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REVIEW AND PERSPECTIVES

The molecular pathogenesis of penile carcinoma—current developments and understanding

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Abstract

Penile squamous cell carcinoma is a rare malignancy with various distinct histological subtypes, each with distinct appearances, histotypic specific associations with human papillomavirus (HPV) and clinical behaviour. Despite a wealth of pathological knowledge, there still remains a limited understanding of the fundamental molecular drivers that govern penile carcinogenesis with their underlying prognostic and therapeutic importance. However, recent work has improved our fundamental understanding of the molecular pathogenesis of penile cancer: commonly divided into the HPV-dependent and HPV-independent pathways. This review aims to summarise current developments in the histopathology and the molecular pathogenesis of penile cancer, with the advent of next-generation sequencing, and the opportunities for the targeting of molecular drivers of metastatic disease.

Keywords Penile squamous cell carcinoma · Human papillomavirus · Penile intraepithelial neoplasia · Histotype · Carcinogenesis · Genetics

Introduction

Penile squamous cell carcinoma (PSCC) is a rare disease, representing approximately 0.5% of all male malignancies. The incidence of PSCC in Europe varies geographically ranging from 0.5 to 1.6 per 100,000 population [1]. This malignancy occurs predominantly in elderly men, with an increasing incidence with age, with the highest rate being between 50 and 70 years [2].

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. 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 [3, 4].

The aetiology of PSCC is multifactorial with a multitude of risk factors identified. One of the most important and extensively studied is infection with human papillomavirus (HPV). Around 33% of all PSCC cases are associated with HPV infection [5], similar to vulvar [6] and head and neck cancers [7]. Other risk factors include phimosis, chronic inflammatory states, poor penile hygiene, penile trauma, high number of sexual partners and smoking [8, 9].

Although rare, a small number of PSCC are known for early loco-regional and angiolymphatic spread with a poor prognosis. 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 PSCC [10].

One of the main challenges in advanced PSCC 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. With no molecular biomarkers currently validated for clinical practice, the management of patients with clinically node-negative disease is primarily dependent on risk stratification with clinico-pathological parameters to either surveillance or

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invasive lymph node staging by bilateral modified inguinal lymphadenectomy or dynamic sentinel lymph node biopsy [1, 2].

Management of advanced PSCC remains difficult as most are chemo/radio-resistant with limited treatment options available, when first-line therapeutic options fail [1, 10]. Radiotherapy is only used for palliation in those with advanced and may improve loco-regional control in patients with extensive metastases and/or extra-nodal spread [2]. Chemotherapy for PSCC 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. Current first-line chemotherapy for PSCC consists of taxane, cisplatin, and 5-fluouracil or ifosfamide (TPF or TIP) as these regimes demonstrated good objective tumoural response [11, 12]. There are limited second-line chemotherapeutic options for the systemic treatment of PSCC, once first-line therapy eventually fails. 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 monoclonal antibodies such as panitumumab [13] and receptor tyrosine kinase inhibitors such as sorafenib and sunitinib [14].

Due to the short-lived response rates of standard chemotherapy in advanced PSCC, there is increased interest in discovering newer targeted therapies to improve the outcome of this debilitating disease. However, the molecular mechanisms underlying penile carcinogenesis are still largely unknown and seldom studied. Supra-regional PSCC networks have been established in the UK, and organisation of national and international research collaborations will undoubtedly improve patient management, our understanding of this disease and potentially leading to identification of biomarkers and novel treatment strategies in PSCC. This paper provides a summary of the current understanding of the pathology and molecular pathogenesis of PSCC.

Histological classification of penile cancer

Squamous cell carcinoma accounts for around 95% of all malignant disease of the penis. The remaining 5% comprise of various rare tumour entities, from clear cell, medullary and basal cell carcinomas to melanomas and sarcomas among others [9, 15].

Despite PSCC being the most common penile neoplasm, various distinct histological subtypes exist, including those with a mixed histological background. The International Society of Urological Pathology (ISUP) has recently reviewed the World Health Organization 2016 classification of penile carcinomas with various expert-driven recommendations made [16]. One such recommendation was to classify the large number of variants of PSCC in accordance with their relationship with HPV, which are given in Table 1. Generally, HPV-negative tumours are preferentially of lower grade and keratinizing maturing neoplasms, with the exception of the adenosquamous and sarcomatoid variants [16, 17].

