UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Mânlio Tasso de Oliveira Mota

Identificação e validação de genes diferencialmente expressos em carcinoma de pênis

São José do Rio Preto
2013

INSTITUTO DE BIOCIÊNCIAS, LETRAS E CIÊNCIAS EXATAS – IBILCE
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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, Área de Concentração - Virologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientadora: Profª. Drª. Paula Rahal

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IDENTIFICAÇÃO E VALIDAÇÃO DE GENES DIFERENCIALMENTE EXPRESSOS EM CARCINOMA DE PÊNIS

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Homenagem a ti que é Rā quando te levantas e Temu quando te pões. Levantas-te, Levantas-te, brilhas, brilhas, és coroado rei dos deuses. Senhor do céu, [és] o senhor da terra; criador dos que habitam nas alturas e dos que habitam nas profundezas. [És] o Deus Uno surgido no começo do tempo. Criaste a terra, afeiçoaste o homem, fizeste o abismo aquífero do céu, formaste Hapi, criaste o homem, fizeste o abismo das águas, e dás vida a tudo o que há dentro dele. Coseste as montanhas umas às outras, fizeste a humanidade e os animais do campo tomarem forma, fizeste os céus e a terra. Sê adorado, ó tu que a deusa Maāt abraça de manha e ao entardecer. (...) Ó Uno, poderoso, de miríades de forma e aspectos, rei do mundo, príncipe de Anu, senhor da eternidade e soberano da perpetuidade, a companhia dos deuses exulta quando te levantas e navegas Pelo céu (...).

Do Papiro de Hu-nefer (1.370 a.C.) (Museu Britânico, 9.901)

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RESUMO

O carcinoma de pênis (CEP) é um tumor epitelial invasivo raro com alta morbidade decorrente da própria doença e/ou de seu tratamento. O perfil socioeconômico e cultural dos pacientes dificulta o diagnóstico precoce, tratamento e seguimento dos enfermos. Pacientes sem tratamento geralmente morrem dentro de dois anos após diagnóstico devido à proliferação celular regional ou a metástases distantes. Não há padrão nos sistemas de estadiamento e nas condutas clínicas, resultando em dificuldades na abordagem terapêutica. Comparado ao câncer cervical, poucos estudos moleculares em CEP foram realizados.

O presente projeto teve como objetivo geral identificar genes diferencialmente expressos em tecidos penianos tumorais e normais e o possível papel do vírus do papiloma humano (HPV) no desenvolvimento de CEP.

Diferenças na expressão gênica entre tecidos tumorais e normais foram verificadas pela metodologia de RaSH (rapid subctration hybridization), que selecionou 5 genes possivelmente relacionados à carcinogênese (*KIAA1033*, *NAMPT*, *RPL6*, *CDKN2A* e *ANXA1*) com expressão alterada em tecidos tumorais. A validação das alterações de expressão destes genes foi realizada por polymerase chain reaction (reação em cadeia da polimerase, PCR) em tempo real. Tanto os genes como as proteínas de ANXA1 como CDKN2A apresentaram aumento de expressão em pacientes com presença de HPV de alto risco em comparação com tecidos negativos para HPV. Por imunohistoquímica foi possível estabelecer correlação entre alterações na expressão da proteína anexina A1 e a presença de HPV de alto risco.

Pacientes portadores de CEP provenientes de duas regiões (São Paulo e Pará) foram avaliados para a presença de HPV por PCR convencional. Amostras positivas foram genotipadas por hibridização reversa em linha (INNO-LiPA). Observou-se que a prevalência geral de HPV em infecções simples ou múltiplas é significantemente maior no Pará (81,67%) que em São Paulo (64.10%). Foram encontrados mais genótipos no Pará, sugerindo que os genótipos circulando na população paraense

são mais diversos e podem estar contribuindo para maior prevalência de CEP no Pará. Também foram identificados dois pacientes portadores de CEP que desenvolveram um segundo tumor primário, atribuível a HPV, anos após a penectomia sugerindo a possibilidade de múltiplos tumores consecutivos devidos à infecção por HPV.

Os dados deste trabalho trazem contribuições importantes no entendimento dos mecanismos moleculares envolvidos no CEP e do papel do HPV nesta patologia podendo auxiliar em diagnósticos e prognósticos mais precisos, em abordagens clínicas e terapêuticas mais adequadas e guiar o desenvolvimento de programas de vacinação mais eficientes.

Palavras-chave: *Câncer de pênis; vírus do papiloma humano; marcadores de câncer; múltiplos tumores.*

ABSTRACT

Penile carcinoma is a rare epithelial tumor with high morbidity due to own disease and/or treatment. The socio-economic and cultural profile of the patients hampers early diagnosis, treatment and follow-up. Without treatment patients usually dies within two years after first diagnosis due to regional cellular proliferation or distant metastasis. There is no standard in staging systems or in clinical procedures, resulting in difficulties in the therapeutic approach. Compared to cervical cancer, there are few molecular studies about this tumor.

The present work aimed to identify differentially expressed genes in tumor and normal penile tissues and the role of human papillomavirus (HPV) in the development of this tumor.

Differences in gene expression between tumoral and normal tissues were accessed by RaSH (rapid subctration hybridization) methodology, which selected five possibly carcinogenesis related genes (KIAA1033, NAMPT, RPL6, CDKN2A and ANXA1) with altered expression in tumor tissues. The validation of gene expression changes was performed by real time polymerase chain reaction (PCR). The ANXA1 and CDKN2A, both genes and proteins, showed superexpression in patients with high-risk HPV compared to HPV negative tissues. Immunohistochemical assays established a correlation between alterations in the superexpression of annexin A1 and the presence of high risk HPV.

Penile cancer harboring patients from two regions (São Paulo and Pará) was assessed for HPV presence by conventional PCR. Positive samples were genotyped by reverse hybridization-based line probe assay (INNO-LiPA). The overall HPV prevalence in single or multiple infections was significantly greater in Pará (81.67%) than in São Paulo (64.10%). A wilder range of genotypes were found in Pará, suggesting that the genotypes circulating in the population of Pará are more diverse and may be contributing to higher prevalence of penile cancer in Pará. It was also observed two patients harboring penile cancer which developed a second primary

tumor attributable to high-risk HPV years after penectomy, arisen the possibility of occurrence of multiple consecutive primary tumors as a result of high risk HPV infection.

Data from this study bring important contributions to the understanding of the molecular mechanisms involved in the penile cancer and the role of HPV in this pathology and may help in more accurate diagnoses and prognoses, more appropriate clinical and therapeutic approaches and may guide the development of more effective vaccination programs.

Keywords: *Penile cancer, Papillomavirus, cancer markers, multiple tumors*.

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ABREVIATURAS

ACTB	β-actina
AJCC	American Joint Committee on Cancer
ANXA1	Anexina A1
CDKN2A	Quinase inibidora dependente de ciclina 2A/p16
CEP	Carcinoma de células escamosas de pênis
E6AP	E6 associated protein, proteína associada a E6
DEPC	Dietil pirocarbonato
GAPDH	Gliceraldeído-3-fosfato desidrogenase
GSTP1	Glutationa S-transferase 1
HPV	*Human Papillomavirus*, vírus do papiloma humano
LR-HPV	*Low-risk HPV*, HPV de baixo risco
HR-HPV	*High risk HPV*, HPV de alto risco
KIAA1033	Gene também conhecido como *WASH7* (subunidade 7 do complexo WASH)
PBEF1	Fator 1 de aumento de colônias de células pré-B
PCR	*Polymerase chain reaction*, reação em cadeia da polimerase
RaSH	*Rapid subtraction hybridization*
Rb	Família retinoblastoma de proteínas
RPL6	Proteína ribossomal L6e da fração 60S
RT-PCR	*Reverse transcription - Polymerase chain reaction*, transcrição reversa - reação em cadeia da polimerase
TNM	Tumor, linfonodo, metástase (sistema de estadiamento de tumores)
OMS	Organização mundial da Saúde
ORF	*Open reading frame*, janela aberta de leitura
qPCR	*Quantitative PCR*, PCR quantitativo (PCR em tempo real)
TUBA	α-tubulina

INTRODUÇÃO

1. Carcinoma de Pênis

O câncer de pênis é um tumor com grande variação na prevalência, com os maiores índices de prevalência em países menos desenvolvidos. Corresponde mundialmente a menos de 0,5% de todos os tumores que afetam homens (Parkin e Bray, 2006), mas em alguns países subdesenvolvidos ou em desenvolvimento da África, Ásia e América do Sul estão as maiores taxas de incidência (2-4/100.000 habitantes) e este tumor pode representar até 16% dos tumores malignos masculinos (Rocha, Ignacio, Jordan *et al.*, 2012). As menores taxas de prevalência se encontram nos Estados Unidos e Europa e Israel (0.3-1/100.000 habitantes) (Afonso, Moyses, Alves *et al.*, 2012; Chaux e Cubilla, 2012a). Os dados sobre a prevalência de carcinoma epidermóide de pênis (CEP) em diversos países é sumarizada na **figura 1**. No Brasil, o CEP responde por 2% de todos os tipos de tumores masculinos e a incidência varia entre 2,9 a 6,8/100.000 habitantes. É mais frequente nas regiões Norte e Nordeste e nestas regiões costuma suplantar o câncer de próstata e de bexiga (Favorito, Nardi, Ronalsa *et al.*, 2008).

Em 2004 a Organização Mundial de Saúde (OMS) reconheceu seis tipos histológicos de tumores penianos. A grande maioria destes tipos histológicos (95%) são carcinomas epidermóides ou carcinomas de células escamosas. A OMS também reconhece que o CEP é dividido em sete subtipos que estão descritos na **tabela 1** (Chaux e Cubilla, 2012a; Chaux, Velazquez, Barreto *et al.*, 2012). O CEP é um tumor epitelial invasivo que se origina da pele ou mucosa peniana e acredita-se que é precedido por lesões pré-malignas como cornos cutâneos, papulose bowenóide, *balanitis xerotica obliterans* (*lichen sclerosus et atrophicus*), eritroplasia de Queyrat e doença de Bowen (Pizzocaro, Algaba, Horenblas *et al.*, 2010). Os sítios anatômicos preferenciais para o surgimento do CEP são a glande (34,5%), seguida pelo prepúcio (13,2%), corpo peniano (5,3%), com sobreposições (4,5%) e outros locais não específicos (42,5%) (Deem, Keane, Bhavsar *et al.*, 2011).

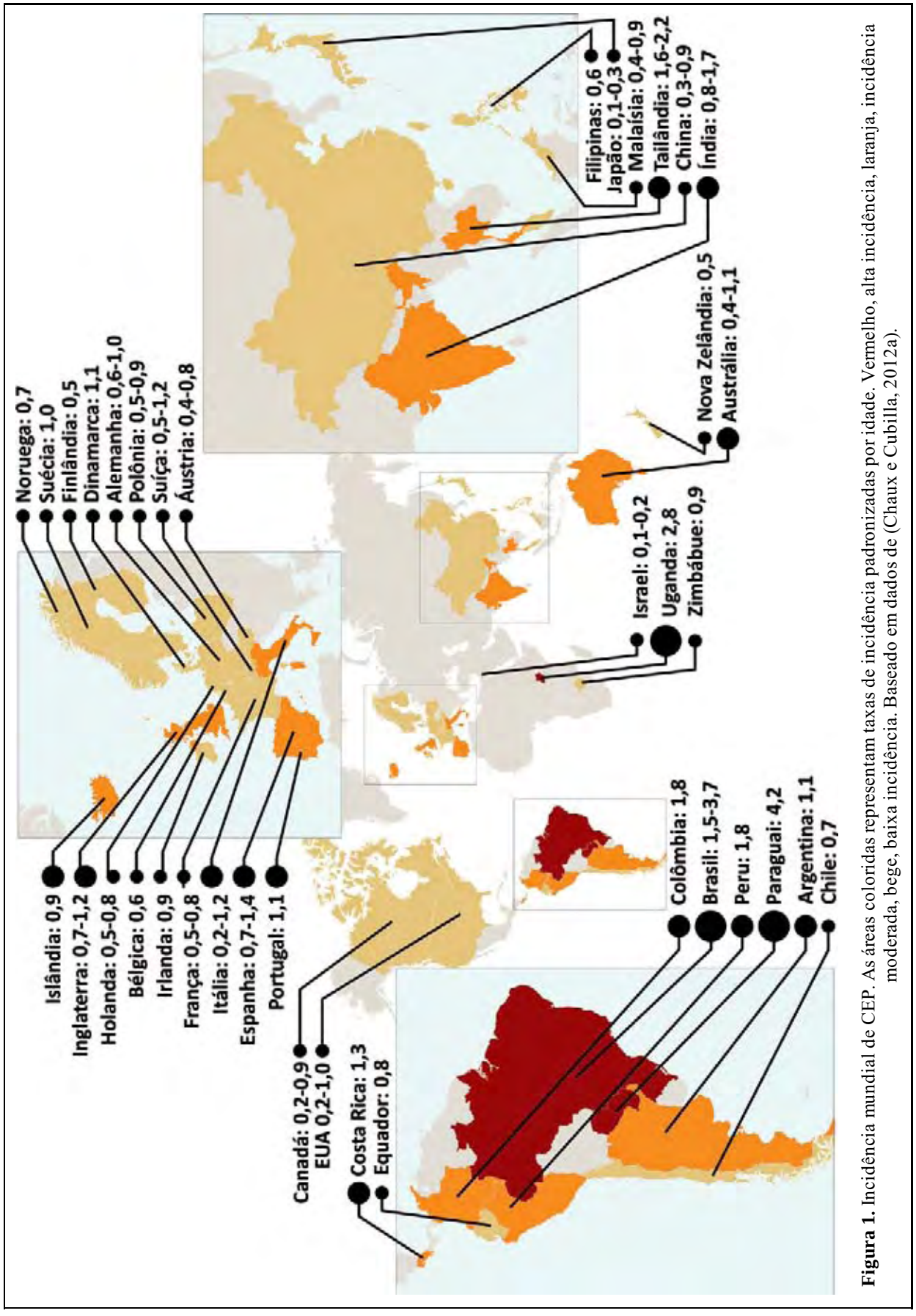

Figura 1. Incidência mundial de CEP. As áreas coloridas representam taxas de incidência padronizadas por idade. Vermelho, alta incidência, laranja, incidência moderada, bege, baixa incidência. Baseado em dados de (Chaux e Cubilla, 2012a).

Tabela 1. Classificação dos tumores malignos epiteliais de pênis segundo a OMS (2004)

Carcinoma escamocelular[a]
Carcinoma basalóide
Carcinoma condilomatoso (warty)
Carcinoma verrucoso
Carcinoma papilífero, sem outras especificações
Carcinoma sarcomatóide
Carcinomas mistos[b]
Carcinoma adenoescamoso
Carcinoma de células de Merkel
Carcinoma de células pequenas do tipo neuroendócrino
Carcinoma sebáceo
Carcinoma de células claras
Carcinoma de células basais

a. É o tipo mais usual.
b. Designa que há mais de um tipo histológico envolvido.

O CEP pode apresentar três padrões distintos de crescimento: superficial, nodular (ou vertical) e verrucoso. Tem progressão locorregional, drenando primariamente para os nódulos linfáticos inguinais e pélvicos (Pizzocaro, Algaba, Horenblas *et al.*, 2010). Os fatores histopatológicos de relevância prognóstica para a dispersão locorregional incluem o grau, o estágio, a profundidade da invasão, presença de invasão linfovascular e o tamanho do tumor. Os sítios anatômicos mais comuns para metástases distantes são pulmão, cérebro e ossos (Kayes, Ahmed, Arya *et al.*, 2007).

Quando diagnosticado nos estágios iniciais, o CEP apresenta elevada taxa de cura, entretanto diversos fatores dificultam o diagnóstico precoce, o tratamento e o seguimento dos enfermos como o *status* sócio-econômico-cultural dos pacientes, o estigma social associado a este tumor e a possibilidade das lesões estarem escondidas por fimose (Deem, Keane, Bhavsar *et al.*, 2011). No Brasil, muitos pacientes costumam demorar mais de um ano, após o inicio dos sintomas, para procurar auxílio médico, por questões culturais ou dificuldade de acesso ao serviço médico. A maioria dos casos de CEP em estádio avançado necessita de tratamento cirúrgico mutilante causando graves repercussões psicológicas e funcionais desfavoráveis, o que dificulta a reabilitação e a reintegração social (Guimaraes, Rocha, Zequi *et al.*, 2011).

O CEP está associado à alta morbidade decorrente da própria doença e/ou de seu tratamento e os pacientes sem tratamento geralmente morrem devido à inanição, sepse hemorragias devido à erosão dos vasos femurais causadas por metástases inguinais vegetantes (Kayes, Ahmed, Arya *et al.*, 2007).

O tratamento do CEP depende de diagnóstico e de estadiamento adequados e o diagnóstico precoce é essencial para o controle local. O diagnóstico de lesões pré-malignas pode ajudar a evitar a progressão maligna e a mutilação do órgão devida a penectomia (Deem, Keane, Bhavsar *et al.*, 2011). Entretanto o sistema de estadiamento do CEP é um processo em desenvolvimento, ainda sem padronização definitiva. Atualmente o sistema do American Joint Committee on Cancer (AJCC) é um dos mais utilizados. Os sistemas de gradação TNM e estadiamento estão descritos na **tabela 2** e **tabela 3** respectivamente (Edge, Byrd, Compton *et al.*, 2010).

Em comparação a outros tumores, há poucos estudos com CEP, refletindo sua baixa incidência em países desenvolvidos. Existem poucos estudos prospectivos e randomizados e a amostragem ainda não é suficiente para estabelecer a melhor conduta nos diversos estádios desta enfermidade.

A etiologia do CEP e os fatores moleculares que influenciam no seu desenvolvimento ainda não são totalmente conhecidos. Alguns fatores de risco já foram descritos como presença de fimose, fumo, lesões penianas, balanite crônica, verrugas genitais higiene pessoal inadequada, fotoquimioterapia com psoraleno-UVA (usada no tratamento de doenças de pele como psoríase, vitiligo e linfoma cutâneo de células T), infecção por HIV e infecção por HPV, (Minhas, Manseck, Watya *et al.*, 2010; Pow-Sang, Ferreira, Pow-Sang *et al.*, 2010; Calmon, Tasso Mota, Vassallo *et al.*, 2011; Annunziata, Buonaguro, Buonaguro *et al.*, 2012; Chaux e Cubilla, 2012b).

