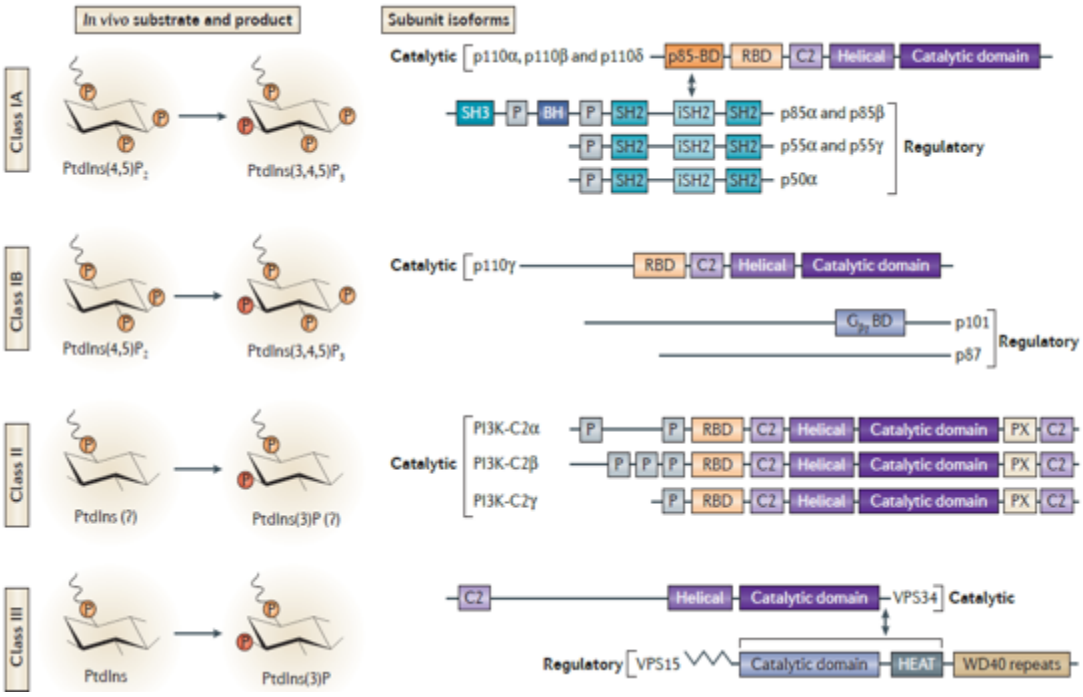

Figure 1.6 Different Classes, Subunit Isoforms and Functions of the Phosphatidylinositol 3-Kinase (PI3K) Family. Class IA and IB PI3Ks - in vivo, phosphorylate PIP_2 into PIP_3 with diverse cellular functions, which include cell motility, growth, transformation, metabolism and survival. **Class IA PI3Ks** - have a p110 catalytic subunit isoforms (α, β, δ), a helical domain, a membrane-interacting (C2) domain, a RAS-binding domain (RBD) and a p85-binding domain (p85-BD). They have multiple heterodimeric regulatory subunit isoforms which have, inter-SH2 (iSH2) domain that binds to class IA catalytic subunits, flanked by SH2 domains that bind to phosphorylated YXXM motifs on RTK or insulin receptor substrates. The longer isoforms, p85α and p85β, additionally have amino-terminal SH3 and breakpoint cluster homology (BH) domains, which are thought to have a negative regulatory effect on the catalytic activity of the p110 subunit. **Class IB PI3Ks** - are heterodimers with a p110γ catalytic subunit and a p101 or p87 regulatory subunit. The structure of the regulatory subunits p101 and p87 are not fully known, but a carboxy-terminal domain (Gβγ binding domain) in p101 has been shown to bind Gβγ, which recruits the p110γ subunit to the plasma membrane. **Class II PI3Ks** - are monomeric and only comprise a catalytic subunit. Their cellular functions include glucose transport, endocytosis, cell migration and survival. Three isoforms exist for this class, which are PI3K-C2α, PI3K-C2β and PI3K-C2γ. **Class III PI3K** - consists of a vacuolar protein sorting 34 (VPS34) subunit, which only has a catalytic, C2 and helical domain. This subunit forms a heterodimer with VPS15 that consists of a catalytic domain, a HEAT domain and WD40 repeats of which the latter two likely mediate protein-protein interaction. They have crucial roles in phagocytosis, autophagy and endosomal trafficking. Adapted from Thorpe LM *et al.* (125)

The Class IA PI3Ks are well known to be involved in oncogenesis and have been implicated in several human cancers. Of the three class IA PI3K catalytic isoforms, the p110α catalytic subunit is the most frequently found isoform to be altered via mutations, amplifications and/or copy number gain in human cancer (124, 125).

