DOCTORADO EN MEDICINA

DEPARTAMENTO DE MEDICINA

Universitat Autònoma de Barcelona

PENILE SQUAMOUS CELL CARCINOMA:

Study of clinicopathological and molecular factors implicated in its pathogenesis and prognosis

DOCTORAL THESIS

Carla Ferrándiz Pulido

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The present study has been possible thanks to the financial support of the following funding sources:

- *Fundació de Dermatologia, Hospital Universitari Vall d'Hebron, Barcelona, España.*
- *Sección Catalana de Dermatología de la AEDV y Societat Catalana de Dermatologia de l'Acadèmia de Ciències Mèdiques i de la Salut de Catalunya, Barcelona, España.*
- *Fondo de Investigación Sanitaria (FIS), Instituto de Salud Carlos III, Ministerio de Sanidad, España.*
- *Fundación Mutua Madrileña, España.*

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The clinical and microscopic images appearing in this work have been edited without previous treatment, in the same format and photographical parameters they had been captured and transmitted, proceeding in all cases from patients included in the study.

A mis padres, a mi marido Santi
y a mi pequeño Alejandro.

Acknowledgements

Me gustaría expresar mi más sincero agradecimiento a todas aquellas personas que han colaborado desinteresadamente en la elaboración de este trabajo.

A mis Directores de Tesis por su empuje, inspiración y dedicación. Al Profesor Vicente García-Patos Briones por su entusiasmo en enseñarme el mundo de la Dermatología desde que empecé la residencia, por todas las horas dedicadas y por su apoyo en los momentos difíciles. Al Dr. Agustí Toll Abelló por acogerme hace unos años sin apenas conocernos y ayudarme a crear este proyecto, por sus siempre rápidas y resolutivas respuestas. Y a la Dra. Inés de Torres Ramírez por su constante ánimo, por toda su enseñanza y su capacidad de trabajo.

Al Prof. Santiago Ramón y Cajal por abrirme las puertas de su servicio, por haberme apoyado en este proyecto desde el principio y por transmitirme su conocimiento sobre vías moleculares.

Al Dr. Javier Hernández Losa por coordinar todas las técnicas de laboratorio y por su paciencia infinita, dedicándome horas para introducirme en el complejo mundo de la biología molecular.

Al Dr. Ramón M. Pujol por facilitarnos el trabajo multicéntrico, por sus correcciones y aportaciones al trabajo.

A la Dra. Belén Lloveras y al equipo del Institut Català d'Oncologia por su imprescindible colaboración en la detección del virus del papiloma humano y por transmitirme sus conocimientos en este campo.

A los urólogos del Hospital Vall d'Hebron y Hospital del Mar, en especial los Dres. José Placer, Carlos Salvador y Juan Morote, por permitirnos esta incursión en su especialidad y colaborar en la elaboración del proyecto.

A Sergi Mojal por su ayuda con el análisis estadístico y por facilitarme la comprensión de esta materia.

Gracias también a los técnicos de laboratorio del Hospital Vall d'Hebron y del Hospital del Mar-IMIM, en especial a Teresa Moliné por la realización de las tinciones inmunohistoquímicas y Tania Lobato por la excelente elaboración de los *arrays* tisulares.

Un especial agradecimiento a Emili Masferrer Niubò, mi “co-doctorando” y también sufridor de este proyecto, por demostrarme su grandísima capacidad de trabajo. Un casual encuentro en la primera Reunión del Grupo Español de Investigación en Dermatología nos llevó a unir fuerzas y desarrollar este proyecto de forma conjunta. Creo que cada uno en su campo, nos hemos sabido complementar muy bien, sacando el máximo partido a este proyecto. Hemos compartido momentos duros, pero también alegrías. Espero haberle correspondido.

A la Dra. Amaya Virós por sus críticas constructivas de nuestro trabajo y sus magníficos consejos que ayudaron a mejorar la calidad de los artículos.

A mis compañeros del Servicio de Dermatología del Hospital Vall d’Hebron por acogerme y darme siempre apoyo moral, y en especial al Dr. Ramón Bartralot Soler, porque fue la primera persona que me dio la idea y me ayudó a empezar a investigar en este campo.

A los pacientes, sin cuya presencia y colaboración sería imposible llevar a cabo estos estudios.

Gracias a todos los maestros que he tenido desde la infancia que me han enseñado a pensar y a trabajar con entusiasmo, rigor y humanidad, entre ellos mis abuelos, el Dr. Rafael Pulido Cuchí y el Dr. Luis Ferrándiz Arjonilla.

A mis amigas, porque aunque no pertenecen a este “mundo” de la investigación, siempre han sabido comprender mis largas horas de dedicación a este campo, dedicándome siempre palabras de ánimo y apoyo.

A mi cuñado, el Dr. David Moreno Ramírez, por sus constantes consejos para mejorar la calidad de este trabajo.

A mi hermana, la Dra. Lara Ferrándiz Pulido, por ser un ejemplo para mí. Sin su ayuda no habría alcanzado ni la mitad de este camino.

A mi padres, la Dra. M Victoria Pulido Clarasó y el Prof. Carlos Ferrándiz Foraster, por su tiempo, su cariño, su apoyo incondicional, por la educación que me han dado y por transmitirme el entusiasmo por alcanzar esta meta profesional.

Finalmente dar las gracias a mi marido Santiago de Abadal Gámiz y a mi hijo Alejandro por su paciencia infinita y por estar siempre a mi lado, apoyarme en mis momentos bajos y por todo ese “tiempo libre” que les correspondía y lo dediqué a la elaboración de esta Tesis.

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Abbreviations

4E-BP1	Eukaryotic Translation Initiation Factor 4E- Binding Protein 1
AJCC	American Joint Committee on Cancer
CIN	Cervical Intraepithelial Neoplasia
EGFR	Epidermal Growth Factor Receptor
eIF4E	Eukaryotic Translation Initiation Factor 4E
EMT	Epithelial-Mesenchymal Transition
ERK	Extracellular-Regulated Kinase
FFPE	Formalin-Fixed, Paraffin Embedded
HPV	Human Papillomavirus
Hr	High-risk
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloproteinase
MNK1/2	MAPK-Interacting Protein Kinase 1 and 2
mTOR	Mammalian Target of Rapamycin
p4E-BP1	Phosphorylated-4E-BP1
P53	Tumor Supresor Protein 53
PCR	Polymerase Chain Reaction
peIF4E	Phosphorylated-eIF4E
PeIN	Penile Intraepithelial Neoplasia
pERK	Phosphorylated-ERK
PI3K	Phosphatidyl-Inositol 3-Kinase
PIK3-CA	Phosphatidyl-Inositol 3-Kinase Alpha-Catalytic subunit
pmTOR	Phosphorylated-Mtor
pRB	Retinoblastoma Protein
SCC	Squamous Cell Carcinoma
TMA	Tissue Microarrays
TP53	Tumor Protein 53 gene
VIN	Vulvar Intraepithelial Neoplasia

Introduction

"Precision of language is precision of though."

AB Ackerman,

Professor of Dermatology and Dermatopathology, 1936-2008.

1. Epidemiology

Penile carcinoma is rare in the developed world, and most cases (98%) correspond to squamous cell carcinoma (SCC). In Europe, penile SCC is most common between the sixth and eighth decades of life, with two-thirds of cases occurring in patients aged over 65 years.[1] The global incidence is 0.1 to 0.7 cases per 100.000 males. It is estimated that approximately 4.000 new cases are diagnosed each year; this accounts for less than 0.5% of all cancers.[1] In Spain, penile SCC accounts for approximately 0.7% of all malignant tumors in men, with an annual incidence between 0.7 and 1.5 cases per 100. 000 males.[1] Rates are similar in other parts of western Europe, but in some parts of the world, such as Uganda and Brazil, they are up to 4 times higher (Fig. 1).[1,2] This considerable geographic variation in incidence is probably due to socioeconomic and cultural differences.

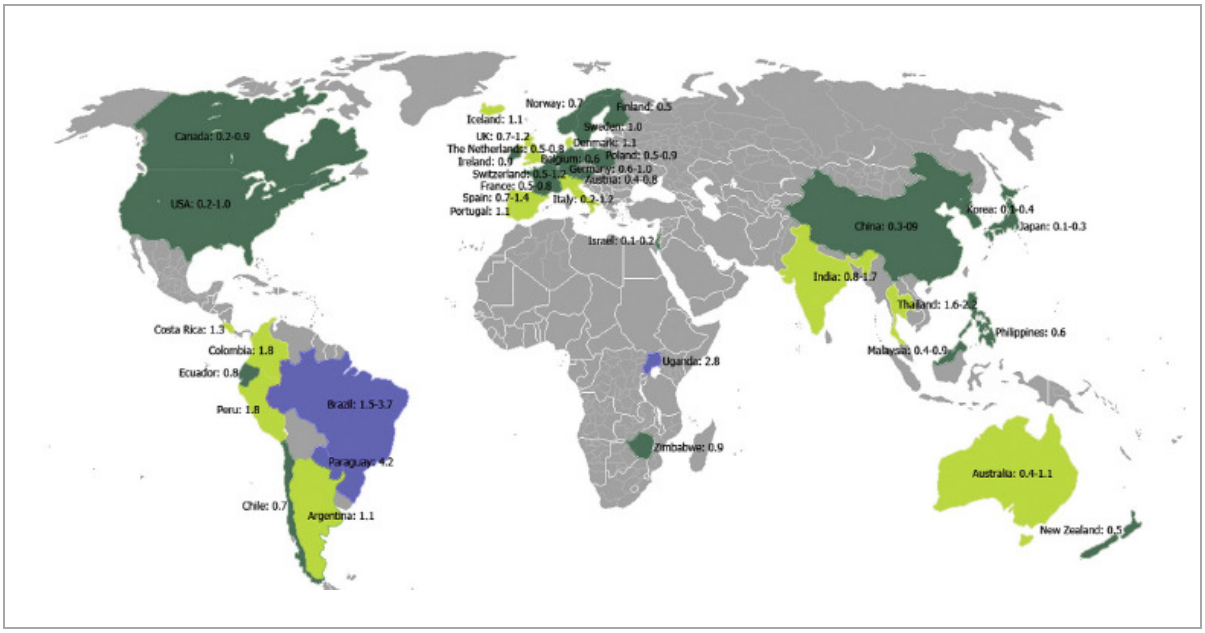

Figure 1. Worldwide distribution of penile cancer incidence. Colored areas represent age-standardized rates of incidence (reported rates in parenthesis): blue, high incidence; light green, moderate incidence; and dark green, low incidence. (*Chaux et al, Human Pathol. 2012*)

2. Risk factors

The main risk factors for the development of penile SCC are poor hygiene, lack of circumcision, human papillomavirus (HPV) infection, and certain chronic inflammatory skin conditions.

2.1 Poor hygiene

Poor hygiene contributes to the development of this tumor through the accumulation of smegma and other irritants in the balano-preputial sulcus, and is also associated with a higher incidence of bacterial and candida infections.[3]

2.2 Lack of circumcision

Most penile SCCs occur in uncircumcised men.[4] Neonatal circumcision exerts a preventive effect,[2,4,5] but it is not known whether a similar effect is achieved with circumcision performed later in life. It is also clear that phimosis, which is present in 40% to 85% of penile SCCs, interferes with adequate hygiene of the glans, contributes to chronic inflammation, and favors the development of this tumor.[6]

2.3 Human papillomavirus infection (HPV)

A direct relationship has been demonstrated between HPV infection and penile SCC. Both conditions are directly linked to the number of sexual partners and early sexual debut.[2] The association with HPV, however, is less common than in cervical cancer, where 95% of patients have this infection. HPV infection is more common in carcinoma *in situ* (70%-100% of cases) than in invasive forms (30%-60%); its occurrence also varies according to histological subtype, with prevalence ranging from just 30% in usual carcinomas to between 70% and 100% in basaloid and warty variants, respectively.[7,8] The most common HPV type involved in the development of penile SCC is HPV-16, but other alpha high-risk (hr) oncogenic types have been isolated; of particular relevance is HPV-18.[9] Even though genital warts are caused by low-risk HPV types, a history of this condition is associated with a 3-fold to 5-fold increased risk of developing penile SCC.[10] This is possibly in part because genital warts often occur in individuals who engage in sexual risk behaviors, placing them at a greater risk of becoming infected by other HPV types. Although Buschke-Lowestein tumor (giant *condyloma acuminatum*) is classified as a warty carcinoma in the literature, it should preferably be considered a separate entity, mainly because it has distinct clinical and pathological features (which resemble those of warts), is associated with HPV-6 and HPV-11 (low-risk types), appears at a relatively young age, and has practically no potential for metastasis.[2,11]

2.4 Pseudoepitheliomatous micaceous and keratotic balanitis

This entity is another risk factor for penile SCC affecting the glans of elderly, uncircumcised men. Like Buschke-Lowestein tumor, this lesion is considered to be premalignant or to have low-grade malignant potential, but it has not been associated with HPV infection. It presents as a plaque covered with silver micaceous scales (similar to those seen in psoriasis) that can form a thick keratotic layer (Fig. 2). Histologically, its appearance can range from that of simple epithelial hyperkeratosis and hyperplasia, with minimal cytologic atypia, to a lesion mimicking a warty carcinoma.

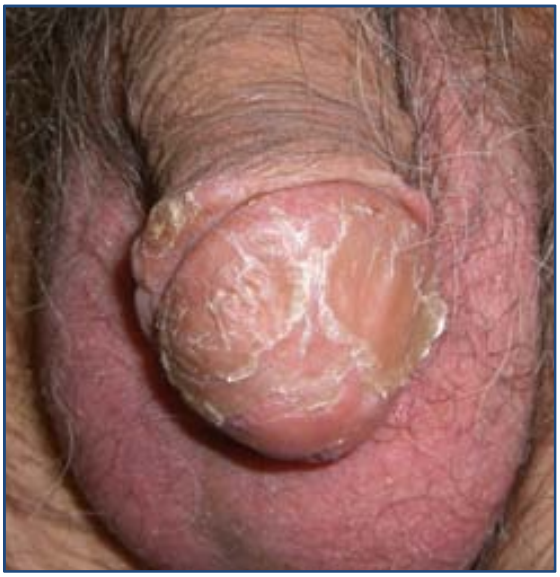

Figure 2: Pseudoepitheliomatous micaceous and keratotic balanitis.

2.5 Inflammatory skin conditions

Other risk factors for penile SCC are certain chronic inflammatory skin conditions, in particular, lichen sclerosus and its more advanced form, balanitis xerotica obliterans, which is characterized by constrictive fibrosis that affects the entire circumference of the prepuce, preventing its retraction. It is estimated, the penile lichen sclerosus can progress to SCC in 6% of cases, but examination of surgical specimens has shown that up to a third of penile SCCs arise in penile lichen sclerosus lesions.[12,13] Accordingly, patients with penile lichen sclerosus should be regularly monitored.

2.6 Others

As seen in other skin cancers, sustained immunosuppression (e.g., in organ transplant recipients or patients infected by human immunodeficiency virus) is closely associated with

increased risk of penile SCC and worse prognosis. Finally, penile SCC, like cancer of the bladder and the oral cavity, has been associated with tobacco use.[2,10,14]

3. Clinical and microscopic features

There are two very distinct clinical and microscopic forms of penile SCC, each with different prognostic and therapeutic implications. These are carcinoma *in situ* and invasive carcinoma.

3.1 Penile SCC *in situ*

Penile SCC arises from a precursor lesion that can be microscopically classified according to the severity and extent of cellular atypia and the presence or absence of HPV infection.[5] The clinical appearance of penile SCC *in situ* is highly variable, ranging from subclinical lesions that can only be seen following the application of acetic acid, to reddish lesions (erythroplasia of Queyrat and genital Bowen disease), white lesions (leukoplakias), and brownish lesions (bowenoid papulosis).

Subclinical lesions can be visualized by peniscopy after the application of acetic acid.[15] These lesions, known as **flat penile lesions** or acetowhite lesions, are associated with hrHPV infection. They are very common and occur in up to 50% to 70% of male sexual partners of women with cervical intraepithelial neoplasia (CIN) and in up to 10% to 20% of men whose partners do not have CIN. These lesions contain large amounts of viral particles and are highly contagious. Histology tends to show varying degrees of epithelial hyperplasia or dysplasia. The majority of lesions resolve either spontaneously or with treatment within a year or two, but a small percentage persist and progress to invasive carcinoma.[16]

Erythroplasia of Queyrat manifests as single or multiple erythematous plaques on the mucosa of the glans or on the inner aspect of the prepuce (Fig. 3A). **Genital Bowen disease** presents as a single, scaly plaque on keratinized skin, generally on the distal third of the penis.[17] **Bowenoid papulosis**, in turn, affects younger men, in their 30s or 40s. It manifests as multiple, small, brown, well-circumscribed wart-like papules affecting the penis, the glans, the prepuce, or the pubic area (Fig. 3B). It is caused by HPV-16 and is highly contagious, meaning that affected individuals' sexual partners will have a high risk of developing CIN.

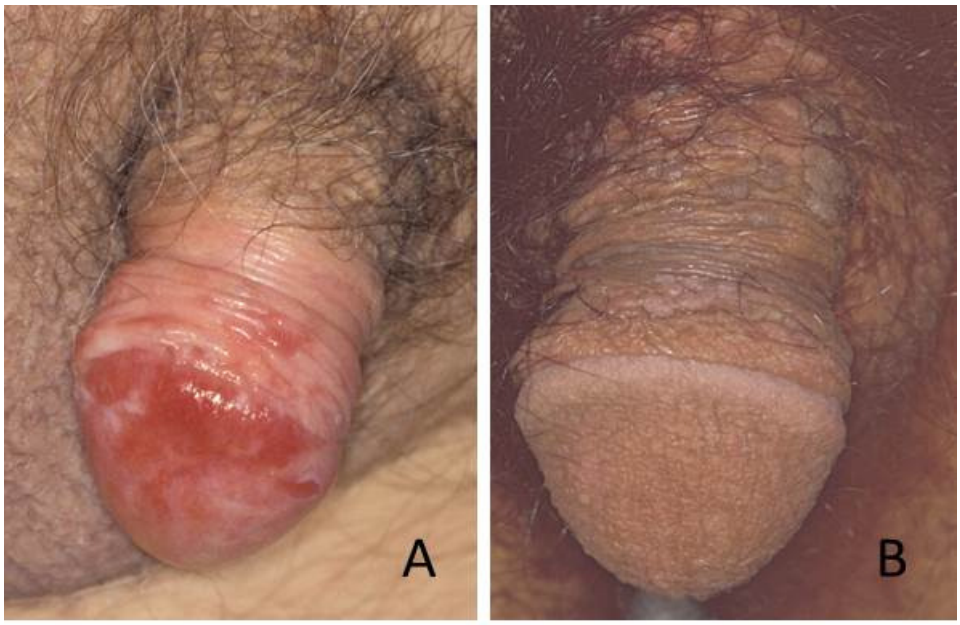

Figure 3: Premalignant or *in situ* lesions. A, Erythroplasia of Queyrat. B, Bowenoid papulosis.

Histology is mandatory in all cases to determine the true nature of penile SCC *in situ*. Histologic alterations seen in penile SCC *in situ* are classified according to grades of penile intraepithelial neoplasia (PeIN), using a similar system to that employed in vulvar and cervical cancer (VIN and CIN grades, respectively). These alterations are often found in the epithelium adjacent to invasive penile SCC. A distinction is also made between differentiated PeIN and undifferentiated PeIN.[18] Differentiated (usual or low-grade) PeIN is characterized by cellular atypia in the basal and suprabasal layers of the epithelium, elongated rete ridges, and conserved architecture in the upper layers (Fig. 4A).[19] It tends to occur in association with lichen sclerosus or epidermal hyperplasia. Differentiated PeIN is the most frequent precursor lesion of penile carcinomas, and usually progresses to usual or verrucous SCC, and, less frequently, to basaloid or warty SCC.[18-21]

Undifferentiated (high-grade) PeIN is further subdivided in warty, basaloid and warty-basaloid subtypes (Fig. 4B and 4C). It is correlated with HPV infection and has cellular atypia in at least the lower two-thirds of the epithelium, as well as basaloid cells or cellular pleomorphism and pleomorphic koilocytosis and abundant mitotic figures.[18-21] It usually progresses to basaloid or warty carcinoma but can also give rise to usual SCC; it almost never progresses to verrucous carcinoma.[18] Erythroplasia of Queyrat, genital Bowen disease, and bowenoid papulosis are clinical manifestations of undifferentiated PeIN and they can all progress to invasive penile SCC; the associated risk is 10% to 30%, 5% to 10% and less than 1%, respectively (Fig. 5).[22]

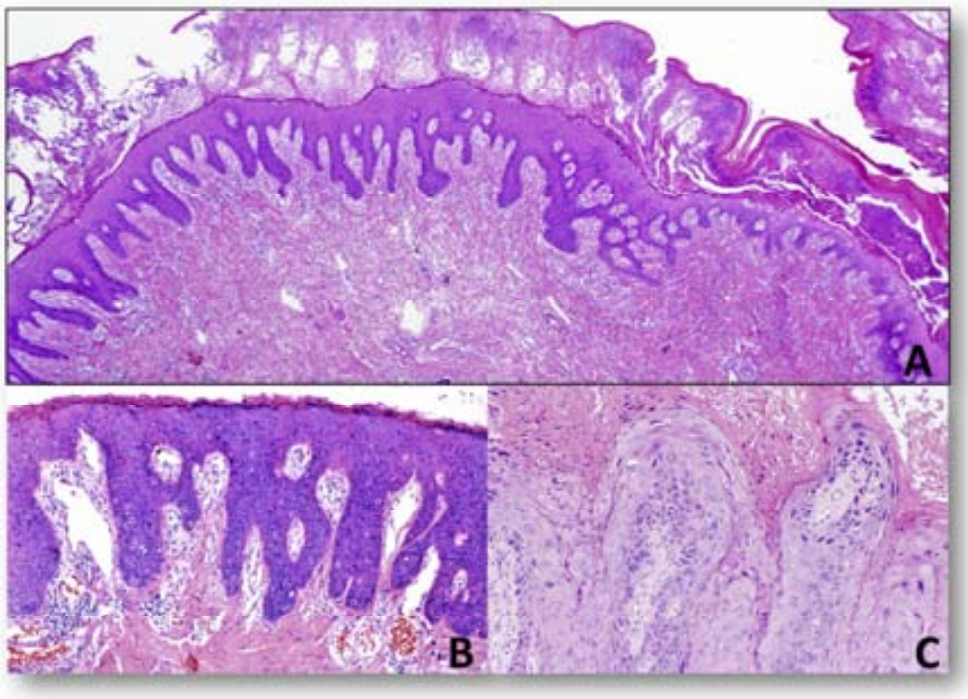

Figure 4: A. Differentiated PeIN, B. Undifferentiated PeIN, basaloid subtype, C. Undifferentiated PeIN, warty subtype.