The major HPV-related PSCC histological subtypes include the warty and basaloid types and the major non-HPV-related PSCC histological subtypes include the usual, verrucous, papillary not otherwise specified (NOS) and sarcomatoid subtypes. These major subtypes and their frequency, gross features and histotype-specific association with HPV and clinical aggressiveness are summarised in Table 2 [18, 19, 21]. Though not related to HPV, verrucous and papillary tumours still show a few tumours with HPV positivity and this reflects the heterogeneity of PSCC and the histopathological difficulty in diagnosing certain variants, especially the rare and mixed histological subtypes. In addition, though the PSCC usual subtype is classified as a non-HPV-related variant, about a third of these tumours are HPV related and usually HPV 16.

The value of $p16^{INK4A}$ in the diagnosis of HPV-related tumours

Multiple methods exist for detecting HPV in research studies from the use of PCR such as the INNO-LiPA HPV Genotyping Extra Amplification Kit or in situ hybridisation technologies. However, as an in-expensive alternative, multiple studies have shown that immunohistochemical overexpression of the $p16^{INK4A}$ protein can be used as a surrogate marker of high-risk HPV (hrHPV) infection (with an overall concordance of 84%). In addition, $p16^{INK4A}$ can also aid in the classification and diagnosis of morphologically challenging cases, diagnosis of different PSCC subtypes and differential diagnosis of low-grade verruciform and high-grade solid penile tumours [16, 22, 23]. Due to this, the use of $p16^{INK4A}$ immunostaining by pathologists has been recommended by the ISUP both to detect HPV infection and to aid in tumour classification [16].

Table 1 PSCC histological variants

Non-HPV related	HPV related	Others
Usual	Basaloid	Mixed
Verrucous	Warty	Unclassified
Papillary NOS	Warty-basaloid	
Cuniculatum	Papillary-basaloid	
Pseudoglandular	Clear cell	
Pseudohyperplastic	Lymphoepithelioma-like	
Sarcomatoid		
Adenosquamous		

Table 2 Characterisation of common PSCC histotypes

PSCC subtype	Gross features	Frequency (%)	HPV positivity (%)	Metastatic rate (%)	Prognosis
Usual	Variable from white to grey, nodular exophytic to flat ulcerated tumours	45–65	24–59	28–39	Intermediate
Basaloid	HPV-related ulcerated, non-exophytic, irregular grey to reddish mass that is deeply invasive	10	70–100	50–100	Poor
Warty	HPV-related cauliflower-like, exophytic verruciform tumours	8–10	22–78	17–18	Good
Verrucous	Low-grade white to grey verruciform exophytic tumour, invading superficial anatomical levels only	3–8	0–23	Nil	Excellent
Papillary	Large, irregular and exophytic verruciform tumour with poor differentiation between the tumour and stroma	5–10	8–15	≤ 12	Good
Sarcomatoid	Bulky, ulcerated and haemorrhagic, fungating or rounded polypoid masses composed of spindle cells	1–4	0–17	75–89	Very poor

Data from the following references: [17–20]

Though a useful adjunct, $p16^{INK4A}$ immunohistochemical staining does have some underlying issues. The first is this methodology only distinguishes samples into hrHPV positivity or negativity, without the possibility of HPV genotyping. Though a high concordance is noted with $p16^{INK4A}$ immunohistochemical staining, a small number of hrHPV-positive tumours could still be missed, such as those with simultaneous inactivation of the $p16^{INK4A}$ gene, either by loss of heterozygosity or promoter hypermethylation. In addition, interpretation of the various $p16^{INK4A}$ staining patterns and the best cut-off point for defining a positive result has proved controversial, though previous studies have shown that a strong full thickness $p16^{INK4A}$ staining pattern is best associated with HPV-related PSCC subtypes and hrHPV [22, 24] (Fig. 1).