1.4.1 Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*) Activation, Downstream Effects and Genetic Alterations

The *PIK3CA* oncogene is located on chromosome 3q26.3 and encodes the p110α protein. This protein forms a heterodimer with the p85 regulatory subunit to create the PI3Kα lipid kinase which is expressed ubiquitously (124). The p110α protein phosphorylates its target lipid substrate, PIP_2 to PIP_3 and the p85 regulatory subunit negatively regulates the catalytic activity of p110α via the helical domain and mediates receptor binding, activation and membrane localisation of PI3Kα enzyme (124, 127). (Figure 1.7)

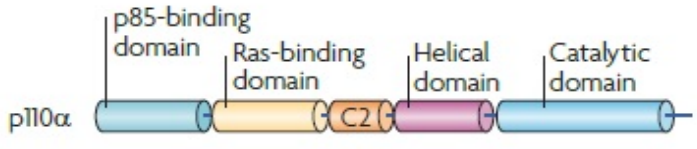

Figure 1.7 Schemata of p110α Protein Structure. The p110α protein is encoded by the *PIK3CA* oncogene. This protein is made up of 5 domains: the p85-binding domain, the RAS-binding domain, the membrane interacting C2 domain, the helical domain and the catalytic kinase domain. Adapted from Vanhaesebroeck *et al.* (128)

The PI3Kα complex resides in the cytoplasm in its inactive form. Activation occurs directly or indirectly via growth factor RTK (129). In direct activation, ligand-mediated activation of the kinase activity and trans-phosphorylation of the RTK cytoplasmic tail is followed by PI3Kα recruitment to the membrane via interaction of the SH2 domain of the p85 subunit with tyrosine phosphate motifs on activated receptors directly (e.g. EGFR) or to adaptor proteins associated with the receptors (e.g. insulin receptor substrate 1, IRS1). RTKs can also activate PI3Kα, indirectly though RAS, which can bind and activate the p110α subunit. Both methods lead to a conformational change in PI3Kα, which relieves the p85 subunit inhibitory effect on the p110α subunit, resulting in membrane localisation and stabilisation of PI3Kα (122, 123, 129). (Figure 1.8)

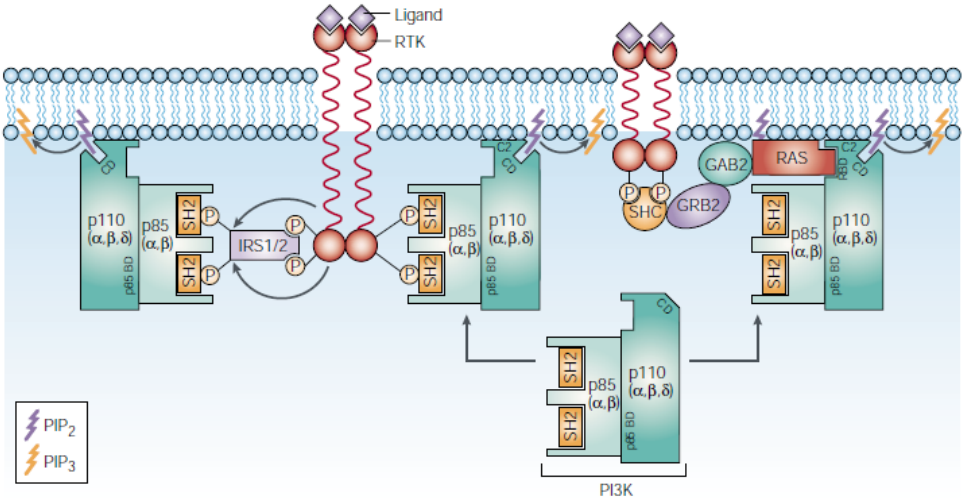

Figure 1.8 Modes of PI3K Activation. Ligand-mediated activation of the RTKs is followed by PI3K recruitment to the membrane via interaction of the SH2 domain of the p85 subunit with tyrosine phosphate motifs on activated receptors directly or to adaptor proteins associated with the receptors such as IRS1/2. These SH2–phosphotyrosine interactions bring PI3K in close proximity to its substrate at the plasma membrane and relieve the inhibitory action of p85 on the p110 catalytic subunit, which converts PIP_2 into PIP_3. RTKs can also activate PI3K, indirectly though RAS, which can bind, stabilise and activate the p110 subunit. This occurs by recruitment of the adaptor proteins SHC, GRB2 and GAB2 to activated RTKs. Vivanco *et al.* (129)