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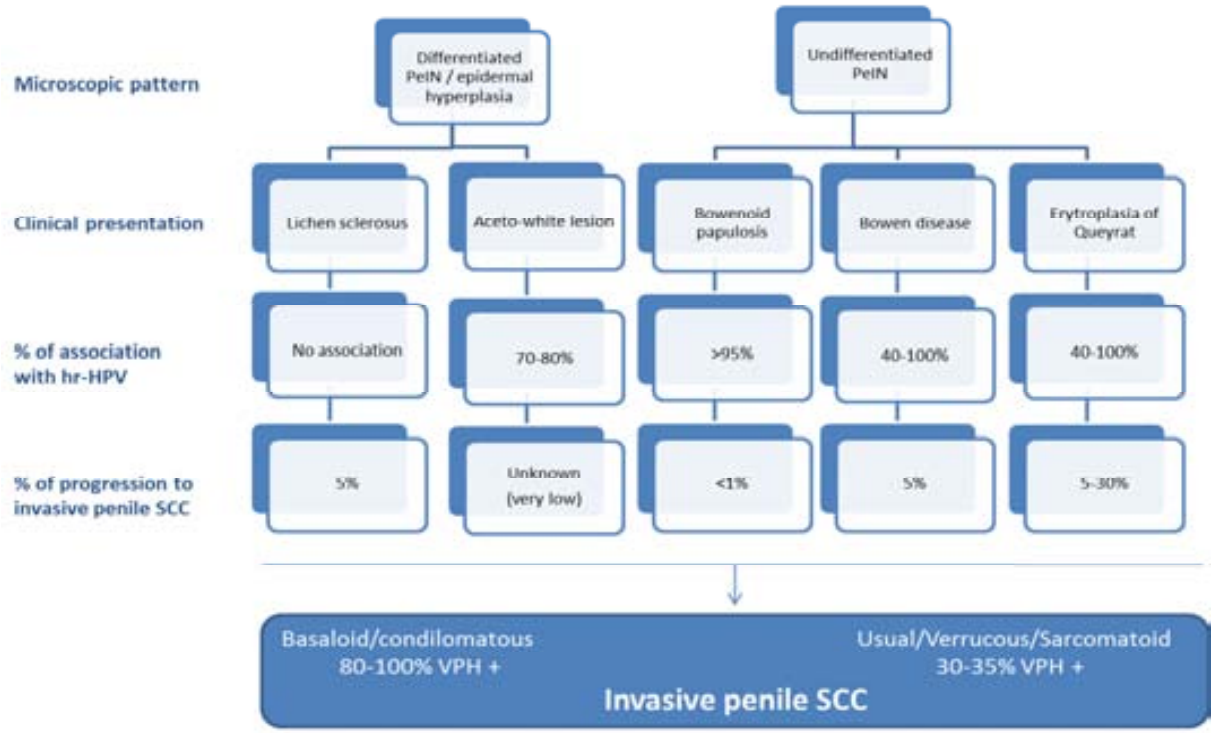

Figure 5: Relationship between microscopic and clinical findings in penile SCC *in situ*, the presence of human papillomavirus (HPV), and progression to invasive SCC. PeIN indicates penile intraepithelial neoplasia.

3.2 Invasive penile SCC

The clinical presentation of invasive penile SCC is highly variable, with manifestations ranging from an erythematous plaque or ulcer to an exophytic or verruciform tumor. The lesions, which can measure up to several centimeters in diameter, can have a stony hard or friable consistency and may bleed (Fig. 6). They tend to be solitary lesions and can be located on any part of the penis, although they are more common on the anterior third (glans, prepuce and/or balano-preputial sulcus). They are found on the shaft of the penis in fewer than 5% of cases. During the clinical evaluation, it is essential to note the number of lesions, their color, morphology, and maximum diameter, and their relationship to other structures (invasion of the external urethral orifice or the corpus spongiosum or cavernosum). It is also important to measure the length of the penis to calculate the approximate length that would remain after a penectomy, should this be necessary.[10]

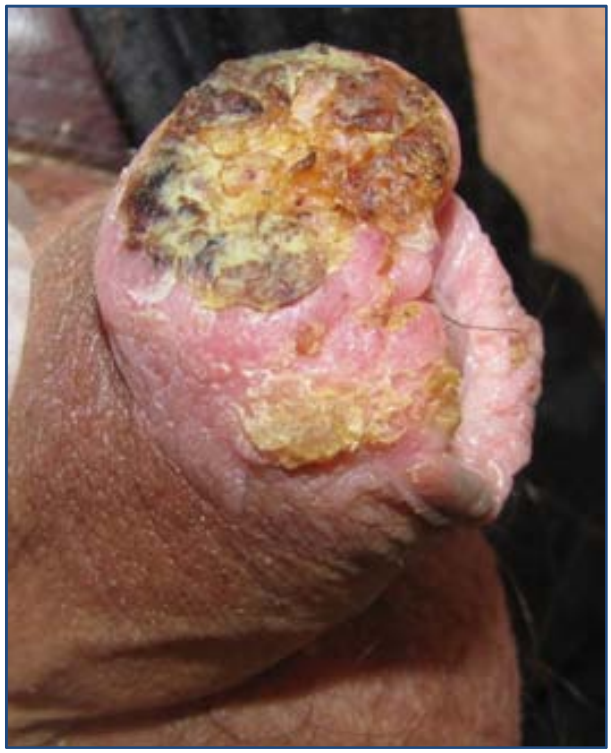

Figure 6: Invasive squamous cell carcinoma in a patient with a history of lichen sclerosus and a liver transplant 1 year before the diagnosis of the tumor.

Several histologic subtypes have been identified on the basis of architectural and cytologic features, including up to 12 subtypes with distinctive clinicopathologic and outcome features (Table 1). **Keratinizing**, or usual SCCs are the most common forms of invasive penile SCC and account for 50% to 60% of all cases; they generally follow an infiltrative growth pattern

and can be well or poorly differentiated (Fig. 7A). **Verrucous** penile SCC is an extremely differentiated neoplasm, architecturally similar to a wart and follows an expansive growth pattern (Fig. 7B). **Basaloid** penile SCC is characterized by the presence of nests of clearly basaloid cells with an infiltrating pattern and peripheral palisading.[23] Solid or centrally necrotic nests (comedonecrosis) are common (Fig. 7C). **Warty** (condilomatous) penile SCC is an exoendophytic tumor that resembles a wart with easily identifiable pleomorphic koilocytosis, large cells and a prominent central fibrovascular core. The interface between the tumor and the stroma is usually jagged.[24] In our experience, some tumors with a usual-type architecture contain a clear basaloid carcinoma component; they are much more common than pure basaloid carcinomas and are related to hrHPV infection. **Papillary** carcinoma is another verruciform tumor that is diagnosed after excluding a verrucous or a warty carcinoma; it shows a jagged interface with low-grade histology and no koilocytosis. **Sarcomatoid** penile SCC is a biphasic epithelial-spindle cell neoplasm that is very poorly differentiated and, a specific immunohistochemical staining for cytokeratins is necessary to demonstrate the true nature of the spindle cells (Fig. 7D). Finally, **mixed variants** account for 10% to 15% of all forms of invasive penile SCC.[25]

Table 1: Microscopic classification of penile SCCs

Subtype	*Frequency*
Usual SCC	48-65%
Basaloid carcinoma	4-10%
Warty carcinoma	7-10%
Verrucous carcinoma	3-8%
Papillary carcinoma	5-15%
Sarcomatoid carcinoma	1-3%
Mixed carcinomas	9-10%
Adenosquamous carcinoma	1-2%
Pseudohyperplastic carcinoma	<1%
Carcinoma cuniculatum	<1%
Pseudoglandular carcinoma	<1%
Warty-basaloid carcinoma	9-14%

The histologic subtypes of penile SCC can also be classified by prognosis. Verrucous and warty types have the best prognosis and metastases are very rare. Carcinoma cuniculatum, a subtype of low-grade verrucous SCC, is also associated with good prognosis. Carcinoma cuniculatum has a verruciform architecture characterized by a deeply penetrating, burrowing

growth pattern. Basaloid, sarcomatoid, and undifferentiated usual tumors are all associated with a high risk of dissemination. Most of them are poorly differentiated and invade the deep dermis. Intermediate subtypes include usual penile SCCs, several mixed forms, and the pleomorphic variants of warty penile SCCs.[25]

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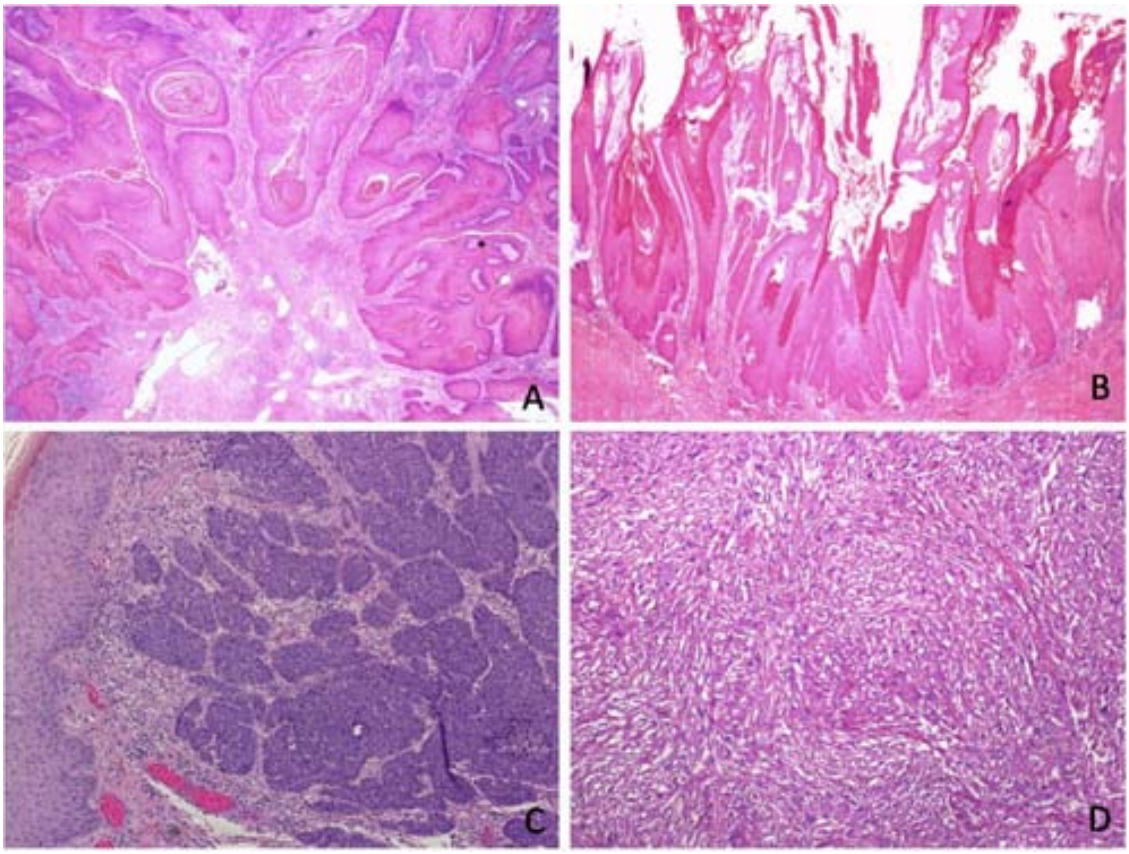

Figure 7: Histologic subtypes of penile squamous cell carcinoma. A, usual (hematoxylin-eosin [H&E], original magnification ×20). B, verrucous (H&E, original magnification ×20). C, Basaloid (H&E, original magnification ×100). D, Sarcomatoid (H&E, original magnification ×100).

4. Staging

In 2009, the American Joint Committee on Cancer (AJCC) proposed a new TNM staging system for penile cancer in which considerable importance was placed on lymph node involvement as a prognostic factor (Table 2).[26] In the revised system, the T1 category is divided into 2 subcategories: T1a, for well differentiated tumors and absence of lymphovascular invasion, and T1b, for tumors that are either poorly differentiated or have lymphovascular invasion. Prostatic invasion has been removed from the T3 category as it is very rare and occurs when many other structures are already affected. The T2 category was not modified, but several authors have indicated that prognosis is much worse when there is

corpus cavernosum invasion than when there is only corpus spongiosum invasion.[26] The revised system also specifies that N1 only refers to unilateral inguinal involvement with mobile lymph nodes. N2 refers to bilateral inguinal involvement with mobile lymph nodes while N3 refers to the presence of 1 or more fixed lymph nodes or metastasis in the pelvic lymph nodes.

Table 2. TNM Classification of penile squamous cell carcinoma according to the European Association of Urologists Guidelines on Penile Cancer 2009.

T: Primary Tumor
TX: Primary tumor cannot be assessed
T0: No evidence of primary tumor
Tis: Carcinoma in situ
Ta: Noninvasive verrucous carcinoma
T1: Tumor invades subepithelial connective tissue
T1a: No lymphovascular invasion and the tumor is well differentiated or moderately differentiated (T1-G1/G2)
T1b: Lymphovascular invasion and the tumor is poorly differentiated or undifferentiated (T1-G3/G4)
T2: Tumor invades corpus spongiosum/corpora cavernosa
T3: Tumor invades urethra
T4: Tumor invades other adjacent structures
N: Regional lymph nodes (p: pathologic classification)
NX: Regional lymph nodes cannot be assessed
N0: No palpable or visibly enlarged inguinal lymph nodes
N1: Palpable mobile unilateral inguinal lymph node
N2: Palpable mobile multiple or bilateral inguinal lymph nodes
N3: Fixed inguinal nodal mass or pelvic lymphadenopathy unilateral or bilateral
M: Distant metastasis
M0: No distant metastasis
M1: Lymph node metastasis outside the true pelvis in addition to visceral sites

5. Pathogenesis

It seems clear that alterations in different molecular pathways are involved in the etiology and pathogenesis of penile SCC. While little is known about the impact and interrelationship of each of these pathways, there is enough evidence to propose a bimodal etiopathogenic pathway in its carcinogenesis, one related to hrHPV infection and another which is hrHPV-independent (Fig. 8) .[6]

Penile SCC caused by hrHPV infection arises from a precursor lesion produced by the virus via a carcinogenic pathway similar to that involved in cervical cancer (usually an undifferentiated PeIN).[27] Nevertheless, tissue-specific and hormonal mechanisms also appear

to exert an influence, as penile SCC and cervical cancer are caused by the same infectious agents but differ substantially in terms of incidence and age of onset.

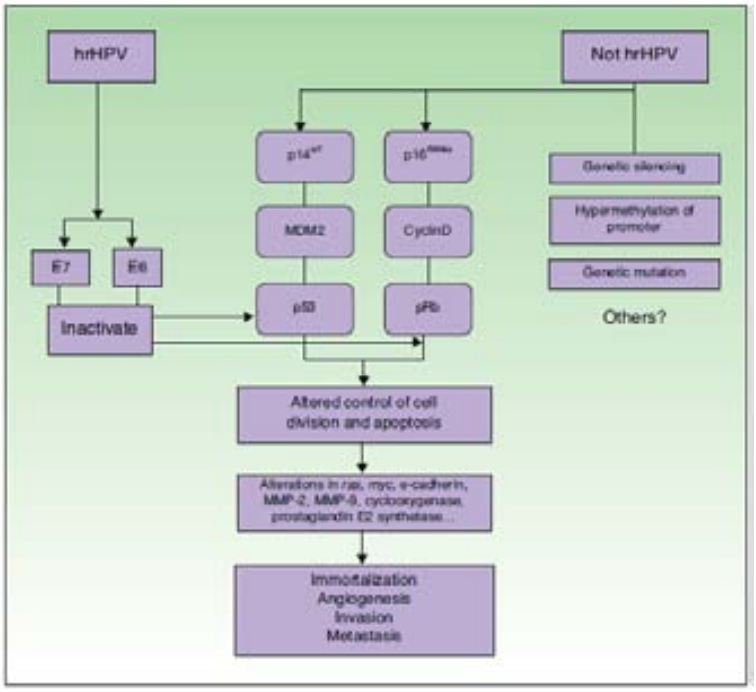

Figure 8: Pathogenesis of penile squamous cell carcinoma. Schematic overview of molecular events that take place in the initial and late phases of carcinogenesis.
HPV indicates human papillomavirus; hr, high-risk; MMP, matrix metalloproteinase.

The initiating event is persistent infection of the squamous epithelium by hrHPV, followed by a series of epigenetic alterations that lead to the malignant transformation of the infected cell. HrHPV expresses the oncoproteins E6 and E7, which bind to and inactivate the tumor suppressor gene products p53 and retinoblastoma protein (pRb).[28-30] E6 and E7 play a key role in inducing and maintaining the transformed phenotype of the infected cell. HrHPV types alter the $p14^{arf}$/MDM2/p53 and $p16^{INK4a}$/cyclin D/Rb pathways and interfere with the control of cell division and apoptosis. The functional inactivation of pRB by E7 leads to overexpression of $p16^{INK4a}$ due to the lack of negative feedback; accordingly, $p16^{INK4a}$ overexpression can be used as a marker of HPV infection.[31] ProxExC can also be used to indirectly detect HPV infection,[32] but this marker has not yet been used in penile SCC.

The pathways involved in initiation of hrHPV-negative penile SCC are not clear, but the following mechanisms might be involved: silencing or mutation of tumor suppressor genes, hypermethylation of promoter genes, and overexpression of oncogenes.[2,31]

The molecular mechanisms involved in more advanced penile SCC are probably the same in both types of tumors (HPV-positive or HPV-negative). Alterations in the expression of the *ras* and *myc* genes, E-cadherin, matrix metalloproteinase (MMP) 2 and MMP-9, cyclooxygenase, and prostaglandin E2 synthase have also been identified in penile SCC.[33-36] These are probably late events and would therefore be involved in disease progression mechanisms such as angiogenesis, invasion, and metastasis. Some of the above factors are considered to be predictive factors of lymph node metastasis. It has also been observed that epidermal growth factor receptor (EGFR), HER3 and HER4 overexpression is associated with penile carcinogenesis.[37,38] Moreover, a high frequency of mutations in the *PIK3CA (*phosphatidylinositol 3-kinase alpha-catalytic subunit*)*, *HRAS* and *KRAS* genes in penile carcinoma have been described.[39]

6. Prognostic Factors

6.1 Clinical

Inguinal lymph node involvement and the number of affected nodes are the most important prognostic factors of survival in penile SCC. Five-year disease-free survival is as high as 80% when one or more superficial, unilateral inguinal lymph nodes are involved (N1), but just 10% to 20% when the involvement is bilateral or pelvic (N2/3), and less than 10% when there is extranodal involvement.[40]

6.2 Microscopic

Perineural invasion, lymphovascular permeation, and a poor grade of differentiation are the most important microscopic indicators of the risk of lymph node metastasis and disease specific death.[25,41-44] Other factors that appear to influence prognosis are tumor depth, growth pattern, histologic subtype, and urethral invasion.

6.3 Molecular

The relationship between HPV infection and prognosis is controversial,[45,46] and there are also contradictory results regarding whether or not p53 expression is an independent predictor of lymph node metastasis.[47,48] Nonetheless, it does appear that strong expression of p53 in T1 tumors is correlated with a higher risk of metastasis and shorter survival rate.[48] High expression levels of ki67 are associated with an increased risk of lymph node metastasis but do not influence survival.[49] Studies by Campos et al[33] and Zhu et al[48] have shown that low E-cadherin levels are correlated with a higher risk of lymph node metastases and worse survival.[33,48] Campos et al[33] also showed overexpression of MMP-9 to be a risk factor for tumor recurrence.

7. Tissue microarrays in penile SCC

Tissue microarrays (also TMAs) consist of paraffin blocks in which up to 1,000 separate tissue cores are assembled in array fashion to allow multiplex histological analysis. The major limitations in molecular clinical analysis of tissues include the cumbersome nature of procedures, limited availability of diagnostic reagents and limited patient sample size. The technique of TMA was developed to address these issues. The TMA allows a rapid screening to detect immunohistochemical alterations as far as they allow the evaluation of hundreds of cases in a unique assay.

Multi-tissue blocks were first introduced by H. Battifora in 1986[50] with his so-called “multitumor (sausage) tissue block" and modified in 1990[51] with its improvement, "the checkerboard tissue block". In 1998, J. Kononen and collaborators developed the current technique, which uses a novel sampling approach to produce tissues of regular size and shape that can be more densely and precisely arrayed.[52]

In the TMA technique, a hollow needle is used to remove tissue cores as small as 0.5 - 2 mm in diameter from regions of interest in paraffin-embedded tissues such as clinical biopsies or tumor samples. These tissue cores, corresponding to up 1000 different samples are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut using a microtome, mounted on a microscope slide

and then analyzed by any method of standard histological analysis. Each microarray block can be cut into 100 – 200 sections, which can be subjected to independent tests (Fig. 9). Tests commonly employed in TMA include immunohistochemistry and fluorescent *in situ* hybridization. TMAs are particularly useful in analysis of cancer samples. When working with TMAs, we can make cuts and stain them with hematoxilin and eosin, allowing us to distinguis tumoral cells from normal cell, making it possible to read the variables of interest directly on the target tissue, avoiding the non-tumoral tissue.

The TMA technique is relatively new, and there are therefore only a few studies focused on penile SCC.[52] Stankievicz et al[53] evaluated immunoexpression of RB, $p16^{INK4A}$, p53 and p21 proteins of 148 penile SCC on TMA. The same authors studied later on the expression of pEGFR, HER2, HER3, HER4, pAkt, Akt1 and PTEN proteins in the same TMAs.[37] Chaux et al[54] also constructed a TMA with 112 cases of penile SCC that were used for $p16^{INK4a}$ immunostaining and HPV *in situ* hybridization. Finally, Bethune et al[55] also selected 43 patients with penile SCC and TMAs were constructed for analysis of immunohistochemical stains $p16^{INK4a}$, p53, and Ki-67.