Precursor penile lesions

PeIN are also classified by their association with HPV, into differentiated (non-HPV related) and undifferentiated (HPV related) PeIN. Differentiated PeIN accounting for around 75% of penile precursor lesions are clinically flat or slightly raised pale/white or erythematous lesions and are associated with lichen sclerosis. As they are unrelated to HPV, they are usually $p16^{INK4A}$ negative and may give rise to the usual and verruciform type tumours. Conversely, undifferentiated PeIN, which make up 25% penile precursor lesions, are usually flat erythematous lesions found on the penile glans and/or foreskin with basaloid/warty morphological features often seen. It is associated with high-risk HPV subtypes, usually $p16^{INK4A}$ positive and may give rise to basaloid, warty or usual type penile tumours [18, 25] (Fig. 1).

Molecular pathogenesis of penile cancer

The molecular mechanisms underlying penile carcinogenesis are still largely unknown; 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. With establishment of PSCC centres and various collaborative endeavours more is known, but a greater understanding of the molecular mechanisms of penile carcinogenesis will lead to identification of biomarkers and novel treatment strategies in PSCC.

Our fundamental understanding of the underlying aetiology of PSCC is in recognition of its heterogeneity and two distinct molecular pathways of carcinogenesis: the HPV- and non-HPV-mediated pathways. Detailed overviews of these pathways are discussed below (Fig. 2).

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 [26]. These mucosal HPVs are classified into 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 (Table 3).

High-risk HPVs are associated with around 33% of all penile cancer cases, with HPV 16 the most prevalent subtype [5]. Low-risk HPVs (lrHV), such as HPV 6 and HPV 11, are more commonly associated with benign lesions. 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 [27]. However, these lrHPV variants are commonly found in PSCC with HPV 6 the second most common HPV subtype found in this tumour (3.7%) [5].

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 [28]. Persistent epithelial HPV infection and

