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TOPIC PAPER

Molecular prognostic factors in penile cancer

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Abstract
Objectives Penile cancer is a rare tumour in developed countries but more common in South America and East Africa. Although pathological prognostic factors have been established, there is great interest in evaluating molecular markers which correlate with prognosis and outcome.
Methods We have reviewed the current status of our understanding of the molecular biology of penile cancer in order to identify established and potential prognostic factors in penile cancer. We have conducted an extensive literature search to review the current understanding of the role of prognostic markers in penile cancer.
Results Although several markers have been evaluated, currently the clinical application of these markers is limited. HPV positive tumours show a variable prognostic outcome. P53 status may correlate with survival in T1 disease but further studies are required to establish the link to lymph node spread.
Conclusions Pathological variables are well-established but further work is required to investigate the role of molecular markers. The development of molecular prognostic markers is important for the surveillance of patients and prediction of lymph node involvement as well as a prognostic marker for survival.

Keywords Prognostics · Squamous cell carcinoma · Penile · Molecular

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Introduction

Penile cancer is a rare tumour with the predominant histological subtype being squamous cell carcinoma (SCC) accounting for over 95% of cases [1]. The rarer subtypes are listed in Table 1.

There are several risk factors which predispose to the development of penile cancer including the presence of a foreskin, balanitis xerotica obliterans and smoking. The prevalence of penile cancer in patients with BXO has been reported at between 2.6 and 5.8% [2]. Smoking has also been shown to increase the relative risk of penile cancer. However, the most extensively studied risk factor is HPV infection. Studies conducted by the International Agency for Research on cancer (IARC) have shown a reduced prevalence of HPV infection in circumcised men and an increased prevalence of cervical cancer in partners of uncircumcised men [3, 4]. Pre-malignant lesions include erythroplasia de Queyrat, leukoplakia, pseudoepitheliomatous micaceous and condyloma acuminatum. Buschke–Löwenstein tumours are locally invasive tumours. Sporadic association has been reported with Bowenoid papulosis of the penis and cutaneous horn of the penis.

The management of occult disease in inguinal lymph nodes is a subject of debate. The current available imaging modalities have limitations in detecting locoregional spread of the disease. A proportion of patients therefore undergo overtreatment of their inguinal lymph nodes with the added complications of lymphoedema, wound breakdowns and lymph fistulae. Identification of patients with a higher risk for lymph node disease would help to reduce unnecessary lymphadenectomies and the technique of dynamic sentinel node biopsy has partly addressed this. Currently there are several histopathological features which have been identified as prognostic indicators. The most important one of

Table 1 Histological subtypes of penile cancer

Squamous cell carcinoma
Adenocarcinoma
Lymphoma
Melanoma
Kaposi sarcoma
Leiomyosarcoma

these is lymph node involvement. Alternative features which can predict locoregional spread include the grade of the tumour, stage, depth of invasion, tumour size and lymphovascular invasion [5, 6]. There is limited data on alternative prognostic markers and markers predicting occult lymph node disease due to the rarity of this neoplasm. We have reviewed the current literature in order to identify the potential prognostic markers and predictors of occult lymph node disease.

The role of HPV in the pathogenesis of penile cancer

HPV is associated with anogenital tumour formation and is an important factor in the development of in situ and invasive epithelial tumours. The prevalence of HPV DNA in penile carcinoma ranges between 20 and 80% and varies depending on the methods utilised for the detection and the geographical variance [7–10]. Techniques using PCR quote a range of 40–45% [11]. A correlation between the penile cancer tumour subtype and HPV infection has been demonstrated. Our understanding of HPV DNA integration in to the human genome stems from work investigating SCC of the cervix and from the development of a HPV specific quadrivalent vaccine [12]. Basaloid and warty subtypes are almost always associated with HPV as is carcinoma in situ (90%) which is in contrast to keratinising and verrucous tumours where only one-third of the tumours are associated with HPV [7]. Studies have demonstrated that the most common type of HPV infection is HPV 16 and 18 which occur in 60–75% of in situ and invasive tumours [13]. Low risk lesions such as Buschke–Lowenstein and condylomas are associated with HPV 6 and 11. Verrucous tumours are not generally associated with HPV involvement. The viral genes *E6* and *E7* are overexpressed in HPV transformed cells and are involved in cellular differentiation and proliferation through their interaction with the retinoblastoma Rb/E2F and p53 tumour suppressor pathways. Although these viral genes have been implicated in the phenotypic expression of cervical cancer, a similar mechanism probably exists in penile cancer.