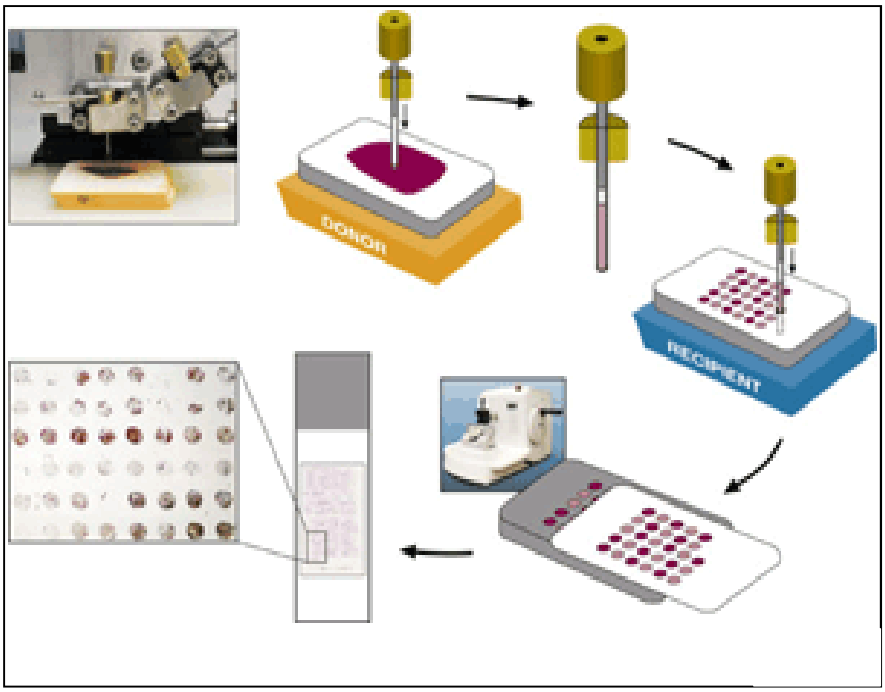

Figure 9: Squematic proposal of TMA construction.

8. HPV assays

The gold standard method for HPV detection in tissue samples is the polymerase chain reaction (PCR) assay. PCR technique based on MY11/MY09 consensus primers and general GP5+/GP6+ PCR primers, followed by cycle sequencing is frequently used. This technique is able to detect 0.05^5 HPV-DNA copies, has a sensitivity of 10 fg to 1 pg, and covers all known HPV types.[56] At the Infection and Cancer Laboratory, Duran i Reynals, Institut Català d'Oncologia from Barcelona, they are developing an international study for HPV detection in vulva, vagina, anus, penis and oropharynx carcinomas (HPV VVAPO). In order to detect HPV infection they use a PCR with SPF-10 broad-spectrum primers followed by DNA enzyme immunoassay and genotyping with a reverse hybridization line probe assay. Further, sequence analysis is done to characterize HPV-positive samples with unknown HPV types. Paraffin blocks are always processed under strict conditions to avoid contamination and four paraffin sections are systematically obtained from each block (sandwich method). First and fourth sections are used for histopathological assessment after hematoxylin and eosin staining, and the second and third sections are used for detection of and genotyping HPV DNA.[57]

9. Present and future perspectives in carcinogenesis of penile SCC

The most important advances in penile SCC in recent years have been the identification of risk factors, improved knowledge of the pathogenesis of this tumor and updating of staging criteria and microscopic classifications. Progress has also been made in the area of treatment, with an increasing tendency towards conservative surgery, whose aim is to minimize the risk of recurrence while preserving sexual and urinary function. However, there is still lacking full knowledge of molecular and genotypic alterations involved in tumor carcinogenesis and prognosis that will open new therapeutic options.

On one hand, there are still limited data about the role of HPV in penile SCC, and only a few studies on small series of patients from the Mediterranean area have focused in this topic.[58-61] Depending on the detection method, geographic area, and tumor type, the infection rates of HPV in penile SCC range from 20% to 80%.[2,35,45,46] However, technical requirements and associated costs preclude the worldwide use of PCR assays on a routine basis, and other diagnostic approaches are required. A close association between hrHPV infection and $p16^{INK4a}$ overexpression has repeatedly been reported for several tumors, such as SCC of the

head and neck region and cervical cancer.[62] Nevertheless, limited data are available regarding $p16^{INK4a}$ immunodetection in penile SCC, its relationship with HPV detection, and its prognostic significance.[53, 62-64]

On the other hand, in a high proportion of human cancers a deregulation of the PI3K-Akt pathway, which regulates mammalian target of rapamycin (mTOR), has been demonstrated, and specific molecular alterations are detected in squamous cell tumors such as head and neck, lung and non-melanoma skin cancers.[65] It is worldwide known the role of this pathway in cutaneous SCC, above all in immunosupressed patients, who received a solid organ transplantation. Interestingly, clinical data have shown that switching from calcineurin inhibitors to sirolimus had an antitumoral effect among kidney transplant recipients with previous cutaneous SCC.[66] Several of the molecules of this pathway are targets for cancer therapy. In head and neck carcinomas, the PI3K/Akt/mTOR pathway has been identified as a potential therapeutic target, and the role of mTOR inhibitors has been successfully evaluated as part of a molecular-targeted metastasis preventive strategy.[66] The therapeutic role of mTOR inhibitors is also being tested in other epithelioid squamous cell tumors such as cervical carcinoma.[67] There is very limited data about the role of mTOR signaling pathway in penile SCC carcinogenesis, but all these data in other similar tumors led us to believe that deregulation of the mTOR pathway may have a significant role in the development and progression of penile carcinoma.

Finally, another crucial factor in epithelial carcinogenesis is p53. P53 is a tumor suppression protein and regulates the cell cycle by integrating numerous signals that control cell life and death. This protein inhibits cancer development and tumor growth through its ability to efficiently inhibit cell proliferation and promote apoptotic cell death in solid tumors.[47,48,60] P53 is a transcription factor for many genes including the pro-apoptosis and anti-apoptosis genes, e.g., Bax, mdm2. P53 is short-lived and expressed at very low levels in normal cells, but it becomes stable and accumulates if it is mutated, being easily detected by immunohistochemistry. In penile cancer, rates of p53 expression range from 41.5 to 89%, and although still controversial, those cases seem to have a worse outcome.[47,48] As previously commented, HPV-E6 protein alters the $p14^{arf}$/MDM2/p53 pathways and interferes with the control of cell division and apoptosis, and although *TP53* mutations are frequent in cutaneous SCC, they have shown to be rare in hrHPV infected head and neck tumors. The relationship

between *TP53* mutation and hrHPV infection in penile SCC remains unclear. Moreover, interactions between the p53 and *PI3K/AKT/mTOR* pathway play a significant role in the determination of cell death/survival. In benign cells these pathways are interrelated through the transcriptional regulation of *PTEN* by p53, which is required for *p53*-mediated apoptosis. *PTEN* exerts its effects by decreasing the phosphorylated *AKT* fraction, thereby diminishing pro-survival activities. Another interaction between signaling pathways is that wild-type p53 can modulate the eukaryotic translation initiation factor 4E- binding protein 1 (4E-BP1), a final effector of mTOR, through cdc2 or other unidentified phosphatases. The relationship between p53 and mTOR pathways in hrHPV-positive and negative penile tumors needs further investigations (Fig. 10).

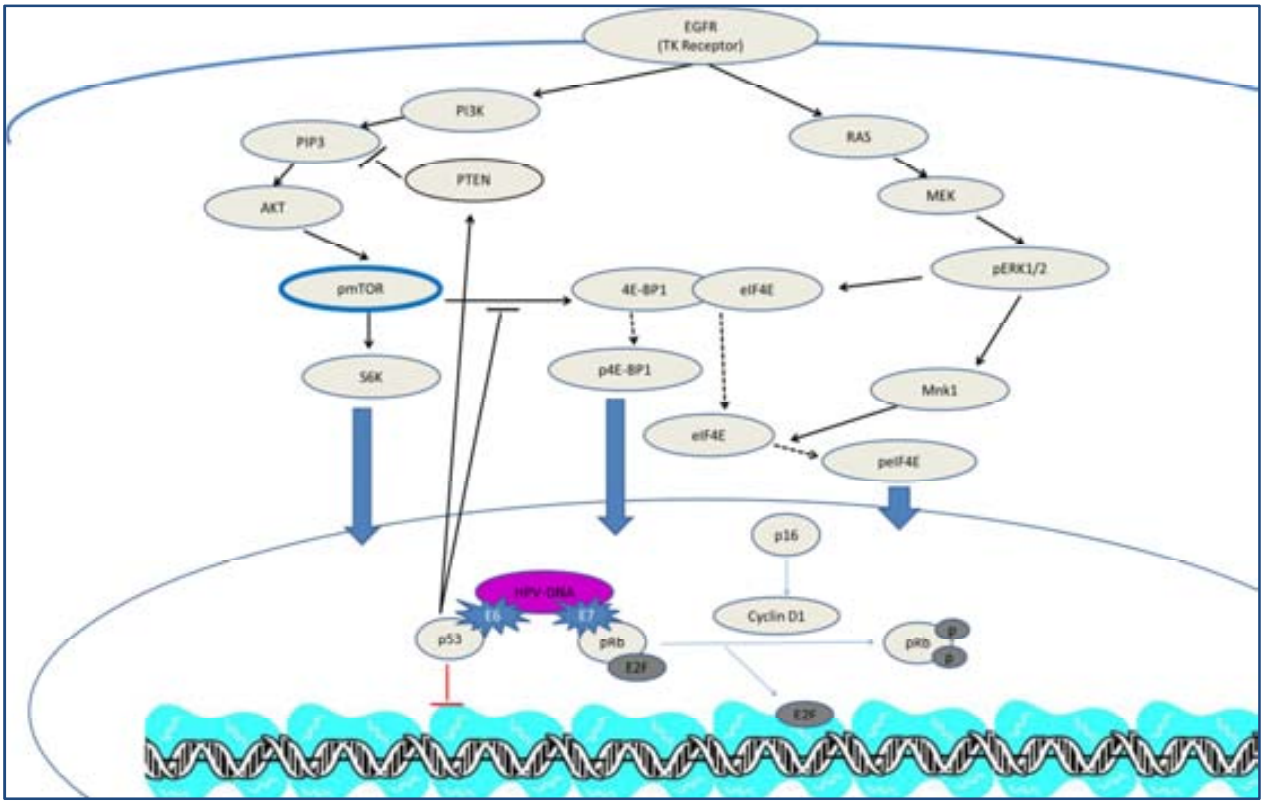

Figure 10: Schematic diagram illustrating molecular pathways that might be implicated in penile cancer and merits further investigation.

Aims

"To believe is very dull, to doubt is intensely engrossing. To be on the alert is to live, to be lulled into security is to die."

Oscar Wild,

Iris writer and poet, 1854-1900.

1. **Provide novel data about the prevalence of HPV in PeIN and invasive penile SCC in a Spanish Mediterranean population.**

    a. Determine the HPV status in a retrospective cohort of PeIN and invasive penile SCCs using a polymerase chain reaction with SPF-10 broad-spectrum primers followed by DNA enzyme immunoassay and genotyping with a reverse hybridization line probe assay under strict conditions.

    b. Correlate the HPV status results with $p16^{INK4a}$ immunostaining on TMAs.

    c. Correlate the HPV and $p16^{INK4a}$ status with different microscopic features, clinical variables and patients' outcome.

2. **Evaluate the role of mTOR signaling pathway in the pathogenesis of invasive penile SCC.**

    a. Evaluate the immunohistochemical expression on TMAs of several factors implicated in mTOR signaling pathway in a retrospective cohort of invasive penile SCCs.

    b. Correlate the immunohistochemical results with microscopic features, clinical variables, HPV status and clinical outcome.

    c. Identify prognostic biomarkers in penile SCC and possible therapeutic targets.

3. **Evaluate the role of p53 in the pathogenesis of invasive penile SCC.**

    a. Evaluate the immunohistochemical expression of p53 on TMAs in a retrospective cohort of invasive penile SCCs.

    b. Correlate the immunohistochemical results with microscopic features, clinical variables, HPV status and clinical outcome.

Results

"Who are you going to believe, me or your own eyes?"

Groucho Marx,

American comedian, 1890-1977.

The results of this Thesis are shown in the following two articles:

1. **Ferrándiz-Pulido C**, Masferrer E, de Torres I, Lloveras B, Hernández-Losa J, Mojal S, Salvador C, Morote J, Ramón y Cajal S, Pujol RM, García-Patos V, Toll A.

   Identification and genotyping of human papillomavirus in a Spanish cohort of penile squamous cell carcinomas: correlation with pathologic subtypes, $p16^{INK4a}$ expression and prognosis.

   Journal of the American Academy of Dermatology. 2013;68:73-82. (2012 IF 4,906)

2. **Ferrándiz-Pulido C**, Masferrer E, Toll A, Hernández-Losa J, Mojal S, Pujol RM, Ramón y Cajal S, de Torres I, García-Patos V.

   mTOR signaling pathway in penile squamous cell carcinoma: pmTOR and peIF4E overexpression correlate with aggressive tumoral behavior.

   Journal of Urology. Accepted for publication June 5th, 2013. In Press (2012 IF 4,02)

Identification and genotyping of human papillomavirus in a Spanish cohort of penile squamous cell carcinomas: Correlation with pathologic subtypes, $p16^{INK4a}$ expression, and prognosis

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Background: Penile squamous cell carcinoma (PSCC) is a tumor with a high metastatic potential. In PSCC the attributable fraction to human papillomavirus (HPV) is not well established.

Objective: We sought to provide novel data about the prevalence of HPV in a large series of penile intraepithelial neoplasia (PeIN) and invasive PSCC, correlating the results with the histologic subtype, $p16^{INK4a}$ immunostaining, and prognosis.

Methods: A total of 82 PSCC were included in the study, 69 invasive and 13 PeIN. HPV detection was performed by polymerase chain reaction with SPF-10 broad-spectrum primers followed by DNA enzyme immunoassay and genotyping with a reverse hybridization line probe assay. $P16^{INK4a}$ immunohistochemical expression on tissue microarrays was also analyzed.

Results: HPV DNA was identified in 31 of 77 (40.2%) PSCC (22 of 67 invasive and 9 of 10 PeIN). In 25 of 31 (80.6%) cases HPV-16 was identified. HPV detection was significantly associated with some histologic subtypes: most basaloid and warty tumors were high-risk HPV (hrHPV) positive, whereas only 15% of usual PSCC were hr-HPV positive. All hrHPV-positive PSCC had an adjacent undifferentiated PeIN. Strong $p16^{INK4a}$ immunostaining correlated with hrHPV infection. Most undifferentiated PeIN showed $p16^{INK4a}$ immunohistochemical overexpression. Both hrHPV-positive and $p16^{INK4a}$-positive tumors showed a better overall survival without reaching statistical significance.

Limitations: This was a retrospective study.

Conclusions: Our results suggest that most hrHPV-positive PSCC develop from undifferentiated hrHPV-positive PeIN. $P16^{INK4a}$ immunostaining may be useful in identifying both etiologically related hrHPV-positive tumors and those with better outcome. The routine use of $p16^{INK4a}$ staining should be incorporated in histologic evaluation of PSCC. (J Am Acad Dermatol 2013;68:73-82.)

Key words: human papillomavirus; $p16^{INK4a}$; penile intraepithelial neoplasia; penis; squamous cell carcinoma; survival.

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Dr Toll and Garcia-Patos equally contributed to the work.

Supported in part by the grants PI04/1728, RD09/0076/00036, and PI10/00785 from Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Sanidad, Federación Española de Enfermedades Raras (FEDER), Spain. The funder was not involved in study design, data collection, data analysis, manuscript preparation, or publication decisions.

Conflicts of interest: None declared.

Presented in part as a poster at the 41st Annual European Society for Dermatological Research (ESDR) Meeting, Barcelona, Spain, September 7-10, 2011.

Accepted for publication May 31, 2012.

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Published online August 6, 2012.

0190-9622/$36.00

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http://dx.doi.org/10.1016/j.jaad.2012.05.029

Penile squamous cell carcinoma (PSCC) is infrequent in developed countries, representing 0.3% to 0.5% of male tumors in Europe.[1] Its incidence ranges from 1.0 per 100,000 people in developed countries to 4.4 per 100,000 people in South America, Asia, and Africa.[1] PSCC may be histologically divided into usual type (70%-90%), followed by a basaloid (10%) and low-grade verruciform tumors that include verrucous, warty, and papillary carcinomas. Rare variants include an aggressive sarcomatoid subtype, and mixed tumors of usual type and another variant.[2] Penile intraepithelial neoplasia (PeIN) may be classified into undifferentiated PeIN, which can be low or high grade, as in the uterine cervix, and differentiated PeIN.[3-5] In light of recent studies, undifferentiated PeIN are human papillomavirus (HPV)-related and would only be precursor lesions to a subset of invasive tumors, such as basaloid and warty carcinomas.[3] In contrast, usual or verrucous carcinomas would arise from HPV-unrelated precursor lesions such as differentiated PeIN, squamous cell hyperplasia, and/or lichen sclerosus, or other still not well-established entities.[3-5]

CAPSULE SUMMARY

- In penile squamous cell carcinoma the attributable fraction to human papillomavirus (HPV) is not well established.
- We provide novel data about the prevalence of HPV in penile squamous cell carcinoma in a large series of a Mediterranean population. HPV seems to play a special role in a subset of usual-type penile squamous cell carcinoma that progress from undifferentiated penile intraepithelial neoplasia and has a better prognosis.
- $P16^{INK4a}$ immunostaining identifies both etiologically related high-risk HPV-positive tumors and those with better outcome.

There are limited data about the role of HPV in PSCC, and only a few studies on small series of patients from the Mediterranean area have focused in this topic.[6-9] HPV genotypes are currently classified according to their oncogenic potential in low- and high-risk subtypes, being the high-risk genotypes those usually found in premalignant and malignant lesions from the mucosal areas.[10] Depending on the detection method, geographic area, and tumor type, the infection rates of HPV in PSCC range from 20% to 80%.[1,11-13]

A close association between high-risk HPV (hrHPV) infection and $p16^{INK4a}$ overexpression has repeatedly been reported for several tumors, such as squamous cell carcinoma (SCC) of the head and neck region and cervical cancer.[14] However, limited data are available regarding $p16^{INK4a}$ immunodetection in PSCC, its relationship with HPV detection, and its prognostic significance.[14-19]

In this study we aimed to provide novel data about the prevalence of HPV in PeIN and invasive PSCC in a Spanish Mediterranean population. A possible correlation between both HPV status results and $p16^{INK4a}$ immunostaining with different histologic subtypes and survival was also assessed.

METHODS

General design

To estimate the prevalence of HPV DNA types in men with either PeIN or PSCC in a Spanish Mediterranean population (1987-2010), a retrospective study was designed. A cohort of 82 patients was selected from the files of the pathology departments of 2 tertiary centers from Barcelona participating in the study (Hospital Vall d'Hebron and Hospital del Mar). From this series, 69 and 13 patients presented PSCC and PeIN, respectively. For each patient, representative formalin-fixed, paraffin-embedded tissue samples were collected. Samples of PeIN/epidermal hyperplasia adjacent to tumors (adjacent penile intraepithelial neoplasia [adjPeIN]) were also selected in 53 PSCC. We also obtained normal-appearing foreskin from 15 healthy individuals as control samples. This study was approved by the local ethics committees from both centers participating in the study, and in accordance with the guidelines of the Helsinki Declaration of 1975, as revised in 1983.

Following a systematized protocol, the following data were recorded for each patient: age, localization of the tumor, immunosuppression status, lymph node metastases, and clinical evolution. The follow-up time was defined as the time from surgery until the last recorded clinical follow-up or death and poor outcome was defined as disease-related death.

Histologic evaluation

All hematoxylin and eosin–stained sections from complete resections were evaluated in all PSCC. In PeIN biopsy specimens or complete resections, when available, were reviewed. General histologic features, including histologic subtype, grade of differentiation, and tumor stage, were evaluated in each representative sample, according to the World Health Organization and

Abbreviations used:

adjPeIN: adjacent penile intraepithelial neoplasia
HPV: human papillomavirus
hrHPV: high-risk human papillomavirus
PeIN: penile intraepithelial neoplasia
PSCC: penile squamous cell carcinoma
SCC: squamous cell carcinoma

TNM classification (American Joint Committee on Cancer 2009). PSCC were classified as usual, verrucous, basaloid, warty, sarcomatoid, or mixed subtype, as previously reported.[2] PeIN and adjPeIN were subclassified as undifferentiated PeIN or differentiated PeIN/epidermal hyperplasia.[3-5]

HPV detection

HPV detection and typing was performed at the Infection and Cancer Laboratory, Duran i Reynals, Institut Català d'Oncologia, Barcelona, Spain, as a part of an ongoing international study (HPV VVAPO [vulva, vagina, anus, penis, oropharynx]). Paraffin blocks were processed under strict conditions to avoid contamination. A section from each specimen (a volume of 50 μL containing 10 μL of extracted DNA) was analyzed for the presence of HPV DNA by a highly sensitive polymerase chain reaction technique based on SPF-10 primers, followed by a general enzyme immune assay for HPV types. Positive samples were subsequently genotyped by $LiPA_{25}$ (Version 1, Laboratory Biomedical Products, Rijswijk, The Netherlands) using the previously described protocol by Kleter et al.[20] $LiPA_{25}$ can be used to detect 25 HPV types, all of them belonging to the α-papillomavirus genus. HPV genotypes were classified into low- and high-risk groups following previous published criteria.[10]

Tissue microarray construction

For tissue microarray construction, we used hematoxylin and eosin–stained slides from each tumor block to select, when available: 2 representative invasive tumor areas, 2 preinvasive lesion areas (for both PeIN and adjPeIN), 2 normal tissue areas, and 2 representative metastatic tissues from a lymph node (8 cases). We punched 2-mm tissue cylinders from the marked tumor area of each block and brought them into a recipient paraffin block using a precision instrument (Arrayer Punch 2.00 mm, ATA200, Advanced Tissue Arrayer, Chemicon International, Temecula, CA).

$P16^{INK4a}$ immunohistochemistry

We cut 4-μm sections from tissue microarrays and immunostained them with $p16^{INK4a}$ using standard heat-induced antigen retrieval methods and the ABC kit (mtm laboratories AG, Heidelberg, Germany) according to the manufacturer's instructions. Immunostaining was evaluated by 2 independent observers (C. F-P. and I. d. T.), and no discordant result was obtained. Sections were scored semiquantitatively by H-score in accordance with previously published data.[16,17] The H-score is based on the percentage of positively stained neoplastic cells (between 0%-100%) and the immunointensity measured as: 1 (weak), 2 (moderate), and 3 (strong). The final H-score was deduced by multiplying the percentage of staining by intensity to give an expression score from 0 to 300. Our cut-off point for positive cases was arbitrarily decided to be H-score of 50 because we found no cases with H-score between 50 and 150.

Statistical analysis

Categorical variables were described with relative frequencies and percentages, and quantitative variables with median and range. The Mann-Whitney U test was used to assess differences in age. The χ^2 test or the Fisher exact test were used, as appropriate, to evaluate the relationship between categorical variables. Kaplan-Meier plots were used with a log rank test to evaluate disease-specific survival. In all cases, P values less than .05 were considered statistically significant. Statistical analysis was performed using software (SPSS, Version 18, IBM Corp, Armonk, NY).