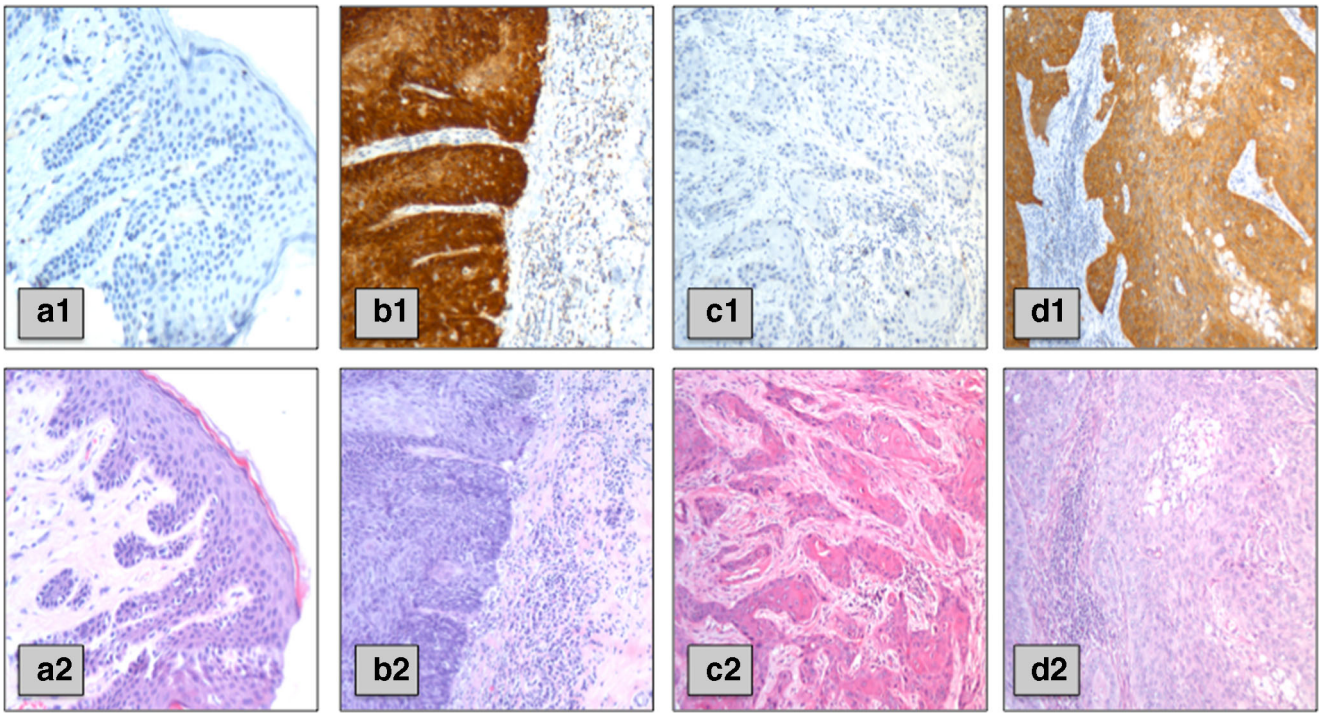

Fig. 1 Examples of $p16^{INK4A}$ IHC in PeIN and PSCC samples. (**A**) Negative $p16^{INK4A}$ immunostaining in PeIN (1) with corresponding H&E staining (2). (**B**) Strong uniform $p16^{INK4A}$ immunostaining in PeIN (1) with corresponding H&E staining (2). (**C**) Negative $p16^{INK4A}$ immunostaining in PSCC (1) with corresponding H&E staining (2). (**D**) Strong positive $p16^{INK4A}$ staining in PSCC (1) with corresponding H&E staining (2)

integration of HPV DNA into the host cell genome lead to a transformed malignant phenotype. 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 [26].

hrHPV E7 oncoprotein associates with and inactivates pRB, releasing the transcription factor E2F to activate genes involved in DNA synthesis and results in uncontrolled cell cycle progression [29]. This process leads to the overexpression of $p16^{INK4A}$, due to the disruption of the negative feedback loop between $p16^{INK4KA}$ and pRB [20, 21, 30]. This allows $p16^{INK4KA}$ overexpression to be used as a surrogate marker of transcriptionally active HPV infection in PSCC, which has been shown by multiple groups [22, 23, 31].

hrHPV E6 oncoprotein targets the p53 tumour suppressor protein for proteasome-mediated degradation. 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 [21].

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 human telomerase reverse transcriptase (hTERT) expression [32] and degradation of the NFX1-91, a repressor for the hTERT promoter [33] 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 [26].

HPV-independent penile carcinogenesis

The genetic mechanisms behind HPV-negative penile carcinogenesis are less well understood. However, significant advances in genetic sequencing have allowed the discovery of multiple genomic alterations, which occur during penile carcinogenesis and these are classified and discussed below.

Chronic inflammation

Chronic inflammation is a known risk factor for PSCC with conditions causing chronic irritation/injury and hence inflammation such as phimosis, balanoposthitis and lichen sclerosis heavily linked with the development of this malignancy [1, 9].

A key mediator of inflammation, cyclooxygenase-2, has been shown to be strongly expressed in early-stage PeIN, invasive PSCC and lymph node metastasis, but not in normal tissue [34]. The overexpression of cyclooxygenase-2 leads to overproduction of thromboxanes and prostaglandins (esp. prostaglandin E2), which play a pivotal role in proliferation,