The HPV family are generally sexually transmitted and consists of double-stranded DNA viruses (8,000 base pairs). The high risk HPV types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 66, 68 and 69. The low risk types are 6, 11, 26, 30, 34, 40, 42–44, 53, 55, 57, 61, 62, 64, 67, 70, 71, 73, 74, 79, 81, 82, 83 and 84 [14]. Within the viral genotype is an early (E) region encoding the proteins which are required for replication, regulation and modification of the host nucleus and cytoplasm together with a late (L) region that encodes for capsid proteins.

HPV as a prognostic indicator in penile cancer

HPV infection has been identified as an aetiological factor in the development of penile cancer. Although studies have found that HPV infection is related to the tumour subtype, the correlation with prognostic variables is still unclear despite the extensive research conducted in this particular area. Evidence from other SCC sites indicate that not all high risk HPV infections manifest as clinically significant infections as transcriptional activity of the *E6*/*E7* oncogene region may not occur [15]. Bezerra et al. [16] analysed tissue from 82 patients treated for penile carcinoma using PCR. Of these 42 patients had lymph node metastases. HPV DNA was detected in 30.5% (25/82) samples of which 13 were positive for HPV-16, four contained HPV-18 with the remaining samples positive for a variety of HPV DNA including HPV-45, HPV-6/11, HPV-31,-33 and 35. There was no correlation between HPV DNA status and 10 year survival. With a mean follow-up of 88.7 months (range 0.1–453 months) the study concluded that HPV status was related only to lymphatic embolisation and had no correlation with the prognosis. Disease specific survival was only significantly related to the lymph node status. A smaller study which retrospectively analysed specimens from 29 patients also found that there was no correlation between HPV DNA status and Kaplan–Meir estimates of survival [17]. In this study HPV infection was found in 9 of the 29 patients of which HPV 16 (8 out of 9 patients) was the predominant subtype.

In contrast to these two studies, Lont et al. [18] investigated the survival outcome in 176 patients with SCC of the penis with a mean follow-up of 95 months. HPV DNA detection was performed using PCR with genotyping for all the high risk HPV subtypes. The study showed the presence of high risk HPV DNA in 50 of the 171 patients (29%) of which 38 (76%) was HPV-16. Multiple logistic regression analysis found that the only factor related to the HPV status was morphea-like growth whereby HPV-positive tumours showed less morphea-like growth. The 5 year disease specific survival was 92% in the HPV positive group compared to 78% in the HPV negative group indicating a survival advantage for patients who are HPV DNA positive. This

may indicate a different disease entity and genetic imbalances for HPV positive patients and is an area that requires further evaluation.

Genetic imbalances and ploidy status in penile cancer

There has been very little data linking the chromosomal abnormalities to the biological behaviour and outcome. This is partly due to the relatively low incidence of the cancer and also due to the technical difficulties posed by the associated necrosis, inflammation and poor growth in culture of this particular cell type. By utilising genomic hybridisation techniques and cytogenetic analysis some of the genetic imbalances have been investigated in penile cancer [19, 20]. A further group found that DNA sequence copy-number alterations were detected and were specifically found to be 8q24, 16p11–12, 20q11–13, 22q, 19q13, and 5p15. Deletions were seen in 13q21–22, 4q21–32, and also along the X-chromosome [21]. The ploidy status of penile cancer has been investigated using flow cytometry [22]. Although this study reported that no additional prognostic data was obtained from performing DNA flow cytometry, analysis of the pathology specimens from 46 patients did show that patients with T1 lesions have a very low risk of lymph node involvement [22]. Studies have also demonstrated that verrucous cancers are associated with a diploid status [23, 24]. The aneuploidy varies according to the grade of tumour with a high DNA index correlating with an increased metastatic risk. Increased telomerase activity has also been detected by Alves et al. [25] in 41 out of 48 invasive penile cancers. This increased telomerase activity within the cells allows neoplastic cells to overcome programmed cell death and thus immortalising the cell. The increased telomerase activity has also been detected in adjacent tissue indicating a generalised field change associated with the tumour. Measurement of telomerase activity may be potentially used as a marker for metastatic risk.