RESULTS

Clinicopathological data

The patients' median age was 69 years (range, 35-96 years), with no differences between patients with hrHPV-positive and -negative tumors (P = .511). Grossly, the majority of tumors were located in the distal part of the penis (glans, coronal sulcus, or foreskin), with only 10% of them affecting the penile shaft. Three patients were immunosuppressed because of HIV infection and another patient was a liver transplant recipient.

Thirteen patients (16.7%) had a stage-0 tumor, 25 patients (32.1%) were stage I, 28 patients (35.9%) stage II, 5 patients (6.4%) stage IIIA, and 7 patients (9%) stage IIIB. In 4 patients the tumor stage could not be evaluated.

The vast majority of invasive tumors was usual type (68%), followed by an equal distribution (7.2%) of verrucous, pure basaloid, and mixed basaloid/usual-type tumors. The less frequent types were warty (5.8%) and sarcomatoid carcinomas (4.3%). Twenty-three cases (33.3%) corresponded to histologically grade-1 tumors, 23 cases (33.3%) to grade 2, 21 cases (30%) to grade 3, and 2 cases (2.9%) to grade 4. Nineteen patients (23.2%) developed lymph node

Table I. Association of types of penile intraepithelial neoplasia with subtypes of invasive penile squamous cell carcinoma

		Undifferentiated PeIN		Differentiated PeIN/epidermal hyperplasia	
	n	N	%	N	%
PSCC histologic subtype					
Usual type	37	9	24	28	76
Verrucous	5	0	0	5	100
Basaloid	3	3	100	0	0
Warty	4	3	75	1	25
Sarcomatoid	0	0		0	
Mixed basaloid and usual type	4	4	100	0	0
					$P < .001$

PeIN, Penile intraepithelial neoplasia; *PSCC*, penile squamous cell carcinoma.

Table II. Prevalence of human papillomavirus infection in penile squamous cell carcinoma

		HPV positive		HrHPV infection		LrHPV infection		HPV X-type* infection	
	n	N	%	N	%	N	%	N	%
PeIN	10	9	90	9	100	0	0	0	0
PSCC	67	22	33	19	86.5	2	9	1	4.5
Total	77	31	46.3	28†	90	2	6.5	1	3.5

HPV, Human papillomavirus; *hrHPV*, high-risk human papillomavirus; *lrHPV*, low-risk human papillomavirus; *PeIN*, penile intraepithelial neoplasia; *PSCC*, penile squamous cell carcinoma.
*Unidentified HPV type.
†Includes 26 single hrHPV infections and 2 multiple HPV infections.

metastases and 11 patients (13.4%) died from the disease (67% of the patients with sarcomatoid subtype, 20% with basaloid and verrucous, 15% with usual subtypes, and none with a warty tumor). The median follow-up was 29.4 months (range 0.26-247).

Regarding pure PeIN, 11 (84.6%) were undifferentiated and 2 (15.4%) differentiated. Nineteen (35.8%) and 34 (64.2%) adjPeIN corresponded to undifferentiated PeIN and differentiated PeIN/epidermal hyperplasias, respectively. The proportion of adjPeIN that were undifferentiated varied significantly depending on the histologic subtype of the corresponding adjacent PSCC, ranging from 100% in basaloid and mixed tumors to none of the verrucous subtypes ($P < .001$) (Table I).

HPV DNA detection and typing

Of the 82 cases diagnosed, only 77 cases were suitable for HPV analysis (67 PSCC and 10 PeIN). Results are summarized in Table II. In all, 26 of 31 HPV-positive tumors (84%) corresponded to single hrHPV infections and 2 specimens (6.5%) were positive for single low-risk HPV infections; 1 sample contained an unidentified HPV type. Only 2 cases (6.5%) harbored 2 HPV types: 1 case had 2 hrHPV types and the other had both hrHPV type and a low-risk HPV type. For statistical analysis both of them were considered hrHPV infections, so we found 36.4% hrHPV-positive tumors (28.4% of PSCC and 90% of PeIN). The most prevalent HPV was type 16, which was present in 25 of 31 (80.6%) HPV-positive tumors (Table III).

Table III. Distribution of human papillomavirus genotypes

Genotype	N
HrHPV	
16	23
58	2
33	1
16 and 6	1
16 and 45	1
LrHPV	
6	1
74	1
HPV X type*	1

HrHPV, High-risk human papillomavirus; *lrHPV*, low-risk human papillomavirus.
*Unidentified HPV type.

The prevalence of hrHPV was significantly associated with some histologic subtypes ($P < .001$): 100% of pure or mixed basaloid carcinomas and 75% of warty tumors were hrHPV positive, whereas only 15% of usual types and none of the verrucous or sarcomatoid subtypes yielded positive results (Table IV). HrHPV-positive tumors were significantly less differentiated than HPV-negative tumors ($P = .03$):

Table IV. High-risk human papillomavirus infection and p16^{INK4a} immunostaining in invasive penile squamous cell carcinoma with regard to histologic subtypes, differentiation grade, tumor stage, lymph node metastases, and death

		HrHPV infection		LrHPV infection/HPV negative		P16 positive		P16 negative		
	n	N	%	N	%	N	%	N	%	*P*
PSCC histologic subtype										.001
Usual type	46	7	15	39	85	11	24	35	76	
Verrucous	5	0	0	5	100	0	0	5	100	
Basaloid	5	5	100	0	0	5	100	0	0	
Warty	4	3	75	1	25	3	75	1	25	
Sarcomatoid	3	0	0	3	100	0	0	3	100	
Mixed basaloid and usual type	4	4	100	0	0	4	100	0	0	
Differentiation grade										.03
G1/G2	45	9	20	36	80	11	25	34	75	
G3/G4	22	10	45	12	55	12	55	10	45	
Tumor stage										>.05
I	24	6	25	18	75	7	29	17	71	
>I	39	11	28	28	72	14	36	25	64	
Lymph node metastases										>.05
Yes	19	4	21	15	79	5	26	14	74	
No	48	15	31	33	69	18	37,5	30	62,5	
Died of disease										>.05
Yes	11	1	9	10	91	2	18	9	82	
No	56	18	32	38	68	21	37,5	35	62,5	

G1/G2, Well-differentiated (G1) and moderately differentiated (G2); *G3/G4*, poorly differentiated (G3) and undifferentiated (G4); *HPV*, human papillomavirus; *hrHPV*, high-risk human papillomavirus; *lrHPV*, low-risk human papillomavirus; *PSCC*, penile squamous cell carcinoma.

Table V. Association of high-risk human papillomavirus infection and p16^{INK4a} immunostaining with penile intraepithelial neoplasia (and adjacent penile intraepithelial neoplasia)

		HrHPV infection		LrHPV infection/HPV negative		P16 positive		P16 negative		
	n	N	%	N	%	N	%	N	%	
PeIN										
Undifferentiated PeIN	28	25	89	3	11	22	79	6	21	
Differentiated PeIN/epidermal hyperplasia	35	0	0	35	100	1	3	34	97	$P < .001$

HPV, Human papillomavirus; *hrHPV*, high-risk human papillomavirus; *lrHPV*, low-risk human papillomavirus; *PeIN*, penile intraepithelial neoplasia; *PSCC*, penile squamous cell carcinoma.

80% of G1 (well-differentiated)/G2 (moderately differentiated) tumors were hrHPV negative. The significant association between poor differentiation and HPV infection was lost in the usual-type PSCC, the most prevalent histologic subtype. All hrHPV-positive PeIN (both PeIN and adjPeIN) were undifferentiated ($P < .001$) (Table V). Interestingly, when analyzing only the usual-type PSCC, we observed that the presence of an adjacent differentiated PeIN or epidermal hyperplasia was a sign of HPV negativity (Table VI).

P16^{INK4a} immunoexpression

P16^{INK4a} immunohistochemical expression was evaluated in 78 patients (67 PSCC + 52 adjPeIN and 11 PeIN) and, when positive, it showed both nuclear and cytoplasmic immunoreactivity in a continuous pattern (Fig 1). The control group was always p16^{INK4a} negative.

With regard to PSCC, p16^{INK4a} was positive in 23 (34%) samples. When the invasive tumor was p16^{INK4a} positive, the adjPeIN was also positive ($P < 0.001$), as well as the metastatic lymph node, if present ($P = .048$). PeIN were p16^{INK4a} positive in 8 cases and negative in 3 (Table VII).

Positive p16^{INK4a} staining showed an excellent correlation with hrHPV detection ($P < .001$) (Tables VIII and IX). The 2 low-risk HPV tumors were p16^{INK4a} negative. HPV-negative tumors also tended to be p16^{INK4a} negative, but 3 cases strongly stained for p16^{INK4a} (score 150). Strong p16^{INK4a} expression also correlated with some histologic subtypes, being

Table VI. Association of human papillomavirus infection with penile intraepithelial neoplasm adjacent to usual penile squamous cell carcinoma

		HrHPV infection		LrHPV infection/HPV negative		
	n	N	%	N	%	
Usual PSCC						
Undifferentiated PeIN	9	6	66	3	33	
Differentiated PeIN/ epidermal hyperplasia	28	0	0	28	100	$P < .001$

HPV, Human papillomavirus; *hrHPV*, high-risk human papillomavirus; *lrHPV*, low-risk human papillomavirus; *PeIN*, penile intraepithelial neoplasia; *PSCC*, penile squamous cell carcinoma.
Most usual PSCC do not have an undifferentiated adjacent PeIN; in those cases, hrHPV infection would not be etiologically related.

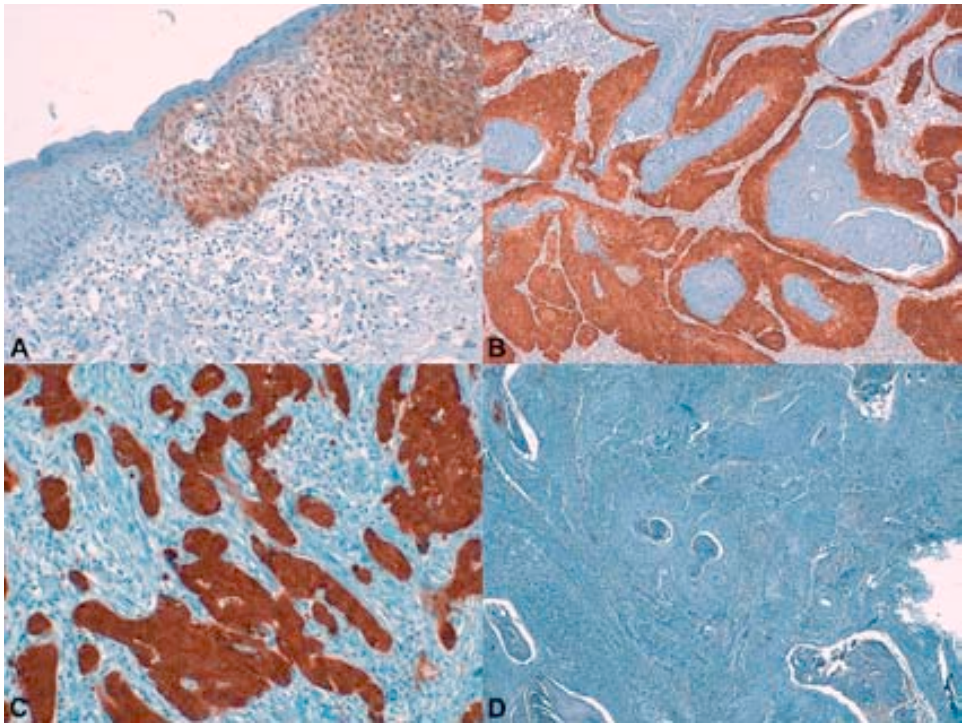

Fig 1. $P16^{INK4a}$ immunoexpression in penile squamous cell carcinoma (PSCC). **A**, Negative $p16^{INK4a}$ immunoexpression in normal epithelia and strong immunostaining in undifferentiated penile intraepithelial neoplasia. **B**, Strong immunostaining in usual-type PSCC. **C**, Strong immunostaining in basaloid subtype PSCC. **D**, Negative $p16^{INK4a}$ expression in usual-type tumor. (**A** to **D**, $P16^{INK4a}$ staining; original magnifications: ×100.)

positive in all pure and mixed basaloid types, 75% of warty types, and 24% of usual types ($P < .03$) (Table IV). $P16^{INK4a}$-positive PeIN and adjPeIN were mostly undifferentiated (79%, $P < .001$) (Table V). Moreover, we also found a significant correlation between $p16^{INK4a}$ expression and poor differentiation grade ($P = .03$) that was lost when considering only the usual subtype.

HPV/$p16^{INK4a}$ overexpression and prognosis

Patients lacking HPV infection and those with no $p16^{INK4a}$ overexpression had a trend toward higher tumoral stages, development of lymph node metastases, disease-related death, or a combination of these, without reaching statistical significance (Table IV and Fig 2). None of the patients with hrHPV-positive usual-type PSCC died from the tumor.

DISCUSSION

In this study, we detected hrHPV in 28% of PSCC and in 90% of PeIN. Similar hrHPV prevalence rates have recently been obtained by Cubilla et al[15] using a similar technical approach. These results allow identification of a subset of PSCC in which HPV would

Table VII. P16^{INK4a} immunoexpression in penile squamous cell carcinoma

		P16 positive		P16 negative	
	n	N	%	N	%
PeIN	11	8	73	3	27
PSCC	67	23	34	44	66
Total	78	31	40	47	60

PeIN, Penile intraepithelial neoplasia; *PSCC*, penile squamous cell carcinoma.

Table VIII. Human papillomavirus infection and p16^{INK4a} status in penile intraepithelial neoplasm

	n	P16 positive	P16 negative
HrHPV infection	7	6	1
LrHPV infection/HPV negative	1	0	1
			$P = .064$

HPV, Human papillomavirus; *hrHPV*, high-risk human papillomavirus; *lrHPV*, low-risk human papillomavirus.

Table IX. Human papillomavirus infection and p16^{INK4a} status in invasive penile squamous cell carcinomas

	n	P16 positive	P16 negative
HrHPV infection	19	19	0
LrHPV infection/HPV negative	47	3	44
			$P < .001$

HPV, Human papillomavirus; *hrHPV*, high-risk human papillomavirus; *lrHPV*, low-risk human papillomavirus.

play a triggering role and give support to the bimodal etiopathogenic hypothesis that distinguishes 2 different subsets of PSCC (HPV-related PSCC and PSCC nonrelated to HPV).[2,12,15,17-19,21-25]

Several studies have shown that HPV-16 is the predominant hrHPV in PSCC both in developed and undeveloped countries.[25-29] However, variable prevalence rates have been observed in different geographic populations.[1] HPV-18 represents the second most prevalent HPV type in PSCC in countries such as Thailand and Brazil,[29-31] but its detection is uncommon in European countries. In contrast to other studies detecting multiple HPV subtypes in a significant number of cases (from 20%-50%),[12,16,31,32] we have observed such phenomenon in only 6.5% of cases. The significance of multiple HPV subtypes is not fully understood, and the potential role of the different detected HPVs as carcinogens or co-infectors has not been elucidated.

We have observed significant differences in the prevalence of HPV infection depending on the histologic subtype: basaloid, mixed usual/basaloid,

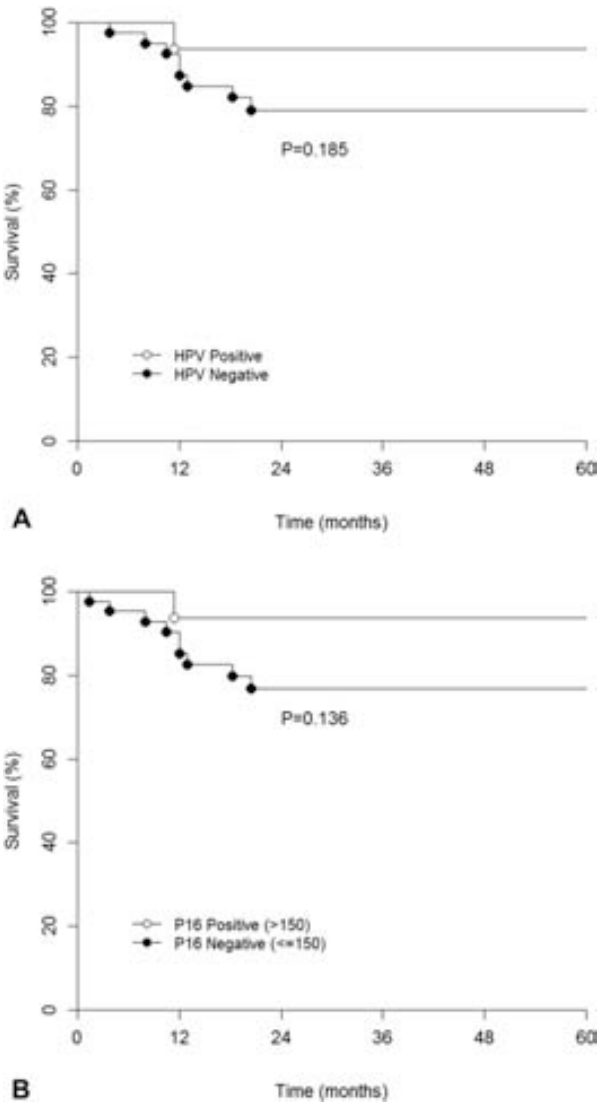

Fig 2. Overall survival depending on high-risk human papillomavirus (*HPV*) infection status **(A)** and p16^{INK4a} immunoexpression **(B)**.

and warty carcinomas were found to be consistently associated with the presence of hrHPV infection. Conversely, hrHPVs were rarely detected in verrucous and sarcomatoid carcinomas. Our results are consistent with other studies on PSCC and on anal and vulvar carcinomas.[26,33-35] However, in the review article by Miralles-Guri et al,[25] the reported prevalence of hrHPV in the usual type was significantly higher than in our population, probably because of geographic differences, heterogeneous detection methods, or both.

Several authors have reported an association between high histologic–grade PSCC and hrHPV positivity.[15,36] In our series, 77% of basaloid or mixed tumors were high-grade tumors and were also positive for hrHPV. When considering only

usual-type tumors in the analysis, no association between hrHPV and high histologic grade could be noted.

The role of hrHPV as a marker of good prognosis in other SCC of the genital and extragenital mucosal areas has previously been suggested.[12,13,37-43] We have also detected a trend toward a better prognosis in the hrHPV-positive group, although not reaching statistical significance. Interestingly, all patients with usual-type PSCC-related death had hrHPV-negative tumors.

HrHPV's E7 oncogene promotes the degradation of the retinoblastoma protein, leading to a $p16^{INK4a}$ overexpression by the loss of a negative feedback mechanism. $P16^{INK4a}$ overexpression correlates with hrHPV in some anatomic locations such as the cervix, oropharynx, and sun-exposed skin.[12,14,37,39,44] Overexpression of p16 is also frequent in PSCC, especially in hrHPV-positive tumors.[15-19] The best method to evaluate $p16^{INK4a}$ overexpression is still an issue of controversy. Several methodologic approaches have been postulated, such as staining topography (nuclear and/or cytoplasmic), percentage and/or distribution of positive cells, staining intensity, and pattern score, but no comparative studies have been reported.[15] Good interobserver correlation was demonstrated and a clear cut-off point using the H-score system was defined. In addition, we have been able to establish good correlation between $p16^{INK4a}$ overexpression and hrHPV infection, as previously reported, which argues in favor of the reliability of this method.

Tumors that were negative for hrHPV but showed $p16^{INK4a}$ overexpression were uncommon. Possible explanations for such cases may include a technical failure to detect low or degraded HPV DNA in tumor samples or an activation of the p16 pathway through a mechanism not related with HPV infection. On the other hand, the low number of hrHPV-positive cases with an absence of $p16^{INK4a}$ expression suggests that allelic loss and/or mutations in the $p16^{INK4a}$ gene were uncommon phenomena in our series, unlike previously reported.[14] The frequent observation of $p16^{INK4a}$ negativity in control samples and normal epithelial areas adjacent to the tumors also supports the usefulness of $p16^{INK4a}$ immunohistochemical expression as a possible surrogate for hrHPV infection.

The positivity for $p16^{INK4a}$ has been proposed as a good prognostic marker in head and neck and in oropharynx SCC.[45,46] Some authors have suggested that $p16^{INK4a}$ plays a major role not only in suppression of cell division but also in suppression of lymphangiogenesis and lymphatic metastasis.[45] As far as we are concerned, the role of $p16^{INK4a}$ immunoexpression as a marker for survival in PSCC has only been evaluated in 1 previous study and an association with a better overall survival was detected.[18] In our study, $p16^{INK4a}$-positive tumors were significantly less differentiated than $p16^{INK4a}$-negative tumors, but showed a better overall survival, suggesting a protective role of $p16^{INK4a}$ in PSCC. It is tempting to hypothesize that hrHPV may be a triggering factor in the development of a subset of PSCC, but would concomitantly induce a $p16^{INK4a}$-mediated protection mechanism.

Transformation of PeIN into invasive PSCC has been reported in 5% to 33% of cases. The prevalence of HPV infection in PeIN ranges from 75% to 100%.[26,30,31,47-49] We have analyzed a large series of PeIN (both isolated and adjacent to invasive PSCC) and several conclusions can be outlined: (1) PeIN can easily be divided histologically into undifferentiated and differentiated; (2) most undifferentiated PeIN show p16 overexpression and harbor hrHPV infection; (3) basaloid or warty PSCC are frequently associated with undifferentiated adjPeIN; and (4) a subgroup of usual-type PSCC (24%) have undifferentiated adjPeIN and most of these invasive tumors are hrHPV positive. Our findings suggest that hrHPV-positive usual-type PSCC probably develop over undifferentiated hrHPV-positive/p16-positive PeIN.

Our study had some limitations that should be pointed out. First, there are inherent biases and potential errors of a retrospective study and a 5-year follow-up period was not achieved in all cases. Charting errors and omissions may also have occurred, especially considering that our study involved 2 different centers. Secondly, the rarity of PSCC hampers the collection of a large series of cases. Thirdly, we could not evaluate HPV infection in all patients because of degradation of DNA in some cases. Finally, immunohistochemistry was performed in 2-mm cylinders, which could render nonrepresentative results in tumors with heterogeneous areas of $p16^{INK4a}$ expression.

In conclusion, we have observed hrHPV infection in 36.4% of PSCC. This infection is more frequent in some histologic subtypes, such as basaloid PSCC. HrHPV infection seems to play a special role in a subset of usual-type PSCC that progress from undifferentiated PeIN. This group of usual-type PSCC seems to have a better prognosis than hrHPV-negative tumors. HrHPV infection in PSCC can be reliably predicted by the observation of $p16^{INK4a}$ overexpression. Both hrHPV and $p16^{INK4a}$ overexpression correlated with good outcome. Considering the aforementioned results, $p16^{INK4a}$ immunohistochemical expression seems to represent a useful and informative diagnostic feature in PSCC. Because this technique can easily be performed in most

pathology laboratories, we consider that it should be incorporated as a routine diagnostic tool in the diagnosis and evaluation of PSCC samples.