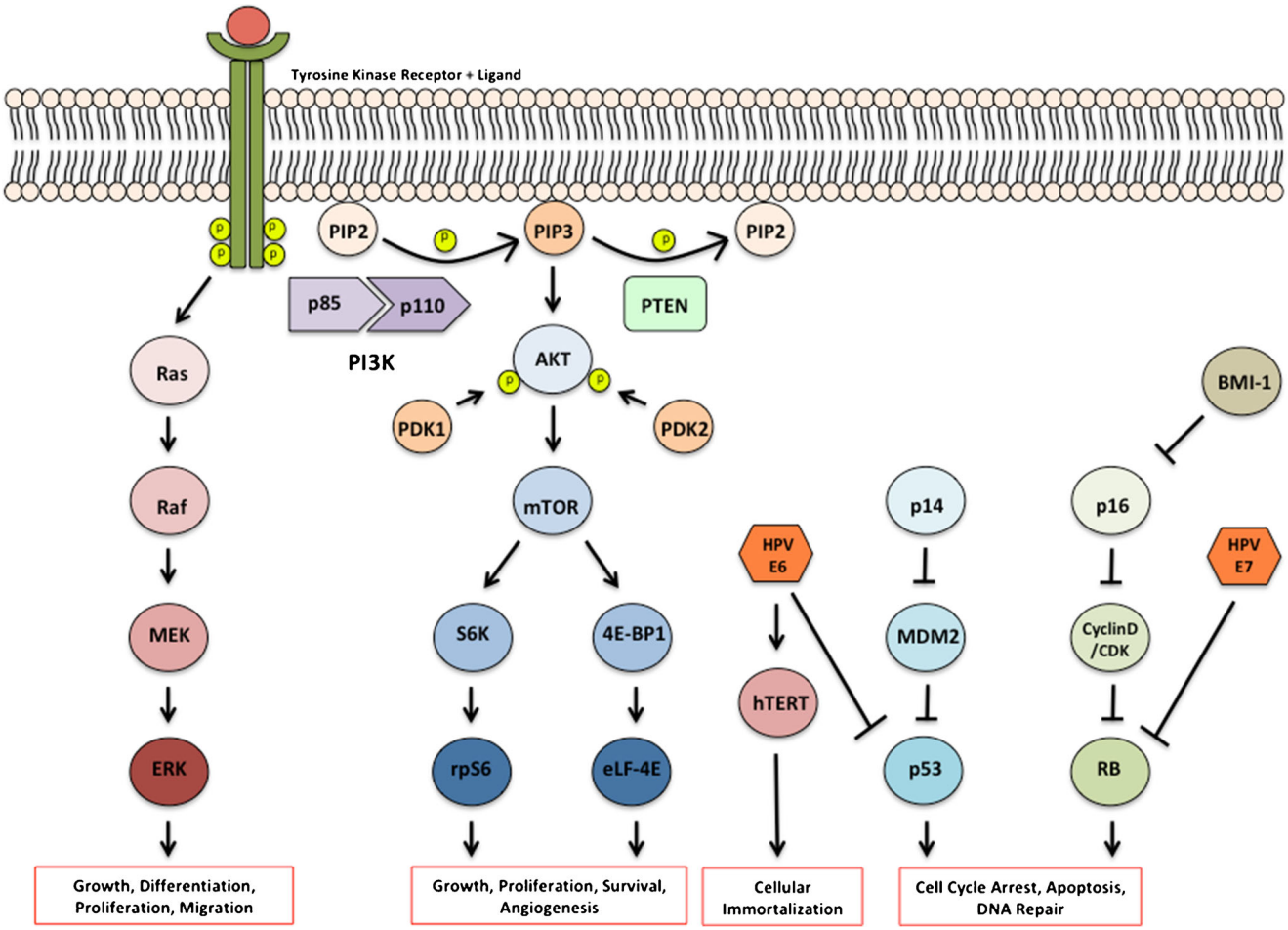

Fig. 2 Schematic diagram illustrating both HPV- and non-HPV-mediated molecular pathways implicating in penile cancer

invasion and angiogenesis via activation of multiple pathways once of which being the PI3K-AKT pathway [35].

Somatic genetic alterations

Multiple genetic alterations from loss of heterozygosity (LOH), mutations, deletions, copy number gain/amplications and epigenetic modifications have been identified in various pathways in PSCC as a mechanism for carcinogenesis.

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 LOH and silencing via DNA methylation with loss of $p16^{INK4A}$ immunoexpression has been significantly associated with lymph node metastasis and disease recurrence [36, 37]. In addition, overexpression of BMI-1 polycomb gene, which results in downregulation of $p16^{INK4A}$ and $p14^{ARF}$, has been found in PSCC [30].