Prognostic value of p53 in penile cancer

Penile carcinogenesis is a multistep process in which genetic events lead to a disruption in the normal regulatory pathways controlling cell division, differentiation and cell death. Oncogenes are altered growth promoting regulatory genes that govern the cells' signal transduction pathway. Mutations of these genes can lead to an overexpression or increased function of excitatory proteins. Where tumorigenesis occurs in HPV independent neoplasms, additional cellular changes are required to induce full malignant potential. The interaction between viral and host DNA is paramount to these changes and involves various tumour suppressor genes and proapoptotic proteins.

Tumour suppressor genes are most often inactivated by point mutation, deletions and rearrangement of both gene copies and is a crucial step in cell transformation. The tumour suppressor gene p53 is known to be mutated in approximately 70% of adult solid tumours [26]. Several studies have investigated the relationship between the TP53 gene, situated on chromosome 17p13, and its functional protein p53 expression with HPV infection and prognosis [27–31].The binding of the HPV oncogenic product *E6* to the oligomerisation region of wild type p53 leads to its degradation by 26S proteasome [32]. The interaction of *E6* to p53 results in dysregulation of the cell cycle and uncontrolled cellular proliferation, loss of cellular differentiation and decreased apoptotic control. A mutation of the TP53 gene leads to either absence of the protein or expression of a mutant protein, the latter being the common scenario. The regulatory protein MDM2 (the human homologue to the murine double minute 2 oncogene) regulates the intracellular p53 concentration through a negative feedback loop. However, mutant p53 does not bind to MDM2 thus resulting in an overexpression. The expression of p53 in invasive penile cancer varies between 40 and 89% and there is no definite correlation with grade or stage of the disease. Although there have been suggestions of an association between polymorphisms at codon 72 of TP53 in SCC models, studies performed by Humbey et al. [29] have shown no correlation between TP53 polymorphism at codon 72 and the presence of HPV DNA.

Studies which have analysed p53 expression and HPV infection in penile cancer patients have shown that p53 immunostaining is an independent predictor of lymph node metastases [18, 27]. Those patients who were negative for p53 had a significantly better 5 and 10 year survival. Outcome was worse for those who were p53 and HPV DNA positive whereas HPV positive and p53 negative patients had the best survival. The study by Lopes et al. [27] reviewed 82 patients with $\geq$T2 disease. In all, 34 of the 82 samples showed nuclear accumulation of p53. Although the p53 status did not correlate with pathological variables such as the grade, T or N stage, tumour depth, lymphatic or venous embolisation or HPV status, the p53 immunoreactivity and p53 grade did correlate with a higher incidence of lymph node metastasis. With a mean follow up of 88.7 months univariate analysis showed that p53 positive cases had a lower overall survival. Martins et al. [33] evaluated p53 expression together with another tumour marker, proliferating cell nuclear antigen (PCNA) in 50 patients. Results showed that PCNA had no prognostic value for disease progression. However, p53 overexpression was associated with regional lymphatic spread, recurrence and disease specific mortality.

A more recent study by Zhu et al. [34] comprising 73 patients of whom 30 had nodal disease showed that p53 expression correlated with disease specific survival in T1 disease but not ≥T2 disease. In this study, p53 immunostaining was positive in 33% of patients and subdivided into high and low expression. Those with high expression of p53 had a 2.3 times risk of metastatic disease. In those patients with positive nodal disease, the 3 year disease specific survival was 71% in the low p53 expression group compared to 11% in the high expression group. However, the variability in the sampling methods and lack of standardisation of the p53 positive expression threshold in these studies means that there is no uniformity amongst the data available. Until this is defined the use of p53 expression as a prognostic marker is subject to debate.