The authors thank Tania Lobato for her contribution in the construction of tissue microarrays; Teresa Moliné for her technical support with immunostains; Eva Lopez for statistical support; and the RIS HPV TT and HPV VVAPO study group from Institut Catala d'Oncologia (ICO) (particularly F. Xavier Bosch, Silvia de Sanjosé, and Jo Ellen Klaustermeier).

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mTOR Signaling Pathway in Penile Squamous Cell Carcinoma: pmTOR and peIF4E Over Expression Correlate with Aggressive Tumor Behavior

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Purpose: Penile squamous cell carcinoma is a rare neoplasm associated with a high risk of metastasis and morbidity. There are limited data on the role of the mTOR signaling pathway in penile squamous cell carcinoma carcinogenesis and tumor maintenance. We assessed a possible role for mTOR signaling pathway activation as a potential predictive biomarker of outcomes and a therapeutic target for penile cancer.

Material and Methods: A cohort of 67 patients diagnosed with invasive penile squamous cell carcinoma from 1987 to 2010 who had known HPV status were selected for study. Tissue microarrays were constructed with 67 primary penile squamous cell carcinomas, matched normal tissues and 8 lymph node metastases. Immunohistochemical staining was performed for p53, pmTOR, pERK, p4E-BP1, eIF4E and peIF4E. Expression was evaluated using a semiquantitative H-score on a scale of 0 to 300.

Results: Expression of pmTOR, p4E-BP1, eIF4E and peIF4E was increased in penile tumors compared with matched adjacent normal tissues, indicating activation of the mTOR signaling pathway in penile tumorigenesis. Over expression of pmTOR, peIF4E and p53 was significantly associated with lymph node disease. peIF4E and p53 also correlated with a poor outcome, including recurrence, metastasis or disease specific death. In contrast, pERK and p4E-BP1 were associated with lower pT stages. pmTOR and intense p53 expression was associated with HPV negative tumors.

Conclusions: Activation of mTOR signaling may contribute to penile squamous cell carcinoma progression and aggressive behavior. Targeting mTOR or its downstream signaling targets, such as peIF4E, may be a valid therapeutic strategy.

Abbreviations and Acronyms

4E-BP1 = eukaryotic translation initiation factor 4E-binding protein 1

eIF4E = eukaryotic translation initiation factor 4E

ERK = extracellular regulated kinase

HPV = human papillomavirus

hrHPV = high risk HPV

MAPK = mitogen activated protein kinase

MNK = MAPK-interacting protein kinase

mTOR = mammalian target of rapamycin

p = phosphorylated

PI3K = phosphatidylinositol 3-kinase

SCC = squamous cell carcinoma

TMA = tissue microarray

TP53 = tumor protein 53 gene

Accepted for publication June 3, 2013.

Study received local ethics committee approval at each participating center.

Supported by Grants PI11/00185, PI04/1728, RD09/0076/00036 and PI10/00785 from the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Sanidad, Federación Española de Enfermedades Raras, FMM-2011-11 from Fundación Mutua Madrileña and RD06/002/0104 from Redes Cancer, Spain, a grant from the Sección Catalana de Dermatología de l'Acadèmia Española de Dermatología y Venereología i la Societat Catalana de Dermatologia de l'Acadèmia de Ciències Mèdiques i de la Salut de Catalunya, and the Xarxa de Bancs de Tumors de Catalunya sponsored by Pla Director d'Oncologia de Catalunya (XBTC), supported by RETICS de Biobancos (ISCIII).

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0022-5347/13/1906-0001/0
THE JOURNAL OF UROLOGY®
© 2013 by American Urological Association Education and Research, Inc.
http://dx.doi.org/10.1016/j.juro.2013.06.015
Vol. 190, 1-8, December 2013
Printed in U.S.A.

Dochead: Investigative Urology FLA 5.2.0 DTD ■ JURO10474_proof ■ 27 August 2013 ■ 3:26 pm ■ EO: JU-13-551

Key Words: penis; carcinoma, squamous cell; biological markers; mTOR protein, human; immunohistochemistry

PENILE carcinoma is rare and 95% of cases are histologically classified as SCC. Several risk factors have been identified, such as lichen sclerosus, poor hygiene, phimosis, the lack of circumcision during childhood and hrHPV infection.[1] Little is known about the carcinogenesis of penile SCC. Currently, there is enough evidence to propose a bimodal etiopathogenic pathway in its carcinogenesis, including one related to hrHPV infection and another that is hrHPV independent.[2,3]

Penile carcinoma is associated with a high risk of metastasis and morbidity. The presence of inguinal lymph node metastasis is the most important clinical prognostic factor for survival.[1] Due to the rarity of this tumor, there is a lack of conclusive data on histological or molecular prognostic markers predicting occult lymph node disease. However, several histological factors predictive of lymph node metastasis and death were proposed, such as differentiation grade, perineural invasion and lymphovascular invasion,[4,5] as well as some molecular factors, including p53, Ki67, matrix metalloproteinase 9 over expression, E-cadherin downregulation and *MYC* copy number gains.[6–9] The prognostic role of HPV infection must be elucidated but hrHPV positive tumors seem to be associated with a better survival rate.[3]

In a high proportion of human cancers deregulation of the PI3K-AKT pathway, which regulates mTOR, was noted. Specific molecular alterations were detected in squamous cell tumors, such as those of the head, neck and lung, and nonmelanoma skin cancer.[10] Interestingly, clinical data show that switching from calcineurin inhibitors to sirolimus had an antitumor effect in kidney transplant recipients who previously had cutaneous SCC.[11] Several molecules of this pathway are targets for cancer therapy. In head and neck carcinoma the Akt-mTOR pathway was identified as a potential therapeutic target and the role of mTOR inhibitors was successfully evaluated as part of a molecularly targeted, metastasis preventive strategy.[12] The therapeutic role of mTOR inhibitors is also being tested for other epithelioid squamous cell tumors, such as cervical carcinoma.[13]

There are limited data on the role of the mTOR signaling pathway in penile SCC carcinogenesis. It was suggested that EGFR, HER3 and HER4 over expression is associated with penile carcinogenesis.[14,15] Moreover, a high frequency of *PIK3CA*, *HRAS* and *KRAS* gene mutations was described in penile carcinoma.[16] This led us to believe that deregulation of the mTOR or MAPK pathway has a significant role in penile carcinoma development and progression.

Our main objective was to evaluate the expression of several factors implicated in the mTOR signaling pathway in a penile SCC series (fig. 1). We also correlated immunohistochemical results with histopathological features, HPV status and clinical outcome. [F1]

MATERIALS AND METHODS

General Design

We retrospectively studied the records of patients diagnosed with invasive penile SCC in a Spanish Mediterranean population from 1987 to 2010. A cohort of 67 patients treated with complete resection of the primary tumor was selected from the files of the pathology departments of 2 tertiary centers in Barcelona (Hospital Vall d'Hebron and Hospital del Mar) that participated in the study. For each patient we collected representative formalin fixed, paraffin embedded tissue samples as well as all hematoxylin and eosin stained sections from complete tumor resections. This study was approved by the local ethics committees at each participating center and done in accordance with the guidelines of the Helsinki Declaration of 1975, as revised in 1983.

Following a systematized protocol, we recorded certain data on each patient, including age, immunosuppression status, presence or absence of lymph node metastasis and clinical evolution. Followup time was defined as time from surgery to the last recorded clinical followup or death. Poor outcome was defined as disease related death. Histological diagnosis was reevaluated in all penile SCC cases by light microscopy. General histological features, including histological subtype, differentiation grade and TNM stage, were evaluated in each representative sample according to WHO and the 2009 American Joint Committee on Cancer TNM classification.

TMA Generation

Ten TMAs were constructed using the semiautomated 2.0 mm Arrayer Punch tissue arrayer for the Advanced Tissue Arrayer (ATA200, Chemicon®). They contained punches of the 67 tumors under study, adjacent normal epithelium and 8 lymph node metastases. Each tissue array was assembled as previously described.[17] Briefly, 2 tissue cylinders (2 mm) per case were punched from morphologically representative tumor areas and 2 matched normal tissue areas of each donor tissue paraffin block. They were brought into a single 3 × 2.5 cm recipient paraffin block. We also included 2 representative tumor areas from the 8 lymph node metastases. Each

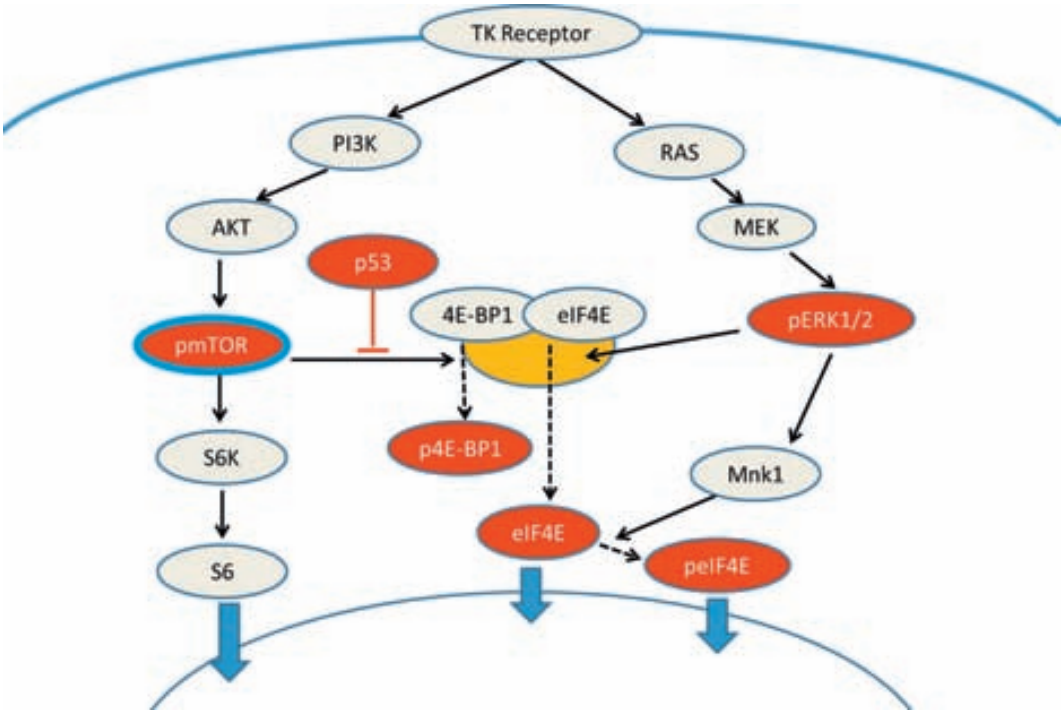

Figure 1. Most important signaling pathways proposed to be implicated in cell growth, including PI3K-AKT pathways, which regulate mTOR and RAS-RAF-MAPK. Pathways converge via several inputs and to 4E-BP1, which controls eIF4E and cap dependent RNA translation. Phosphorylation of 4E-BP1 may be due to many oncogenic events in several biochemical pathways. In quiescent cells or cells with low growth factor levels unphosphorylated 4E-BP1 binds to eIF4E, impeding cap dependent initiation complex formation and key protein synthesis, including c-MYC, cyclin D1 or vascular endothelial growth factor, which favors apoptosis.

TMA was stained with hematoxylin and eosin to verify histopathological findings.

Antibodies and Immunohistochemistry

Immunohistochemical staining using the avidin-biotin-peroxidase technique was performed for each antibody (pmTOR, pERK, p4E-BP1, eIF4E, peIF4E and p53). The anti-p53 antibody was DO-7 clone, which mainly detects mutated p53 but may also detect wild-type p53 when it is highly expressed in cells. Sections (5 μm) were cut from the TMAs and placed on poly-L-lysine coated glass slides. Sections were deparaffinized by xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked by immersing sections in 0.1% hydrogen peroxidase in absolute methanol for 20 minutes.

For antigen retrieval the tissue sections were heated in a pressure cooker in 10 mmol/l citric acid monohydrate, pH 6.0, for 5 minutes and then incubated with primary antibody at room temperature. Table 1 lists primary antibodies, dilutions and incubation times. Immunohistochemistry was performed with the Benchmark® XT. All slides were counterstained with hematoxylin, dehydrated and mounted. Positive and negative controls were used as previously reported.[18,19] [T1]

Each slide was assessed independently by two of us (CFP and IdT). Discrepancies were resolved by concurrent reexamination by the 2 investigators using a 2-headed microscope. Protein expression was evaluated in a semi-quantitative manner with expression levels shown as the percent of positive cells and staining intensity using the equation, H-score = 1 × (weak staining percent) + 2 × (moderate staining percent) + 3 × (intense staining percent) with ranking ranging between 0 and 300.[20] The core with the highest score was selected for analysis.

pmTOR protein and eIF4E were diffusely expressed in the cytoplasm of neoplastic epithelial cells. In contrast,

Table 1. *Primary antibodies and dilutions*

Protein	Phosphorylation Site	Source	Antibody	Dilution	Incubation (mins)
pmTOR	Ser2448	Cell Signaling Technology®	Rabbit monoclonal	1/100	60
pERK	Thr202/Tyr204	Cell Signaling Technology	Rabbit polyclonal	1/200	60
p4E-BP1	Thr70	Cell Signaling Technology	Rabbit polyclonal	1/50	60
eIF4E	—	Cell Signaling Technology	Rabbit polyclonal	1/75	60
peIF4E	Ser209	Abcam®	Rabbit monoclonal	1/50	60
p53	—	Ventana®	Mouse monoclonal	Prediluted	16

pERK, p4E-BP1 and peIF4E were observed in the cytoplasm or nucleus of neoplastic epithelial cells. Immunohistochemical staining for p53 was nuclear only (fig. 2). [F2]

Statistical Analysis

Differences in expression between normal samples and matched tumors were tested using the nonparametric Wilcoxon test. Immunohistochemical H-scores of different tumor characteristics were compared by the Mann-Whitney U test. The chi-square test was used to assess the association between p53 (categorized as 150 or less vs greater than 150) and the presence of hrHPV infection. Kaplan-Meier plots with the log rank test were used to evaluate cause specific survival. Univariate and multivariate analyses were done of prognostic variables for recurrence, lymph node metastasis or cause specific death using the Cox proportional hazards method. For these tests over expression was considered when the H-score of each marker was greater than the median H-score of that marker for all invasive penile SCCs. Statistical significance was considered at $p < 0.05$ and statistical analysis was done using SPSS®, version 18.

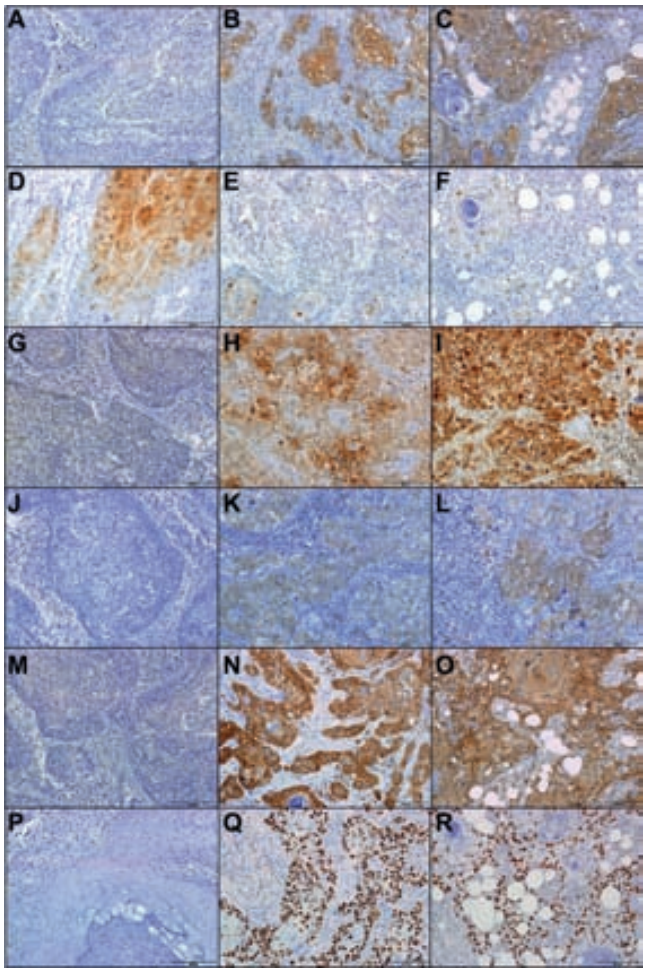

Figure 2. Representative penile SCC immunohistochemical results. Low grade pmTOR negative penile SCC (*A*). High grade pmTOR positive penile SCC (*B*) and matched metastatic lymph node (*C*). pERK positive staining of well differentiated penile SCC (*D*). low pERK expression in poorly differentiated penile SCC (*E*) and matched metastatic lymph node (*F*). Negative p4E-BP1 penile SCC (*G*). Positive p4E-BP1 penile SCC (*H*) and matched metastatic lymph node (*I*). Negative eIF4E tumor (*J*), same as *G*. Positive eIF4E penile SCC (*K*) and matched metastatic lymph node (*L*). Low grade negative peIF4E penile SCC (*M*). Positive peIF4E high grade penile SCC (*N*) and matched metastatic lymph node (*O*). Low grade p53 negative penile SCC (*P*). High grade p53 positive penile SCC (*Q*) and matched metastatic lymph node (*R*). Reduced from ×200.

RESULTS

Clinicopathological Data

Median patient age was 70 years (range 40 to 96). Two patients were immunosuppressed due to HIV infection and another was a liver transplant recipient on tacrolimus and mycophenolic acid treatment. Tumors were pT1 in 30 patients (48.4%), pT2 in 15 (24.2%) and pT3 in 17 (27.4%). In 4 patients the pT category could not be evaluated. Grade was 1, 2, 3 and 4 in 23 (34.3%), 22 (32.8%), 20 (29.9%) and 2 cases (3%), respectively. Lymph node metastasis was noted at diagnosis or during followup in 18 patients (26.9%). By the end of the study 10 patients (14.9%) had died of the disease. Median followup was 27 months (range 1 to 247).

Activated pmTOR Signaling Pathway Was Increased During Penile SCC Tumorigenesis

To assess the possible role of the mTOR molecular pathway in penile carcinogenesis we evaluated the immunohistochemical expression of several activated factors implicated in this pathway (pmTOR, pERK, p4E-BP1, eIF4E and peIF4E) in penile carcinoma and normal penile tissues. Expression of these molecules was evaluated in 67 penile carcinomas and compared with matched adjacent nontumor samples. The expression of pmTOR, p4E-BP1, eIF4E and peIF4E was significantly increased in carcinoma samples. In contrast, pERK expression did not differ between carcinoma specimens and adjacent normal tissues. p53 was negative in all normal tissues but positive in 32 cases (48%) with a wide range of staining intensities (table 2). [T2]

Activated pmTOR, peIF4E and p53 Expression Was Associated with Aggressive Tumors

H-scores of all 67 tumors were compared with different clinicopathological parameters. pT1 tumors had lower pmTOR expression than tumors greater than pT1 ($p = 0.028$). In contrast, pERK and p4E-BP1 expression was significantly stronger in pT1 tumors than in tumors greater than pT1 (table 2). p53 expression was stronger in poorly differentiated tumors ($p = 0.016$, table 2).

Table 2. *Active immunohistochemical expression of markers in 67 penile tumor samples with matched adjacent normal tissues containing normal keratinized epithelium, and pT stage and differentiation grade immunohistochemical H-scores*

	Median H-score (p25-p75)					
	pmTOR	pERK	p4E-BP1	eIF4E	peIF4E	p53
Nontumor epithelium	0 (0-0)	0 (0-20)	50 (0-90)	0 (0-0)	0 (0-60)	0 (0-0)
Invasive penile SCC	50 (11-80)	5 (0-35)	140 (100-200)	50 (10-80)	90 (60-130)	5 (0-42)
p Value (Wilcoxon matched pairs test)	<0.001	0.447	<0.001	<0.001	<0.001	<0.001
pT1	30 (5-70)	20 (0-50)	160 (115-222)	50 (20-90)	100 (50-140)	1.5 (0-32)
pT greater than 1	70 (28-90)	0 (0-5)	100 (50-147)	40 (10-60)	90 (60-125)	12.5 (0-85)
p Value (Mann-Whitney U test)	0.028	0.001	0.012	0.191	0.876	0.195
G1/G2	60 (25-80)	10 (0-50)	140 (92-185)	40 (10-60)	80 (50-111)	0 (0-37)
G3/G4	30 (4-100)	2 (0-18)	130 (100-217)	50 (20-90)	100 (60-135)	20 (2-205)
p Value (Mann-Whitney U test)	0.385	0.185	0.843	0.221	0.190	0.016

A significant positive association was noted for pmTOR, peIF4E and p53 expression in 18 primary tumor cases in which lymph node disease developed compared to the 46 primary tumors without metastasis ($p = 0.05$, 0.006 and <0.001, respectively, fig. 3). This suggested that the 3 molecules may be predictive markers of metastatic potential in primary penile SCC. In support we found significant co-expression of these markers in primary tumors and their corresponding lymph node metastases in 8 cases (fig. 2).

p53 and peIF4E over expression was associated with disease related death on Kaplan-Meier plots ($p <0.001$ and 0.053, respectively, fig. 4). Univariate analysis of clinicopathological and immunohistochemical variables associated with poor prognosis, including recurrence, lymph node metastasis or cause specific death, revealed that pT stage, p53 and peIF4E were statistically significant (p = 004, 0.002 and 0.016, respectively). Only pT stage and p53 remained in the multivariate model ($p = 0.009$ and 0.016, respectively), while peIF4E was marginally significant ($p = 0.086$, table 3).

pmTOR and Intense p53 Expression Were Associated with HPV Negative Tumors

We previously noted the presence of HPV DNA using a highly sensitive polymerase chain reaction technique based on SPF10 primers, followed by a general enzyme immunoassay for HPV types in our cohort.[3] Of 67 invasive penile SCCs 18 (27%) were positive for hrHPV infection, including subtype 16 in 16. We also detected 2 cases positive for HPV58 and 1 positive for HPV33. We compared the H-scores of all studied molecules with the presence of hrHPV infection and only pmTOR expression significantly correlated with HPV negativity ($p = 0.006$). Interestingly, intense expression of p53 (H-score greater than 150), which has been used as a surrogate marker of *TP53* mutation,[21] also correlated inversely with hrHPV infection ($p = 0.043$).