Tabela 2. Estadiamento TNM de câncer de pênis baseado no AJCC

Tumor primário (T)	
TX	Tumor primário não pode ser acessado
T0	Sem evidência de tumor primário
Tis	Carcinoma *in situ*
Ta	Carcinoma verrucoso não-invasivo[a]
T1a	Tumor invade tecido conectivo subepitelial sem invasão linfovascular e não é pouco diferenciado
T1b	Tumor invade tecido conectivo subepitelial com invasão linfovascular ou é pouco diferenciado
T2	Tumor invade corpo esponjoso ou cavernoso
T3	Tumor invade a uretra
T4	Tumor invade outras estruturas adjacentes
Linfonodos regionais (N)	
Estágio clínico[b]	
cNX	Linfonodos regionais não podem ser acessados
cN0	Sem linfonodos inguinais aumentados palpáveis ou visíveis
cN1	Linfonodo inguinal unilateral móvel palpável
cN2	Linfonodos inguinais palpáveis múltiplos ou bilaterais
cN3	Massa nodal inguinal fixa palpável ou linfoadenopatia pélvica unilateral ou bilateral
Estágio patológico[c]	
pNX	Linfonodos regionais não podem ser acessados
pN0	Sem metástases em linfonodos regionais
pN1	Metástase em um único linfonodo
pN2	Metástase em linfonodos múltiplos ou bilaterais
pN3	Extensão extranodal de metástase linfonodal, linfonodo pélvico uni ou bilateral
Metástases distantes (M)	
M0	Sem metástases distantes
M1	Metástases distantes[d]

a. Penetração de borda ampla (invasão) é permitida; invasão destruidora é contra este diagnóstico
b. Baseado em palpação e imagem.
c. Definição de estágio patológico baseada em biópsia ou excisão cirúrgica.
d. Metástases linfonodais for a da pelve em adição a sítios ósseos e viscerais.

Tabela 3. Estágios anatômicos/grupos de prognóstico

Estágio 0	Tis	N0	M0
	Ta	N0	M0
Estágio I	T1a	N0	M0
Estágio II	T1b	N0	M0
	T2	N0	M0
	T3	N0	M0
Estágio IIIa	T1-3	N1	M0
Estágio IIIb	T1-3	N2	M0
Estágio IV	T4	Qulaquer N	M0
	Qualquer T	N3	M0
	Qualquer T	Qualquer N	M1

Vários estudos têm sugerido a existência de duas vias etiológicas para o surgimento do CEP: uma relacionada à infecção por HPV de alto risco e outra relacionada a estados de inflamação crônica, fimose e líquen escleroso (Chaux e Cubilla, 2012b).

Diversos fatores prognósticos já foram estudados. Descobriu-se que a presença de linfonodos inguinais metastáticos é, isoladamente, o fator mais importante para predição de sobrevivência de pacientes com CEP (Chaux e Cubilla, 2012c).

Além de fatores anatomopatológicos, alterações gênicas como mudanças na expressão ou mutações podem ser úteis como fatores prognósticos. A determinação de genes expressos diferencialmente entre tecidos normais e tumorais é uma importante ferramenta que permite determinar quais destes estão envolvidos com o desenvolvimento e progressão de determinados tumores e avaliar a ação de drogas sobre estes. Diversas técnicas que permitem a detecção de genes diferencialmente expressos foram descritas nas últimas décadas, como a hibridação subtrativa (Hedrick, Cohen, Nielsen *et al.*, 2005), "mRNA *differential display*" (Liang e Pardee, 1992) análise da diferença representativa (RDA) (Lisitsyn e Wigler, 1993; Hubank e Schatz, 1994), análise seriada da expressão gênica (serial analysis of gene expression, SAGE) (Velculescu, Zhang, Vogelstein *et al.*, 1995), "*small amplified* RNA-SAGE" (SAR-SAGE) (Vilain e Vassart, 2004), cDNA *microarrays* (Schena, Shalon, Davis *et al.*, 1995), sequenciamento de próxima geração (next generation sequencing, NGS) (Cullum, Alder e Hoodless, 2011), *microRNA profiling* (Pritchard, Cheng e Tewari, 2012), "*high-throughput sequencing*" (Prickett e Oakey, 2012) entre outras, todas elas apresentando vantagens e desvantagens.

Uma modificação na técnica de hibridação subtrativa é a metodologia denominada RaSH ("*rapid subtraction hybridization*"). Nessa técnica os processos de subtração de cDNAs são simplificados, apresentando vantagens sobre outras técnicas, como uma grande eficiência na subtração e significativa redução dos custos (Jiang, Kang, Alexandre *et al.*, 2000)

principalmente quando comparada a técnica de *microarray*. Essa metodologia tem sido utilizada para a identificação de genes diferencialmente expressos em diferentes situações, como por exemplo, na carcinogênese hepatocelular (Andrade, Guerra, Jardim *et al.*, 2011), na endometriose (Meola, Silva, Dentillo *et al.*, 2010), na progressão tumoral cerebral (Emdad, Sarkar, Su *et al.*, 2007), em melanomas (Boukerche, Su, Kang *et al.*, 2004), na resistência à infecção pelo HIV (Simm, Su, Huang *et al.*, 2001) e também para a avaliação dos efeitos do tratamento de algumas drogas como interferon, mezerina (Jiang, Kang, Alexandre *et al.*, 2000) e anexina-1 (Rodrigues-Lisoni, Mehet, Peitl *et al.*, 2006). Em todos estes estudos os dados sobre a expressão gênica diferencial revelados pelo RaSH foram validados por RT-PCR semiquantitativo, PCR em tempo real ou Northern Blot, confirmando a eficiência da metodologia.

Algumas alterações moleculares sabidamente presentes em outros tumores foram também encontradas em pacientes portadores de CEP, bem como alterações no número de cópias de genes e aneuploidias também já foram descritas. Entretanto ainda não há consenso entre os diversos autores sobre o real papel que cada alteração exerce no CEP. Algumas das alterações mais comuns encontradas estão sumarizadas na **tabela 4**.

Cerca de 20% de todos os tumores humanos estão relacionados a agentes infecciosos e a maioria destes são oncovírus (Martin e Gutkind, 2008; Hernandez-Lopez e Graham, 2012). Entre estes vírus o HPV é um dos mais importantes, com cerca de 560.000 novos casos de tumor anualmente no mundo associados a ele diretamente (Chaux e Cubilla, 2012b). Entre os diversos fatores de desenvolvimento do CEP, a infecção por HPV tem sido analisada em diversos estudos. Entretanto a incidência de HPV nos tumores penianos tem grande variação, dependendo do tipo histológico analisado. DNA de HPV é, em geral, detectado em cerca de 40 a 50% de todos os tumores de pênis.

Em 2009 dois grupos revisaram vários estudos de prevalência de HPV no CEP, chegando a resultados similares. Backes e cols. (Backes, Kurman, Pimenta *et al.*, 2009) revisaram 30 estudos totalizando 1266 amostras e encontraram prevalência de 48% de infecções por HPV, variando de 22% nos carcinomas verrucosos a 66% nos carcinomas basalóide e condilomatoso. Miralles-Guri e cols. (Miralles-Guri, Bruni, Cubilla *et al.*, 2009) revisaram 31 estudos com um total de 1466 casos e encontraram de 47% de infecções por HPV, variando de 24% nos carcinomas verrucosos a 76% nos carcinomas basalóides.

Tabela 4. Alterações moleculares observadas em CEP

Alterações moleculares	Gene/cromossomo/proteína	Referências
Aumento do número de cópias	8q24, 16p11-12, 20q11-13, 22q, 19q13, e 5p15	(Guerrero, Guarch, Ojer *et al.*, 2008)
Deleções	13q21-22, 4q21-32 e ao longo do cromossomo X	(Guerrero, Guarch, Ojer *et al.*, 2008)
Aneuploidia	Vários genes e cromossomos	(Kayes, Loddo, Patel *et al.*, 2009)
Aumento de expressão em pacientes HPV-positivos	*p53*, *CDKN2A*, *p21*, KI-67, HER3, *c-myc*	(Lam, Chan, Chan *et al.*, 1995; Sastre-Garau, Favre, Couturier *et al.*, 2000; Stankiewicz, Prowse, Ng *et al.*, 2011; Stankiewicz, Ng, Cuzick *et al.*, 2012)
Diminuição de expressão em pacientes HPV-positivos	pEGFR, RB	(Stankiewicz, Prowse, Ktori *et al.*, 2011; Stankiewicz, Prowse, Ng *et al.*, 2011)
Aumento de expressão	*p53*, *CDKN2A*, *p21*, *RB* e *NF-κB*, HER3, HER4, Akt1, PGE2, COX, Topo II, MMP9	(Golijanin, Tan, Kazior *et al.*, 2004; Campos, Lopes, Guimaraes *et al.*, 2006; Berney, Stankiewicz, Adlan *et al.*, 2008; Senba, Buziba, Mori *et al.*, 2009; Stankiewicz, Kudahetti, Prowse *et al.*, 2009; Stankiewicz, Prowse, Ktori *et al.*, 2011; Stankiewicz, Prowse, Ng *et al.*, 2011; Rocha, Ignacio, Jordan *et al.*, 2012)
Diminuição de expressão	*CDKN2A*, E-caderina	(Campos, Lopes, Guimaraes *et al.*, 2006; Stankiewicz, Kudahetti, Prowse *et al.*, 2009)
Mutações em genes	*p53*, *c-ras*^{Ha}, *PIK3CA*, *HRAS* e *KRAS*	(Leis, Stevens, Baer *et al.*, 1998; Andersson, Kolaric, Windahl *et al.*, 2008)
Expressão desbalanceada	Bcl-2/Bax,	(Saeed, Keehn, Khalil *et al.*, 2005)
Hipermetilação do DNA na região promotora do gene	*DAPK*, *FHIT*, *MGMT*, *CDKN2A*, *p14*^{ARF}, *RAR-β*, *RASSF1A*, *RUNX3* e *TSP-1*	(Guerrero, Guarch, Ojer *et al.*, 2008; Kalantari, Villa, Calleja-Macias *et al.*, 2008; Yanagawa, Osakabe, Hayashi *et al.*, 2008)

Os produtos gênicos virais E6 e E7, expressos durante a infecção, têm papel preponderante na gênese tumoral. A oncoproteína E7 é capaz de evitar o ponto de checagem G1-S através da inativação da proteína supressora de tumor retinoblastoma (pRB1), criando condições para a replicação do DNA viral. Uma das funções da oncoproteína E6 é inibir a ação da proteína supressora de tumor p53 (White, Kramer, Tan *et al.*, 2012). Entretanto a grande maioria das infecções por HPV são assintomáticas, sendo, portanto necessário distinguir os tipos com maior potencial oncogênico como HPV16 e HPV18 (Gregoire, Cubilla, Reuter *et al.*, 1995; Khan, Castle, Lorincz *et al.*, 2005; Chaux e Cubilla, 2012b) dos demais. As interações moleculares entre HPV e oncogenes, que levam ao desenvolvimento do tumor, ainda não são totalmente compreendidos.

2. Vírus do papiloma humano (HPV)

Os HPVs pertencem à família *Papillomaviridae*, uma família muito antiga de patógenos que parece ter coevoluído com seus hospedeiros (anfíbios, répteis, aves e mamíferos). São muito específicos em relação aos seus hospedeiros e não infectam outras espécies mesmo que sejam intimamente relacionadas. Até o presente já foram descritos cerca de 160 genótipos de HPVs (Chow, Broker e Steinberg, 2010; Kovanda, Kocjan, Luzar *et al.*, 2011; Kovanda, Kocjan, Potocnik *et al.*, 2011; White, Kramer, Tan *et al.*, 2012), que se agrupam em 5 gêneros: α, β, γ, μ e ν. Cada gênero é subdividido em espécies que podem ser compostas por um ou mais genótipos (**figura 2**). Estes vírus têm forte tropismo para tecidos epiteliais estratificados podendo infectar mucosas ou a pele e causar diversas patologias nestes tecidos. Os genótipos do gênero α infectam mucosas e pele enquanto os genótipos dos demais gêneros infectam apenas a pele (Crow, 2012).

O genoma viral é constituído por uma única molécula de DNA circular de dupla fita com 8kb, tendo uma pequena variação de acordo com o genótipo de HPV. O genoma pode se dividido em três regiões funcionais, com aproximadamente 8 ORFs (*open reading frames*, janelas abertas de leitura): a região precoce (*early region*, proteínas E1 a E7) que codifica

proteínas essenciais à replicação, a região tardia (*late region*, proteínas L1 e L2) que codifica proteínas estruturais e uma região de controle, em grande parte não codificadora, chamada LCR (*long control region*, região longa de controle) ou URR (*untranslated regulatory region*, região não traduzida regulatória), que contém os elementos *cis* necessários para replicação e transcrição do DNA viral (Doorbar, 2006; Iarc, 2007). Dois promotores: um precoce (p97) e outro tardio (p670) controlam a expressão das regiões precoce e tardia (**figura 3**).

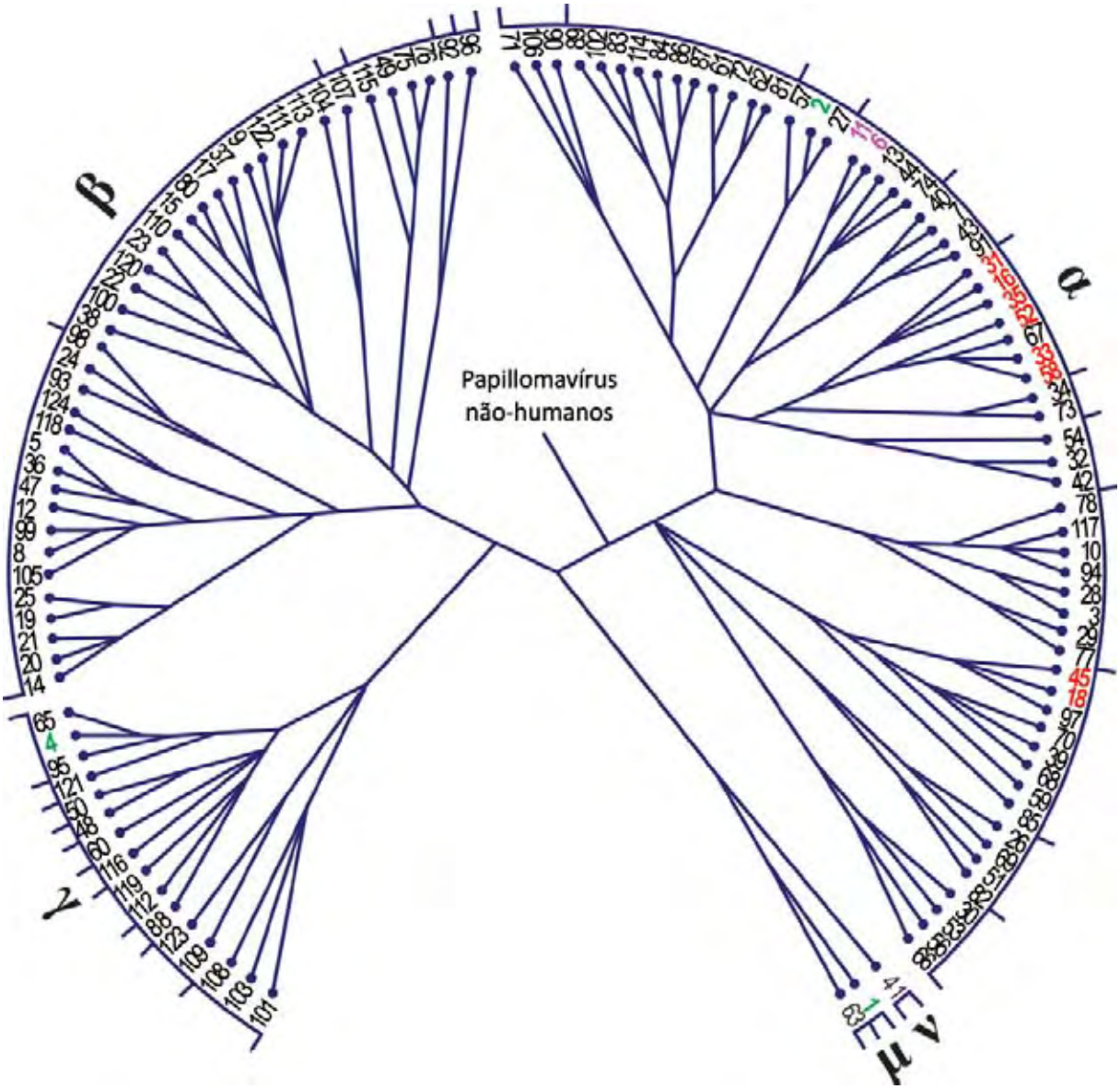

Figura 2. Árvore filogenética (baseada em sequencias de L1) mostrando o agrupamento dos HPVs em 5 gêneros: α, β, γ, μ e ν. Os genótipos do grupo α-*papillomavirus* são os mais associados ao desenvolvimento de tumor. Em vermelho, os genótipos mais associados ao desenvolvimento de câncer. Em verde, os genótipos mais associados a verrugas e em rosa os genótipos que estão associados a 90% das verrugas genitais. Os arcos externos indicam os gêneros, as linhas externas indicam as espécies. Baseado em (Crow, 2012).

Os HPVs não são envelopados. O capsídeo, com diâmetro entre 52 e 55nm, é composto pelas proteínas tardias L1 e L2, cuja expressão é controlada pelo promotor tardio p670 após uma mudança no padrão de *splicing* e no uso do sítio de poliadenilação (do sítio de poliadenilação precoce (PAP) para o tardio (PAT)) (Doorbar, 2006).

A proteína L1 é altamente conservada entre os diversos genótipos de HPV e 360 cópias desta proteína formam 72 capsômeros pentaméricos de simetria icosaédrica (T=7). A proteína L2 é mais interna e desempenha múltiplas funções participando da encapsidação, estabilização do capsídeo, escape dos vírions do endossomo e transporte nuclear do genoma viral (Schiller, Day e Kines, 2010).

