The RB/ p16^{INK4A} pathway but not p53 is disrupted by human papillomavirus in penile squamous cell carcinoma

Short title: HPV and protein expression in penile SCC

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Abbreviations: HPV, human papillomavirus; PCR, polymerase chain reaction; PSCC, penile squamous cell carcinoma

Abstract

Aims: The Pathogenesis of penile squamous cell carcinoma (PSCC) is not well understood. Human papillomavirus (HPV) may be involved in carcinogenesis, but few studies have compared the cell-cycle protein expression in HPV positive and negative cancers of this type. The aim was to determine the extent of HPV infection in different histological subtypes of PSCC and its impact on the expression of key cell cycle proteins: p53, p21, $p16^{INK4A}$ and RB.

Methods and Results: We examined 148 PSCC samples immunohistochemically for RB, $p16^{INK4A}$, p53 and p21 protein expression. 102 cases were typed for HPV by PCR. HPV DNA was detected in 56% of tumours with HPV16 present in 81%. Marked differences were observed in HPV prevalence and RB and $p16^{INK4A}$ expression between tumour subtypes. RB protein negatively ($p<0.0001$) and $p16^{INK4A}$ ($p<0.0001$) and p21 ($p=0.0002$) positively correlated with HPV infection. *Conclusions:* HPV infection is present in over half of penile cancers and it is responsible for RB pathway disruption in those tumours. However, no link between HPV and p53 immunodetection was found. Different histological subtypes of PSSC express HPV DNA at different levels, confirming possible separate aetiologies for those tumours.

Introduction

Penile carcinoma is rare in developed countries, representing 0.3-0.5% of male malignancies in Europe and the USA [1]. In the UK there are approximately 600 (rate 1 per 100 000) new cases each year, mostly after the sixth decade [1, 2]. The vast majority (95%) are squamous cell carcinomas [3]. These may be divided into usual type (70%), followed by more aggressive basaloid (10%) and a slow growing, low grade group of 'verruciform' tumours (20%). Verruciform lesions include verrucous carcinoma, warty carcinoma and papillary squamous cell carcinomas [4]. Mixed tumours of usual type and one or more of other subtypes of SCC also exist.

Risk factors for penile cancer include lack of circumcision during childhood, phimosis and cigarette smoking [5]. There is some confusion on the role of infection with human papillomavirus in penile cancer [3], as contrary to cervical cancer [6] the incidence rate varies significantly from 15% to 77.5% [7, 8], depending on detection range, population studied and tumour type [9]. HPV16 is most prevalent infection [7], similar to other HPV-related ano-genital cancers [10]. Basaloid and warty tumours have been shown to be strongly associated with HPV infection [11]. Therefore, penile cancer may resemble vulvar cancer, which has two different aetiologies, one related to HPV infection and one that is not [12].

The carcinogenic abilities of high-risk HPV types are well known due to extensive studies on cervical cancer. Viral oncoproteins E6 and E7 can disrupt cell cycle checkpoints and apoptosis by respectively interacting with tumour suppressor proteins, RB and p53. RB regulates cell cycle progression, protecting the cell from uncontrolled proliferation and is regulated by cyclin dependent kinase phosphorylation. $p16^{INK4A}$ can inhibit cyclin dependent kinase mediated RB phosphorylation, preventing cell cycle progression. In cells infected with high-risk HPV, viral E7 protein binds directly to RB causing its inactivation and downregulation, which prevents cell-cycle control by $p16^{INK4A}$ [13, 14]. In these circumstances accumulation of $p16^{INK4A}$ can occur, which is an indication of HPV infection [15]. p53 can also

inhibit cancer development and tumour growth through its ability to efficiently inhibit cell proliferation and promote apoptotic cell death [16]. In cancers harbouring high-risk HPV, expression of viral E6 protein can inactivate p53 through its downregulation and an inverse correlation between HPV positivity and p53 overexpression has been found in some cancer sites but not others [17]. HPV E7 protein can additionally overcome the inhibitory function of p21. p21 is a p53-responsive protein and arrests cell cycle in presence of DNA damage. E7 protein binds to p21 and abrogates its inhibitory functions, therefore overcoming a DNA damage-induced cell cycle arrest despite high levels of p21 [18].

The mechanisms of oncogenesis in penile cancer are not fully understood. There are no data on RB expression in penile cancer and results on p53 in relation to HPV are inconclusive. Limited data is available on $p16^{INK4A}$ immunodetection [19, 20] and p21 expression [21] in penile tumours. Therefore, we investigated HPV infection type in one of the largest series of penile SCC to test the hypothesis that HPV type and its association with key cell cycle proteins had differential effects on the tumour subtypes, in order to elucidate their role in tumour pathogenesis.