At the plasma membrane, PI3Kα phosphorylates its substrate PIP_2 to PIP_3. This lipid product acts as a second messenger, recruiting a subset of signaling proteins with pleckstrin homology domains such as PDK1 and AKT to the membrane leading to activation of multiple downstream signaling pathways. The phosphatase and tensin homolog (PTEN), a lipid phosphoinositide 3-phosphatase, acts to dephosphorylate PIP_3 to PIP_2, to inactivate PI3Kα signalling (129, 130).

The serine/threonine kinase, AKT, is the foremost pleckstrin homolog domain containing protein, activated by PIP_3. Recruitment to the membrane by PIP_3, results in a conformational change in AKT, which exposes two critical amino acid residues for phosphorylation. Phosphorylation at threonine 308 by PDK1 is necessary and sufficient for AKT activation; however, an additional phosphorylation at serine 473 by PDK2 (currently thought to be mTORC2; the mTOR/rictor complex) is required for maximal activation of AKT. Activated AKT phosphorylates numerous protein targets and regulates a wide range of key cellular process involved in protein synthesis, cell survival, proliferation, growth, angiogenesis and metabolism.

One of the primary targets of AKT is mTORC1 (mTOR/raptor complex), which is a central regulator of cellular metabolism and biosynthesis and essential for the oncogenicity of PI3Kα and AKT (123, 124, 131, 132). Aoki *et al.* demonstrated that mTOR inhibition by the antibiotic rapamycin strongly and specifically reduced PI3K- and AKT-induced cellular transformation of chicken embryo fibroblasts (133).

Additionally PI3Kα and PIP_3 have also been shown to regulate the activity of other downstream effector molecules through poorly characterized mechanisms. These include RAC1 and serum and glucocorticoid-inducible kinase (SGK) of which, their activities are thought to involve a role in cell cytoskeleton rearrangement and survival, respectively (134, 135). (Figure 1.9)