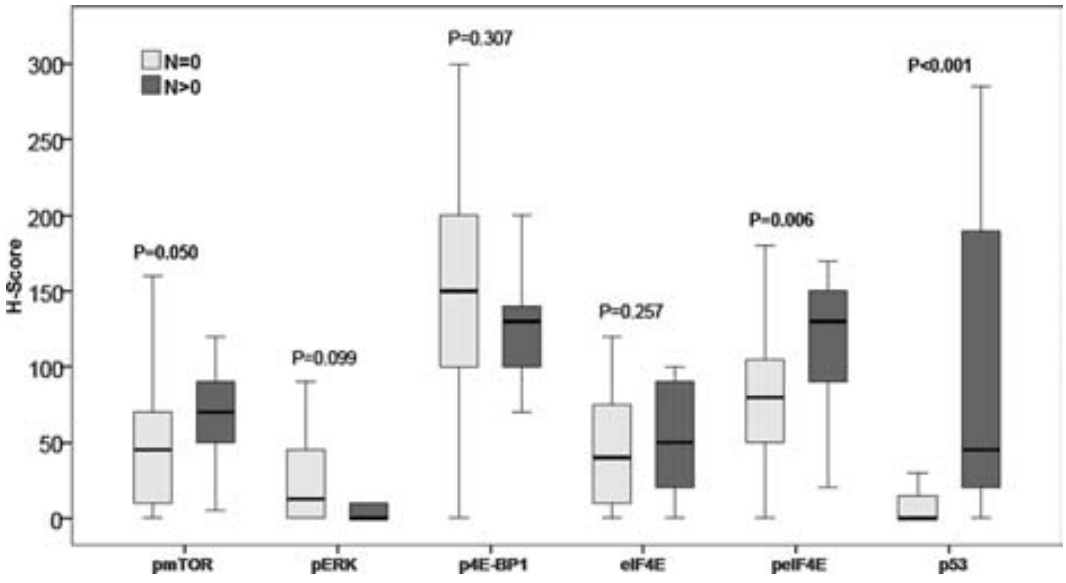

Figure 3. Immunohistochemical marker expression in primary tumors associated with lymph node disease (*N>0*) and primary tumors without metastasis (*N=0*) by Mann-Whitney U test (median p25-p75).

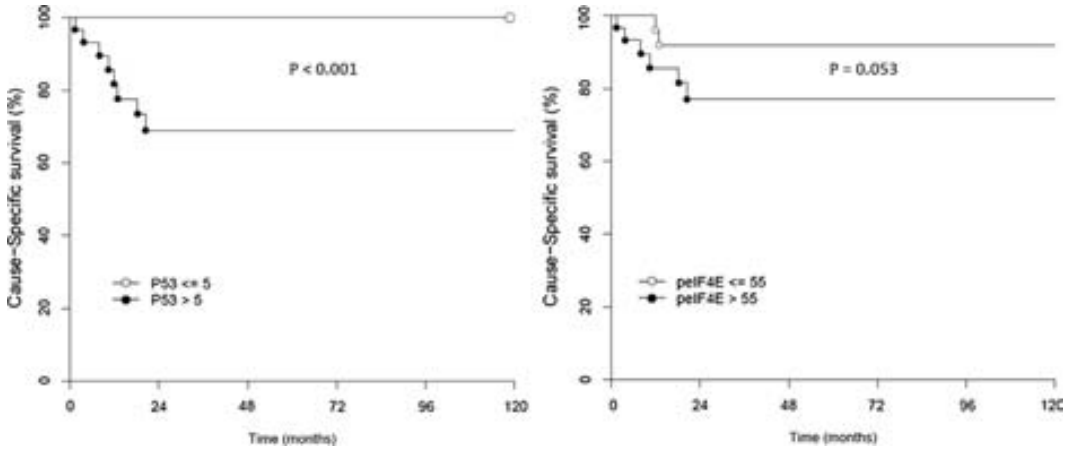

Figure 4. Cause specific survival curves in relation to p53 and peIF4E expression (Kaplan-Meier and log rank tests)

DISCUSSION

The regulation of mTOR signaling is frequently altered in tumors.[10] Therefore, mTOR is currently under investigation as a potential target for anticancer therapy.[22] Groups investigated the biological role of pmTOR in the carcinogenesis and development of head and neck SCC but evidence is lacking of its role in penile SCC.[12] We found that the activated mTOR signaling pathway is over expressed during penile SCC tumorigenesis. We also found that pmTOR expression was associated with higher tumor stage and a higher risk of lymph node disease. Our studies suggest that pmTOR might be a candidate biomarker for aggressive penile SCC and a therapeutic target in patients at high risk for disease progression. However, confirmation of our results is needed in larger series.

pERK1/2 expression is the hallmark of activated MAPK signaling. In the current study the ERK-MAPK pathway was not activated in tumor cells compared to normal samples. In normal endometrium MAPK signaling has physiological importance, contributing to normal cell proliferation.[23] We found no studies of MAPK signaling in normal squamous cell epithelium. However, in accordance with our results it seems that MAPK signaling would be more important for normal proliferation than cancer progression in penile epithelium.

HRAS or *KRAS* gene mutations were reported in 10% of penile carcinomas.[16] Deregulated Ras proteins usually induce constitutive activation of downstream kinase cascades. However, studies of endometrial cancer showed that MAPK activation does not necessarily result from *RAS* mutations.[23] To our knowledge our novel finding in this study is that pERK is more expressed at earlier tumor stages. Although increased pERK expression is associated with poor prognosis in several tumor types, such as breast and prostate cancer,[24] it is also associated with favorable prognosis in some tumors, including endometrial, breast and ovarian cancer.[23,25] Different immunohistochemical assay conditions could be responsible for these discrepancies among tumor types.

It was proposed that 4E-BP1 is a final funnel factor of signaling pathways leading to cell growth.[18] While 4E-BP1 is mainly phosphorylated through the mTOR pathway, it has another 6 phosphorylation sites where other kinases have a role, such as pERK.[18] The level of p4E-BP1 expression and its downstream effector eIF4E are also associated with the aggressiveness of some tumors, such as breast, ovary, prostate, cervix and colon carcinoma.[18,26] However, our data showed no p4E-BP1 over expression at advanced stages of penile SCC. Most tumors had p4E-BP1 and eIF4E over expression, indicating that the 2 molecules are involved in invasive penile SCC carcinogenesis, although we could not determine whether they were associated with aggressive behavior. p4E-BP1 expression was higher in pT1 tumors, suggesting that 4E-BP1 phosphorylation is an early event in penile carcinogenesis.

Table 3. *Univariate and multivariate analysis of clinicopathological and immunohistochemical variables associated with poor prognosis*

	Univariate		Multivariate	
	HR (95% CI)	p Value	HR (95% CI)	p Value
pT	20.3 (2.67–154.16)	0.004	15.2 (1.96–118.1)	0.009
Grade	2.09 (0.83–5.28)	0.120	–	–
hrHPV	0.51 (0.15–1.76)	0.286	–	–
pmTOR	2.06 (0.79–5.35)	0.140	–	–
pERK	0.41 (0.14–1.16)	0.094	–	–
p4E-BP1	0.36 (0.12–1.1)	0.073	–	–
eIF4E	1.15 (0.44–3.03)	0.778	–	–
peIF4E	3.99 (1.29–12.34)	0.016	2.79 (0.87–9.03)	0.086
p53	23.37 (3.08–177.19)	0.002	12.32 (1.6–95.11)	0.016

The oncogenic activity of eIF4E correlates with its ability to become phosphorylated. MNK1 phosphorylates eIF4E at Ser209 upon activation by mitogenic and/or stress stimuli mediated by ERK1/2 and p38-MAPK. peIF4E function has not been fully addressed but it also seems to be associated with poor outcome in some tumors, such as prostate and lung carcinoma.[27,28] While p4EB-P1 and eIF4E were not associated with a worse prognosis, we observed a significant association between peIF4E over expression and indicators of aggressiveness, suggesting that eIF4E phosphorylation may be induced via different molecular pathways that were not evaluated in this study.

A distinct subset of penile SCCs has been linked to hrHPV infection, which leads to deregulation of the retinoblastoma pathway and p16 over expression.[3] There is also evidence that wild-type p53 is transcriptionally inactive in keratinocytes that express hrHPV E7 oncoprotein.[29] When there is functional p53, cells can enter cell cycle arrest. Although a strong, diffuse staining pattern is considered indicative of the mutant p53 encoding gene *TP53*,[21] wild-type p53 may also be slightly expressed in response to an oncogenic stress such as HPV infection. In penile cancer the p53 expression rate is 41.5% to 89%.[6,7,30] In accordance with several previous reports, we found detectable p53 staining in 47% of tumors and those cases strongly correlated with higher metastatic risk and a poor survival rate, corroborating that p53 is an excellent marker of poor outcome in penile SCC.[8] When we considered only cases with intense p53 expression, we detected an inverse correlation with hrHPV infection, suggesting that *TP53* mutations may have a role in hrHPV negative tumors. This may also explain the better prognosis associated with hrHPV positive tumors, which probably do not harbor a *TP53* mutation.[3] Interestingly, we also found an inverse correlation between pmTOR expression and hrHPV infection, suggesting that the PI3K/Akt/mTOR pathway would also be a signaling pathway mainly involved in HPV negative tumors.

Our study has some limitations. 1) A retrospective study has inherent biases and potential errors, and a 5-year followup was not achieved in all cases. Charting errors and omissions may also have occurred, especially considering that our study involved 2 centers. 2) The rarity of penile SCC hampers the collection of a large series and the low number of patients who died may have been responsible for the limited statistical power of the study. 3) Immunohistochemical evaluations were performed using 2 mm cylinders, which could render nonrepresentative results in tumors with heterogeneous areas of protein expression.

Penile SCC is a rare but aggressive tumor with no optimal treatment when metastasis develops. There is a great need for novel, effective therapeutic strategies to improve the overall survival of patients with advanced penile SCC. A promising approach consists of receptor mediated, carcinoma targeted therapy. In our study pmTOR, peIF4E and p53 had prognostic significance for aggressive behavior. Targeted therapy, such as the MNK inhibitor CGP57380,[29] may be a potential treatment for aggressive penile SCC. The role of other pmTOR targeting molecules, including the rapamycin analogues deforolimus, everolimus and temsirolimus, also merit further investigation.[22]

CONCLUSIONS

Overall, our results suggest that mTOR signaling may contribute to penile SCC progression and aggressive behavior. Over expression of peIF4E and p53 is a prognostic factor of poor survival in penile SCC cases. p53 and pmTOR seem to be especially over expressed in hrHPV negative tumors. The role of mTOR inhibitors or chemical compounds that prevent eIF4E phosphorylation, such as MNK inhibitors, warrants further investigation to treat aggressive penile SCC.

ACKNOWLEDGMENTS

Tania Lobato assisted with TMAs. Teresa Moliné assisted with immunohistochemistry. Clinical care was provided by B. Lloveras, F. Xavier Bosch, Silvia de Sanjosé and Jo Ellen Klaustermeier, the RIS HPV TT and HPV VVAPO study groups from ICO, and Jose Placer, Carlos Salvador and Juan Morote, Hospital Vall d'Hebron and Hospital del Mar. The Tumor Bank of Vall d'Hebron University Hospital Biobank provided half of the study samples.

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Discussion

"Believe those who are seeking the truth. Doubt those who find it."

Andre Gide,

Nobel Prize in Literature, 1947.

In our first article we aimed to provide novel data about de prevalence of HPV infection in penile carcinomas from a Spanish Mediterranean population, including PeIN and invasive SCCs. In order to achieve this objective, we designed a retrospective study. A cohort of 82 patients was selected from the files of the pathology departments of 2 tertiary centers from Barcelona participating in the study (Hospital Vall d'Hebron and Hospital del Mar). From this cohort, 69 and 13 patients presented penile SCC and PeIN, respectively. We also obtained normal-appearing foreskin from 15 healthy individuals as control samples. Following a systematized protocol, the following data were recorded for each patient by reviewing their clinical charts: age, immunosuppression status, recurrence, presence or absence of lymph node metastases and clinical evolution.

For each patient, representative formalin-fixed, paraffin embedded (FFPE) tissue samples and all the slides available were collected. Those slides were reviewed and histological diagnosis was reevaluated in all cases by light microscopy examination. General histologic features, including histologic subtype, grade of differentiation, and TNM stage, were evaluated in each representative sample, according to the World Health Organization and TNM classification 2009.

Before handling those FFPE blocks, we sent them for HPV detection and typing at the Infection and Cancer Laboratory, Duran i Reynals, Institut Català d'Oncologia from Barcelona because they were developing an international study for HPV detection in vulva, vagina, anus, penis and oropharynx carcinomas (HPV VVAPO) that was performed under strict conditions to avoid contamination and false positives.

Later on, ten TMAs were constructed. For TMA construction, we used hematoxylin and eosin stained slides from each tumor block to select, when available: 2 representative invasive tumor areas, 2 preinvasive lesion areas (for both PeIN and adjacent PeIN/epidermal hyperplasia to invasive SCCs [adjPeIN]), 2 normal tissue areas, and 2 representative metastatic tissues from a lymph node when available (8 cases). We punched 2-mm tissue cylinders from the marked tumor area of each block and brought them into a recipient paraffin block using a precision instrument. From those TMAs we cut 4 mm sections and immunostained them with $p16^{INK4a}$ using standard heat-induced antigen retrieval methods and the ABC kit. Immunostaining was evaluated and sections were scored semiquantitatively by H-score from 0 to 300. Our cut-off point for positive

cases was arbitrarily decided to be H-score of 50 because we found no cases with H-score between 50 and 150.

In this study, we detected hrHPV in 28% of penile SCCs and in 90% of PeIN. Similar hrHPV prevalence rates have recently been obtained by Cubilla et al[63] using a similar technical approach. These results suggest that there is a group of penile SCC in which HPV would play a triggering role and give support to the bimodal etiopathogenic hypothesis that distinguishes two different subsets of penile SCC (HPV-related penile SCC and penile SCC non-related to HPV).[25,31,45,63,64,68-72]

Several studies have shown that HPV-16 is the predominant hrHPV in penile SCC both in developed and undeveloped countries.[8,73,74] However, variable prevalence rates have been observed in different geographic populations. HPV-18 represents the second most prevalent HPV type in penile SCC in countries such as Thailand and Brazil,[6,9,75] but its detection is uncommon in European countries. HPV-16 was the most frequent serotype detected in our cohort (80%), while HPV18 was absent. In contrast to other studies detecting multiple HPV subtypes in a significant number of cases (from 20%-50%),[6,45,53,76] we have observed such phenomenon in only 6.5% of cases. The significance of multiple HPV subtypes is not fully understood, and the potential role of the different detected HPVs as carcinogens or coinfectors has not been elucidated.

We have also observed significant differences in the prevalence of HPV infection depending on the histologic subtype: basaloid, mixed usual/basaloid, and warty carcinomas were found to be consistently associated with the presence of hrHPV infection. Conversely, hrHPVs were rarely detected in verrucous and sarcomatoid carcinomas. Our results are consistent with other studies on penile SCC and on anal and vulvar carcinomas.[73,77-79] However, in the review article by Miralles-Guri et al[8] the reported prevalence of hrHPV in the usual type was significantly higher than in our population, probably because of geographic differences, heterogeneous detection methods, or both.

Several authors have reported an association between high histologic grade penile SCC and hrHPV positivity.[63,80] In our series, 77% of basaloid or mixed tumors were high-grade tumors and were also positive for hrHPV. When considering only usual-type

tumors in the analysis, no association between hrHPV and high histologic grade could be noted.

The role of hrHPV as a marker of good prognosis in other SCC of the genital and extragenital mucosal areas has previously been suggested.[45,46,81-87] We have also detected a trend toward a better prognosis in the hrHPV-positive group, although not reaching statistical significance. Interestingly, all patients with usual-type penile SCC-related death had hrHPV-negative tumors.

HrHPV's E7 oncogene promotes the degradation of the pRB, leading to a $p16^{INK4a}$ overexpression by the loss of a negative feedback mechanism. $P16^{INK4a}$ overexpression correlates with hrHPV in some anatomic locations such as the cervix, oropharynx, and sun-exposed skin.[45,62,81,83,88] Overexpression of $p16^{INK4a}$ is also frequent in penile SCC, especially in hrHPV-positive tumors.[31,63,53] The best method to evaluate $p16^{INK4a}$ overexpression is still an issue of controversy. Several methodological approaches have been postulated, such as staining topography (nuclear and/or cytoplasmic), percentage and/or distribution of positive cells, staining intensity, and pattern score, but no comparative studies have been reported.[31] Good interobserver correlation was demonstrated in our study and a clear cut-off point using the H-score system was defined. In addition, we have been able to establish good correlation between $p16^{INK4a}$ overexpression and hrHPV infection, as previously reported, which argues in favor of the reliability of our method.

Tumors that were negative for hrHPV but showed $p16^{INK4a}$ overexpression were uncommon. Possible explanations for such cases may include a technical failure to detect low or degraded HPV DNA in tumor samples or an activation of the p16 pathway through a mechanism not related with HPV infection. On the other hand, the low number of hrHPV-positive cases with an absence of $p16^{INK4a}$ expression suggests that allelic loss and/or mutations in the $p16^{INK4a}$ gene were uncommon phenomena in our series, unlike previously reported.[62] The frequent observation of $p16^{INK4a}$ negativity in control samples and normal epithelial areas adjacent to the tumors also supports the usefulness of $p16^{INK4a}$ immunohistochemical expression as a possible surrogate for hrHPV infection.

The positivity for $p16^{INK4a}$ has been proposed as a good prognostic marker in head and neck and in oropharynx SCC.[89,90] Some authors have suggested that $p16^{INK4a}$ plays a major role not only in suppression of cell division but also in suppression of lymphangiogenesis and lymphatic metastasis.[89]As far as we are concerned, the role of $p16^{INK4a}$ immunoexpression as a marker for survival in penile SCC has only been evaluated in one previous study and an association with a better overall survival was detected.[68] In our study, $p16^{INK4a}$-positive tumors were significantly less differentiated than $p16^{INK4a}$-negative tumors, but showed a better overall survival, suggesting a protective role of $p16^{INK4a}$ in penile SCC. It is tempting to hypothesize that hrHPV may be a triggering factor in the development of a subset of penile SCC, but would concomitantly induce a $p16^{INK4a}$-mediated protection mechanism.

Transformation of PeIN into invasive penile SCC has been reported in 5% to 33% of cases. The prevalence of hrHPV infection in PeIN ranges from 75% to100%.[4,6,9,22,73,91] We have analyzed a large series of PeIN (both isolated and adjacent to invasive penile SCC) and several conclusions can be outlined: (1) PeIN can easily be divided histologically into undifferentiated and differentiated; (2) most undifferentiated PeIN show $p16^{INK4a}$ overexpression and harbor hrHPV infection; (3) basaloid or warty penile SCC are frequently associated with undifferentiated adjPeIN; and (4) a subgroup of usual-type penile SCC (24%) have an undifferentiated adjPeIN and most of these invasive tumors are hrHPV positive. Our findings suggest that hrHPV positive usual-type penile SCC probably develop over undifferentiated hrHPV-positive/$p16^{INK4a}$ -positive PeIN.

The second part of our research work was focused on molecular pathways implicated in the development and progression of invasive penile SCC. For this issue, we evaluated immunohistochemical expression of several molecules implicated in mTOR pathway on TMA (phosphor-mTOR [pmTOR], phosphor-extracellular-regulated kinase [pERK], phosphor- 4E-BP1 [p4E-BP1], eukaryotic translation initiation factor 4E [eIF4E], peIF4E and p53). The expression of different markers was also evaluated in a semiquantitative manner obtaining a H-score between 0 and 300.

The regulation of mTOR signaling is frequently altered in tumors[10] and its pathway is currently under investigation as a potential target for anti-cancer therapy.[92] Some studies

have investigated the biological role of mTOR in the carcinogenesis and the development of head and neck SCC, but there is lacking evidence of its role in penile SCC.[93] We found that activated mTOR signaling pathway is overexpressed during squamous cell penile tumorigenesis. Moreover, we also found pmTOR expression to be associated with higher tumoral stages and a higher risk of lymph node disease development. Therefore, our studies suggest that pmTOR might be a candidate biomarker of aggressive penile SCC, and may be a therapeutic target in patients at high risk of disease progression, although confirmation of our results with larger series are necessary. Interestingly, we also found an inverse correlation between pmTOR expression and hrHPV infection, which suggests that the PI3K/Akt/mTOR pathway would also be a signaling pathway mainly involved in hrHPV-negative tumors.

The expression of pERK1/2 is the hallmark of activated mitogen-activated protein kinase (MAPK) signaling, and its immunohistochemical expression was also analyzed in our study. In the present study, we found that ERK-MAPK pathway was not activated in tumoral cells when compared to normal samples. It has been demonstrated in normal endometrium that MAPK signaling has a physiological importance contributing to normal proliferation of cells.[94] We haven't found studies focused on MAPK signaling in normal squamous cell epithelium, but, in accordance with our results, it seems that MAPK signaling would be more important for normal proliferation than cancer progression in penile epithelium. Mutations of *HRAS* or *KRAS* genes have previously been reported in 10% of penile carcinomas.[39] Usually, deregulated RAS proteins induce constitutive activation of downstream kinase cascades. However, studies on endometrial cancer have shown that MAPK activation does not necessarily result from *RAS* mutations.[94] The novel finding we obtained in the present study is that pERK is more expressed in earlier tumoral stages. Although increased pERK expression has been associated with poor prognosis in several tumor types, such as breast and prostate cancer,[95] it has also been associated with favorable prognosis in some tumors such as endometrial, breast or ovarian cancers.[94,96] Different assay conditions of immunohistochemistry could be responsible for these discrepancies between different tumor types.

4E-BP1 has been proposed as a final funnel factor of signaling pathways leading to cell growth.[97] 4E-BP1 is mainly phosphorylated through the mTOR pathway, but it has other

6 phosphorylation sites where other kinases such as pERK play a role.[97] The level of p4E-BP1 expression and its downstream effector eIF4E have also been associated with aggressiveness of some tumors such as breast, ovary, prostate, cervix and colon carcinomas.[97,98] However, although we found p4E-BP1 to be significantly overexpressed in tumoral cells, when comparing with normal tissues, our data does not show overexpression of p4E-BP1 in advanced stages of penile SCC. Most tumors had p4E-BP1 and eIF4E overexpression, indicating that both molecules are involved in invasive penile SCC carcinogenesis, but we could not demonstrate that they were associated with aggressive behavior. Contrary, expression of p4E-BP1 was higher in pT1 tumors, suggesting that 4E-BP1 phosphorylation is an early event in penile carcinogenesis.