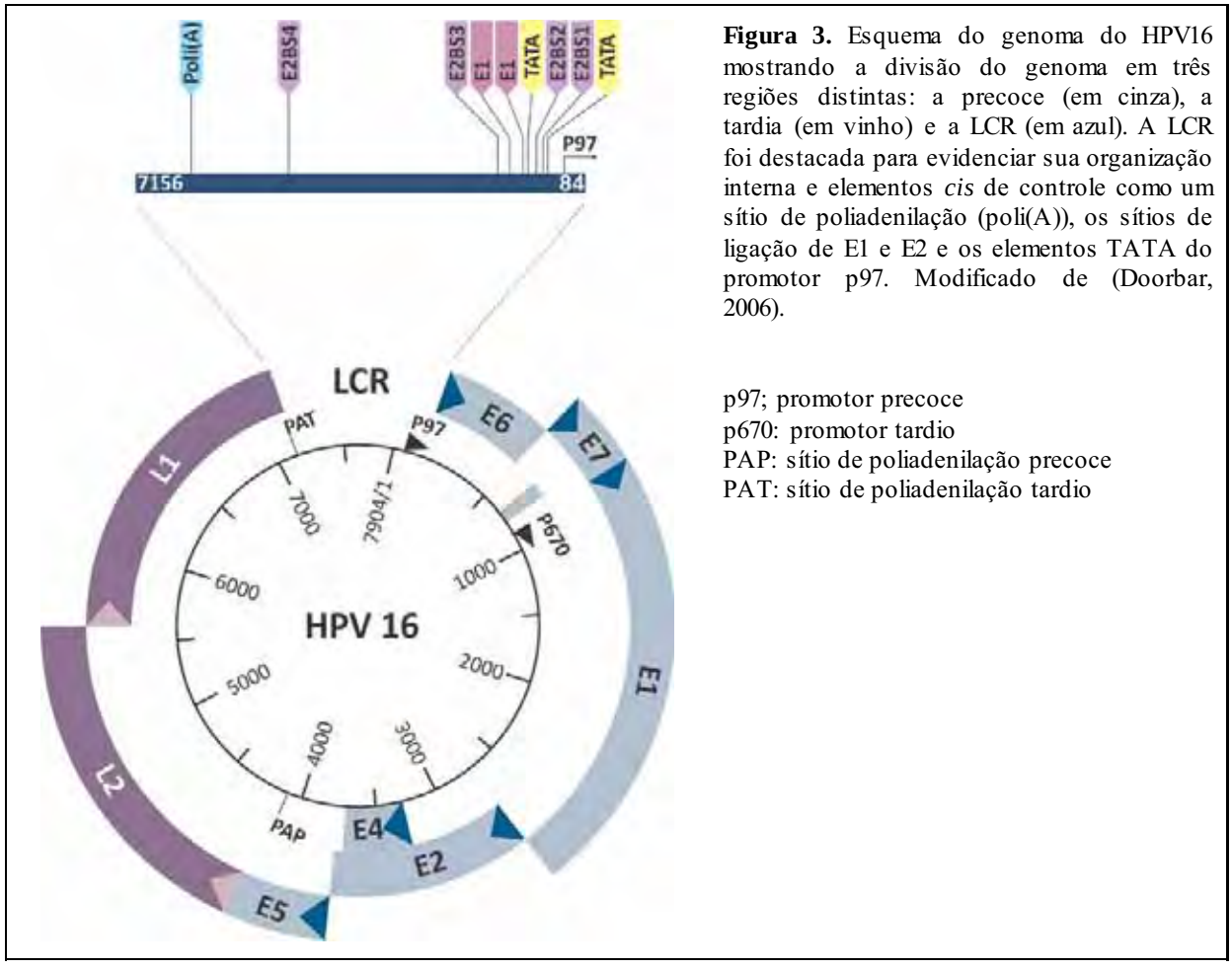

Figura 3. Esquema do genoma do HPV16 mostrando a divisão do genoma em três regiões distintas: a precoce (em cinza), a tardia (em vinho) e a LCR (em azul). A LCR foi destacada para evidenciar sua organização interna e elementos *cis* de controle como um sítio de poliadenilação (poli(A)), os sítios de ligação de E1 e E2 e os elementos TATA do promotor p97. Modificado de (Doorbar, 2006).

p97; promotor precoce
p670: promotor tardio
PAP: sítio de poliadenilação precoce
PAT: sítio de poliadenilação tardio

Os dois promotores, o precoce (p97) e o tardio (p670) controlam a expressão das proteínas precoces em diferentes estágios da maturação dos queratinócitos (Hansen, Nielsen e Norrild, 2010).

A expressão dos genes E1 e E2 é necessária para manutenção do genoma viral na forma de um elemento extracromossomal superenovelado chamado episssomo (Doorbar, 2006). A proteína E2 reconhece motivos palidrômicos AACCg(N4)cGGTT na região LCR do genoma vira, e esta ligação é fundamental na inicialização da replicação e segregação do DNA viral nas células da camada basal do epitélio. Esta ligação é necessária para o recrutamento da helicase viral E1 e outras proteínas celulares (como a proteína de replicação A e a DNA polimerase α primase) à origem de replicação viral. (Dell, Wilkinson, Tranter *et al.*, 2003). Além de colaborar com a replicação a proteína E2, quando em altas concentrações, também é capaz de exercer atividade supressora sobre as oncoproteínas virais E6 e E7 (Doorbar, 2006).

Algumas das funções das proteínas virais são antagônicas. Por exemplo, E4 tem a capacidade de realocar o complexo ciclina B/Cdk2 para o citoplasma, o que impede a mitose e assim mantém a célula na fase G2 do ciclo celular. Assim ela antagoniza os efeitos da proliferação celular mediada por E7. E4 é expressa tanto na fase precoce como na tardia a infecção (Nakahara, Peh, Doorbar *et al.*, 2005). O acúmulo de E4 nas células superficiais também facilita a liberação das partículas virais (Doorbar, 2006). E5 é considerada uma oncoproteína juntamente com E6 e E7. É uma proteína transmembrana que coopera com E6 e E7 para induzir hiperproliferação das células hospedeiras através da modulação de EGFR (*epidermal growth factor receptor*, receptor do fator de crescimento epidermal). E5 inibe a acidificação de endossomos o que diminui a reciclagem de EGFR, mantem um sinal constitutivo de crescimento para a célula (Maufort, Shai, Pitot *et al.*, 2010; Moody e Laimins, 2010) e provavelmente facilita a progressão maligna.

Os produtos das ORFs E6 e E7 são considerados oncoproteínas, pois podem se ligar a múltiplos alvos celulares e inativar os mecanismos que controlam o ciclo celular e a apoptose. Entretanto estas proteínas são expressas em quantidade apenas quando o genoma de HPV se

integra ao genoma da célula hospedeira. Para que o HPV consiga induzir transformação maligna é preciso que haja estabelecimento de infecção persistente, integração do genoma viral ao genoma da célula hospedeira e expressão das oncoproteínas E6, E7 e E5. Durante uma infecção normalmente os HPVs mantém-se na forma episssomal e as infecções são autolimitantes e se resolvem em meses. Entretanto em alguns casos o genoma viral se integra de forma truncada ao genoma celular e estima-se que 10% dos casos desenvolvem câncer. A integração leva à quebra da ORF de E2 retirando a repressão que esta proteína exerce sobre as ORFs de E6 e E7 causando aumento de sua expressão (Schmitz, Driesch, Jansen *et al.*, 2012).

A proteína E7 é um dos principais fatores de transformação maligna em HPV. Tem a capacidade de se ligar a membros da família retinoblastoma (Rb) como p105, p107 e p130 (e outros fatores como p21, p27, ciclina A, p300 entre outros) e marcá-los para degradação resultando na liberação e ativação do fator de transcrição E2F que direciona a expressão de genes da fase S, o que leva a hiperproliferação celular. Antagonicamente a ligação de E7 a pRb pode levar a inibição do crescimento celular e desencadeamento da cascata de apoptose através do gene supressor de tumores p53. A apoptose é bloqueada por E6 que inibe a ação de p53 ligando-se a ele ou induzindo sua degradação proteassomal. Isto permite que as células hospedeiras, diferenciadas ou não, continuem crescendo (Moody e Laimins, 2010; Pim e Banks, 2010).

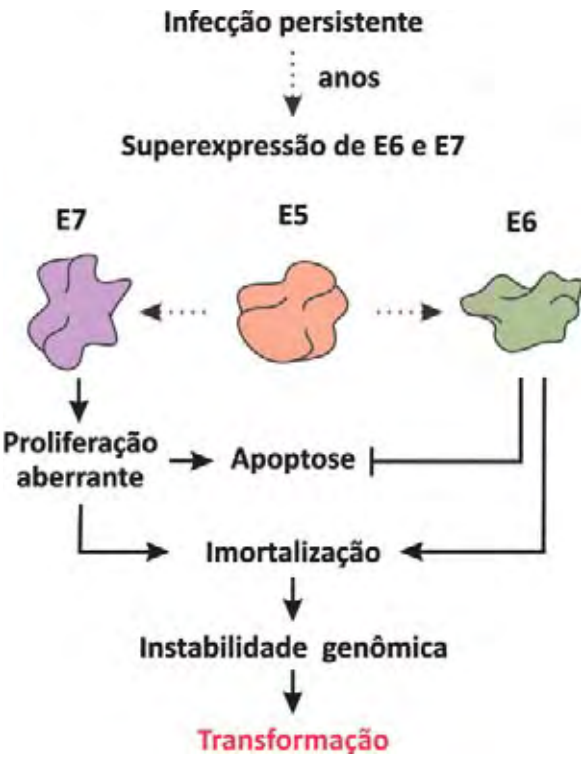

Figura 4. Mecanismo molecular de cooperação entre as proteínas virais para indução de transformação maligna. Infecção persistente por HPV leva a superexpressão de E6 e E7. Modificado de (Moody e Laimins, 2010).

A E6 é capaz de se ligar a diversas proteínas celulares, entre elas fatores celulares como p300, Bak, myc e proteínas PDZ, alterando assim funções celulares como trafego de sinais, polaridade e ciclo celular (Pim e Banks, 2010; White, Kramer, Tan *et al.*, 2012).

A cooperação entre E6 e E7 leva a imortalização da célula hospedeira (**figura 4**). Estas proteínas têm habilidade de se ligar a reguladores da proliferação, apoptose, imortalização e estabilidade genômica o que conjuntamente promove o surgimento de populações clonais com vantagens de crescimento e maior propensão à transformação e progressão maligna (Dimaio e Mattoon, 2001; Moody e Laimins, 2010).

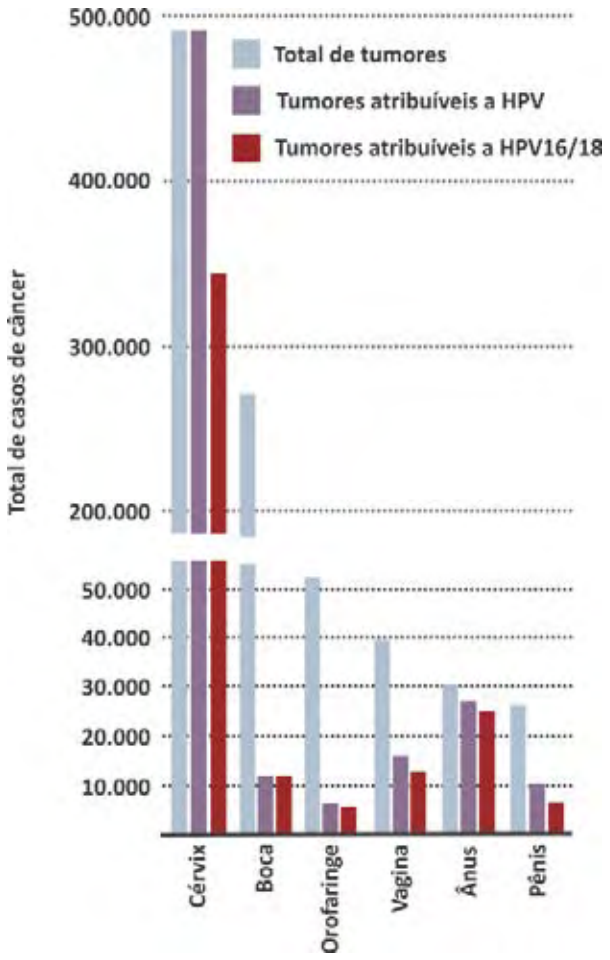

Figura 5. Incidência de infecção por HPV, em diversos tumores. Modificado de (Crow, 2012).

Os HPVs são altamente prevalentes, mas com grande variação na incidência nos diversos tumores aos quais estão associados. No câncer cervical, DNA de HPV é detectado em mais de 99,5% dos casos, sendo considerado uma causa necessária para o desenvolvimento deste câncer. Em outros tumores a incidência varia grandemente e em alguns tipos de tumor o envolvimento deste vírus ainda é controverso (Grulich, Jin, Conway *et al.*, 2010). As **figuras 5** e **6** mostram as incidências de HPV em diferentes tipos de tumores e a incidência global de HPV e os genótipos mais prevalentes em cada região do planeta respectivamente.

As infecções podem permanecer subclínicas ou induzir hiperproliferações epiteliais benignas como verrugas, papilomas ou condilomas. De acordo com sua capacidade de induzir transformações malignas, são classificados como HPV HR (*high-risk* HPV, HPV de alto risco) (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82) ou HPV LR (*low-risk* HPV, HPV de baixo risco) (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, e CP6108) (Chelimo, Wouldes, Cameron *et al.*, 2012). No homem a maioria das infecções são subclínicas e só são diagnosticadas por meios moleculares (Giuliano, Lazcano, Villa *et al.*, 2009).

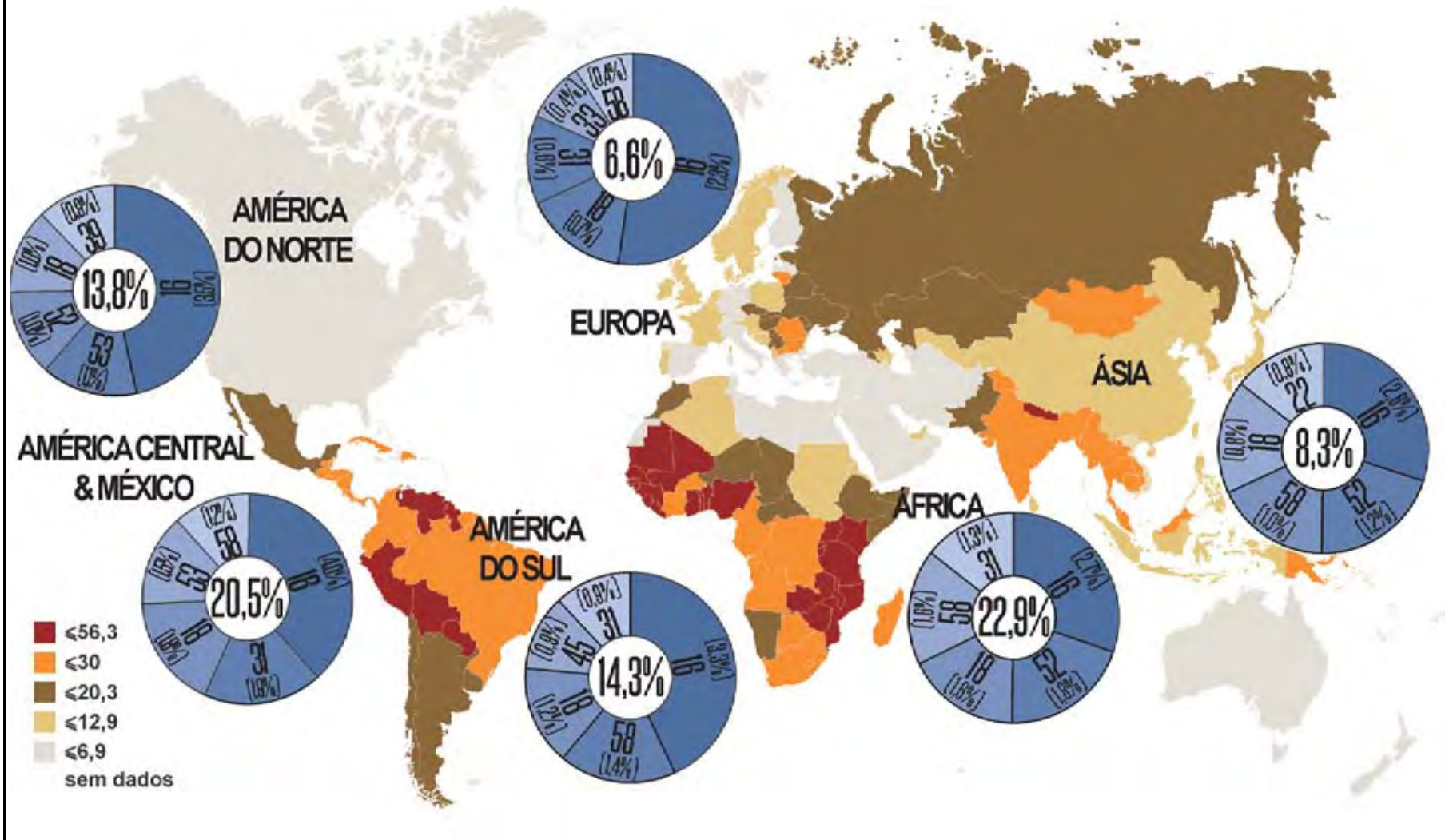

Figura 6. Incidência mundial de HPV. Dentro dos círculos a porcentagem de mulheres sadias portadoras de HPV. Os números externos são os genótipos mais prevalentes e entre parênteses sua porcentagem na prevalência da região. No mapa estão indicadas as taxas de câncer cervical, padronizadas por idade (casos/100.000habtantes por ano). Baseado em dados de (Crow, 2012).

O ciclo de vida dos HPVs está intimamente relacionado à maturação dos queratinócitos. É necessário que estas células sofram mitose e alguma das células filhas se diferencie (Chow, Broker e Steinberg, 2010). Ainda há muita controvérsia sobre os receptores do HPV, mas o proteoglicano heparan-sulfato parece ter papel na ligação inicial ou captação do HPV pelas células (Joyce, Tung, Przysiecki *et al.*, 1999; Shafti-Keramat, Handisurya, Kriehuber *et al.*, 2003).