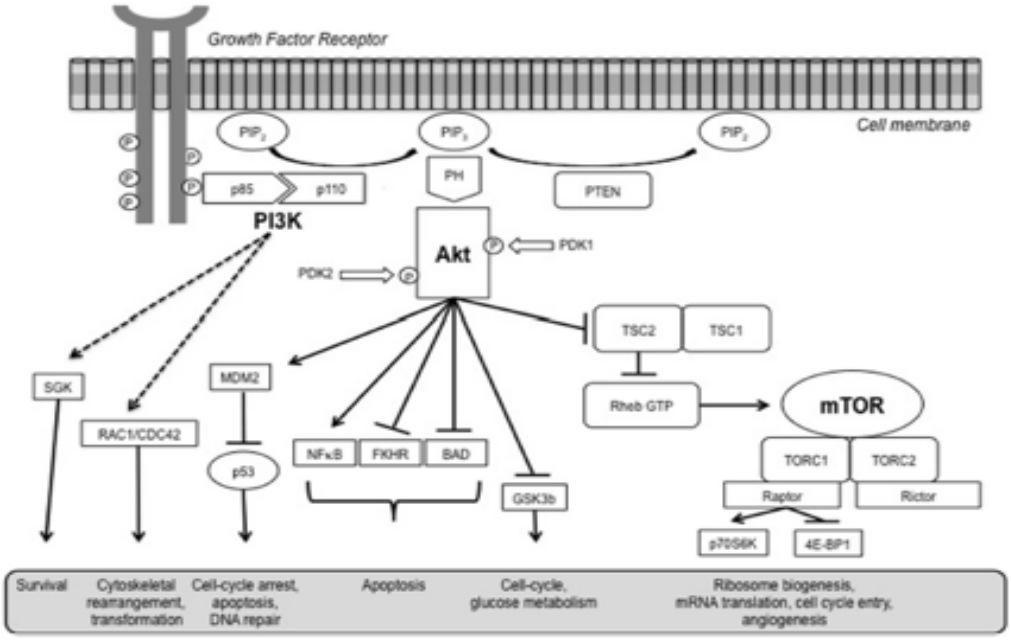

Figure 1.9 Class IA PI3K Signalling Pathways. Activation of PI3Ks leads to phosphorylation of the p85 subunit of the PI3K heterodimer, which subsequently activates the catalytic subunit, p110. This phosphorylated heterodimer catalyses the conversion of PIP_2 into PIP_3. This step is negatively regulated by PTEN, which dephosphorylates PIP_3 into PIP_2. PIP_3 generation on the inner leaflet of the plasma membrane recruits AKT by direct interaction with their pleckstrin homology domain. At the membrane PDK-1 phosphorylates AKT at threonine T308 and PDK-2 (mTOR2) phosphorylates AKT at serine residue S473, for maximal AKT activation. Activated AKT then mediates the activation or inhibition of multiple targets, such as mTOR, which results in cellular growth, survival and proliferation. In addition, PI3K have also been shown to regulate the activity of other cellular targets, in an AKT-independent manner such as SGK and the small GTP-binding proteins RAC1 and CDC42. The activity of which also leads to cellular survival, transformation and motility. Adapted from Porta *et al.* (123)

Somatic mutations in *PIK3CA* occur in up to 30% of common epithelial cancers, such as breast, endometrial and colon cancers (125). Missense mutations occur in all domains of p110α, and 80% of all mutations in the *PIK3CA* gene occur in two 'hot spot' regions, the most common being E542K and E545K in the helical domain and H1047R in the kinase domain. The two helical domain mutations reduce inhibition of p110α by the p85 regulatory subunit and the H1047R kinase domain mutation induces a conformational change in the activation loop leading to less upstream signal transduction dependent activation and thus access to its lipid substrate in the membranes (130, 136). Other rare cancer-specific mutations are also seen in the *PIK3CA* gene such as C420R which occurs in the C2 domain and increases the basic positive surface charge of the domain and hence likely increases p110α recruitment to the cell membrane (137).

In vitro studies and genetically engineered mouse models have confirmed that these *PIK3CA* hotspot mutations lead to cellular transformation, tumour initiation and progression by the constitutive activation of p110α (128, 138, 139). Rare *PIK3CA* mutations also have similar transformative capabilities, however not to the same extent as the hotspot mutations and this superior activity of the hotspot mutants likely explains their higher occurrence in cancer (137).

PIK3CA gene amplification and copy number gain are also common in cancer (128). In cervical and ovarian cancer cell lines, increased *PIK3CA* gene copy number is associated with increased p110α transcription, translation and total PI3Kα activity. This overexpression of a structurally normal PI3Kα protein likely contributes to malignant transformation via activation of AKT-dependent and AKT-independent downstream signaling pathways (129, 140, 141).

1.4.2 PI3K-AKT-mTOR Pathway in Penile Carcinogenesis

The deregulated PI3K-AKT-mTOR pathway plays a pivotal role in many human cancers leading to cellular proliferation, survival and angiogenesis. In addition, it provides an attractive target for cancer therapeutics and biomarker identification (142).

Multiples studies have taken place looking into PI3K-AKT-mTOR pathway in PSCC. Stankiewicz *et al.* (23) found activated EGFR (phospho-EGFR), HER3 and HER4, all of which are transmembrane RTKs which stimulate PI3K-AKT-mTOR signalling, are overexpressed in a cohort of 148 PSCC cases. They also reported HPV negative

tumours expressed significantly more phospho-EGFR than HPV positive tumours and this expression correlated with activated AKT (phospho-AKT, p=0.002). Conversely, HER3 expression was significantly more common in HPV positive cases, which correlated with AKT1 protein expression. Limited data on targeted inhibition of EGFR by anti-EGFR antibodies such as panitumumab have shown promising results in patients with advanced penile cancers (73).

The *PIK3CA* oncogene was found to be mutated in 8/28 (29%) penile cancer cases by Andersson *et al.* (114) and Ferrandiz-Pulido *et al.* (115) more recently found a lower prevalence of *PIK3CA* mutations in 6/65 (9%) PSCC cases. The PI3K pathway can also be activated by the RAS oncogene. Activating mutations in RAS are common in cancer and have been found in 1-18% of penile cancers (114, 115, 117).