The oncogenic activity of eIF4E correlates with its ability to become phosphorylated. Upon activation by mitogenic and/or stress stimuli mediated by ERK1/2 and p38-MAPK, MAPK interacting protein kinase 1 (MNK1) phosphorylates eIF4E at Ser209. The function of peIF4E has not been fully addressed, but it also seems to be associated with poor outcome in some tumors, such as prostate and lung carcinomas.[99,100] While p4EB-P1 and eIF4E were not associated with a worse prognosis, we observed a significant association between peIF4E overexpression and indicators of aggressiveness, suggesting that phosphorylation of eIF4E may be induced through different molecular pathways that were not evaluated in this study.

As we have previously reported, there is a distinct subset of penile SCCs which has been linked to hrHPV infection leading to deregulation of the pRB pathway and $p16^{INK4a}$ overexpression. There is also evidence that in keratinocytes expressing hrHPV E7 oncoprotein, wild type p53 is transcriptionally inactive.[101] In cases where there is functional p53, cells are able to enter cell cycle arrest. Although a strong and diffuse staining pattern is considered as indicative of the mutant p53 encoding gene *TP53,*[102] wild-type p53 may also be slightly expressed in response to an oncogenic stress such as HPV infection. In penile cancer, rates of p53 expression range from 41 to 89%.[47,103,104] In accordance with several previous reports, we found detectable p53 staining in 47% of tumors, and those cases strongly correlated with a higher metastatic risk and poor survival rate, corroborating that p53 is an excellent marker of poor outcome in penile SCC.[105] In accordance with other authors, when we considered only those cases with an

intense expression of p53, an inverse correlation with hrHPV infection was detected, suggesting that *TP53* mutations may play a role in hrHPV-negative tumors.[106] This may also explain the better prognosis associated to hrHPV-positive tumors, which probably do not harbor *TP53* mutations.

Penile SCC is an infrequent but aggressive tumor with no optimal treatment when metastases develop. There is a great need for novel and effective therapeutic strategies to improve overall survival for patients with advanced penile SCC. A promising approach consists of receptor mediated carcinoma-targeted therapy. In our study, pmTOR, peIF4E and p53 had prognostic significance for aggressive behavior. Targeted therapies, such as the MNK inhibitors to prevent eIF4E phosphorylation, may turn out to be potential treatments of aggressive penile SCC. The role of other pmTOR targeting molecules, such as rapamycin analogues deforolimus, everolimus and temsirolimus, also merit further investigation.[92]

Finally, another aspect of the pathogenesis of penile SCC is the possible role of somatic gene mutations, an issue that has been only rarely studied. The role of *KRAS, PIK3CA* status or other molecular biomarkers as prognostic or predictive factors in penis cancer patients remains unclear. A cohort of 29 penile SCCs were found to harbor somatic mutations of the PI3K and RAS pathways in 29% and 10% of tumors, repectively.[39] Results based on this study also showed that *KRAS* mutations were found in pathologically characterized advanced tumors, while *PIK3CA* mutations were found distributed across all stages. However, in another cohort of 150 cases, only one *KRAS* and no *BRAF* mutations were found.[107] A large sample size is required to establish that a gene mutation has a significant impact for the patient prediction of prognosis. Since the effect of anti-EGFR treatment highly depends on intact *KRAS*, these findings are very meaningful for penile cancer treatment. Depending in this line of investigation, we are developing new studies on this Mediterranean population of penile SCCs. We are analyzing somatic mutations of genes downstream of EGFR in this cohort of PeINs and penile SCCs, especially the PI3K and RAS pathways. Those activations could be associated with resistance to EGFR inhibitors as well as with prognosis. Conducting such large studies with the standard sequencing technologies is too time consuming and too expensive to be practical for clinical studies. We are therefore using the Sequenom technique, a cost-effective and high throughput methodology that detects frequent and

infrequent cancer mutations genes in a large number of samples, that works with small amounts of degraded DNAs isolated from FFPE samples. These results have been recently presented in the AEDV Congress 2013.

Another important biological process of tumor progression includes invasion and metastatic spread which are believed to be highly correlated to changes within the microenvironment of the tumor and the epithelial-mesenchymal transition (EMT). The essential part of the invasion is the breakdown of the cell-to-cell adhesion in the tumor invasion front. This enables tumor cells to invade through the basement membrane. Until now, there is only limited data about the role of EMT in penile carcinoma.[33] Our group has also worked on this issue, and a manuscript with the main results is under second review in the Journal of Human Pathology (Emili Masferrer et al).

Our studies had some limitations that should be pointed out. First, there are the inherent biases and potential errors of retrospective studies and a 5-year follow-up period was not achieved in all cases. Charting errors and omissions may also have occurred, especially considering that our studies involved two different centers. Secondly, the rarity of penile SCC hampers the collection of a large series of cases. Thirdly, we could not evaluate HPV infection in all patients because of degradation of DNA in some cases. Finally, immunohistochemistry was performed in 2-mm cylinders, which could render non representative results in tumors with heterogeneous areas of protein expression.

Conclusions

"When you make the finding yourself – even if you're the last person on Earth to see the light – you'll never forget it."

Carl Sagan,

Astronomer, 1934-1996.

1. HrHPV infection is present in 36.4% of our Spanish Mediterranean cohort of patients with penile SCC (28% of invasive penile SCC and in 90% of PeIN). This infection is more frequent in some histologic subtypes, such as basaloid or warty penile SCC. hrHPV infection play a special role in a subset of usual-type penile SCC that progress from undifferentiated PeINs and have a better prognosis.

2. HrHPV infection in penile SCC can be reliably predicted by the observation of $p16^{INK4a}$ overexpression. $P16^{INK4a}$ immunohistochemical expression represents a useful and informative diagnostic feature in penile SCC, that should be incorporated as a routine diagnostic tool in the diagnosis and evaluation of penile SCC samples.

3. mTOR signaling may contribute to tumor progression, with a special role in hrHPV-negative tumors. This signaling pathway also seems to lead to a more aggressive penile SCC (advanced pT stages and lymph node development). Immunohistochemical expression of peIF4E has shown to be a predictor of poor outcome (lymph node development, recurrence or death).

4. The role of mTOR inhibitors or chemical compounds that prevent the phosphorylation of eIF4E, such as MNK inhibitors, to treat aggressive penile SCC deserves further investigation.

5. Abnormal function of p53 is the best marker of poor outcome and its strong expression correlates with hrHPV-negative tumors.

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Annex

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REVISI N

Carcinoma escamoso de pene

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Recibido el 30 de abril de 2011; aceptado el 12 de agosto de 2011
Disponible en Internet el 17 de enero de 2012

PALABRAS CLAVE
Carcinoma escamoso;
Carcinoma epidermoide;
Carcinoma *in situ*;
Eritroplasia de Queyrat;
Enfermedad de Bowen;
Pene;
Revisi n

Resumen El carcinoma escamoso de pene (CEP) es una neoplasia infrecuente en Europa, suponiendo un 0,7% de los tumores en varones. La mala higiene, no estar circuncidado, la infecci n por el virus del papiloma humano (VPH) y algunas dermatosis in amatorias cr nicas son los principales factores de riesgo. El VPH se detecta en un 70 100% de los CEP *in situ* y en un 30 60% de las formas invasivas, sobre todo en los tumores basaloides y condilomatosos. Los tumores *in situ* pueden tratarse de forma conservadora, pero requieren un seguimiento estricto, puesto que del 1 al 30% evolucionan a formas invasivas. En los CEP invasivos el tratamiento de elecci n es la cirug a. La irradiaci n pro l ctica de los ganglios inguinales est actualmente desaconsejada. Parece que el uso de la biopsia selectiva de ganglio centinela podr a ser til para disminuir la morbilidad asociada a la linfadenectom a inguinal pro l ctica. La supervivencia se relaciona directamente con la presencia de met stasis ganglionares. El conocimiento de las alteraciones moleculares y genot picas subyacentes abrir nuevas v as terap uticas.
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KEYWORDS
Squamous cell carcinoma;
Carcinoma in situ;
Erythroplasia of Queyrat;
Bowen disease;
Penis;
Review

Penile Squamous Cell Carcinoma

Abstract Penile squamous cell carcinoma (SCC) is uncommon in Europe, where it accounts for approximately 0.7% of all malignant tumors in men. The main risk factors are poor hygiene, lack of circumcision, human papillomavirus (HPV) infection, and certain chronic in ammatory skin diseases. HPV infection is detected in 70% to 100% of all penile in situ SCCs and in 30% to 50% of invasive forms of the disease, mainly basaloid and warty SCCs. In situ tumors can be treated conservatively, but close monitoring is essential as they become invasive in between 1% and 30% of cases. The treatment of choice for penile SCC is surgery. Inguinal lymph node irradiation is no longer recommended as a prophylactic measure, and it appears that selective lymph node biopsy might be useful for reducing the morbidity associated with prophylactic inguinal lymph node dissection. Survival is directly related to lymph node involvement. Improving our knowledge of underlying molecular changes and their associated genotypes will open up new therapeutic pathways.
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10.1016/j.ad.2011.08.008

Introducción

El cáncer de pene es excepcional en países desarrollados. La mayoría de tumores (98%) corresponden a carcinomas escamosos. Los principales avances sobre el carcinoma escamoso de pene (CEP) en los últimos años se han centrado en la identificación de factores de riesgo, en el conocimiento de las vías moleculares implicadas en su desarrollo y en la actualización de su estadificación. También hay avances en su manejo terapéutico, con tendencia a realizar una cirugía cada vez más conservadora que, minimizando el riesgo de recidiva, preserve la función sexual y urinaria del pene.

En nuestro medio el CEP predomina entre la sexta y la octava décadas de la vida, apareciendo dos tercios de los casos en mayores de 65 años[1]. La incidencia global oscila entre 0,1 y 0,7 casos por 100.000 varones. Se estima que en todo el mundo se diagnostican unos 4.000 nuevos casos cada año, lo que representa menos del 0,5% de todos los cánceres[1]. Concretamente, en España, la incidencia anual se sitúa entre el 0,7 y 1,5 casos/100.000 varones, representando aproximadamente un 0,7% de los tumores malignos en hombres. En otras zonas de Europa Occidental la incidencia es similar, pero en países como Uganda y Brasil es hasta 4 veces superior[1,2]. Estas amplias variaciones de incidencia estarían justificadas por diferencias socioeconómicas y culturales.

Factores de riesgo

Los principales factores de riesgo implicados en el desarrollo del CEP serían la mala higiene, no estar circuncidado, la infección por el virus del papiloma humano (VPH) y algunas dermatosis inflamatorias crónicas.

La mala higiene contribuye al desarrollo del CEP a través de la acumulación de esmegma y otros irritantes en el espacio balanoprepucial, así como a una mayor incidencia de infecciones bacterianas y/o candidiásicas[3].

La mayoría de CEP aparecen en varones no circuncidados[4]. La circuncisión neonatal tiene un papel preventivo[2,4,5], pero no se ha demostrado que realizada más tardíamente tenga el mismo efecto beneficioso. También es evidente que la fimosis, presente en un 40-85% de estos tumores, impide una higiene correcta del glande, contribuye a la inflamación crónica y favorece su desarrollo[6].

Se ha demostrado una relación directa entre la infección por el VPH y el CEP. Ambos se relacionan directamente con el número de parejas sexuales y la edad temprana de las primeras relaciones sexuales[2]. Sin embargo, mientras que un 95% de los cánceres de cérvix se relacionan con esta infección viral, esto es menos usual en los CEP. Es más frecuente en los carcinomas *in situ* (70-100% de los casos) que en las formas invasivas (30-60%) y varía de uno a otro subtipo histológico: alcanza el 70-100% en los CEP basaloides y condilomatosos y se sitúa solo en el 30% de los comunes[7,8]. El serotipo implicado con mayor frecuencia es el VPH 16 (69%)[7], si bien se han detectado otros serotipos oncogénicos, con especial relevancia del VPH 18[9].

La historia de condilomas acuminados, aunque estén causados por VPH de bajo riesgo oncogénico (VPHbr), aumenta la probabilidad de padecer un CEP entre 3 y 5 veces[10]. A este hecho posiblemente contribuyen las conductas sexuales de riesgo que muchas veces coexisten, con posibilidad de reinfectarse por otros VPH.

El tumor de Buschke-Löwestein, o condiloma gigante, a pesar de que en la literatura se ha clasificado como un carcinoma verrucoso, es mejor considerarlo una entidad separada. Las principales razones para ello son que tiene características clínico-patológicas distintivas, simulando clínica e histológicamente un condiloma, se relaciona con VPHbr (6 y 11), aparece a edades relativamente tempranas y prácticamente no tiene capacidad metastásica[2,11].

Otro proceso excepcional es la balanitis queratósica, micácea y pseudoepiteliomatosa de Lortat-Jacob. Afecta el glande de varones de edad avanzada no circuncidados. Al igual que el tumor de Buschke-Löwestein, se considera una lesión premaligna o de bajo grado de malignidad. No se ha relacionado con la infección por el VPH. Se inicia como una placa cubierta de escamas micáceas, plateadas, con un aspecto psoriasiforme, que puede hacerse francamente queratósica (fig. 1A). Histológicamente se observa desde una simple hiperplasia epitelial hiperqueratósica con mínima atipia celular hasta una lesión que simula un verdadero carcinoma verrugoso.

Otros factores de riesgo a tener en cuenta son las enfermedades inflamatorias crónicas del pene. Destacan el liquen escleroso (LE) y su forma más avanzada, la balanitis xerótica *obliterans*, caracterizada por una fibrosis constrictiva que afecta toda la circunferencia del prepucio, impidiendo su retracción. Un 6% de los LE de pene pueden desarrollar un CEP y, al revisar piezas quirúrgicas, hasta una tercera parte de los CEP asientan sobre un LE[12,13]. Por tanto, los pacientes con LE del pene deben seguir controles periódicos.

Como en otros carcinomas cutáneos, la inmunosupresión mantenida que sufren los pacientes trasplantados o los infectados por el virus de la inmunodeficiencia humana desempeña un papel fundamental en el aumento del riesgo y empeoramiento del pronóstico del CEP. Finalmente, existen estudios que han relacionado esta neoplasia con el hábito tabáquico, al igual que ocurre con el cáncer de vejiga y de cavidad oral[2,10,14].

Clínica e histología

Desde el punto de vista clínico-patológico hay dos situaciones bien diferenciadas, con implicaciones pronósticas y terapéuticas: el carcinoma *in situ* y el carcinoma infiltrante.

Carcinoma escamoso de pene *in situ*

El CEP se origina a partir de la progresión de una lesión precursora que puede subclasificarse según la intensidad y extensión de la atipia citológica y la presencia o no de VPH[5]. El aspecto clínico de estos carcinomas *in situ* puede ser muy variable, oscilando desde lesiones inaparentes que solo se desenmascaran si se aplica ácido acético hasta lesiones rojizas (eritroplasia de Queyrat [EQ] y enfermedad de Bowen genital [EBG]), blancas (leucoplasias) o marronáceas (papulosis bowenoide [PB]).

Las lesiones subclínicas se visualizan mediante penescopia tras aplicar ácido acético[15]. Se denominan lesiones planas del pene o lesiones «acetoblancas» y están

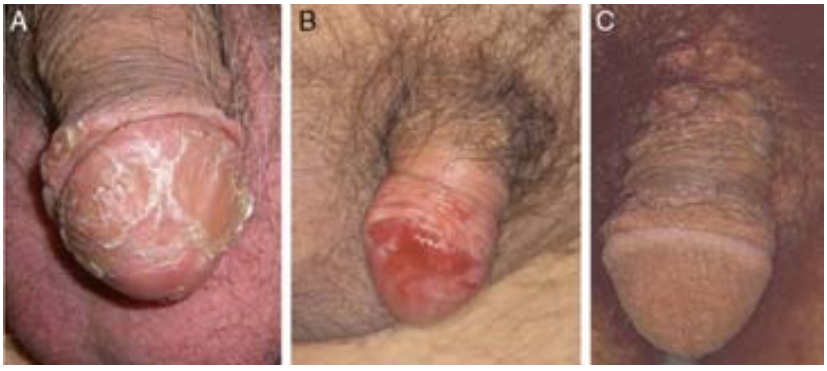

Figura 1 Aspecto de lesiones premalignas o *in situ*: A. Balanitis micácea pseudoepiteliomatosa. B Eritroplasia de Queyrat. C. Papulosis bowenoide.

relacionadas con la infección por VPH de alto riesgo oncogénico (VPHar). Son muy frecuentes, pues están presentes hasta en el 50-70% de las parejas sexuales masculinas de mujeres con neoplasia intraepitelial cervical (CIN) y en un 10-20% de los varones cuyas parejas no tienen CIN. Contienen grandes cantidades de virus y son muy contagiosas. La histología suele mostrar una hiperplasia epitelial o displasia variable. La mayoría se curan espontáneamente o con tratamiento en uno o dos años, y solo un pequeño porcentaje persiste e incluso pueden progresar hacia un carcinoma invasor[16].

La EQ se manifiesta en forma de una placa eritematosa, única o múltiple, en la mucosa del glande o en la cara interna del prepucio (fig. 1B). La EBG se presenta como una placa única y escamosa, localizada en la piel queratinizante, generalmente en el tercio distal del pene[17]. La PB aparece en varones más jóvenes, entre la tercera y cuarta década de la vida. Clínicamente se trata de pápulas verrucosas, múltiples, pequeñas y bien delimitadas, de color marrón, localizadas en el pene, el glande, el prepucio o el pubis (fig. 1C). Están producidas por el VPH 16 y son muy contagiosas, lo que implica un elevado riesgo de tener una CIN para la pareja sexual.

En todas estas lesiones es imprescindible realizar un estudio histológico para confirmar su verdadera naturaleza. Las alteraciones observadas se engloban bajo la denominación de neoplasia intraepitelial de pene (PIN), por analogía con la neoplasia intraepitelial vulvar (VIN) o cervical (CIN). Estas mismas alteraciones a menudo continúan presentes en el epitelio adyacente de los carcinomas. Se distinguen la PIN diferenciada y la PIN indiferenciada[18].

La PIN diferenciada, común o de bajo grado, es aquella en la que se observa atipia citológica en las capas basal y suprabasal de la epidermis, crestas elongadas y una arquitectura conservada en las capas superiores[19]. Suele asociarse a la presencia de LE o hiperplasia epidérmica.

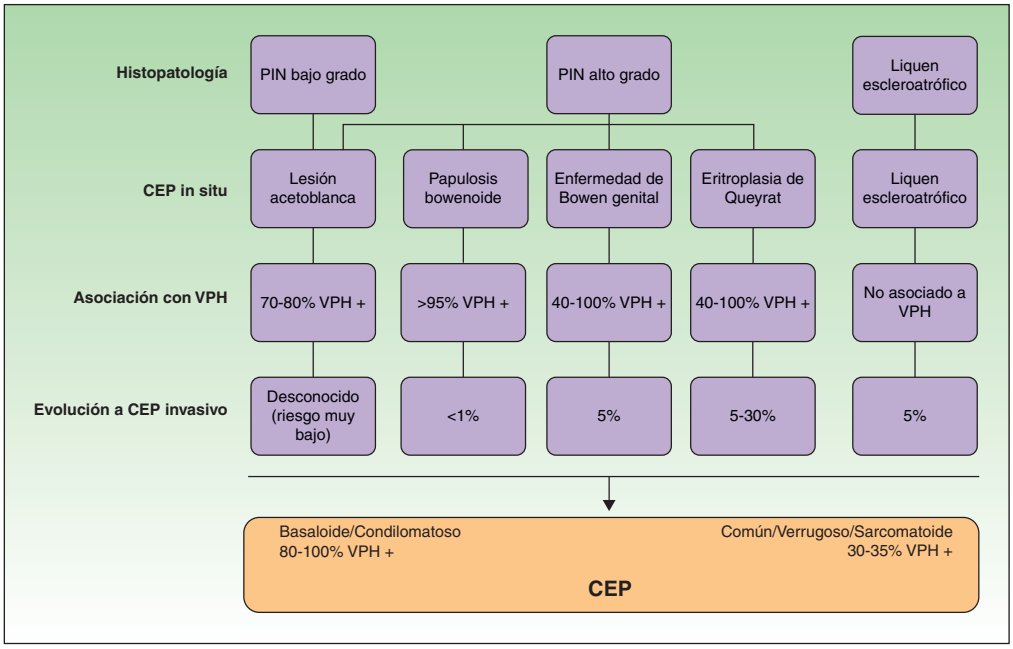

Figura 2 Relación entre la histología y la clínica en el carcinoma escamoso *in situ*, la presencia de VPH y la evolución a carcinoma escamoso invasivo.

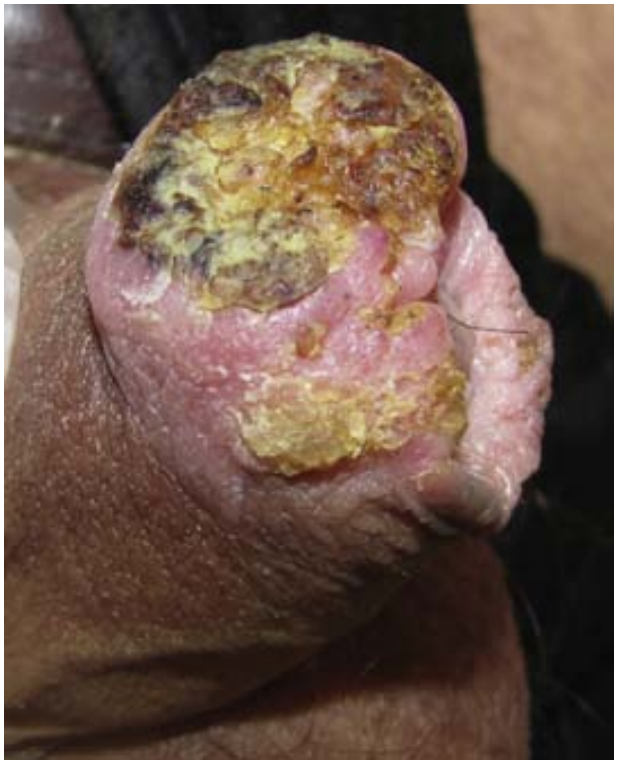

Figura 3 Carcinoma escamoso infiltrante en paciente con antecedente de liquen escleroso y trasplante hepático un año antes del diagnóstico de la lesión.

Puede evolucionar a un CEP común, verrugoso o, con menor frecuencia, basaloide o condilomatoso[18–21].

La PIN indiferenciada, bowenoide o de alto grado, tiene atipia citológica que afecta a más de los dos tercios inferiores del epitelio, células de aspecto basaloide, abundantes mitosis y se correlaciona con la infección por el VPH[18–21]. Suele evolucionar a un CEP basaloide o condilomatoso, con menos frecuencia hacia un CEP común, y casi nunca hacia un carcinoma verrugoso[18]. La EQ, la EBG y PB son manifestaciones clínicas de la PIN indiferenciada, con un riesgo decreciente de evolucionar hacia un CEP invasivo (10-30%, 5-10% y menor al 1%, respectivamente)[22] (fig. 2).