Materials and Methods

The study was conducted upon approval from East London and The City Research Ethics Committee. We retrospectively reviewed the Cellular Pathology Department Registry of St George's Hospital to identify patients treated for penile SCC between 2001 - 2007. We retrieved 148 penile SCCs. 97 samples were usual type SCCs, 17 basaloid, 15 pure verrucous carcinomas, 7 mixed verrucous/usual type, 7 mixed verrucous/warty, 2 warty and 3 warty/usual types. 21 cases were obtained from excision biopsies/circumcisions, 82 from glansectomies and 45 from partial/total penectomies. All cases were re-reviewed by an expert uropathologist (C.C.) including subtyping, grading and staging by standard methodologies [4], [22].

Polymerase chain reaction (PCR).

102 wax blocks from penile SCC cases were suitable for DNA extraction with a QIAamp DNA Mini kit (51304; Qiagen, Crawley, U.K.). Beta-Globin polymerase chain reaction was performed using primers B1 and B19 to confirm the adequacy of the extracted DNA. Validated samples were tested for the presence of HPV DNA by a broad-spectrum HPV PCR method using SPF10 primers which amplify a 65-bp fragment of the L1 open reading frame and HPV genotypes identified by the INNO-LiPA line probe assay (Innogenetics NV, Ghent, Belgium) [19].

Immunohistochemistry.

Tissue microarray blocks were prepared using a manual microarrayer. Three x 1mm tissue cores were taken from each tumour. Four um sections were cut and immunostained using standard heat-induced antigen retrieval methods and the ABC kit (Vector Laboratories, PK-6200), according to manufacturer instructions [23]. Primary antibodies dilutions were: 1:50 for RB (Novocastra, NCL-RB-358), 1:100 for $p16^{INK4A}$ (Neomarkers, MS-1064-PO), 1:1000 for p53 (Dako, M7001) and p21 (Dako, M7202). Positive controls included CIN III for $p16^{INK4A}$, placenta for p21, anaplastic thyroid cancer for p53 and tonsil for RB. The staining pattern of RB, p53 and p21 was nuclear. $p16^{INK4A}$ showed both nuclear and cytoplasmic staining.

Sections were scored semiquantitatively by a consultant genitourinary pathologist (D.B.). For nuclear positivity each core was given an estimated visual score between 0 – 100%, representing the percentage of positively stained neoplastic nuclei. The intensity of staining was also measured as: 1 (weak), 2 (medium) and 3 (strong). The final score was deduced by multiplying the percentage of staining by intensity to give an expression score from 0-300. p53 expression was always strong; therefore nuclear score alone was applied. Cytoplasmic expression of $p16^{INK4A}$ was determined by intensity of staining alone. The core with highest score was selected for analysis. Statistical analysis was performed using StatsDirect software, version 2.60.6000. The correlations between antibodies were evaluated using Spearman's rank correlation test and the tumour type or HPV infection was evaluated by Chi-Square test or Fisher's exact probability test. Comparisons between antibodies expression in different histological subtypes of SCCs were restricted to usual type, verrucous and basaloid only. The warty group of tumours was too heterogeneous and included only 2 pure warty samples. The cut-off points selected for antibody positivity were: >0 for $p16^{INK4A}$, ≥5 for p53, ≥10 for p21 and ≥240 for RB. All analyses were 2-sided, $p<0.05$ was considered to be significant.

Results

148 tumours were analysed which comprised 97 usual type SCCs, 17 basaloid, 15 verrucous, 7 mixed verrucous/usual type and 12 mixed warty and other SCC subtypes. The histopathological features of the tumours are listed in Table 1. Of these, 102 cases were also suitable for HPV analysis.

HPV infection

HPV DNA was detected in 57/102 (56%) penile SCCs. Of these HPV positive tumours, 39/57 (68%) were single and 18/57 (32%) multiple HPV type infections containing up to 6 low and high risk HPV types (Table 2). High-risk type 16 was the most prevalent type, present in 46/57 (81%) of HPV positive tumours. HPV18 was not detected. In the majority of HPV positive tumours, 33/57 (58%) HPV16 was the only HPV type detected (Table 2). Differences in the HPV infections were observed between the histological subtypes of PSCC. For the usual type, HPV DNA was detected in 38/64 (59%) tumours, with high risk HPV16 present in 33/38 (87%) cases. Mixed warty subtypes were positive for HPV DNA in 6/11 (55%) cases, with HPV16 present in 3/6 (50%) cases. Basaloid tumours showed higher positivity, 10/13 (77%) for HPV DNA, with HPV type 16 present in 100% of these cases. In contrast, HPV was detected in only 3/13 (23%) verrucous tumours and HPV16 was not found (0%).