On the other hand, decreased immunoexpression of PTEN, a tumour suppressor gene and repressor of the PI3K pathway, is a common event in PSCC present in 62-75% of cases (23, 117). However, loss of PTEN protein is not due to genetic events such as DNA copy number loss or silencing mutations and does not correlate with activated phospho-AKT immunoexpression as described by Stankiewicz *et al.* (23). This may mean that epigenetic mechanisms such as DNA methylation could be attributed to this reduction in PTEN immunoexpression. Or alternatively, other factors may have a greater impact on the activity of the PI3K-AKT-mTOR pathway in PSCC than PTEN such as *PIK3CA* for example (23, 114, 143).

Few studies, have investigated the downstream components of the PI3K-AKT-mTOR pathway in penile cancer. Ferrandiz-Pulido *et al.* (144) in 67 PSCC cases found phospho-mTOR (activated mTOR) and phospho-eIF4E (a downstream effector protein of mTOR) immunoexpression was significantly increased in PSCC compared to adjacent normal tissues and associated with lymph node metastasis (p=0.05 and p=0.006, respectively). Secondly, Chaux *et al.* (143) found that in 112 PSCC cases phospho-S6 (a downstream effector protein of mTOR) immunoexpression, was significantly higher in low-grade tumours (p=0.001). This suggests that mTOR plays a role both in early and late stage penile carcinogenesis and possibly represents an attractive therapeutic target.

HrHPV has also been shown to utilise the PI3K-AKT-mTOR pathway as it develops and establishes a malignant phenotype. Examples include the E5 viral oncoprotein, which is expressed in the early stages of carcinogenesis and induces the expression of VEGF through activation of EGFR and phosphorylation of AKT and MAPK (145).

HrHPV oncoprotein E6 has been shown to reduce *PTEN* and increase *PIK3CA* gene transcription levels by degradation of hDlg and p53 proteins, respectively (146, 147). And finally, the E7 oncoprotein has been shown to activate the PI3K-AKT pathway by inhibition of the phosphatase, PP2A, which acts to suppress AKT (148).

1.4.3 PIK3CA Copy Number Gain in Other HPV Induced Squamous Cell Carcinomas and Activation of the PI3K-AKT-mTOR pathway

There are distinct similarities in the genetic alterations between PSCC and other HPV induced squamous cell carcinomas (HISCCs). One such shared feature is the amplification or copy number gain of chromosome arm 3q, including the 3q26.3 region, which harbours *PIK3CA*. This has been shown to be one of the most consistent aberrations in cervical (140), head and neck (149) and oesophageal cancers (150).

Akagi *et al.* (151) found 12/45 (26.7%) tumours with *PIK3CA* amplification in oesophageal squamous cell carcinoma (OSCC) and Fenic *et al.* (152) found 16/33 (48.5%) with *PIK3CA* copy number gain in head and neck squamous cell carcinoma (HNSCC). Recent work by La-Touche *et al.* (97) found copy number gains in 55/64 cases (86%) within the 3p12.3-q29 loci, similar to Busso-Lopes *et al.* (98) in PSCC. Within this chromosomal region resides the *PIK3CA* oncogene, at the 3q26.3 locus, which was found to harbour a high prevalence of copy number gains, 79/258 (31%) in PSCC (97).

These genetic alterations have been shown to increase the activity of the PI3K-AKT-mTOR pathway in HISCCs. For example, Bertelsen *et al.* (153) found that *PIK3CA* amplification was significantly associated with increased immunoexpression of activated AKT (phospho-AKT (Ser473)) in cervical squamous cell carcinoma (CSCC) ($p=0.006$). And Clark *et al.* (154) identified that HNSCC cases had significantly higher levels of activated mTOR (phospho-mTOR (Ser2448)) than normal uvula cases via western blot ($p=0.026$) and immunohistochemistry (IHC) (81.9% vs. 0%; $p<0.001$).