Este vírus ganha acesso a células da camada basal através de pequenas soluções de continuidade no epitélio como microlesões ou microabrasões. Após a amplificação do genoma ocorre a síntese das proteínas do capsídeo, montagem e liberação de novas partículas virais (Chow, Broker e Steinberg, 2010; Moody e Laimins, 2010). As principais etapas estão esquematizadas na **figura 7**.

As classificações de tumores, baseadas em características histológicas e imunohistoquímicas, fornecem algumas informações dos tipos celulares envolvidos e do índice proliferativo destes tumores. Entretanto os contínuos desenvolvimentos tecnológicos e as novas descobertas sobre os complexos mecanismos moleculares de muitas doenças tornaram a patologia molecular uma ferramenta fundamental nos estudos clínicos de tumores bem como no desenvolvimento farmacológico de novas drogas anticarcinogênicas. A patologia molecular pode fornecer informações mais precisas que os métodos imunohistoquímicos, pois através de análises da expressão gênica de um tumor são obtidos novos dados sobre a ativação de vias de sinalização celular, evasão de apoptose, angiogênese, resposta a drogas e potencial para metastização. Estes dados permitem entender melhor a patogênese e progressão do CEP além de permitir classificá-lo de forma mais coerente. Considerando que a biópsia ainda constitui o principal meio de confirmação de diagnóstico para CEP (Horenblas, 2012; Sadeghi, Gholami, Zakavi *et al.*, 2012) avanços no entendimento do CEP que permitam diagnósticos e prognósticos mais rápidos e precisos são necessários.

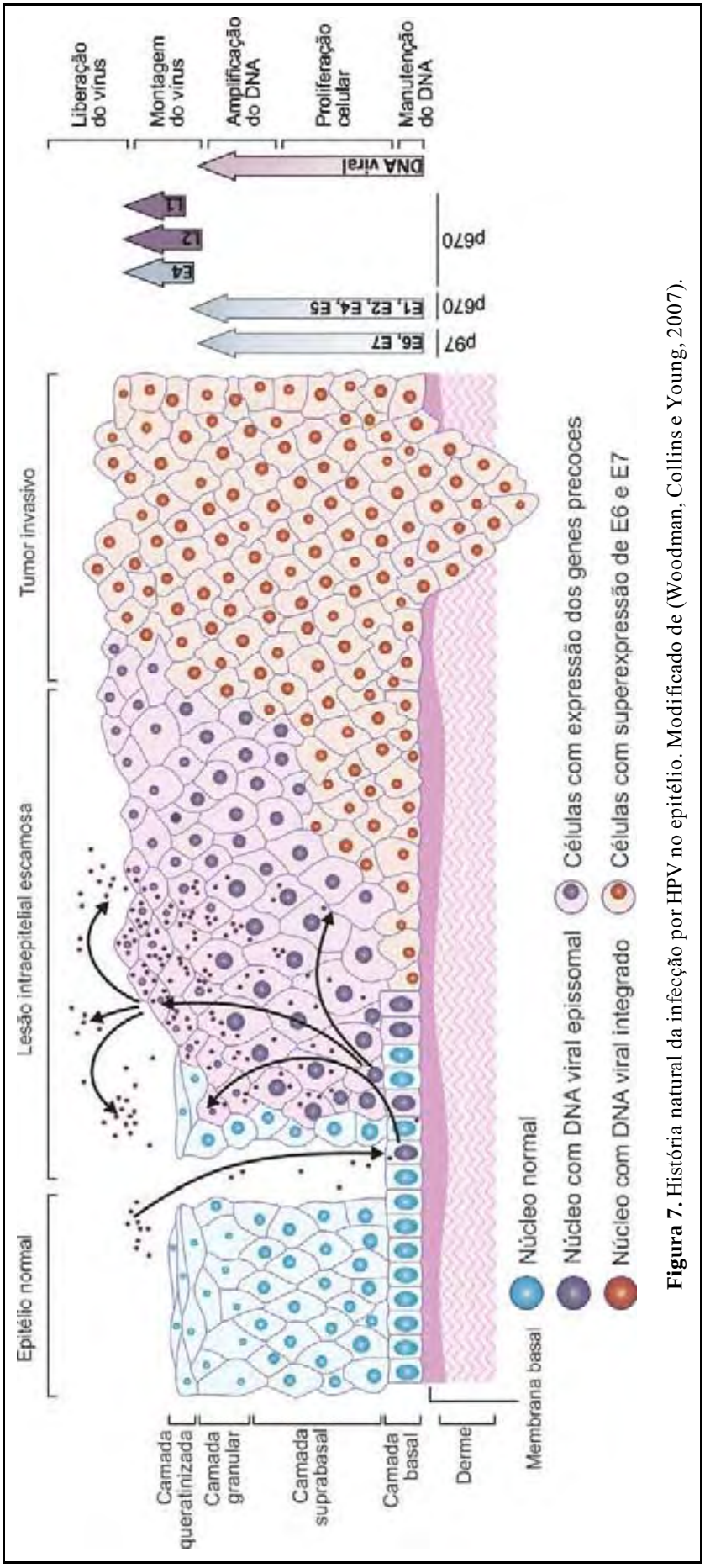

Figura 7. História natural da infecção por HPV no epitélio. Modificado de (Woodman, Collins e Young, 2007).

Poucos estudos têm enfocado os mecanismos moleculares da infecção do HPV com relação ao CEP. Esta escassez evidencia a necessidade de se investigar os mecanismos moleculares envolvidos nesta patologia identificando genes cuja expressão seja diferencial entre tecidos normais e tumorais. O papel do HPV na gênese e progressão tumoral, que permitam auxiliar no diagnóstico e tratamento do carcinoma de pênis.

A caracterização molecular e genética pode levar a uma correlação mais específica entre a expressão gênica e a gênese do processo tumoral além de esclarecer o possível papel do HPV na carcinogênese. Estes dados contribuirão para diagnósticos e prognósticos mais precisos da doença além de resultar em abordagens clínicas e terapêuticas mais adequadas para essa neoplasia.

OBJETIVOS

O presente trabalho teve como objetivos:

1. Caracterizar possíveis diferenças na expressão gênica entre amostras de carcinoma de pênis e de tecido normal;
2. Verificar a presença de HPV e identificar seus genotipos em tecidos tumorais;
3. Verificar se há correlação entre a presença de HPV e genótipos com os possíveis genes diferencialmente expressos;
4. Correlacionar a presença de HPV e genótipos com os parâmetros clínicos dos pacientes portadores de CEP.

CAPÍTULO I

(Penile carcinoma - risk factors and molecular alterations)

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Penile Carcinoma: Risk Factors and Molecular Alterations

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Penile carcinoma is a rare, male cancer. Although the incidence of penile carcinoma is very low in Western countries, in some countries, the incidence is significantly greater, with penile carcinoma accounting for ≤10% of all male malignancies. Greater insight has been gained in recent years as to its pathogenesis, the risk factors associated with its development, and the clinical and histological precursor lesions related to this disease. In this review, risk and conditions factors for penile carcinoma, molecular alterations in this type of cancer, histological types, and prognostic factors will be discussed in order to further our understanding of the biology and behavior of this cancer.

KEYWORDS: penile, carcinoma, risk factors, molecular alterations

INTRODUCTION

The incidence rate of penile carcinoma varies enormously among different populations; some developing countries are most seriously affected. The disease can reach as high as a 10% incidence rate in men in some African, Asian, and South American countries (4.2 and 4.4/100,000 in Paraguay and Uganda, respectively[1,2]). This tumor represents 2% of all cancer cases in Brazilian men, being more frequent in the North and Northeast regions than in the South and Southeast regions. In the regions of high incidence, the number of cases is higher than those of prostate and bladder cancer[3]. In Western Europe and the U.S., age-standardized incidence rates range from 0.3 to 1.0/100,000, accounting for 0.4–0.6% of all malignancies in these parts of the world[4]. The substantial worldwide variation in penile carcinoma incidences may be linked to differences in socioeconomic and religious conditions[5].

Although squamous cell carcinoma (SCC) of the penis can occur at any age[6], most cases affect individuals of advanced age, with a peak of incidence ~60 years old[7,8]. Familial occurrence has rarely been documented[9].

Penile carcinomas usually have a squamous epithelial origin and include carcinomas *in situ*. The latter are known as erythroplasia de Queyrat and Bowen disease. These diseases have similar histological appearance and biological behavior and, therefore, are frequently considered as different aspects of a single preneoplasic disorder[10,11]. Invasive carcinoma is represented by SCC and its variant of low-grade well-differentiated verrucous carcinoma, frequently reported as giant condyloma acuminatum or Buschke-Loewenstein tumor, although the classification of these diseases remains controversial.

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Therefore, the best classification is giant condyloma acuminatum or Buschke-Loewenstein tumor as verrucous carcinomas of the anogenital mucosae[12,13].

The clinical presentation of penile carcinoma varies from a subtle swelling; a small lump, pustule, or warty growth; to an extensive carcinoma with peeling. The earlier symptoms of penile carcinoma include itching or burning under the foreskin of the glans or ulceration of the foreskin. The carcinoma originates mainly in the glans (80%), followed by the prepuce (15%), the balanopreputial membrane (5%), and more rarely in the shaft. Macroscopically, it may present as vegetative or ulcerated lesions. The vegetative lesions have a better prognosis because they tend to grow out, whereas ulcerated lesions are more infiltrative and metastasize more frequently[14].

The progression of penile carcinoma occurs in a locoregional manner, with sequential involvement of the sentinel (inguinal and pelvic) lymph nodes before the development of distant metastasis. The common sites of metastases include lung, brain, and bone. Tumor recurrence may develop in the lymphatic system with discrete or distant nodes. Death often occurs as a result of starvation and sepsis or bleeding as a result of erosion caused by femoral venous ulceration of inguinal metastatic involvement[14].

This review looks primarily at the risk and conditions factors for penile carcinoma, as well as the possible molecular alterations that lead to histologically distinct tumor, and into prognostic factors to get a better understanding of its biology and behavior.

METHODOLOGY

A Medline search using PubMed with the search terms "penile/penis cancer/carcinoma" revealed 5004 published papers between 1956 and 2010. A manual search for publications with strictly direct relevance to the risk factors, the molecular and genetic changes, and potential HPV involved in the molecular genetics of its etiology resulted in 80 papers being identified. These form the basis of the following review, being augmented by other articles on the epidemiology and management of penile carcinoma where appropriate.

RISK CONDITIONS

The etiology of penile carcinoma seems to be multifactorial, but some risk conditions and risk factors have been identified. Penile tear, rash (defined as chronic dermatitis lasting >1 month), injury, and inflammation were associated with an increased risk for cancer development[15]. Some studies have also associated penile carcinoma with inflammation together with injury, as many penile carcinomas arise at sites of infection, chronic irritation, or injury[16,17].

Another risk condition in the development of SCC is PUVA therapy (photochemotherapy using psoralen and ultraviolet [UV] A irradiation) used in the treatment of skin diseases such as psoriasis, vitiligo, and cutaneous T-cell lymphoma[18,19]. Injection of mineral oil into the skin of the corpus cavernosum region can also be a risk condition. This injection may cause an inflammatory reaction, confirmed by the presence of lipogranulomas and foreign body reactions[20].

Hispanics have the highest rates of primary malignant penile carcinoma compared to any other racial/ethnic group, followed by Alaskan Native/American Indians, Africans, Caucasians, and non-Hispanics[21]. It is possible that these racial/ethnic effects are due to differences in sexual and other behavior patterns that may lead to HPV exposure. Because genital HPV infection is spread by sexual contact, behavioral differences between racial and ethnic groups could partially explain these variations in rates. Indeed, high school students of Asian-Pacific ancestry are significantly less likely to have ever engaged in sexual intercourse than African, Caucasian, or Hispanic students[5,22].

Madsen et al.[23] suggest that the transmission of etiological factors responsible for penile carcinogenesis, presumably HPV16 and others hrHPVs, frequently occur by intimate contact. There is a significant trend of increased risk with an increase in the number of female sexual partners. Notably, the

number of partners of <20 years of age seems to differ between the patients diagnosed with SCC of the penis and the controls. This study showed a strong and significant association between oral-penis sex and the risk of SCC, suggesting that oral sex, a common sexual practice in almost half of the control population in Denmark, may have an underestimated role in viral transmission from the oral cavity/pharynx of the female partner to the penis.

RISK FACTORS

Use of Tobacco

Although an association with smoking has been repeatedly observed, the exact role that smoking plays in the development of this disease remains unknown. There is an association between men who smoked when they were diagnosed with penile carcinoma, both *in situ* and invasive[15,17]. Hellberg et al. found a clear dose-response relation, with smokers of >10 cigarettes/day having a significantly greater risk than light smokers[24]. Maden et al. found that the risk of penile carcinoma among men who smoked at diagnosis was 2.8 times that of men who had never smoked, and lifetime smoking of >45 pack-years of cigarettes elevated the risk to 3.2 times that of men who had never smoked[16]. Tobacco might act through its metabolites or directly after systemic absorption[25]. Smoking seems to have an important role in cases where cigarette smokers had been diagnosed with penile carcinoma, although it may be more important in the advanced stages of progression[15].

Human Papillomavirus (HPV)

The HPV is a family of small, double-stranded DNA viruses (8000 bp) of distinct pathogenetic types. There are >100 known variants of HPV. Muñoz et al. have classified HPV types according to their oncogenic potential (Table 1)[26]. Sexual transmission is the most common route for viral propagation, although both oral and vertical transmission have been described[27].

TABLE 1
HPV Types and Oncogenic Potential

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

Modified from Muñoz et al.[26].

Positivity for HPV DNA is strongly associated histopathologically with the basaloid/warty subtypes and is weakly associated with keratinizing/other SCCs. There is only a weak or no association between verrucous carcinoma of the penis and HPV positivity, but this depends on the origin of the population under review[2,28,29,30].

The common HPV types are HPV16 and HPV18. HPV16 is more prevalent in North America, Europe, South America, and India[30,31]. Although HPV18 is the second type more usually identified in the samples of SCC, it has a low prevalence among all the histological types. HPV infection may influence prognosis in patients with penile carcinoma. Traditionally, high-risk HPV types, especially with

HPV16 and 18, were thought to be associated with aggressive variants and, therefore, to give rise to poorer survival than non-HPV–associated penile carcinoma.

Penile carcinoma is a heterogeneous disease and a proportion of these carcinomas can be attributed to HPV infection, whereas a number of other molecular mechanisms causing penile carcinoma independently of HPV probably represent the most common events. However, HPV16 remains an important etiologic factor[15,24].

Cancer of the penis associated with HPV appears from a progression of precursor lesions caused by the viral infection. Some researchers believe that the pathway is similar to carcinogenesis of the uterine cervix mediated by HPV. Carcinogenesis induced by HPV involves several steps: resistant infection caused by HPV is the initial causal event, with genetic[32] and epigenetic alterations[33,34,35,36]. It is necessary for a HPV-infected cell to acquire a malignant phenotype.

The HPVs exert their oncogenic effects through expression of the E6 and E7 oncoproteins, which bind and inactivate p53 and pRB proteins, products of tumor-suppressor genes[37,38]. Their expression is needed to induce and maintain the neoplastic phenotype of cervical cancer cells, and similar mechanisms may therefor apply in penile carcinoma. By means of imbalance of the $p14^{ARF}$/MDM2/p53 and $p16^{INK4A}$ pathways, the oncogenic HPVs interfere with cell cycle control, affecting both proliferation and apoptosis. Functional disruption of pRB by E7 of the HPV results in the reciprocal overexpression of $p16^{INK4A}$, due to a negative feedback between pRB and $p16^{INK4A}$[39,40]. The genetic and epigenetic events subsequent to the cell-host interaction remain poorly understood.

Until today, some studies identified nonviral mechanisms that can lead to an imbalance of both the $p14^{ARF}$/MDM2/p53 and $p16^{INK4A}$/cyclin D1/RB pathways in a subset of penile carcinoma. There are at least two possible mechanisms that can disrupt the latter pathway during carcinogenesis in the absence of HPV. One mechanism is the silencing of the $p16^{INK4A}$ gene through hypermethylation of its promoter region. Another mechanism is the overexpression of the gene BMI-1 from the polycomb group, by which the $p16^{INK4A}$ pathway may be disturbed[41]. The INK4A/ARF locus, encoding both $p16^{INK4A}$ and $p14^{ARF}$, was identified as a critical downstream target of BMI-1. In mice, BMI-1 overexpression results in decreased expression of the respective genes.

Human Immunodeficiency Virus (HIV)

Sexual transmission of HIV requires the virus to penetrate epithelial tissue. The inner lining of the prepuce provides such an access route. This is because it is a mucosal epithelium with a protective keratin layer that is very much thinner than in the outer prepuce and glans penis[42]. The uncircumcised penis is more susceptible to minor trauma and ulcerative disease, and the preputial sac serves as a reservoir for pathogenic organisms present in the pool of smegma (a whitish film consisting of neutral lipids, fatty acids, sterols, and exfoliated cells) that accumulates beneath the prepuce. Because of the potential for infection these pose, the mucosal epithelium has a high prevalence of immune system cells, including CD4+ T cells, Langerhans cells, and macrophages.

The mechanism by which the immunosuppression related to HIV plays a role in the increased risk of cancer associated to HPV is not yet clear. Among the individuals infected with HIV, the incidence of invasive cancers associated to HPV does not seem to have a relationship with CD4-T-cell count, used to measure the immune pattern[43,44]. Comprehension of associations of immunosuppression and an active antiretroviral therapy (HAART) used against the HIV virus with the risk of cancer associated to HPV is important because the recovery of the immune system from HAART is partial. Prolonged survival of individuals infected with HIV along with a compromised immune system could lead to an increased incidence of cancers weakly related to immunosuppression[44,45].

Chaturvedi et al.[46] observed that there is a higher risk of patients infected with HIV developing penile carcinoma compared to the general population in the U.S. A higher risk of developing penile carcinoma *in situ* is found in patients infected with HIV than invasive cancer associated to HPV. Studies reported a higher incidence of penile carcinoma in individuals infected with HIV in the U.S. and Uganda[47,48].