Immunohistochemistry

The positive expression of proteins and mean values are listed in Table 3. High RB expression (Figure 1A) was detected in 85/147 (58%) of penile SCC and significant differences were observed between histological groups ($p<0.0001$). A high percentage of verrucous cases (87%), an intermediate number of usual type (60%) and few basaloid cancers (12%) expressed high RB levels. The mean RB expression was two-fold lower in basaloid than in verrucous and usual subtypes.

$p16^{INK4A}$ demonstrated both nuclear and cytoplasmic staining (Figure 1B). Overall 47% of PSCCs are positive for cytoplasmic $p16^{INK4A}$ expression, with significant differences between histological groups: 13% of verrucous, 48% usual and 94% of basaloid cases were $p16^{INK4A}$ positive. Basaloid samples had very high mean expression of nuclear and cytoplasmic $p16^{INK4A}$, while mean expression of $p16^{INK4A}$ in verrucous samples was very low and usual type showed intermediate values. There was a significant inverse correlation between RB and $p16^{INK4A}$ expression ($p<0.0001$) in penile SCCs.

p53 immunodetection (Figure 1C) showed no significant difference between histological subtypes of SCC and was present in 79% of cases overall. Intensity of p21 staining was weak (Figure 1D) and present in 62%, with no difference in the expression between different SCC subtypes. No relationship was detected between p53 and p21 expression. There was a positive correlation between p21 and $p16^{INK4A}$ expression (Spearman's $\rho = 0.658793$, $p<0.0001$) and negative correlation with RB (Spearman's $\rho = -0.499952$, $p<0.0001$).

There was also a strong positive correlation between HPV infection and p21 ($p=0.0002$) and $p16^{INK4A}$ ($p<0.0001$) immunodetection and negative correlation with RB expression ($p<0.0001$) in penile SCC. p53 did not show any correlation with HPV infection ($p=0.5682$).

Discussion

The rate of HPV infection in penile cancer varies widely, depending on the population studied and sensitivity and specificity of the method used. Our results suggest that in a developed country, unlike cervical cancer, penile cancer has at least two aetiologies: one HPV related and one unrelated (similar to vulvar cancer) as we detected HPV DNA in 56% (57/102) of PSCC cases. This is consistent with our previous report of HPV prevalence in PSCC of 54% [19] and the recent review that found 48% of 1,266 cases from 30 studies of invasive penile cancer were HPV positive [24]. We confirm existence of differences in HPV infection between histological subtypes [11]. HPV prevalence in usual type SCC varies between 11% and 71% [7, 8, 25], and 59% (38/64) reported by us falls well within this range. As previously reported by our group , verrucous tumours were mostly HPV negative confirming the lack of HPV involvement in this neoplasm [26], while basaloid carcinomas showed strong correlation to HPV infection [11, 25].

HPV16 is the most prevalent type in our study and was detected in 81% (46/57) of positive samples and in over half of these as a single infection suggesting that this HPV genotype is more likely to contribute to the carcinogenic process.

Comparing these results with protein expression reveals that basaloid tumours have an aetiology related to high-risk HPV infection, which manifests itself in high $p16^{INK4A}$ and decreased RB expression, as has been shown in cervical cancer [27]. The aetiology of usual type SCC can only be attributed to HPV infection in approximately half of the tumours, showing corresponding loss of RB and gain of $p16^{INK4A}$ protein expression. This is analogous to the involvement reported for HPV in carcinogenesis of anal SCC [28] and tonsillar carcinoma [29].

Detection of p53 protein by immunohistochemistry in penile cancer varies between 41.5% and 89% [21, 30, 31] and there is a lack of reports comparing different histological types. We detected p53 immunostaining in 79% (103/143) of penile SCCs and the value was very similar regardless of histology (Table 3). There was no correlation between high-risk HPV

infection and p53 immunostaining, which is in agreement with previous reports on penile SCC [30, 31].

p21 was expressed in 62% (88/143) of penile SCC with no significant difference between tumour subtypes. Lam and Chan [21] showed lower p21 expression in penile cancer but on much smaller cohort. Interestingly p21 did not correlate with p53 and some cases expressed high levels of p21 despite low or absent p53 protein, suggesting p53-independent activation of p21. Surprisingly, similar to tonsilar SCC [29], we found positive correlation of p21 with HPV infection (p=0.0002). Additionally, p21 positively correlated with $p16^{INK4A}$ (p<0.0001) and negatively with RB expression (p<0.0001). Funk at al [18] reported that high-risk HPV16 E7 protein can directly bind to p21 and abrogate a DNA damage-induced cell cycle arrest, despite high levels of p21. He suggested it is possible that the release of E2F from RB and inactivation of $p16^{INK4A}$ and p21 are all necessary for the ability of E7 to bypass cell cycle arrest signals. On the other hand, there is emerging evidence that p21 in certain cancers may itself act as an oncogene and actually promote proliferation [32].