$P16^{INK4a}$ expression in penile cancer

Another common cancer pathway in humans involves the $P16^{INK4a}$/cyclin D/retinoblastoma pathway. $P16^{INK4a}$ is a cyclin-dependent kinase inhibitor. Dysregulation at several points in the pathway has been implicated in several human cancers. The retinoblastoma tumour suppressor gene regulates the cell cycle by the hypophosphorylated protein pRb preventing cells from transition across the G1 checkpoint by sequestering transcription factors such as E2F which activate S phase genes. The retinoblastoma protein is a target for the viral oncoprotein E7 and therefore may contribute to carcinogenic events in HPV-dependent tumours. Immunohistochemical techniques have been utilised to analyse penile cancer tissue for $P16^{INK4a}$ and BM11[35, 36]. Prowse et al. identified $P16^{INK4a}$ expression in 54% of the penile SCC series and also identified an association with high risk HPV DNA and $P16^{INK4a}$ [35]. The $P16^{INK4a}$ expression was higher than that reported by Ferreux et al. [36]. Polymerase chain reaction (PCR) has also been used to assess $P16^{INK4a}$ methylation, HPV-16 E6 and E7 mRNA [36]. The findings have shown HPV-dependent and HPV-independent mechanisms interacting with this signalling pathway such as E7 oncoprotein inhibiting retinoblastoma function with upregulation of $P16^{INK4a}$. Silencing of the $P16^{INK4a}$ gene by methylation in the absence of HPV infection also occurs. In 10% of cases, overexpression of BM11 which targets $P16^{INK4a}$ and p14ARF in the absence of HPV infection can also occur.

Use of SCC antigen

Squamous cell carcinoma antigen also referred to as TA-4 is a 43 kDa glycoprotein which has been utilised in the diagnosis and management of SCC in various tissues [37, 38]. The exact mechanism accounting for the release of SCC antigen into the serum is not completely understood. There have been limited studies which have evaluated the potential use of SCC antigen in penile cancer. Wishnow et al. [39] analysed serum levels of SCC antigen in 23 patients with penile cancer and found that levels were elevated in those patients with metastatic disease and also found that the levels correlated with disease progression. A smaller study by Laniado et al. [40] comprising 11 men of whom 7 had histological evidence of nodal disease. This sensitivity of the elevated SCC antigen in detecting nodal disease was 57% and the specificity was 100%. Touloupidis et al. [41] sequentially measured SCC antigen in 16 men with penile cancer. This study again showed that SCC antigen may have a role in detecting nodal disease and response to treatment. The largest study was performed by Hungerhuber et al. [42] and comprised 54 men with penile cancer. SCC antigen levels reflected the tumour burden and increased significantly with extensive lymph node involvement or metastatic disease. However, microscopic nodal involvement could not be predicted using SCC antigen.

Until larger prospective trials are conducted with serial measurements, the role of SCC antigen as a marker for prognosis is still limited. It may present a useful tool as an adjunct to sentinel lymph node biopsy in patients placed on surveillance or as a marker for disease response following chemotherapy.

Ki-67 and PCNA as prognostic markers

Ki-67 is a nuclear protein which helps to identify the growth fraction within tumours. It is expressed in all phases of the cell cycle namely G1, S, G2, M. However, it is absent from the cells that exit the cell cycle and enter the senescence or G0 phase. Therefore Ki-67 can identify those cells that remain within the cell cycle. Studies have demonstrated that Ki-67 protein is localised to the growing edge of verrucous tumours of the penis indicating that this area is where proliferative activity is occurring [43]. In this study p53 staining was negative although a separate study has shown that HPV positive tumours have high levels of p53 and Ki-67 [44]. Berdjis et al. [45] suggested that there was a strong correlation of Ki-67 staining to tumour grade and a trend towards greater expression in more advanced tumours. There was no significant association with tumour stage or lymph node involvement [45]. However, in this study only 4 of the 44 patients had grade 3 tumours. A more recent study by Guimaraes et al. [46] which retrospectively evaluated 125 patients with penile SCC. Ki-67 was positive in 53% of cases and negative in 47%. Using a multivariate model the relative risk for lymph node metastasis with Ki-67 immunoreactivity was 3.73. However, there was no

correlation between Ki-67 expression and survival. Similarly the study by Zhu et al. [34] found no correlation between Ki-67 expression and cancer specific survival.

Proliferating cell nuclear antigen has also been extensively studied in other tumours [47, 48] although very little has been performed in penile cancer. Martins et al. [49] performed a semiquantitative analysis and graded the cancers into one of four groups. There was a possible association between the tissue strongly positive for PCNA and lymph node metastatic risk. However, a further study by Martins et al. [33] found no correlation between PCNA immunoexpression and prognosis. The most recent study evaluating the role of PCNA is that already mentioned by Guimaraes et al. [46]. In this study PCNA was an independent risk factor in both univariate and multivariate analysis for lymph node metastasis only. Again there was no correlation with survival.