Smegma

Improved penile hygiene is the main reason for circumcision. Smegma has been cited by men as dirty and infected with micro-organisms. Improved penile hygiene frequently is difficult to achieve, and sometimes the attempts to get a good asepsis can cause dermatologic problems in uncircumcised men. To the parents, it is easier to keep their childrens' penises clean when they have been circumcised[49]. There is a trend in circumcised men to take showers and retract the skin in order to clean the penis more frequently than in uncircumcised men[50].

Production of the smegma increases in younger people, peaking between 20 and 40 years of age. Initially, smegma is a yellow or white lubricant. In time, it transforms chemically from a lubricant to a mixture of epithelial cells, dirt, and micro-organisms that together form aggregates and generate an offensive odor. For a long time, it was believed that smegma could be carcinogenic[51]. Some studies between 1940 and 1960 tried to find such evidence[52,53,54], some of which are still considered valid by several researchers. However, these studies were based in animal models, in which human smegma was introduced into skin tunnels or the vagina of mice. The evidence of association with cancer development is weak and could be explained by the chronic inflammation due to an exogenous antigen in the mucosa. Therefore, this association does not seem to be based on scientific evidence[49].

Phimosis/Circumcision

Phimosis is pathology characterized by the inability to retract the prepuce to expose the gland; it occurs in uncircumcised men or men who were inappropriately circumcised. Several studies have associated the history of phimosis with an increase in risk of the development of penile carcinoma[23,24]. Phimosis is also linked to others pathologies, such lichen sclerosus and giant condyloma (Buschke-Löwenstein tumor)[55]. However, circumcision seems to decrease the risk of penile carcinoma only when it is performed in childhood.

Neonatal circumcision has been well established as an effective prophylactic measure for penile carcinoma. Its protective effect is mainly explained by the fact that certain conditions, such as phimosis, related to the retention of smegma and lichen sclerosus are less prevalent in neonatal circumcised men[56]. Some reseachers observed that men who were not circumcised in childhood have a higher risk of invasive penile carcinoma and phimosis compared to circumcised men[15,57]. On the other hand, Madsen et al.[23] observed that there is no difference in the risk of penile carcinoma between men circumcised in childhood and uncircumcised men.

Apparently, the increase in the risk of penile carcinoma in men who were not circumcised in childhood is related to the increase in the risk of phimosis. This indicates that the risk of penile carcinoma can be reduced by preventing the development of phimosis through circumcision in childhood. In uncircumcised men, phimosis can cause injuries that can facilitate access of HPV to the basal epithelium. Also, phimosis can cause inflammation of the glans or prepuce that may result in chronic infection, which increases the risk of penile carcinoma even with only a few sexual partners[15]. Besides, circumcision may protect against HPV-associated disease by enhancing the resolution of infection[58].

Lichen Sclerosus (LS)/Balanitis Xerotica Obliterans (BXO)

Penile LS was first described in 1928 by Stuhmer[59]. Most authors consider LS as synonymous with BXO. It is a chronic, sclerotic, inflammatory disease leading to phimosis and meatal stenosis. The lesions are initially polygonal, flat-topped plaques of white color that progress to atrophic and sclerotic plaques. The most commonly affected anatomical sites are the glans and foreskin, but other regions (such as the frenulum, urethral meatus, and anterior urethra) may also be involved. Less commonly, LS can involve the shaft of the penis. Hemorrhage is common when there is glans involvement. Additionally,

hemorrhagic bullae, erosions, and fissures may be present. A typical finding is a sclerotic white ring at the tip of the prepuce[55].

The process is benign, but the lesions lead to mucosal contraction and subsequent phimosis with progression of the disease. In advanced cases, meatal stenosis can cause urinary flow obstruction and requires surgical treatment[60]. Symptoms are pruritus, burning, priapism, difficulty in retracting the foreskin, dysuria, and a poor urinary stream. SCC of the penis arising on a background of LS manifests between 36 and 83 years of age, with a mean of 54[61], being more common in uncircumcised men[55]. The etiopathology of LS remains unknown, but some possible causes are autoimmunity, infection (*Borrelia burgdorferi*, HPV, hepatitis C virus [HCV]), hormonal factors, traumas (burnings, radiotherapy, surgery), and meatal stenosis[62].

The association between LS and SCC in women is widely recognized. The risk of malignant transformation with vulvar LS is 3–6%[55]. LS, SCC, and HPV are more common in uncircumcised men, but the association between LS and SCC in men is unproven. The available data in literature seem contradictory. In retrospective studies with patients who progress to SCC, high rates of LS are found. In a study of 20 patients bearing SCC, 50% showed evidence of LS[61]. In another study, the clinicopathologic data of 207 SCC and giant condyloma samples were verified and 68 (32.9%) patients with LS were identified[56]. In a group of 18 patients with SCC, Perceau et al.[63] found eight (44%) with LS.

However, in studies of patients with LS who progress to SCC, only a small percentage led to some kind of malignant transformation. In a review of 130 LS cases, Barbagli et al.[64] found 11 (8.4%) patients who developed malignant or premalignant alterations. In a study of 54 patients with LS, only two (3.7%) cases of tumor were noted[65]. Nasca et al.[66] found malignant alterations in only six of 86 (5.8%) patients with LS; in another study, the same group found eight (9.3%) patients who developed epithelial carcinoma in a cohort of 86 patients[67].

Some studies have demonstrated a wide range in lag time from LS diagnosis to cancer development[66]. The data taken together suggest that LS must act as a catalyst and not as a primary carcinogenic, since the development of SCC from LS is unusual. Perhaps the development of phimosis predisposes the tissue by accumulation of smegma or even allows HPV viability for longer periods[68].

INGUINAL METASTASIS

The survival of the patients bearing penile SCC is associated with the presence and extent of inguinal node metastasis[69]. The presence of metastatic lymph nodes was the best prognostic factor for patient survival found in a study of 202 cases of penile SCC. The rate of inguinal/regional metastatic lymph node involvement in patients with clinical stages N0, N1, and N2 were 24, 70, and 42%, respectively. Patients with negative lymphadenectomy had better survival rates at 10 years than those with positive lymphadenectomy[70]. In another study of 333 cases of penile SCC, half the patients who underwent total or partial penectomy showed inguinal lymph node metastasis. In contrast, patients who underwent circumcision or local excision did not show iguinal lymph node metastasis and had a cure rate of 100%[71].

HISTOLOGICAL TYPES

SCC accounts for 95% of all penile neoplasias. It is classified in the subtypes: usual type, verrucous carcinoma, warty carcinoma, papillary carcinoma, basaloid carcinoma, sarcomatoid carcinoma, carcinoma cuniculatum, pseudohyperplastic carcinoma, adenosquamous carcinoma, and acantholytic carcinoma[72]. Major histological types are given in Table 2.

TABLE 2
Characterization of Penile SCC Histologic Types that have Higher Distributions

Carcinoma Subtype	Distribution (%)	Main Features	Metastatic Rate (%)	Prognosis
Usual	48–65	Most common subtype, morphologically similar to SCC from other sites	28–39	Intermediate
Basaloid	4–10	HPV-related aggressive tumor composed of deeply invasive tumor nests of basaloid cells	50–100	Ominous
Verrucous	3–8	Low-grade verruciform tumor, usually invading only superficial anatomic levels	Null	Excellent
Warty	7–10	HPV-related verruciform tumor similar to warty carcinomas from other sites	17–18	Good
Papillary	5–15	Low-grade verruciform tumor frequently invading superficial erectile tissues	12	Good

Modified from Chaux et al.[72].

SCC can also be divided on morphological grounds into two groups: exophytic (fungating, papillary) and endophytic (infiltrative, ulcerating). The exophytic form is well differentiated and heavily keratinized, and may grow into a large polypoid mass. Exophytic penile carcinoma is commonly located in the glans and may occur in the prepuce. The endophytic form is more undifferentiated, occurring in the foreskin, glans, and more rarely in shaft. Ulcerating tumors have higher rates of lymph node metastasis[73]. Cubilla et al.[74] classified SCC of the penis into five categories: superficial spreading, vertical growth, verruciform, multicentric, and mixed. The superficial growth tumors are the most frequent and inguinal metastasis is present in 42% of the cases.

Metastatic lymph nodes were found in 82% of the deep vertical growth tumors, 33% of the multicentric cases, and none with verrucous pattern. The verrucous carcinoma is responsible for 5% of all penile carcinomas. The tumor can be locally aggressive, but it is biologically benign and, if correctly disgnosed, shows no metastasis[71]. Microscopically, it is an extremely well-differentiated papillary SCC, with the same characteristics of the homonym tumor of the superior aerodigestive tract[73].

Nonsquamous primary malignancies make up <5% of the penile carcinomas. Among them, the sarcomas (including Kaposi's sarcoma) are the more frequent, followed by melanomas, basal cell carcinomas, extramammary Paget's disease, and lymphomas. Carcinomas rarely metastasize to the penis. Priapism is one form of presentation. The most common sources are, in decreasing order of frequency, prostate, bladder, rectosigmoid colon, kidney, and testis.

MOLECULAR ALTERATIONS

The rarity of penile carcinoma, together with the technical difficulties inherent in this tumor because of the presence of inflammation, necrosis, and poor growth in cell culture, have led to few results and reviews about molecular alterations of penile carcinoma[75,76]. Table 3 summarizes the molecular alterations observed in penile carcinoma.

There are alterations in the number of the DNA copies in samples of penile SCC, with similarities with other types of SCCs, such as oral and esophageal carcinoma. There is gain in the number of DNA copies, including 8q24, 16p11-12, 20q11-13, 22q, 19q13, and 5p15, and deletions in 13q21-22, 4q21-32, and along the X chromosome[32]. Ornellas et al. and Masih et al.[77,78] discovered a diploid population in verrucous carcinomas. Ornellas et al. also observed that aneuploidy varied according to grade of the penile SCC. Unfortunately, there were only three poorly differentiated tumors in this study, preventing

TABLE 3
Molecular Alterations Observed in Penile Carcinoma

Molecular Alterations	Gene/Chromosome	Refs.
Copy number gains	8q24, 16p11-12, 20q11-13, 22q, 19q13, and 5p15	[32]
Deletions	13q21-22, 4q21-32 and along the X chromosome	[32]
Aneuploidy	Aneuploidy in invasive penile SCC	[77]
Gene overexpression in HPV-positive patients	*p53*	[83]
Gene overexpression	*p53*, *p21*, *RB*, and *NF-κB*	[87,88]
Gene low expression	$p16^{INK4a}$	[87]
Gene mutations	*p53*, $c\text{-}ras^{Ha}$ *PIK3CA*, *HRAS*, and *KRAS*	[89] [92]
Imbalance protein expression	Bcl-2/Bax	[93,94]
DNA hypermethylation in the promoter region of the gene	*DAPK*, *FHIT*, *MGMT*, $p16^{INK4a}$, $p14^{ARF}$, *RAR-β*, *RASSF1A*, *RUNX3*, and *TSP-1*	[33,34,35]

formal analysis of aneuploidy as a prognostic factor[77]. Nonetheless, there was a tendency towards a high DNA index correlating with increased metastatic risk.

Several studies have investigated the relation between *p53* expression and HPV infection, as well as the relation of *p53* expression to prognosis of penile carcinoma[2,79,80,81]. The HPV oncogenic product, E6, is known to interfere with this pathway by binding to the oligomerization region of wild-type *p53* to mediate its ubiquitination and degradation by 26S proteasome via a pathway that is thought to be analogous to MDM2-mediated p53 degradation. Binding of E6 to p53 causes suppression of normal *p53* inhibitory function at the G1/S transition of the cell cycle, causing uncontrolled cell proliferation, loss of differentiation, and decreased apoptotic control through BCL-2 and BCL-2–associated X protein (BAX) imbalance. Blanton et al.[82] produced an *in vitro* model in which foreskin and ectocervical epithelial cells were infected with retroviral vectors expressing HPV16 oncogenes. Expression of *E7* caused persistent proliferation in the suprabasal domain, but no effect on terminal differentiation. Cells expressing only *E7* were positive for *p53*, whereas cells coexpressing *E6* and *E7* were negative, but still proliferating suprabasally. The authors concluded that *E7*-induced suprabasal proliferation is independent of the amount of baseline *p53*.

The evidence linking *p53* expression and presence of HPV DNA in penile carcinoma is contradictory. Lam et al.[83] reported that 100% of HPV-infected cells also showed positive p53 staining. However, several reports showed an inverse or negative relationship between these two factors, reviewed the expression profile of premalignant conditions (treatment-resistant genital warts, bowenoid papulosis, erythroplasia de Queyrat, and carcinoma *in situ*), and compared this with the profiles of invasive tumors. They concluded that there was no correlation between *p53* expression and HPV status. Overexpression of *p53* does not indicate a *p53* mutation in premalignant disease, which infers that *p53* gene mutations might occur in the later events of male genital carcinogenesis and, thus, describe a subset of patients with alternate disease progression[84,85,86].

Lam et al.[83] developed their initial findings of p53 expression in penile carcinoma to include p21 expression data, so that the interplay between these two proteins could be investigated. Nuclear staining for the p21 protein was found in 40% of tumors, with a suprabasal localization. This staining was also seen in adjacent dysplastic and carcinoma *in situ* areas with no correlation to grade or stage. There was a strong association between HPV16 positivity and positive p21 immunostaining (100%). The authors suggest that, in penile carcinoma, p21 expression is controlled by p53-dependent and p53-independent mechanisms. Stankiewicz et al.[87] failed to observe a significant association between p21 protein expression and histological types of penile carcinoma.

The $p16^{INK4a}$/cyclin D/retinoblastoma pathway is of specific interest in the pathogenesis of penile carcinoma. This pathway is deregulated in several types of human carcinomas, with inactivation of single or multiple steps within the pathway. Retinoblastoma is a target for the viral oncoprotein E7; thus, its inactivation might contribute to carcinogenic events in HPV-dependent tumors.

Ferreux et al.[41] showed that this pathway can be disrupted by three independent mechanisms in penile carcinoma. They described HPV-dependent and HPV-independent mechanisms affecting the normal functioning of this important signaling pathway. These mechanisms included blocking of retinoblastoma function by the E7 oncoprotein with up-regulation of $p16^{INK4a}$ and silencing of the $p16^{INK4a}$ gene by methylation in the absence of HPV infection, with overexpression of $p16^{INK4a}$ in 15% of cases. Overexpression of *BMI-1*, which targets the downstream factors $p16^{INK4a}$ and $p14^{ARF}$, was also noted in 10% of cases in the absence of HPV infection. The authors postulated that $p16^{INK4a}$ might act as a potential prognostic marker and that these results have strong implications on the potential effectiveness of prophylactic HPV vaccines for this tumor[41].

An immunohistochemical analysis of several proteins associated to cell cycle and HPV detection in penile carcinoma samples has been carried out in which it was observed that $p16^{INK4a}$ and Ki-67 expression were significantly lower in verrucous carcinoma than in the common type of SCC, suggesting that low levels of $p16^{INK4a}$ and Ki-67 expression differentiate a verrucous penile carcinoma from a common type of SCC. Besides, HPV infection was detected in only 23% of the samples analyzed. The low level of $p16^{INK4a}$ expression and HPV detection suggests that the pathogenesis of penile verrucous carcinoma is unrelated to HPV infection, neither oncogenes nor suppressor tumors genes classically altered by viral infection[87]. On the other hand, another study of penile carcinoma samples from Kenya failed to find any difference in $p16^{INK4a}$ expression between penile carcinomas that were HPV negative or positive[88].

Proto-oncogene activity in penile carcinoma has been analyzed through $c\text{-}ras^{Ha}$ and *myc* mutations and their relation with HPV involvement. Leis et al.[89] assessed two patients with penile carcinoma, with one developing late relapse from inguinal metastasis in the seventh year. HPV18 was isolated in the primary tumor and nodal metastasis, which was linked to mutations in *p53* and $c\text{-}ras^{Ha}$. Although HPV infection can be involved in early carcinogenesis, mutations in *ras* genes occur later and are linked to disease progression. Couturier et al.[90] and Sastre-Garau et al.[91] investigated the pattern of genomic integration of HPV DNA in a small number of patients with penile carcinoma. These studies highlighted the fact that the integration of HPV types 16 and 18 was localized to sites containing *c-myc* (8q24.1) and *n-myc* (2p24) proto-oncogenes. Another study analyzed possible mutations in *PIK3CA, PTEN, BRAF, HRAS, KRAS,* and *NRAS,* and observed that *PIK3CA* mutations were found in all grades and stages, whereas *HRAS* and *KRAS* mutations were present in larger and more advanced tumors[92].

The complex interaction between the proapoptotic protein BAX, the antiapoptotic BCL-2, and the p53 deregulation is well known in several tumors. Saeed et al.[93] found no difference in BAX expression between the verrucous carcinoma and well-differentiated SCC. Nascimento et al.[94] and Saeed et al.[93] reviewed this area in penile carcinoma. Both studies found an imbalance in BAX/BCL-2 ratios between benign, premalignant, and invasive disease. BCL-2 concentrations were significantly increased in low-grade disease compared with verrucous cancers, whereas BAX concentrations were comparable. The average proportion of BCL-2/BAX was significantly low in verrucous carcinoma compared to well-differentiated SCC, and the proportion of BCL-2/BAX was considered one of the strongest independent predictors of disease progression and prognosis[95,96]. High proportions of BCL-2/BAX in poorly differentiated squamous tumors and an increase in the proportion of BCL-2/BAX are associated to poor outcomes in others types of tumors. Nascimento et al.[94] observed an imbalance in the proportions of BCL-2/BAX between the benign, premalignant, and invasive lesions, suggesting that the imbalance of BCL-2 and BAX is probably associated with the neoplasic process.

Epigenetic mechanisms involving DNA methylation, histone modifications, and noncoding RNAs regulate and maintain gene expression states. Similar to genetic mutations, alterations in epigenetic regulation can lead to uncontrolled cell division; tumor initiation; and growth, invasiveness, and metastasis[97].

The role of DNA methylation in cancer has received much attention; it is accepted that the methylation of the promoter region of many genes is associated with gene silencing[98]. DNA methylation is the addition of a methyl group for the carbon atom 5'-cytosine, present in the dinucleotide CpG, resulting in the formation of a 5-methylcytosine[99]. The methyl groups found mainly on the islands of the CpG promoter regions of genes can influence the expression of DNA into proteins, reducing the binding affinity between the promoter regions and transcription factors through mechanisms involving changes in chromatin structure or levels of histone acetylation[100].