Carcinoma escamoso de pene invasivo

El aspecto clínico del CEP invasivo puede ser muy variable, incluyendo una placa eritematosa, una úlcera o un verdadero tumor exofítico o de aspecto verrugoso. Las lesiones pueden ser pétreas o friables, sangrantes y alcanzar varios centímetros de diámetro (fig. 3). Suelen ser únicas y ubicarse en cualquier parte del pene, si bien lo más frecuente es que estén en el tercio anterior (glande, surco balanoprepucial y/o prepucio). Menos del 5% se originan en el cuerpo del pene. En el momento de la valoración clínica es fundamental recoger la siguiente información: diámetro máximo, número, morfología y color de la lesión, relación con otras estructuras (invasión del meato uretral, del cuerpo esponjoso o del cavernoso) y medir el tamaño del pene para tener una valoración aproximada de la longitud residual tras la penectomía en caso de ser necesaria[10].

Según las características arquitecturales y citológicas se han identificado varios subtipos histológicos de CEP invasivo. El tipo común o queratinizante es el más frecuente, representa un 50-60% de los casos y habitualmente tiene un crecimiento infiltrante, con marcada hiperqueratosis, pudiendo estar bien o mal diferenciado (fig. 4A). El CEP verrugoso (8-10%) tiene una arquitectura que simula una verruga y un crecimiento expansivo (fig. 4B). El CEP basaloide (4-6%) se caracteriza por la presencia de nidos infiltrantes de células claramente basaloides con una empalizada periférica[23] (fig. 4C), y el tipo condilomatoso (6-10%) simula un condiloma, tiene cambios citopáticos fácilmente identificables y células de mayor tamaño[24]. Según nuestra experiencia algunos tumores con una arquitectura de tipo común tienen una citología llamativamente basaloide; son mucho más frecuentes que el basaloide puro y también están relacionados con la infección por VPHar. El CEP sarcomatoide (1%) está muy mal diferenciado, y para demostrar la verdadera naturaleza de las células fusiformes es necesario realizar tinciones inmunohistoquímicas específicas para citoqueratinas (fig. 4D). Por último, existen formas mixtas (10-15%)[25].

Los subtipos histológicos del CEP también pueden estratificarse según su pronóstico, siendo el verrugoso y condilomatoso los de mejor pronóstico, al igual que la subvariedad *cuniculatum* de CEP común. Esta variedad de carcinoma tiene una arquitectura verrugosa con invaginaciones tumorales que característicamente penetran en profundidad remedando las «madrigueras de los conejos». Los tumores con alto riesgo de diseminación son el basaloide, el sarcomatoide y los comunes indiferenciados. La mayoría de ellos están mal diferenciados e invaden la dermis profunda. La categoría intermedia estaría constituida por los CEP comunes y algunas formas mixtas, así como las variantes pleomórficas del CEP condilomatoso[25].

Estadificación

En 2009 la *American Joint Committee on Cancer* (AJCC) propuso una nueva estadificación TNM que da gran importancia al compromiso ganglionar como factor pronóstico de supervivencia[26] (tabla 1). Respecto a la T, se ha subdividido en T1a cuando el tumor está bien diferenciado y no hay invasión linfovascular, y en T1b cuando no se da alguna de estas dos circunstancias. Además, en el T3 se ha eliminado la próstata, puesto que su invasión solo ocurre excepcionalmente, cuando ya están afectadas otras muchas estructuras. La definición de la categoría T2 todavía no se ha modificado, pero algunos estudios han señalado que el pronóstico es mucho peor cuando el tumor invade el cuerpo cavernoso que si solo afecta el cuerpo esponjoso[26].

En cuanto a la N se ha especificado que N1 designa únicamente la afectación inguinal unilateral con ganglios móviles, N2 la afectación inguinal bilateral con ganglios móviles y N3 la presencia de uno o más ganglios fijos e inmóviles o la afectación de ganglios pélvicos.

Patogenia

En relación con la etiopatogenia, parece evidente la alteración de diferentes vías moleculares. Si bien desconocemos el impacto y la interrelación entre cada una de ellas, una

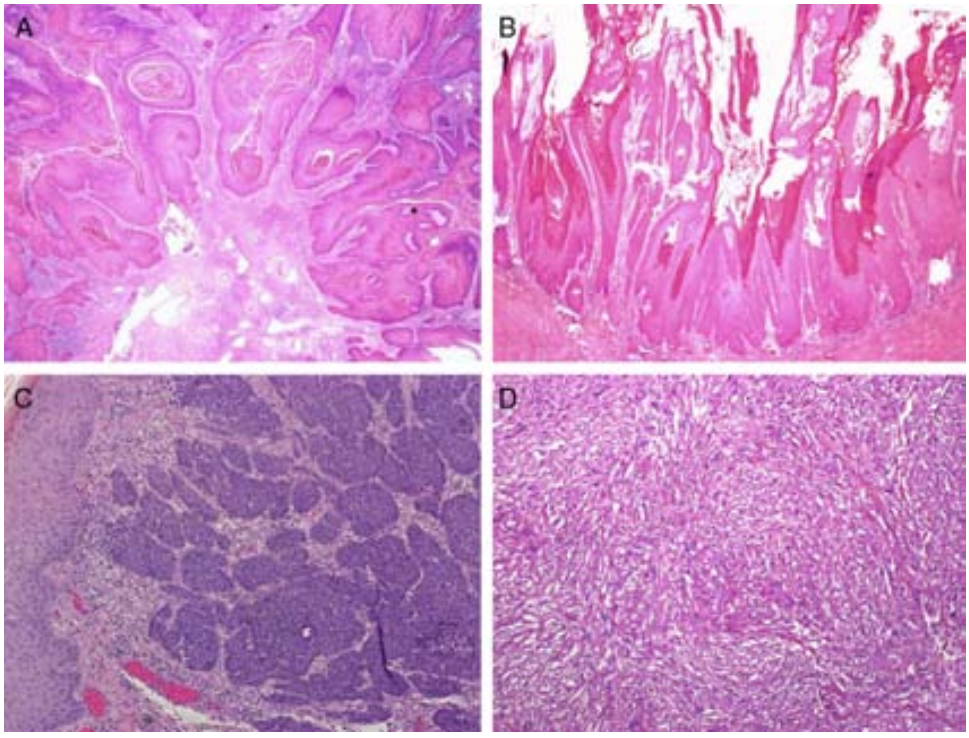

Figura 4 Subtipos histológicos del carcinoma escamoso de pene. A. Común (H&E × 20). B. Verrugoso (H&E × 20). C. Basaloide (H&E × 100). D. Sarcomatoide (H&E × 100).

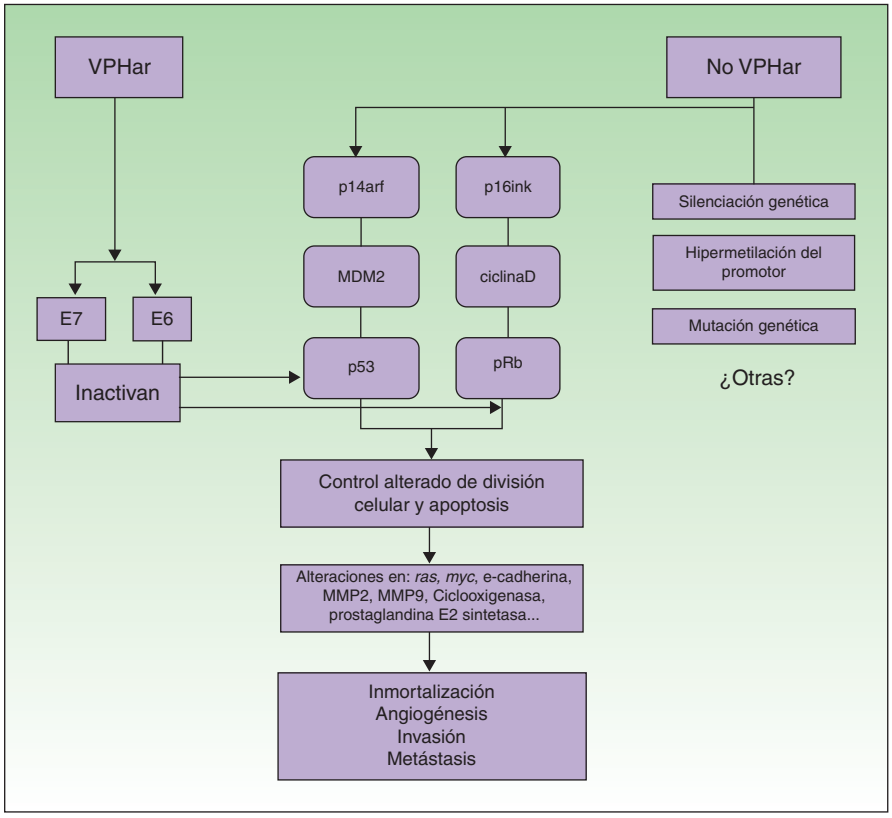

Figura 5 Patogenia en el carcinoma escamoso de pene. Visión esquemática de los eventos moleculares que tienen lugar en las fases inicial y tardía de la carcinogénesis.

Tabla 1 Clasificación TNM en el carcinoma escamoso de pene según la *European Association of Urologists penile cancer guidelines* 2009

T: tumor primario
TX: el tumor primario no puede ser evaluado
T0: no hay evidencia de tumor primario
Tis: carcinoma *in situ*
Ta: carcinoma verrucoso no invasivo
T1: el tumor invade el tejido conectivo subepitelial
T1a: el tumor no tiene invasión linfovascular y es bien diferenciado o moderadamente diferenciado (T1-G1/G2)
T1b: el tumor tiene invasión linfovascular, es pobremente diferenciado o es indiferenciado (T1-G3/G4)
T2: el tumor invade el cuerpo esponjoso o los cuerpos cavernosos
T3: el tumor invade la uretra
T4: el tumor invade otras estructuras adyacentes

N: ganglios linfáticos regionales (p: estadio patológico)
NX: los ganglios linfáticos regionales no pueden evaluarse
N0: ganglios linfáticos inguinales no palpables ni aumentados de tamaño
N1: una metástasis ganglionar inguinal unilateral, palpable y móvil
N2: múltiples metástasis ganglionares inguinales uni o bilaterales, palpables y móviles
N3: metástasis ganglionar inguinal fija o linfadenopatía pélvica unilateral o bilateral

M: metástasis a distancia
M0: no hay metástasis a distancia
M1: metástasis linfáticas fuera de la pelvis o viscerales

parte de los CEP serían atribuibles a la infección por VPHar, mientras que el resto estarían producidos por mecanismos moleculares independientes del VPH[6] (fig. 5).

Los CEP producidos por VPHar surgen a partir de una lesión precursora causada por el virus, siendo la vía carcinogénica similar a la implicada en el cáncer de cérvix[27]. Sin embargo, parece que deben influir mecanismos tisulares u hormonales que hacen que existan diferencias significativas en la incidencia de la enfermedad y en la edad de aparición del CEP respecto al cáncer de cérvix, a pesar de estar ambos producidos por los mismos agentes infecciosos.

El evento inicial es la infección persistente del epitelio escamoso por VPHar, seguida de una serie de alteraciones epigenéticas que confieren características de malignidad a la célula infectada. El VPHar expresa las oncoproteínas E6 y E7, que al unirse a dos genes supresores tumorales (p53 y Rb), los inactivan[28–30]. Las proteínas E6 y E7 del VPH son fundamentales para inducir y mantener la transformación del fenotipo de la célula infectada. Alterando las vías moleculares $p14^{arf}$/MDM2/p53 y $p16^{ink}$/ciclinaD/Rb, los VPH oncogénicos interfieren en el control de la división celular y de la apoptosis. La inactivación funcional de pRb por E7 produce una sobreexpresión de $p16^{ink}A4$, debido a la falta de *feedback* negativo. Por eso, la sobreexpresión de p16 puede utilizarse como un marcador de infección por VPH[31]. Otro marcador que sirve para identificar de forma indirecta la presencia de VPH es el ProxExC, pero todavía no existe experiencia en el CEP[32].

En los CEP no relacionados con la infección por el VPH se alteran las mismas vías de señalización. Los mecanismos serían silenciación de genes supresores tumorales, la hipermetilación de genes promotores o la sobreexpresión de oncogenes[2,31].

Cuando el CEP ya está más avanzado, parece que los mecanismos moleculares serían equivalentes en ambos grupos (VPH positivos y negativos). Se han identificado alteraciones en la expresión de los genes *ras* y *myc*, E-cadherina, metaloproteinasas (MMP) 2 y 9, ciclooxigenasa y prostaglandina E2 sintetasa[33–35]. Serían procesos tardíos que, por tanto, estarían implicados en la progresión de la enfermedad, tales como la angiogénesis y su capacidad invasiva y metastásica. Algunos de estos factores se consideran predictivos de metástasis ganglionares.

Factores pronósticos

La presencia y el número de adenopatías en la región inguinal son los factores pronósticos de supervivencia más importantes. La supervivencia libre de enfermedad a los 5 años llega a ser del 80% cuando hay afectación de uno o dos ganglios inguinales superficiales y unilaterales (N1), descendiendo al 10-20% cuando la afectación es bilateral o pélvica (N2/3) y a menos del 10% cuando hay afectación extranodal[36].

Como parámetros histológicos determinantes del riesgo de metástasis ganglionares y de muerte por el tumor, parece que la invasión perineural, la permeación linfovascular y el grado de diferenciación son los más importantes[25,37–40]. Otros factores que parecen condicionar el pronóstico son la profundidad, el patrón de crecimiento y el subtipo histológico del tumor, así como la invasión uretral.

La implicación del VPH en el pronóstico es controvertida[41,42]. Los resultados también son contradictorios en cuanto a la expresión de p53 como predictor independiente de metástasis ganglionares[43,44]. Sin embargo, sí que parece que en los tumores T1 la expresión intensa de p53 se correlacionaría con un mayor riesgo de metástasis y menor supervivencia[44]. La elevada expresión de ki67 aumenta el riesgo de metástasis ganglionares, pero no influye en la supervivencia[45]. En los estudios de Campos y Zhu se vió que la baja expresión de E-cadherina se correlaciona con mayor riesgo de metástasis ganglionares y peor supervivencia[33,44]. Finalmente Campos et al. demostraron que la sobreexpresión de MMP9 supone un factor de riesgo para la recidiva del tumor[33].

Tratamiento

La baja incidencia del CEP justifica la falta de protocolos estandarizados para su manejo y la ausencia de ensayos clínicos aleatorizados que realmente ayuden en la toma de decisiones.

Tratamiento del tumor primario

Los CEP *in situ* pueden tratarse con exéresis simple o cirugía micrográfica de Mohs[46]; con ambas técnicas la tasa de recidivas es baja y se preserva la función del órgano. La

crioterapia, la terapia fotodinámica, el láser (CO_2 o Nd:YAG) o la aplicación tópica de 5-fluorouracilo al 5% o imiquimod al 5% son otras opciones terapéuticas eficaces[47–51]. A pesar del riesgo relativamente bajo de que estos CEP *in situ* se hagan invasivos, se recomienda un seguimiento clínico tras el tratamiento. La falta de respuesta o la recidiva deben alertar de la posible progresión del tumor.

En los CEP invasivos la cirugía del tumor primario con márgenes de seguridad de 5 a 10 mm es la base del tratamiento. El objetivo es realizar una cirugía curativa que preserve, siempre que sea posible, las funciones urinaria y sexual del miembro. La calidad de vida y la salud sexual son aspectos a considerar y que deben discutirse siempre con el paciente en el momento de decidir la opción terapéutica.

Al plantear la estrategia terapéutica a seguir son determinantes el tamaño y grado de infiltración del tumor, la localización del mismo (glande, prepucio o cuerpo del pene) y la experiencia del cirujano. En tumores extensos pero superficiales pueden plantearse opciones terapéuticas como la decorticación del glande y reconstrucción con un injerto de piel parcial o la combinación de exéresis con láser y reconstrucción quirúrgica, dejando la quimioterapia intralesional (vinblastina, bleomicina, metotrexato) o la radioterapia para casos muy excepcionales, pues tiene mayores efectos adversos[52,53]. Cuando el tumor afecta al prepucio la postectomía ampliada es el tratamiento de elección, y cuando el tumor ya es más infiltrante deben plantearse opciones quirúrgicas más agresivas como la glandectomía o penectomía parcial según el tamaño tumoral[54–57], o incluso la penectomía total si es un tumor localizado en el cuerpo del pene o muy mal diferenciado[56,57]. En tumores T4, muy infrecuentes, la administración de quimioterapia sistémica neoadyuvante seguida de cirugía en pacientes respondedores es el tratamiento de primera línea.

La radioterapia del tumor primario puede ser una alternativa en tumores T1-T2 menores de 4 cm de diámetro, con tasas de curación del 70 al 90% a los 5 años[58,59]. Los mejores resultados se obtienen con braquiterapia o radioterapia con haz de electrones, pero las recurrencias locales son mas frecuentes que si se realiza una penectomía parcial. En estas situaciones la cirugía de rescate puede restaurar el control local del tumor. La estenosis uretral (10-45%), la necrosis del glande (0-23%) o la fibrosis de los cuerpos esponjosos son complicaciones atribuibles a la radioterapia[60].

Manejo de los ganglios linfáticos

Es fundamental realizar una palpación minuciosa de las zonas inguinales en busca de ganglios linfáticos potencialmente infiltrados por el tumor, ya que es donde se localiza con mayor frecuencia el ganglio centinela. A medida que el tumor progresa puede llegar a invadir la fascia de Buck, permear los cuerpos cavernosos y, por vía linfática, alcanzar secuencialmente los ganglios regionales de los territorios inguinales superficial, profundo y pélvico, para finalmente metastatizar en órganos distales. La utilización de la tomografía por emisión de positrones (PET-TC) ayuda a completar la estadificación gracias a su elevada sensibilidad[61]. La punción-aspiración con aguja fina (PAAF) guiada por ecografía es un método rápido y fácil para detectar metástasis cuando se palpan ganglios inguinales[62]. En caso de no detectarse células tumorales tras la punción se puede optar por repetirla, extirpar el ganglio o hacer un nuevo control tras 4 semanas de antibioticoterapia sistémica empírica. En caso de positividad (presencia de células neoplásicas en la PAAF o en la biopsia del ganglio), la linfadenectomía (LDN) es obligatoria.

La LDN es el tratamiento de elección en pacientes con ganglios linfáticos inguinales metastásicos. Sin embargo, es una técnica dificultosa y con un riesgo de complicaciones del 50% (24-87%), entre las que destacan los edemas crónicos en miembros inferiores y escroto, necrosis de los colgajos e infección de la herida quirúrgica, incluyendo una mortalidad del 1-3% de los casos[63]. Con objeto de disminuir la morbilidad atribuible a la LDN se está probando la utilización de LDN inguinal por vía endoscópica con resultados prometedores[64]. La realización de LDN bilateral profiláctica conlleva una alta morbilidad siendo en muchos casos innecesaria, ya que no mejora el pronóstico.

Para aquellos pacientes con tumores moderadamente o mal diferenciados o $\geq$ T3 sin ganglios palpables, no existe ninguna técnica mínimamente o no invasiva que sea 100% informativa del estado de los ganglios linfáticos[65]. Algunos estudios han demostrado que la biopsia selectiva de ganglio centinela (BSGC) aumenta la supervivencia respecto a la conducta expectante y reduce la morbilidad comparada con la LDN inguinal profiláctica, teniendo una especificidad del 100% y una sensibilidad del 95%, pero no se realiza en todos los centros[66]. En su defecto, se han diseñado algoritmos basados en las características histológicas del tumor que predicen el riesgo de metástasis, pero su sensibilidad no supera el 80%[13,67–69].

La radioterapia profiláctica en pacientes N0 ya ha quedado descartada porque no previene la aparición de ganglios linfáticos metastásicos, tiene complicaciones y dificulta el seguimiento de los pacientes por la fibrosis residual[70]. La quimioterapia adyuvante tiene únicamente un papel paliativo en pacientes con metástasis inguinales irresecables o en órganos distales.

Seguimiento

Los pacientes deben seguir un control estricto durante al menos 5 años. Si se ha realizado cirugía conservadora se recomiendan controles trimestrales durante los dos primeros años, y luego semestrales. Si la cirugía ha sido más radical son suficientes los controles semestrales[71]. En tumores T1 o T2, bien diferenciados, sin permeación linfovascular ni adenopatías palpables en el momento del tratamiento es suficiente el seguimiento clínico. En otros casos conviene realizar ecografía inguinal en los controles.

El riesgo de recidiva local global no supera el 5%. La mayoría de recidivas locales con cirugías preservadoras del órgano se producen durante los dos primeros años. La recidiva local suele detectarla el propio paciente, su pareja o el médico habitual, por lo que no suele afectar a la supervivencia.

El riesgo de metástasis ganglionares es mayor (9%) en los pacientes en los que se han valorado los ganglios por palpación que en los estadificados quirúrgicamente (2,3%), ya sea mediante LDN profiláctica o por BSGG. Los pacientes ya tratados por metástasis linfáticas tienen un riesgo de recidiva del 19%[10].

Prevención

El CEP es una enfermedad prevenible. En todos los pacientes con fimosis, que impida una exploración adecuada y una buena higiene del glande, debería realizarse una postectomía.

Dado que la infección por VPH, sobre todo por VPH 16, desempeña un papel etiológico fundamental en algunos subtipos de CEP, las vacunas antipapilomavirus podrían tener efectos beneficiosos si se administrasen en los varones antes de iniciar sus primeras relaciones sexuales. Hasta la fecha existen dos vacunas para el VPH, una bivalente frente a los genotipos 16 y 18 y una tetravalente frente a VPH 16, 18, 6 y 11. Sin embargo, dado que la incidencia de CEP es mucho menor que la de cáncer de cérvix, es difícil que se recomiende de forma sistemática a todos los niños. Además, es una patología mucho más prevalente en países subdesarrollados, donde las posibilidades de vacunar a grandes colectivos son mínimas. Aunque el uso del preservativo no protege al 100% frente a la infección por el VPH, se ha demostrado que reduce el riesgo de transmisión y acorta el tiempo de curación de las lesiones producidas por el virus[72].

Conclusiones

La creación de equipos multidisciplinares con urólogos, dermatólogos, patólogos y biólogos moleculares ayudará a comprender los mecanismos oncogénicos subyacentes del CEP, permitiendo el correcto diagnóstico de las lesiones iniciales, la identificación de factores pronósticos, la implantación de campañas de prevención y la identificación de dianas moleculares que optimizarán su tratamiento, aumento de la supervivencia y reducirán la morbilidad.

Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses.

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