Factors involved in penile cancer progression as prognostic markers

As with all malignant tumours, tumour progression involves the loss of cell to cell interaction and invasion of the extracellular matrix. E-cadherin is one of the epithelial cell adhesion molecules and is expressed in normal epithelia. A study by Campos et al. [50] has shown that low E-cadherin immunoreactivity is associated with a greater risk of lymph node involvement on univariate analysis. In this series of 125 patients, they found that 59.5% of patients with a low expression of E-cadherin had metastases compared to 38.3% of those with high expression of E-cadherin. This was confirmed using univariate analysis in a more recent study by Zhu et al. [34] where 45% of tumours had a low expression of E-cadherin and was associated with poorer cancer specific survival.

Matrix metalloproteinases, specifically MMP-2 (72 kDa type IV collagenase) and MMP-9 (urokinase 92 kDa type IV collagenase) are involved in the breakdown of type IV collagen in the basement membrane. Increased immunoreactivity of MMP is correlated with a higher risk of metastases in other tumours [51, 52]. Campos et al. also analysed MMP-2 and MMP-9 expression in penile cancer and showed that only high MMP-9 expression was an independent risk factor for disease recurrence.

Potential alternative prognostic indicators

The role of protooncogenes in penile cancer has been investigated through *c-ras* and *myc* mutations [53, 54]. Investigations on a small number of patients found that the integration of HPV 16 and HPV 18 was localised to sites containing *c-myc* (8q24.1) and *n-myc* (2p24) proto-oncogene. The *ras* protein has intrinsic intracellular GTPase activity regulated by protein factors thus acting as a focal point for several cell signalling pathways. One study has linked HPV 18 infection to mutations in TP53 and *c-ras* in patients presenting with late relapse [55]. However, the *ras* mutations occur late in the carcinogenesis pathway and linked to disease progression.

The cyclooxygenase isoforms (COX-1 and COX-2) convert arachidonic acid into the prostaglandin (PG) intermediaries PGG2 and PGH2 which then undergo further enzymatic degradation to the final PG products. PGE synthase-1 is responsible for the production of PGE2. PGE2 stimulates cell proliferation, inhibits apoptosis, modulates angiogenesis, cell–cell signalling and suppresses immune surveillance. A small cohort of patients has been used to investigate the expression of COX-2 and PGE synthase-1 [56]. There appears to be up regulation of COX-2 and PGE synthase-1 in dysplastic and invasive samples although the sample size is too small to draw any definitive conclusions and to assess the potential for these factors to act as diagnostic and prognostic markers in the future.

Table 2 Summary of current status of prognostic markers

Marker	Lymph node status	Survival
HPV	Contradictory evidence. Probable link with high risk HPV	Majority of studies show no correlation
p53	Unclear relationship to lymph node status	Correlates with survival in T1 lesions only
$P16^{INK4a}$	Not established	Not established
SCC antigen	Correlates with macroscopic lymph node involvement	No role
Ki-67	Predicts increased risk	No role
E-cadherin	Low expression associated with lymph node involvement	Low expression predicts poorer survival
MMP-9	No role	High expression predicts recurrence

Conclusions

Previously there has tended to be a focus on the role of HPV infection in the development and progression of penile cancer. However, studies have now shown that both HPV dependent and independent tumours occur with conflicting evidence regarding tumour progression and prognosis. By applying the multi-step carcinogenesis pathway to penile cancer, several alternative molecular and genetic factors have been identified which are involved in the pathogenesis of this tumour and are therefore potential prognostic markers in this disease. These markers can potentially be developed into markers of occult disease and could also be used to assess progression and response to chemotherapy. The current status of these markers in summarised in Table 2. Due to the low incidence of penile cancer relative to other urological cancers, large tissue banks and patient numbers have not been available in order to form definitive conclusions. With the development of penile cancer supraregional centres in the UK, coordinated concerted activity on these molecular pathways and factors can be established. This will allow targeted novel chemotherapeutic agents for metastatic disease to be developed as well as the potential for gene therapy.

Conflict of interest statement There is no conflict of interest.

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