Yanagawa et al.[35] analyzed the methylation pattern of several genes in penile carcinoma samples and observed hypermethylation in the genes *DAPK, FHIT, MGMT, p14^{ARF}, p16^{INK4a}, RAR-β, RASSF1A,* and *RUNX3*, suggesting that in the absence of HPV, pathogenesis of SCC may require alterations of a number of genes, including *p53* gene mutations and methylation of suppressor tumor genes, or genes related to tumor than the pathogenesis with HPV infection[35]. Guerrero et al. observed hypermethylation in the genes *TSP-1, RASSF-1,* and p16^{INK4a} in penile SCCs, and the hypermethylation of the gene *TSP-1* was significantly associated with unfavorable histological grade and vascular invasion[33]. Another study analyzed the methylation status of several genes and found that at least one of the genes was hypermethylated in all the samples analyzed. With the exception of the gene *FHIT*, the genes *DAPK, MGMT, p16^{INK4a}, RAR-β,* and *RUNX3* also were hypermethylated in >20% of the samples, suggesting that the methylation of the suppressor tumor genes or genes related to tumor affects the pathogenesis of penile carcinoma as well as others carcinomas[34].

CONCLUSION

Penile carcinoma is a severe, uncommon disease mainly related to poor hygiene, sexual history, and even smoking. Developments in the understanding of the pathogenetic changes that occur in penile carcinoma have been limited, mostly because of the small numbers and paucity of pathological tissue for preclinical research. The comparative rate of progress to other tumor types is fundamentally a result of the rarity of this disease, and only a small number of investigations have focused on the molecular and genetic alterations. This review shows the risk factors related to penile carcinoma, and limited data of the molecular and genetic alterations related to the pathogenesis of this type of cancer. It is essential to develop strategies to inform the population of developing countries, mainly the Brazilian population, about the importance of personal hygiene for men, the risk factors involved in penile carcinoma, and the importance of early diagnosis of this type of cancer in order to improve both mortality and management strategies.

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CAPÍTULO II

(Overexpression of ANXA1 in penile carcinomas positive for high-risk HPVs)

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Overexpression of ANXA1 in Penile Carcinomas Positive for High-Risk HPVs

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Abstract

The incidence of penile cancer varies between populations but is rare in developed nations. Penile cancer is associated with a number of established risk factors and associated diseases including phimosis with chronic inflammation, human papillomavirus (HPV) infection, poor hygiene and smoking. The objective of this study was to identify genes related to this type of cancer. The detection of HPV was analyzed in 47 penile squamous cell carcinoma samples. HPV DNA was detected in 48.9% of penile squamous cell carcinoma cases. High-risk HPV were present in 42.5% of cases and low-risk HPV were detected in 10.6% of penile squamous cell carcinomas. The *RaSH* approach identified differential expression of *Annexin A1 (ANXA1), p16, RPL6, PBEF1* and *KIAA1033* in high-risk HPV positive penile carcinoma; *ANXA1* and *p16* were overexpressed in penile squamous cells positive for high-risk HPVs compared to normal penile samples by qPCR. ANXA1 and p16 proteins were significantly more expressed in the cells from high-risk HPV-positive penile carcinoma as compared to HPV-negative tumors ($p<0.0001$) independently of the subtype of the carcinoma. Overexpression of *ANXA1* might be mediated by HPV E6 in penile squamous cell carcinoma of patients with high-risk HPVs, suggesting that this gene plays an important role in penile cancer.

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Introduction

Penile cancer affects predominantly men aged between 50 and 70 years [1–3]. Penile cancer is associated with several established risk factors and associated diseases including phimosis with chronic inflammation, human papillomavirus (HPV) infection, poor hygiene and smoking [4]. Studies reported an overall HPV prevalence of, approximately, 48% in penile cancer worldwide [5,6]. In penile carcinomas the most common HPV types are HPV 16 and HPV 18. HPV 16 is most prevalent in North America, Europe, South America and India [5,7].

HPV contributes to tumorigenesis predominantly through the action of viral oncoproteins (E6 and E7) [8]. E6 inhibits apoptotic signaling in response to growth-suppressive cytokines by interacting with tumor necrosis factor (TNF)-α receptor TNFR1, FAS-associated protein with death domain (FADD) and caspase 8, and via degradation of pro-apoptotic BAX and BAK. E6-mediated degradation of PDZ proteins leads to a loss of cell polarity and induces hyperplasia [9,10]. Therefore, E6 can interfere with the regulation of expression of genes by interacting with and binding to the proteins mentioned above, prompting the interest of some researchers in identifying new genes whose expression can be disturbed by E6 protein.

One gene that could be subject to regulation by the oncoprotein E6 is ANXA1 as previously suggested by Shimoji et al., 2009 [11]. The annexin superfamily proteins have been implicated in several cellular processes including differentiation, apoptosis, proliferation and inflammation. The expression of *ANXA1* has been studied in various types of cancer, but there is no consensus concerning the role this protein plays during tumor initiation and/or progression. Some studies observed a correlation between the decrease of *ANXA1* mRNA and protein with esophageal, prostate and breast cancers [12–15]. On the contrary, others studies showed the overexpression of *ANXA1* in head and neck and pancreatic cancers [16,17].

Here, we aimed to identify novel genes differentially expressed in penile squamous cell carcinoma positive for high-risk HPVs and evaluate a possible correlation between HPV positivity, the expression of the genes and the subtypes of penile squamous cell carcinoma.

Materials and Methods

Patients

Archival paraffin wax-embedded tissue sections from 47 penile squamous cell carcinoma were obtained and reviewed by a pathologist, with approval from the Research Ethics Committee of the College of Medicine of São José do Rio Preto, Research Ethics Committee of Hospital A. C. Camargo, São Paulo, and Research Ethics Committee of University Hospital João de Barros, Belem, all located in Brazil. Twelve penile squamous cell carcinoma samples and seven normal penile fresh-frozen tissue samples were obtained from the College of Medicine of São José do Rio Preto, and Hospital A. C. Camargo. The use of patient-derived material was approved by the institution's Committee Research Ethics Board and written consent was obtained from all patients. Tissues were obtained at surgery from patients undergoing tumor resection, and the diagnosis of penile squamous cell carcinoma was verified post-operatively using histopathology. All slides were histologically examined accordingly to the TNM classification system (American Joint Comittee on Cancer) [18]. The slides were also classified according to morphologic criteria outlined in the Atlas of Tumor Pathology [19]. The following variants were considered: usual, basaloid, warty, papillary, verrucous, sarcomatoid and mixed squamous cell carcinoma.

DNA Extraction

DNA was extracted from 6 slices of 10 micra of paraffin wax-embedded sections using the QIAamp DNA FFPE Tissue kit (Cat. No. 56404; Qiagen, Crawley, U.K.). The polymerase chain reaction (PCR) was performed on DNA extracted from penile squamous cell carcinoma samples. Purified DNA (1–10%) was subjected to PCR. The amplification of a fragment of the *β-globin* gene served as an internal control to assess the sufficiency of DNA in each specimen.

HPV DNA Detection

Globin positive specimens were analyzed by PCR for the presence of HPV DNA using the consensus primers GP5+/GP6+, which flank a fragment of approximately 140 bp of the *L1 gene*, a highly conserved sequence in HPV genomes, allowing several genital HPV types to be detected [20] The reaction components in a final volume of 50 ul were: 1.0 mM GP5+/GP6+; 2.0U Taq DNA polymerase (Fermentas, California, USA); 20 mM Tris–HCl, pH 8.4; 50 mM KCl; 3.0 mM $MgCl_2$; 200 mM of each deoxyribonucleotide (Amersham Pharmacia Biotech, New Jersey, USA) and between 3.0 and 7.0 µl of DNA from the samples. The PCR conditions were an initial step of five min at 94°C, 40 cycles of one min at 94°C, one min at 45°C, and 90 s at 72°C; the last cycle was five min at 72°C. For each reaction, DNA from HeLa cells, a HPV-18 positive cervical cancer derived cell line, was used as a positive control and water and DNA from C33 cells were used as negative controls. The C33 and HeLa cell lines were a generous gift from Dr. Luisa Lina Villa from University of São Paulo [21,22].

HPV Genotyping by INNO-LiPA

Genotyping was performed with the INNO-LiPA HPV Genotyping Extra test (Innogenetics, Gent, Belgium) allowing the identification of 28 different HPV genotypes as well as the HLA-DPB1 gene as internal control for DNA quality. As recommended by the manufacturer, only samples positive for any HPV and/or for the HLA-DPB1 gene were included in the analysis.

RNA Extraction and RT-PCR

Total RNA was isolated from penile squamous cell carcinoma tissue and normal tissue using TRIzol reagent (solution for extraction of RNA, Life Technologies, Grand Island, USA) according to the manufacturer's instructions. RNA integrity post-purification was ensured using the Agilent 2100-Bioanalyser, giving a minimal RIN value of 5.5.

Rapid Subtractive Hybridization (*RaSH*)

Four fresh-frozen samples of penile squamous cell carcinoma were used to perform RaSH methodology. Tissues adjacent to tumor and tumor tissues from the same patient were reviewed by two pathologists and microdissected aiming to obtain most representative tumoral and morphologically normal tissues. HPV 16 was detected in tumoral cells while normal samples were HPV DNA negative. *RaSH* cDNA libraries were performed as described previously [23], with modifications. From the 25 µg total RNA pool, cDNAs were synthesized and digested with MboI (Invitrogen Life Technologies, California, USA) at 37°C for one hour and extracted with phenol-chloroform followed by ethanol precipitation. The digested cDNAs were mixed with 20 mmol/L of the primers XDPN-14 (5′CTGATCACTCGAGA3′) and XDPN-12 (5′GATCTCTCGAGT3′) in 30 µL of 1X T4 DNA Ligase Buffer (Invitrogen Life Technologies, California, USA), heated at 55°C for one min, and cooled to 14°C within one hour. Ligation was carried out overnight at 14°C after adding nine units of T4 DNA ligase to each sample.

The samples were diluted to 100 µl and 40 ul of the mixture was used for PCR amplification with the primer XDPN-18 (5′CTGATCACTCGAGAGATC 3′). Aliquots (10 µg) of the tester PCR products (penile carcinoma or normal tissue) were digested with 20 units of XhoI (Invitrogen Life Technologies, California, USA) and purified with phenol-chloroform extraction and ethanol precipitation. The fragments were inserted into XhoI-digested pZERO plasmid (1 µg/µl) at 16°C for three hours. The constructs were introduced into TOP10 competent cells. Two *RaSH* cDNA libraries were prepared, one using cDNA from the penile squamous cell carcinoma as a tester and normal tissue of penis as a driver, and the other using cDNA from normal tissue of penis as a tester with cDNA from the penile squamous cell carcinoma as a driver.

Bacterial colonies were analyzed using PCR and the M13 forward and M13 reverse primers to identify those with an insert. The sequences of these clones were determined using a DNA sequencer (ABI PRISM 377, Applied Biosystems, California, USA) and DYEnamic ET Dye Terminator Sequencing Kit (Amersham Biosciences, New Jersey, USA). A total of 230 cDNA clones were sequenced, 27 clones obtained from the reverse library (downregulated genes) and 30 clones obtained from the upregulated genes library. The sequences were analyzed using an annotation pipeline with four steps: (1) quality checking, phred base-calling, cutoff 0.09, minmatch 10 and minscore 20; (2) vector trimming and removal of undesirable sequences such as bacterial, mitochondrial and rRNA sequences; (3) masking of repetitive elements and screening of low-complexity regions by Repeat Masker, using the default settings [24]; (4) annotation against existing databases, using BLASTN with default parameters. Significant hits were determined using an E-value threshold of 10^{-15} for searches against nucleotide sequence databases [25].

qPCR

qPCR was used to assess the expression of genes identified by rapid subtraction hybridization (*RaSH*) in fresh samples of penile

squamous cell carcinoma. For qPCR, 12 fresh samples of penile squamous cell carcinoma positive for high-risk HPVs and a pool of 7 fresh normal penile tissue samples were used; the normal tissues were defined as the normal reference. Gene-specific primers for qPCR were designed for optimal hybridization kinetics using the Primer 3.0 program (provided by the Whitehead/MIT Center for Genome Research, Cambridge, MA).

Quantitative Real-time PCR was performed using an ABI prism 7300 sequencer detector system and SybrGreen PCR Core Reagent (Applied Biosystems, California, USA), following the manufacturer's protocol. In brief, the reaction mixture (20 µl total volume) contained 25 ng cDNA, gene-specific forward and reverse primers for each gene, and 10 µL of 2x Quantitative Sybr Green PCR Master Mix (Applied Biosystems, California, USA). Relative quantification was given by the CT values, determined for triplicate reactions of penile tumor samples and reference samples for each gene and tubulin (*TUBA1A*) for the endogenous control. The primer sequences are available on request.

Therefore, the relative expression of each specific gene was calculated by using the formula: $R = (E\ \text{target})^{\Delta Ct\ \text{target (control - sample)}} / (E\ \text{endogenous})^{\Delta Ct\ \text{endogenous (control - sample)}}$, as previously described [26]. The cut-off for analysis of gene expression was ≥4 for increases and decreases in expression. A value below this cut-off was considered to indicate that the increase/decrease in expression was not significant.

Immunohistochemistry

For histopathological evaluation, two observers that were unaware of the clinical data, reviewed independently the slides, and discrepancies were resolved by joint review of the slides in question. The primary lesion was staged according to the TNM classification system (Americam Joint Committee on Cancer) [18].

Immunohistochemistry was used to evaluate *ANXA1* and *p16* protein expressions in 20 histologically normal tumor margins (10 margins from squamous cell carcinoma of penis high-risk HPV positive samples and 10 margins from squamous cell carcinoma of penis HPV negative samples - control group), 24 squamous cell carcinoma of penis samples without HPV (HPV-negative group), 3 samples of squamous cell carcinoma of penis samples with low-risk HPVs (HPV-low risk group) and 20 squamous cell carcinoma of penis samples positive for high-risk HPVs (HPV-high risk group) (Table 1).

The detection of ANXA1 and p16 were conducted in 4 µm sections of each designated formalin-fixed, paraffin-embedded tissue blocks. After an antigen retrieval step using citrate buffer pH 6.0, the endogenous peroxide activity was blocked and the sections were incubated overnight at 4°C with the primary antibodies: monoclonal anti-p16 (1:1000) (Abcam, Cambridge, UK) or rabbit polyclonal anti-ANXA1 (1:2000) (Zymed Laboratories, Cambridge, UK) diluted in 1% BSA. After washing, sections were incubated with a secondary biotinylated antibody (Dako, Cambridge, UK). Positive staining was detected using a peroxidase conjugated streptavidin complex and colour developed using DAB substrate (Dako, Cambridge, UK). The sections were counterstained with hematoxylin.

The ANXA1 and p16 densitometric analyses were conducted with an Axioskop II microscope (Zeiss, Germany) using the Software Axiovision™ (Zeiss). For these analyses five different fields from each tumor fragments were used and 20 different points were analyzed for an average related to the intensity of immunoreactivity. The values were obtained as arbitrary units (a.u.).

Table 1. Description of penile squamous cell carcinoma patients with clinical parameters and HPV types.

Variable	Number of patients
Age years (median 67)	
≤67	24
>67	23
T stage	
$T_{1a,1b,\ 2}$	42
$T_{3,4}$	5
N stage	
$N_{0,1}$	45
$N_{2,3}$	2
M stage	
M_0	47
M_1	0
HPV Types	
None	24
11	3
16	18
16,11	1
35,11	1

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Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad, California, USA) and data were expressed as means ± SEM. The Mann-Whitney U test was used to assess differences in age. The Wilcoxon Signed Ranks Test was applied to compare the gene expression levels in tumor tissue and normal penile tissue. Data from protein expression detected by immunohistochemistry were statistically examined by Kruskal-Wallis with Tukey's *post hoc* tests for multiple comparisons. The significance level was set at $P<0.05$ for all analyses.

Results

Pathological Findings and HPV Detection

The presence of penile squamous cell carcinoma was confirmed in all samples analyzed using a histopathological revision examination; these samples were subjected to DNA extraction for molecular analysis. All fresh samples were positive for the amplification of a human β-globin gene.

The patient age range was 31 to 95 years (mean 63 years), with no differences between patients with penile squamous cell carcinoma HPV positive and HPV negative ($p = 0.70$). HPV DNA was present in 23 of 47 (48.9%) penile squamous cell carcinoma cases studied. Most commonly only 1 genotype was identified [21 of 23 (91.3%)]. High-risk HPVs were present in 42.5% (20/47) of the cases and low-risk HPVs were identified in 10.6% (5/47) of penile squamous cell carcinoma samples. High-risk type 16 was the most prevalent type, present in 19 of 23 (82.6%) of HPV positive tumours. HPV18 was not detected. In the majority of HPV-positive tumours [18 of 23 (78.2%)] HPV16 was the only HPV type detected. In tumors with multiple viral infections there was a simultaneous presence of low-risk and high-risk HPV (Table 1).

The usual subtype of penile squamous cell carcinoma was the most common subtype present in 83% of the cases, followed by verrucous (8.5%), warty (4.2%), papillary (2.1%) and sarcomatoid (2.1%). For the usual type, HPV DNA was detected in 19 of 39 (48.7%) tumours, with high-risk HPV16 present in 15 of 39 (38.5%) samples. Verrucous and warty subtypes were positive for HPV DNA in 50% of the analyzed samples, with HPV16 present in the HPV positive samples. HPV type 16 was detected in 100% of the papillary tumours. In contrast, HPV was not detected in sarcomatoid tumours. No association of any of the HPV genotypes with subtypes of penile squamous cell carcinoma was found (Table 2).

Identification of Genes Differentially Expressed in Penile Squamous Cell Carcinoma by RaSH

The *RaSH* approach was adopted to identify genes expressed differentially in penile with high-risk HPVs. After alignment with the RefSeq database, sequences that presented >90% of the target sequence length at alignment were selected. These included *ANXA, p16, RPL6, PBEF1* and *KIAA1033*.