Table 3 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

Allelic loss and mutations of TP53, $p14^{ARF}$ mutation and methylation, and overexpression of mouse double minute 2 homolog (MDM2), which is a negative regulator of TP53, have also been shown in PSCC [38, 39]. Mutation of TP53 can also lead to increased expression of an inactive p53 mutant protein (90%) or absent p53 protein expression (10%) [9]. P53 immunoexpression in PSCC varies from 41.5 to 89% and is associated with a poor prognosis in PSCC [9, 20]. Insulin-like growth factor-1 receptor (IGF1R) is known to play a role in cellular growth and transformation and recently it has been shown by Faraj et al. [40] that IGF1R expression is associated with PSCC histological grade, subtype and HPV, with potential prognostic relevance which needs further investigation.

The deregulated PI3K-AKT-mTOR pathway has been shown to play a role in penile carcinogenesis. Stankiewicz et al. [41] found p-EGFR, HER3 and HER4, which stimulate PI3K-AKT-mTOR signalling, are overexpressed in a cohort

of 148 PSCC cases. They also reported that HPV-negative tumours expressed significantly more p-EGFR than HPV-positive tumours and this expression correlated with p-AKT ($p = 0.002$). Limited data on targeted inhibition of EGFR by anti-EGFR antibodies such as panitumumab have shown promising results in patients with advanced PSCC [42] and this correlates with a recent study showing polysomy and amplification of the EGFR gene as an independent risk factor for penile cancer–specific mortality [43].

The PIK3CA oncogene has been found to be mutated in 8–9% of PSCC cases [44, 45]. Activating mutations in RAS are common in cancer and have been found in 1–19% of PSCC [45–47]. 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 [41].

Ferrandiz-Pulido et al. [48] in 67 PSCC cases found p-mTOR and p-elF4E immunoexpression was significantly increased in PSCC compared with adjacent normal tissues and associated with lymph node metastasis (LNM) ($p = 0.05$ and $p = 0.006$, respectively). Secondly, Chaux et al. [49] 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. However, our recent paper downplays the significance of the PI3K-AKT-mTOR pathway as we found normal penile epithelium had higher levels of protein expression of p-AKT ($p = 0.0247$) and p-mTOR ($p = 0.0041$) than PSCC [44] (Table 4).

Table 4 Common genetic alterations in penile cancer

Genetic change	Gene/ chromosome	Frequency	References
Point mutations	*TP53*	13–40%	[9]
	PIK3CA	8–9%	[44, 45]
	CSN1	17%	[50]
	RAS	1–19%	[45]
DNA methylation	$p16^{INK4A}$	42–44%	[36, 37]
Loss of heterozygosity	$p16^{INK4A}$	62%	[36]
	TP53	42%	[36]
Copy number gain/amplification	1p13.3 - q44	88%	[51]
	3q13.3 - q29	42–86%	[51, 52]
	8q21.2 - q24.3	42–84%	[51, 52]
Copy number loss	2q33 - q37.3	86%	[51]
	3p24.3 - q11.1	34–83%	[51, 52]
	11q12.2 - q25	81%	[51]

Genomic copy number variations

Copy number variations (CNVs) are a type of structural variation, where a considerable number of DNA base pairs are duplicated or deleted. Two studies analysed the genomic changes in PSCC by array comparative genomic hybridisation methods looking to map the CNVs in PSCC. La-Touche et al. [51] on 64 PSCC cases reported recurrent gains in chromosomes 1p13.3-q44 (88%) and 3p12.3-q29 (86%) and losses in chromosomes 2q33- q37.3 (86%) and 11q12.2-q25 (81%). Similar patterns of genetic aberrations were found between HPV-positive and HPV-negative PSCC cases, which they suggested to provide evidence that HPV-positive and HPV-negative PSCC target similar molecular pathways and both may utilise a final common pathogenetic pathway.

Busso-Lopes et al. [52] in 46 PSCC cases discovered significantly lower cancer-specific survival (CSS) and disease-free survival (DFS) 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 PSCC. They also noted DCL1 and PPARG losses were also associated with worse prognosis. They also identified 19 specific genomic alterations which were more common in HPV-positive PSCC cases, thus supporting the hypothesis of two distinct PSCC aetiologies: one dependent and the other independent of HPV infection (Table 3).