Currently, three therapeutic agents targeting the PI3K-AKT-mTOR pathway have been approved for clinical use in treating various different cancers. These include the mTOR inhibitors, everolimus and temsirolimus, which have both been licenced for use in renal cell carcinoma among other tumours (123). And lastly, idelalisib, a PI3K p110δ inhibitor, has been approved for the treatment of haematological malignancies

such as chronic lymphocytic leukaemia and non-Hodgkin's lymphoma (155, 156). In addition, a vast number of other biological therapies targeting the PI3K-AKT-mTOR pathway are currently in pre-clinical studies and clinical trials in multiple advanced solid tumours including HISCCs (136) such as cervical (157, 158) and HNSCC (159, 160). Preclinical models have demonstrated some evidence of the predictive capabilities of *PIK3CA* mutations and activated AKT and mTOR proteins assessed by IHC in predicting treatment sensitivity to therapies targeting the PI3K-AKT-mTOR pathway, but have not been validated in the clinical setting (161, 162). Potentially, *PIK3CA* copy number gain could prove a useful marker for the activation and thus, use of biological therapies to target the PI3K-AKT-mTOR pathway in PSCC.

1.5 Aim

Based on our previous findings (97), we hypothesize that *PIK3CA* copy number gain is an oncogenic driver of penile carcinogenesis via activation of the PI3K-AKT-mTOR pathway and has the potential to be used as a marker of this pathways activation that presents an opportunity for targeted therapeutics in PSCC.

The aims of my project are:

1. To evaluate the prevalence of *PIK3CA* copy number gain in evolving disease stages of PSCC with fluorescence *in-situ* hybridisation (FISH) and correlate this with PIK3CA RNA and PI3Kα protein expression.

2. To assess the activity of the PI3K-AKT-mTOR pathway in PSCC using western blot and IHC and correlate to *PIK3CA* copy number status.

3. To evaluate the potential use of *PIK3CA* copy number status as a marker of lymph node metastasis and prognostication in PSCC via correlation with histopathological parameters and clinical outcome data.

CHAPTER TWO

METHODS AND MATERIALS

2.1 Primary Tissue Specimen

2.1.1 Fresh Frozen Clinical Specimen

Twenty-four fresh frozen primary PSCC specimens with corresponding normal adjacent penile epithelium samples available for 15 of these specimen, were obtained under ethical approval from St George's Hospital in London. (Table 2.1 and Figure 2.1A)

Table 2.1 Fresh Frozen Primary PSCC Cohort - Patient and Tumour Characteristics

Patient and Tumour Characteristics	
	N (%)
Age	
Median (Range)	66 (35 - 86)
Pathological T Stage	
T1	3 (12%)
T2	9 (38%)
T3	9 (38%)
T4	3 (12%)
Nodal Stage	
NX	4 (16%)
N0	9 (38%)
N1	1 (4%)
N2	1 (4%)
N3	9 (38%)

2.1.2 Formalin-Fixed Paraffin Embedded (FFPE) Clinical Specimen

Tissue microarray (TMA) blocks containing FFPE penile tissue cores from 244 primary PSCC, 15 normal penile epithelium and 58 PeIN samples were available in our laboratory for FISH and IHC analysis. Tissue samples for TMAs preparation were obtained under ethical approval from St George's Hospital in London (Table 2.2 and Figure 2.1A&B)

Table 2.2 FFPE Primary PSCC Cohort - Patient and Tumour Characteristics

Patient and Tumour Characteristics	
	N (%)
Age	
Median (Range)	65 (28 - 91)
Pathological T Stage	
TX	2 (1%)
T1	80 (33%)
T2	112 (46%)
T3	42 (17%)
T4	8 (3%)
Nodal Stage	
NX	26 (11%)
N0	143 (57%)
N1	31 (13%)
N2	21 (9%)
N3	23 (10%)

Additionally, TMA blocks, containing 27 FFPE advanced primary PSCC were obtained under ethical approval from the national Phase II Trial of docetaxel, cisplatin and 5-fluorouracil (TPF) chemotherapy in penile cancer (67). These 27 FFPE samples of advanced primary PSCC came from patients who had developed aggressive locally advanced or metastatic disease at presentation. (Table 2.3 and Figure 2.1A&B)

Table 2.3 FFPE Advanced Primary PSCC Cohort - Patient and Tumour Characteristics

Patient and Tumour Characteristics	
	N (%)
Age	
Median (Range)	61 (49 - 72)
Pathological T Stage	
TX	9 (33%)
T1	5 (19%)
T2	7 (26%)
T3	6 (22%)
Nodal Stage	
N1	5 (19%)
N2	7 (26%)
N3	15 (55%)
Metastatic Stage	
M0	19 (70%)
M1	8 (30%)