Validation of Identified Genes by qPCR

For the detection of genes expressed differentially in penile tumors, a gene expression profile was performed using 12 fresh samples of primary penile squamous cell carcinoma positive for high-risk HPVs. The relative expression levels of five genes were compared using qPCR, using triple determination and normalization based on the *tubulin* level. In the evaluation of the target genes, penile squamous cell tumor samples were used, and a pool of normal penile tissues was used as a reference (control group).

The expression of the genes *PBEF1, KIAA1033* and *RPL6* did not differ between penile squamous cell carcinoma and normal penile tissue, with fold-change values for gene expression raging from 1.6 to 3.3. *ANXA1* and *p16* were overexpressed in penile squamous cell carcinoma samples compared with the sample reference ($P=0.002$ and 0.0001 respectively) and the fold-change values for gene expression were 7.9 and 8450, respectively (Figure 1). The results obtained for *ANXA1* and *p16* using qPCR were in agreement with the *RaSH* method, providing further evidence that these genes are cancer-related.

Immunohistochemistry

Immunostaining of ANXA1 was mostly weak or negative in the cytoplasm of cells from tumor margins (control group) (Figure 2A) compared to the samples from the HPV-negative ($p<0.01$, Tukey's *post hoc* test) (Figure 2B and H) and HPV-high-risk ($p<0.0001$, Tukey's *post hoc* test) (Figure 2C and H) groups. Low-risk HPV positive squamous cell carcinoma of penis showed decreased expression of ANXA1 compared to the high-risk HPV tumours (data not shown for low-risk HPV positive samples and they were not included in the statistical analysis due to the small number). ANXA1 immunostaining was significantly increased in the cytoplasm of cells from penile squamous cell carcinoma with high-risk HPVs independently of the subtype compared to HPV-negative penile squamous cell carcinoma ($p<0.0001$, Tukey's *post hoc* test) (Figure 2B, C and H). Immunoreactivity for p16 was not detected or presented a weak expression in the nuclei of the non-neoplastic epithelia (control group) (Figure 2D) and increased immunoreactivity was observed in the nuclei of penile squamous cell carcinoma samples negative for HPV ($p<0.0001$, Tukey's *post hoc* test) (Figure 2E and I) compared to non-neoplastic epithelia. Low-risk HPV positive penile squamous cell carcinoma samples showed decreased expression of p16 compared to the high-risk HPV penile squamous cell carcinoma samples (data not shown for low-risk HPV positive samples and they were not included in the statistical analysis due to the small number). The penile squamous cell carcinoma samples with high-risk HPVs showed increased p16 expression observed both in the nuclei and in the cytoplasm indenpendently of the subtype ($p<0.0001$, Tukey's *post hoc* test) (Figure 2F and I) relative to penile squamous cell carcinoma without HPV. Negative control reactions were used for ANXA1 and p16 immunostaining (Figure 2G). ANXA1 and p16 immunodetection showed no significant difference between histological subtypes of penile squamous cell carcinoma since the most prevalent subtype was usual carcinoma (83%).

Table 2. Histological Subtypes of penile squamous cell carcinoma and HPV Genotypes.

Subtype	11	16	35 and 11	16 and 11	Negative	Total
Usual	3	14	1	1	20	39
Verrucous		2			2	4
Warty		1			1	2
Sarcomatoid					1	1
Papillary		1				1
Total	3	18	1	1	24	47

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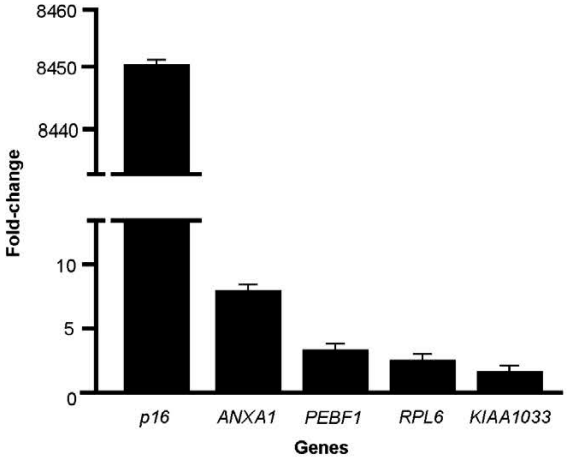

Figure 1. Relative expression media of the selected genes for validation using qPCR.
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Discussion

Overexpression of *ANXA1* mRNA and Annexin-I (ANXA1) protein were detected in squamous cell carcinoma of penis. *ANXA1* was the first member characterized of the annexin superfamily, characterized by the calcium-dependent ability to bind phospholipids. ANXA1 inhibits the activity of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX-2), thus exhibiting anti-inflammatory, anti-pyretic and anti-hyperalgesic activities [27,28]. In addition, ANXA1 is associated with various physiological processes including cellular differentiation [29], cell proliferation and signal transduction [30,31]. Furthermore, de-

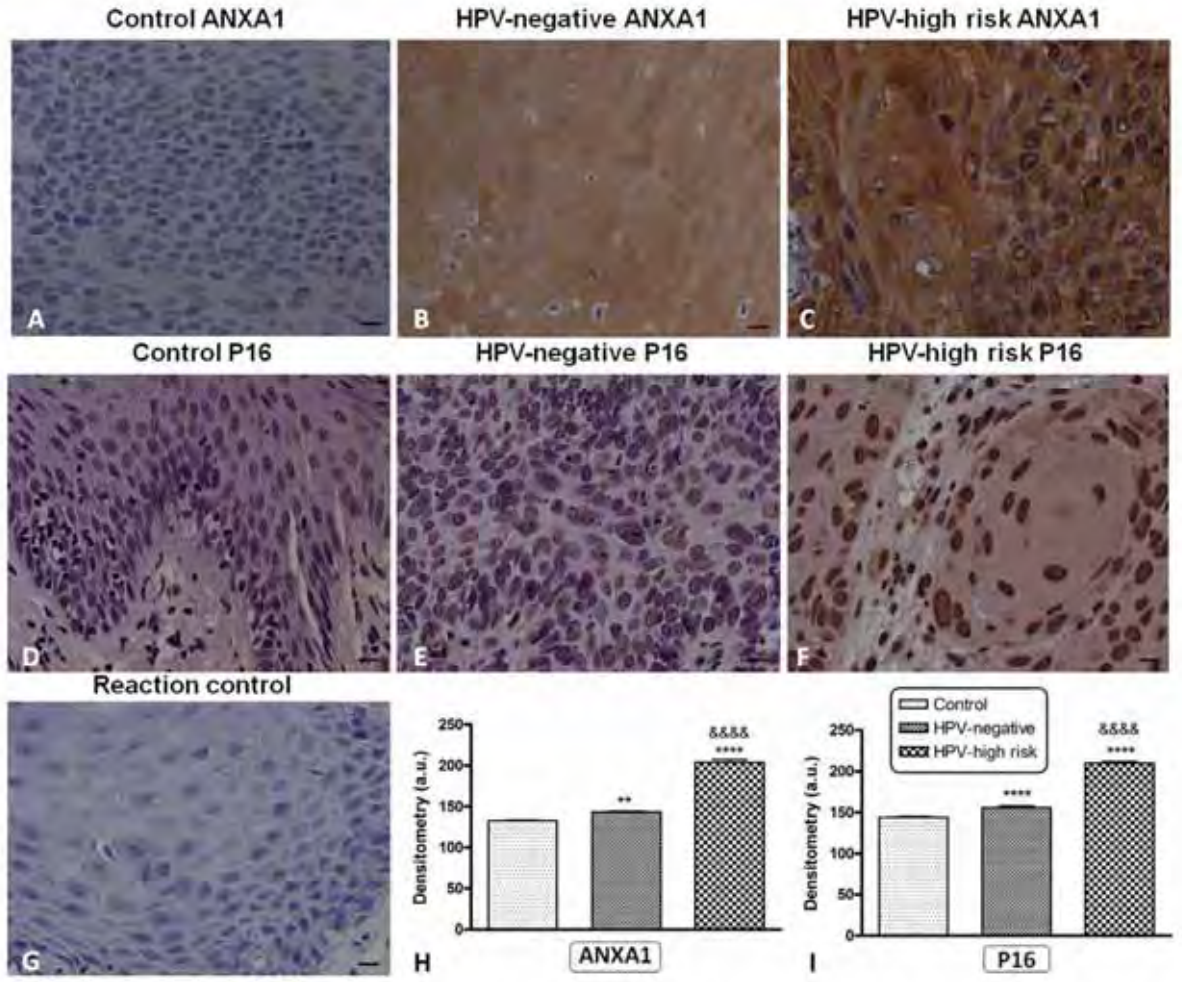

Figure 2. Immunolocalization of annexin A1 (ANXA1) and p16 in human primary penile squamous cell carcinoma and histologically normal tumor margins. ANXA1 immunostaining in A) Histologically normal tumor margins; B) Human primary penile squamous cell carcinoma HPV-negative; C) Human primary penile squamous cell carcinoma positive for high-risk HPV. p16 immunostaining in D) Histologically normal tumor margins; E) Human primary penile squamous cell carcinoma HPV-negative; F) Human primary penile squamous cell carcinoma positive for high-risk HPV. G) Reaction control for ANXA1. H) Graphic of densitometry of the immunostaining of ANXA1 in the samples analyzed. I) Graphic of densitometry of the immunostaining of p16 in the samples analyzed. Bars = 50 µm. (** = $p<0.01$; **** = $p<0.0001$; &&&& = $p<0.0001$, Tukey's *post hoc* test).
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regulation of ANXA1 has been correlated with tumor progression in several types of cancer [16,17,32–39]. One study suggested that ANXA1 appears to be induced in tumor endothelium, and the lack of ANXA1 in ANXA1-KO mice may impair tumor-induced angiogenesis with reduced blood supply explaining retarded tumor growth and metastasis in Lewis Lung carcinoma [40]. Other recent investigation showed that strong cellular and cell surface expression of ANXA1 in tumor cells at the invasion front was significantly associated with the occurrence of metastasis in penile cancer [41]. This finding could be explained by the important role of ANXA1 in regulation of cell invasion and migration. These data corroborate our results that have shown ANXA1 overexpression in all penile squamous cell carcinoma samples analyzed and classified pathologically as stage T3 or T4. Probably, when ANXA1 is expressed, tumors develop more blood vessels and, in consequence, tumors grow faster, suggesting that ANXA1 is a key regulator of pathological angiogenesis and physiological angiogenic balance.

Furthermore, it is the first time in the literature that ANXA1 protein overexpression is associated with HPV related penile cancer. It is known that E6AP binds to ANXA1 *in vivo* and *in vitro* and overexpression of E6AP enhances proteasomal degradation of ANXA1 *in vivo* [11]. Physical and functional association of E6AP with viral proteins, such as HPV16E6 [42] and HCV core protein [43], have also been demonstrated. E6 interaction with E6AP has been reported to be important for skin carcinogenesis in transgenic mouse models [44,45]. it is possible that the viral proteins such as HPV16E6 redirect E6AP away from ANXA1, which increases increasing the stability of ANXA1, and thereby contributes to viral pathogenesis [11]. Our work also corroborated with this hypothesis since ANXA1 protein expression was significantly increased in high-risk HPV squamous cell carcinoma of penis samples in-

dependently of the subtype of penile squamous cell carcinoma compared to the HPV negative squamous cell carcinoma of penis samples. So, probably ANXA1 might have an oncogenic role in penile cancer with high-risk HPVs.

HPV induces cervical cancer through uncontrolled G1-S transition. The E6 and E7 proteins of high-risk HPV inhibit p53 and pRb proteins, cell cycle regulatory proteins that control G1-S transition [46]. $p16^{INK4a}$ (p16) is a protein belonging to the inhibitors of cyclin-dependent kinase (CDK) 4 family (INK4a family). The inactivation of pRb by E7 causes p16 overexpression as p16 is regulated by negative feedback of pRb [47]. Increased p16 expression has been observed in cancer samples of cervix [48], penis [49], head and neck [50], oral [51] and the anorectal region [52] when positive for high-risk HPVs and its overexpression was found to be a reliable marker for high-risk HPV in penile carcinoma [53]. p16 protein expression was significantly higher in penile carcinoma samples positive for high-risk HPVs independently of the subtype of penile squamous cell carcinoma compared to penile carcinoma HPV negative samples in our study. Some studies focused on p16 alterations in penile cancer, but with different emphases. One study found an overexpression of p16 in 29% of penile carcinomas, especially in connection with HPV infection [54]. Prowse et al. detected p16 overexpression in 46% of penile SCCs, which was significantly associated with HPV infection [49]. However, Senba et al. described p16 overexpression in an equal amount of HPV-positive and HPV-negative penile carcinomas from Kenya [55]. Based in our data, we suggested the p16 could be a marker for penile carcinoma, confirming the diagnosis of malignant penile lesions with high-risk HPVs corroborating with previous studies with the same type of cancer [49,53,56].

This study identified two overexpressed genes, *ANXA1* and *p16*, in penile squamous cell carcinoma positive for high-risk HPVs. To the best of our knowledge this report is the first to describe ANXA1 protein overexpression in penile carcinoma with high-risk HPV independently of the subtype. These genes are associated with various physiological processes including cellular differentiation, cell proliferation and signal transduction, suggesting that they have an important role in penile carcinogenesis. However, additional studies are required in order to elucidate their specific role in penile cancer with high-risk HPV.

Author Contributions

Conceived and designed the experiments: MFC LLV SMO PR. Performed the experiments: MTOM EB NMC APG CFM MFC. Analyzed the data: JV FAS MFC APG JLB. Contributed reagents/materials/analysis tools: BMR RVDS JAT GHAM GCG JGFA. Wrote the paper: MFC SMO LLV PR.

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CAPÍTULO III

(High risk human papillomaviruses in two different primary tumors in the same patient)

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Case Report

High-risk human papillomaviruses in two different primary tumors in the same patient

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Abbreviations & Acronyms
FFPE = formalin-fixed, paraffin-embedded tissue
HPV = human papillomavirus
HR = high risk
PCR = polymerase chain reaction
SCCP = squamous cell carcinomas of the penis

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Abstract: Two cases of patients with high-risk human papillomavirus-related squamous cell carcinomas of the penis are reported. In both patients, a second high-risk human papillomavirus-related squamous cell carcinoma, of the same type (genotype 16), was detected: a carcinoma of the oropharynx 2 years after treatment of the squamous cell carcinomas of the penis in the first patient, and a carcinoma of the esophagus 1 year after the treatment of the squamous cell carcinomas of the penis in the second patient. To the best of our knowledge, this is the first time that multiple human papillomavirus-related tumors in the same patient are reported. It is suggested that a careful clinical investigation is necessary in patients with tumors attributable to high-risk human papillomavirus for the early detection of a possible second neoplasm related to this virus in a different organ.

Key words: esophageal cancer, human papillomavirus 16, laryngeal cancer, papillomavirus infections, penile cancer.

Introduction

Some HPV genotypes are classified as high risk because of their oncogenic potential. They are the etiological agents of cervical carcinoma,[1] and have also been implicated in the development of a diverse range of neoplasias, such as cancers of the vulva and vagina, penis, anus, oral cavity, oropharynx, larynx, esophagus, and lung.[2–5] HPV replicates during cell division and maturation of the squamous epithelial cells of the basal layer. HPV are able to establish a persistent infection, and integrate their genome randomly into host DNA and express high levels of the oncoproteins E6 and E7. E6 and E7 interfere with cellular control mechanisms, including those involved in the cell cycle, apoptosis and maintenance of chromosomal stability, and they initiate the malignant transformation of the host cell.[6]

Herein we report the cases of two patients who developed primary tumors of the gastrointestinal tract years after the treatment of SCCP by penectomy. In both cases, after penectomy, a new primary tumor attributable to HR HPV emerged in an organ other than the penis. One patient developed cancer of the larynx and the other developed cancer of the esophagus. Both patients were treated by the Medical Service of the Medical School of São José do Rio Preto, São Paulo state, Brazil.

Archival FFPE tissue samples were obtained from biopsies of the penis and larynx from one patient (case 1) and biopsies of the penis and esophagus from the other patient (case 2). All samples underwent evaluation by a pathologist before being used. The present study was approved by the Committee for Research Ethics (process number 3444/2007) from the Medical School of São José do Rio Preto (FAMERP), São Paulo state, Brazil.

After DNA extraction with QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), HPV was detected by PCR with general primers GP5+/GP6+, which amplifies 150 base pairs (bp) of the L1 viral gene.[7]

Genotyping was carried out using the INNO-LiPA HPV Genotyping Extra kit assay (Innogenetics, Ghent, Belgium). Genotyping results were confirmed by PCR with specific primers that amplify a 109 bp region of E6 gene as described elsewhere.[8] All tests were carried out with controls and repeated on different days to confirm the results.

Case reports

Case 1

The patient was a Caucasian man, aged 66 years, who was illiterate and reported smoking a pack of cigarettes a day. He was admitted to the Urology Department for a cystostomy because of urinary retention. Phimosis and lesions on the glans were observed. Biopsy of the lesions showed moderately differentiated invasive SCCP and balanoposthitis. A total penectomy was carried out because of the large area of tissue that had been affected. Anatomopathological examination of the samples from the penectomy showed an invasive SCCP with extensive areas of carcinoma *in situ* and involvement of the penile urethra. Koilocytic atypia, suggestive of HPV infection, was noted. The tumor was staged as T3N0M0. After surgery, no remaining affected tissue was observed and the patient was discharged.

Two years after penectomy, the patient returned with complaints of chronic hoarseness and was admitted to the intensive-care unit with suspected cancer of the oropharynx. An emergency tracheostomy was necessary. Laryngoscopy showed a vegetative lesion involving the right pyriform sinus. The biopsy showed an invasive squamous cell carcinoma of the larynx, moderately differentiated and staged as T3N0M0. A total of 15 days later, he developed a severe urinary tract infection and pneumonia. During treatment of the infections, a recurrent invasive SCCP was noted. Before the patient recovered from the infections and was able to undergo surgery, he developed a necrotizing fasciitis in the lower limbs and died.

Case 2

The patient was a 74-year-old Caucasian man with cognitive and motor impairment as a result of a previous stroke. The patient complained of difficulty while urinating. At clinical examination, a lesion on the penis and phimosis were noted. The biopsy showed an invasive SCCP, moderately differentiated, ulcerated and staged as T2N0M0. He underwent a partial penectomy. He had no significant difficulties afterwards, and was discharged after a few days.