To our knowledge this is the largest study to examine penile cancer pathogenesis by comparing HPV type with proteins commonly affected by HPV infection. We demonstrated, for the first time, that HPV infection in penile SCC disrupts the RB/ $p16^{INK4A}$ pathway through downregulation of RB and elimination of cell-cycle control from $p16^{INK4A}$, manifesting itself in accumulation of $p16^{INK4A}$, which fails to block cell cycle progression. p21 was widely expressed, consistent with abnormal cell cycle regulation. However, p21 seems to be regulated independently from p53 and may be involved in oncogenic process. We confirm that penile tumours seem to have two different aetiologies: one related to HPV and one unrelated. These data suggest that use of the bivalent HPV16/18 prophylactic vaccine in men could reduce occurrence of penile SCC by about 45%.

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Table 1. Histological grade and stage of different subtypes of penile squamous cell carcinoma

SCC subtype		**Grade (1-3)**			**Stage (1-4)**				
		1	2	3	1	2	3	4	No data
All SCCs	n=148	36	59	54	48	70	16	7	6
Usual type	n=97	13	49	35	28	46	12	6	5
Verrucous	n=15	12	3	0	8	7	0	0	0
Basaloid	n=17	0	2	15	5	7	3	1	1
Mixed warty	n=12	9	2	1	7	4	1	0	0

SCC, squamous cell carcinoma; No data, there was no tumour stage available for six patients, which underwent penile circumcision or excision biopsy.

Table 2. Human papillomavirus DNA detection by polymerase chain reaction method in different histological subtypes of penile squamous cell carcinoma

			HPV genotype distribution																		
Histological subtype of SCC	n	HPV DNA positive	6	11	16	33	6 16	11 16	11 45	16 31	16 35	16 45	16 52	31 33	51 52	6 11 16	11 16 31	6 16 33 45	31 33 45	6 11 16 31 33 45	X
Usual type	64	38 (59%)	2	1	24	0	1	3	1	1	0	2	0	0	0	1	0	1	0	0	1
Basaloid	13	10 (77%)	0	0	9	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Verrucous	13	3 (23%)	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0
Mixed verrucous/usual type	1	0 (0%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mixed warty	11	6 (55%)	0	1	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0

SCC, squamous cell carcinoma; HPV, human papillomavirus; X, unclassified HPV genotype.

Table 3. Positive expression of RB, nuclear and cytoplasmic $p16^{INK4A}$, p53, p21 and Ki67 in all penile squamous cell carcinomas and in regard to histological subtypes

SCC subtype	**RB (≥240)**		**nuc $p16^{INK4A}$ (>0)**		**cyt $p16^{INK4A}$ (>0)**		**p53 (≥ 5%)**		**p21 (≥ 10%)**	
	n (%)	**mean**	**n (%)**	**mean**	**n (%)**	**mean**	**n (%)**	**mean**	**n (%)**	**mean**
All SCCs	85/147 (58)	205.6	65/144 (45)	54.2	67/144 (47)	1.1	113/143 (79)	19.4	88/143 (62)	38.1
Usual type	58/97 (60)	212	48/93 (48)	62.3	48/93 (48)	1.2	75/93 (81)	20.9	58/94 (62)	41.7
Verrucous	13/15 (87)	248	1/15 (7)	2.7	2/15 (13)	0.1	13/15 (87)	15.4	8/13 (62)	17.8
Basaloid	2/17 (12)	118.2	16/17 (94)	102.4	16/17 (94)	2.2	14/17 (82)	17.5	15/17 (88)	47.4
Mixed warty	6/11 (55)	199	2/12 (17)	16.7	3/12 (25)	0.4	7/12 (58)	7.8	5/12 (42)	30.4

nuc $p16^{INK4A}$, nuclear $p16^{INK4A}$; cyt $p16^{INK4A}$, cytoplasmic $p16^{INK4A}$

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Figure 1. Immunostaining results for penile squamous cell carcinoma. (A) Strong RB expression throughout the tumour. (B) $p16^{INK4A}$ expression showing both cytoplasmic and nuclear positivity. (C) Strong positivity of p53 in basal areas of the tumour. (D) p21 expression, showing scattered staining only

Titles and legends to Tables

Table 1. Histological grade and stage of different subtypes of penile squamous cell carcinoma

Table 2. Human papillomavirus DNA detection by polymerase chain reaction method in different histological subtypes of penile squamous cell carcinoma

Table 3. Positive expression of RB, nuclear and cytoplasmic $p16^{INK4A}$, p53, p21 and Ki67 in all penile squamous cell carcinomas and in regard to histological subtypes