Aberrant microRNA expression

microRNAs (miRNA) are small non-coding RNA that function in RNA silencing and post-transcriptional regulation of gene expression. Recently, it has become evident that miRNAs have a role in carcinogenesis as they are frequently dysregulated and aberrantly expressed in human cancers [28]. Hartz et al. [53] found that the loss of three miRNAs (miR-1, miR-101, miR-204) was associated with LNM and poor prognosis in PSCC with a potential clinical utility in developing a miRNA signature panel to aid risk stratification by predicting the formation of PSCC metastasis at an early stage. In addition, it has been found that the downregulation of the SLC8A1 gene, by miR-223, can cause a reduction in calcium levels in PSCC leading to suppressed apoptosis and increased cell proliferation [54].

Zhang et al. [55] performed the first miRNA profile using next-generation sequencing in 10 PSCC and matched adjacent normal tissue samples. They found 56 miRNAs with significantly different expression between the paired tissues and the putative target genes of the aberrant miRNA expression were closely associated with signalling pathways established in carcinogenesis such as the MAPK, p53 and PI3K-AKT pathways. A more recent integrative analysis identified increased MMP1 expression levels as a better predictive marker of LNM in PSCC than usual clinico-pathological data [56].

Aberrant immunological expression

Cancer cells suppress and escape the host immune response via various mechanisms leading to an immunotolerant state. One of the immunotolerance enhancing checkpoints is the program death-1 (PD-1)/PD-1 ligand (PD-L1) axis, as when PD-L1 binds to PD-1, this leads to suppression of T cell activation and proliferation. Recent advances in immunotherapies targeting this pathway have shown great promise in various urological malignancies [57].

Udager et al. [57] were the first to study this axis in PSCC and found 62% (23/37) of tumour were positive for PD-L1 expression and were associated with LNM ($p = 0.024$) and reduced CSS ($p = 0.011$). These results have been confirmed by Ottenhof et al. [58] showing increased levels of PD-L1 expression in 48% (96/200), thus offering promise of future anti-PD-1 and anti-PD-L1 immunotherapeutic options in PSCC.

Genetic profiling and next-generation sequencing

In recent times, more in-depth studies have been carried out and integrated analysis with the arrival of next-generation sequencing (NGS). One of which by, Feber et al. [50] analysing the somatic mutational landscape of PSCC via whole exome sequencing 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%). 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 with HPV-negative tumours. An earlier NGS study found that lack of p16 expression and MYC and CCND1 amplication were significantly associated with shorter time to progression or survival [59] (Table 3).

A recent and first mitochondrial genomic analysis in PSCC by Araujo et al. [60] found increased mitochondrial genomic instability in PSCC due to a higher frequency heteroplasmy and mitochondrial DNA depletion. Yang et al. [61] performed a multiplatform kinase analysis and gene expression platform on 11 PSCC samples and found the PTEN, STAT3, GNRH, IL-* and B cell receptor signalling pathways to be most commonly upregulated in PSCC. However, this study was perfomed in a small number of samples and as such larger and translational studies to validate these findings are needed. A comprehensive integrative analysis by Marchi et al. [62] in 20 PSCC samples identified top driver genes, which include PPARG, RB1, AR and STAT1, and found shorter OS was associated with BIRC5 ($p = 0.026$) and DNMT3B overexpression ($p = 0.002$), highlighting its potential as a novel prognostic marker for PSCC.

Conclusion

Increasingly, more is understood of the underlying molecular mechanisms involved in penile carcinogenesis with the advent of next-generation sequencing methodologies and international/multi-institutional penile collaborations. Clearly, there are multiple drivers involved in PSCC with diverse genetic and epigenetic changes with the potential for prognostic and therapeutic implications identified. In the future, there should be more functional studies to further evaluate these drivers. In addition, translational and clinical research is needed to transform these potentially key drivers and pathways into clinical applicable strategies to improve patient care in this debilitating disease.

Contributions Literature review—AA and JN
Manuscript writing—AA
Final manuscript review—AA, JN, NW and DB

Compliance with ethical standards This study has full adherence and compliance to ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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