One year later, the patient returned with complaints of an inability to swallow. A biopsy showed a squamous cell carcinoma of the esophagus, moderately differentiated, invasive and ulcerated. After aspiration pneumonia, the patient died of respiratory arrest as a result of pleural effusion and emphysema.

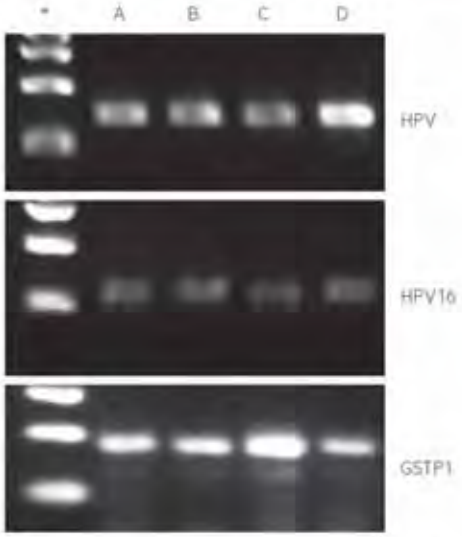

Fig. 1 Results of both PCR with general (superior line, bands of 150 bp) and specific primers for HPV16 (middle line, bands of 109 bp) in the samples of the cases. Columns A and B: penis and larynx of case 1. Columns C and D: penis and esophagus of case 2. *GSTP1* (bottom line, bands of 176 bp) is an endogenous gene used as a control for successful DNA extraction. *Ladder of 100 bp.

The presence of HPV was detected by PCR. INNO-LiPA confirmed the presence of HPV, and genotyping identified the HPV in all samples to be HPV16. The INNO-LiPA genotyping was confirmed by PCR with a set of primers specific for HPV16. The results of both PCR with a general and specific set of primers are shown in Figure 1.

Discussion

SCCP has a very low incidence in developed countries, but is much more common in developing countries. The association between SCCP and HR HPV infection has been addressed by several studies.[1,9] Laryngeal and esophageal cancers have a very high incidence worldwide, and have also been linked to HR HPV infections.[3,4] For example, in India, it was reported that a significantly higher number of SCCP (30%), esophageal cancers (63%) and laryngeal cancers (30–60%) were HPV16 positive,[10] and so HPV-attributable tumors in these organs in the same patients might not be so rare.

This is the first case report of primary tumors attributable to HPV arising in a second organ after an initial HPV-attributable tumor in the same patient. All three types of tumor that the patients in the present report developed (penile, laryngeal and esophageal) are derived from epithelial cells and have been associated with HR HPV infection. These two case reports show the need for more studies on

the occurrence of multiple consecutive primary tumors arising from epithelial tissues in the same patient as a result of HR HPV. The present case report shows that the possibility of HPV vaccination of all children would lower the incidence of cancers attributable to HPV in men in the future.

Because most tumors have a better prognosis when diagnosed early, patients who underwent treatment for squamous cell carcinomas associated with HR HPV infection would benefit from a more careful follow up and a broader investigation of possible tumors arising in other organs.

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Conflict of interest

None declared.

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CAPÍTULO IV

(Human Papillomavirus genotype prevalence in penile carcinoma of patients from two distinct geographic regions of Brazil)

Human Papillomavirus genotype prevalence in penile carcinoma of patients from two distinct geographic regions of Brazil

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3. ABSTRACT

Penile carcinoma is an uncommon tumor in developed countries with higher rates in some underdeveloped or developing regions. There are few studies accessing the HPV genotypes distribution in Brazil. In this work it was analyzed two groups of patients harboring penile carcinoma from distinct cultural and geographic regions of Brazil: one group from Pará State in the North region and one group from São Paulo State in Southeast region, the more industrialized region. The overall prevalence of HPV in single or multiple infections is significantly higher in Pará (81.67%) than in São Paulo (64.10%). Although the prevalence of oncogenic high risk HPV genotypes, mainly HPV16, has been greater in São Paulo (64%) than in Pará (49%). It was also found more genotypes in the samples of patients from Pará State, suggesting that HPV genotypes circulating in Pará population must be more diverse than those circulating in São Paulo State population and may contribute to the higher

prevalence of penile carcinoma in the Pará. These data may be useful in development of guidelines for the implementation of more efficient and less expensive vaccination programs.

4. KEY WORDS

Cancer of the penis; HPV genotyping; genotypes distribution

5. INTRODUCTION

Penile carcinoma is an uncommon tumor with low incidence in developed countries [1] but with much higher rates in some underdeveloped or developing regions were can represent 10-20% of malignant disease in men [2, 3]. In Uganda it is the most commonly diagnosed cancer. Brazil has one of the world's highest incidence rates for this neoplasia with age-adjusted incidence of 8.3 per 1000,000 men in Brazil. [2].

The vast majority (95%) of penile cancers is squamous cell carcinoma of the penis (SCCP). which are classified into histological subtypes: usual type, verrucous carcinoma, warty carcinoma, papillary carcinoma, basaloid carcinoma, sarcomatoid carcinoma, carcinoma cuniculatum, pseudohyperplastic carcinoma, adenosquamous carcinoma, and acantholytic carcinoma [4, 5]. Usually SCCP affects men aged 50-70 years but approximately 19% of the cases occurs in men aged < 40 years old and 7% occurs in men < 30 years old [6].

Despite the understanding of the pathophysiology of this disease remains incomplete many etiologies underlying the development to SCCP has been proposed and many risk factors have been linked to this disease. The most important risk factors identified until now are presence of phimosis, chronic inflammatory conditions such as balanoposthitis and lichen sclerosus et atrophicus, treatment with sporalene and ultraviolet A phototherapy (PUVA), cigarette smoking, poor personal hygiene, history of multiple sexual partners and early age at first intercourse and history of condylomata, [2, 7, 8]. Many works had also access the role of infection by human papillomavirus (HPV) with the development of this tumor. The incidence

of HPV ranges between 1 and 80%, mainly due to the sensitivity of the HPV detection method employed and the SCCP histological subtypes used [4, 9, 10].

SCCP shares some pathological features with others squamous cell carcinomas (SCC) like head and neck, upper digestive tract and anogenital carcinomas [2]. High oncogenic risk HPVs have been found in almost 100% of cervical carcinoma and so persistent HPV infection is regarded as a necessary cause of the development of this cancer [11, 12]. However the data about prevalence of HPV infection in SCCP is not so consistent and it had been observed that they may differ according to the histological subtypes studied [5]. These findings led to some authors to hypothesizes that, unlike cervical cancer, there may be two etiologies for SCCP, one HPV related and the other due to factors unrelated to HPV infection [13].

The presence of phimosis associated to poor hygiene probably responds for the HPV independent pathway for development of SCCP. This association can create the conditions for perpetuation of a chronic inflammatory state, which through metaplastic changes may leads to malignant transformation [1, 14]. The protective effect of childhood circumcision, but not of circumcision in adulthood, seems to be related to the elimination of this inflammatory state [9, 15]. On the other hand exposure to infection with high oncogenic risk HPVs may be responsible for the development of many cases of SCCP [16-18].

HPV belongs to Papillomaviridae, a highly diverse and ubiquitous family of DNA viruses with a high epithelial and mucosal tropism. HPV are non-enveloped, icosaedrical, 55nm diameter, ~8000kb double stranded DNA viruses. More than 150 different genotypes of HPV have been characterized until now [19]. The mucosal HPVs strains can be clinically grouped into non-oncogenic or low-risk (LR) types such as HPV6 and HPV11, and potentially oncogenic or high-risk (HR) viruses, like HPV16 and HPV18 according to their ability to drive host cells into malignant transformation. More than 40 genotypes of mucosal

HPVs are isolated from mucosa and epithelium in the anogenital tract and other areas [20, 21].

The HPV life cycle is well synchronized with keratinocyte differentiation and to establish an infection these viruses must gain access to the basal cell layer of the epithelium where they replicates as the basal cells matures in keratinocytes. HR HPVs are able to integrate their genome to the host chromatin, increasing the transcription of the oncogenes E6 and E7. These viral oncoproteins can modulates the activity of cell cycle regulatory proteins that controls the differentiation program of the keratinocytes forcing these cells to stop their maturation and keeping them in a state of continuous replication [22, 23]. These proteins can also inhibits cellular tumor suppressor proteins like p53 and pRb, resulting in mitogenic stimulus and apoptosis prevention [24] and ultimately driving the malignant transformation of the host cell.

Many types of carcinomas are attributable to HPV. They are responsible for 5.2% of all cancers worldwide [25-27]. However, there are great differences in both geographic distribution and relative incidence of HPV genotypes [28-30].

There are few studies about HPV prevalence in penile cancer reflecting the low incidence of this disease in developed countries. This prompt us to access the prevalence of HPV in SCCP and the clinical staging of this tumor in two geographic regions of Brazil which population has distinct sociocultural patterns in order to verify if there is significant differences in genotypes found and in the clinical staging. One group of SCCP samples came from São Paulo state, in Southeast region, the most industrialized region of the country and the other group came from Pará state, in North region, which has one of the worst socioeconomic indicators of Brazil (based on information from the demographic census from 2000 to 2010 of the Brazilian Institute of Geography and Statistics).

6. MATERIALS AND METHODS

Samples and tumor staging. Ninety-nine archival formalin-fixed, paraffin-embedded (FFPE) penile samples of patients diagnosed with SCCP were used in this work divided in two groups: 39 from patients treated in São Paulo state medical services and 60 treated in Pará state medical services. All patients were treated in their respective districts of origin. For all cases the TNM classification was obtained and the cases were staged according to AJCC Cancer Staging Manual (7^{th} edition) [31]. The present study was approved by the Committee for Research Ethics (process number 3444/2007) from Medicine School of São José do Rio Preto (FAMERP), São Paulo state, Brazil.

DNA extraction. Total DNA was extracted by QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer instructions. Briefly between 3 to 7 paraffin sections of 5µm thick of each sample underwent four washes with xylene followed by two washes with ethanol 100%. Samples was lysed with proteinase K for 1 hour and incubated at 90°C to reverses formalin crosslinking. After addition of buffer AL and ethanol 100% the mix was transferred to the QIAamp MinElute column. The columns were washed with buffers AW1 and AW2 and finally the DNA was eluted from the columns with 100µL of ATE buffer. After extraction 1µL was used for PCR for an endogen gene (GSTP1) to ensure the quality of extracted DNA. All samples are GSTP1 positive, indicating successful DNA extraction.

HPV detection and genotyping. 5µl of the extracted DNA was used for HPV detection. It was conducted with PCR in two rounds with generic set of primers GP5+/GP6+ described elsewhere [32] that annealing to a short conserved region (approximately 170bp according to the genotype) on HPV DNA. 5µL of the total DNA was used for one round of PCR and specific bands were visualized in a 3% agarose gel with ethidium bromide. 5µl of negative samples were subject to another round of PCR with the same conditions of previous PCR. Assays are conducted with positive and negative controls and a tube containing only the PCR mix without DNA are used as control of contamination. All positive tissues for any HPV by

GP5+/GP6+ PCR were genotyped by INNO-LiPA HPV Genotyping Extra kit (Innogenetics) following the manufacturer instructions. The kit is carried out in two steps: first of all an amplification of a 65bp region of the conserved HPV L1 viral capsid gene with the SPF10 set of primers. Then 5μL of amplified products is used in a reverse line blot hybridization assay for HPV genotyping. This methodology allows the discrimination of 28 anogenital HPV genotypes, including 12 HR HPVs, 1 probable HR HPV, 7 possible HR HPVs, and 8 low-risk types [33].

Statistical analysis. Statistical analysis was conducted using the two samples T-test with Minitab 16 software.

7. RESULTS

HPV detection. The overall prevalence of HPV was 74.75% (74 of 99) with a mean age of 62.5. In the samples of the SP group the HPV prevalence is lower than in the samples from PA group. In the SP group, 25 of 39 (64.1%) patients harboring SCCP were positive for HPV while in the PA group 49 of 60 (81.67%) patients were positive for HPV.

HPV genotyping. The HPV genotype distribution found in both groups is very different. The PA group showed a broader spectrum of genotypes than the SP group. Despite the higher HPV prevalence in PA group the prevalence of HR HPV, mainly HPV16 is higher than in SP group. In the SP group 16 (64%) of the samples were positive for any HR HPV genotype in single or multiple infections while in the PA group 24 (48.98%) of the samples were positive for HR HPV. The PA group showed a higher rate of co-infections with 26 (53.06%) against 9 (36%) in SP group.

To further compare the two groups they were subdivided in four different groups: infections with a single HR genotype (HR si) or LR genotype (LR si) and infections with mixed HR and LR genotypes (HR mi) or mixed LR genotypes (LR mi). In the SP group most of the patients harboring infections with only one HR genotype while in the PA group the

patients harboring infections mostly with mixed HR genotypes and with LR in single infections. The results are summarized in **table 1**.

Table 1. HPV prevalence

	SP group n (%)	PA group n (%)
HR si	10 (40)	5 (10.2)
HR mi	6 (24)	19 (38.78)
LR si	6 (24)	18 (36.73)
LR mi	3 (12)	7 (14.29)

In SP group only HPV6, 11, 16 and 18 were found. In PA group, in addition to these genotypes, it was found the HR genotypes 33, 45, 51, 52, 53, 58, and 68, at large, in mixed infections with genotypes 6 and 11. These genotypes are also the most common in mixed infections in SP group. The differences in prevalence of single or mixed infections between SP and PA groups are summarized in **figure 1**.

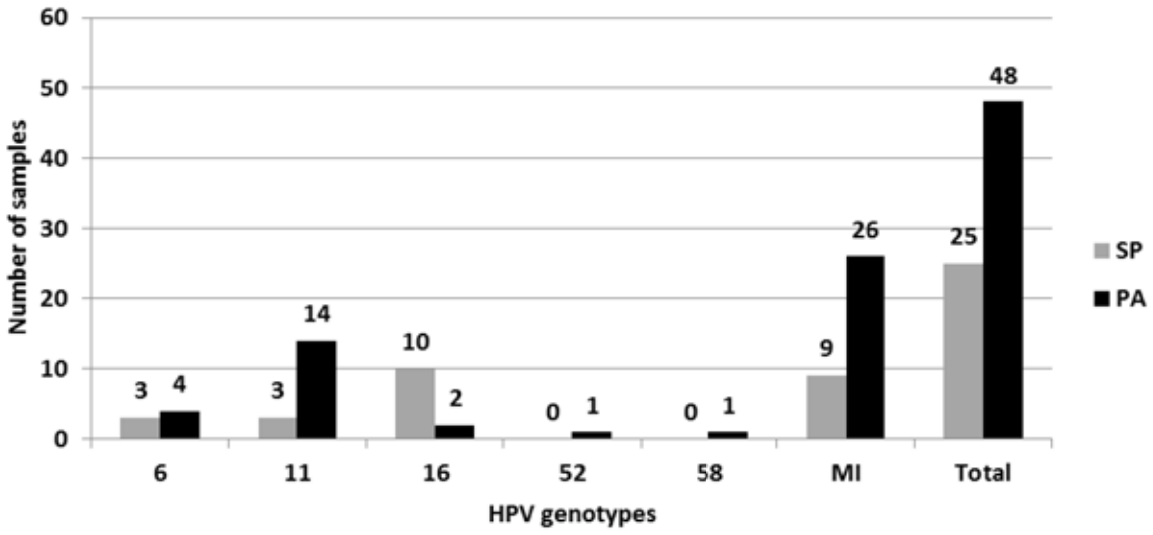

Figure 1. HPV genotype distribution in single or multiple infections in SP and PA groups. Numbered columns represents single HPV genotype infections in SP and PA groups. MI, multiple infections with different HPV genotypes.

Tumor staging. To compare if there are clinical differences between the two groups, all SCCP cases were staged according to the AJCC Cancer Staging Manual.

The clinical staging of tumors in both groups was compared. There are striking differences between the two groups. Nearly 85% of the cases in the SP group were in stages I (43.59%) and II (41.03%). The cases in PA group showed a more diverse staging, with

21.67% of the cases were in stages I, 28.33% in stage II, 6.67% in stage IIIa, 10.0% in stage IIIb and 31.67% were in stage IV. The results are summarized in **figure 2**.

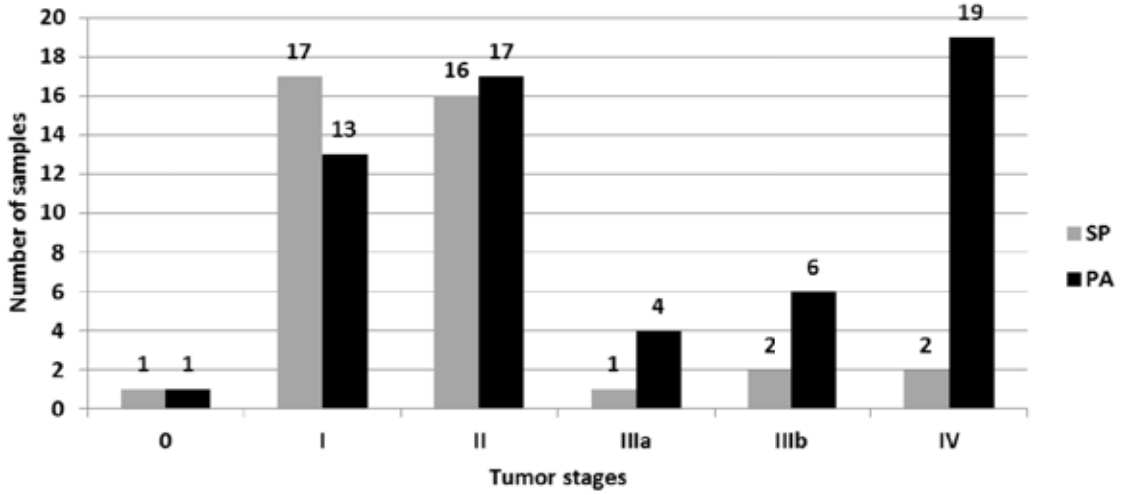

Figure 2. Differences in clinical stages between the SP and PA groups.

The groups were divided in three arbitrary age groups: under 40 years, 41-59 years, and equal to or greater than 60 years. The overall mean age was 62.5, with little difference in mean age between the two groups: 64.7 for SP and 61.1 for PA.

To access the contribution of each group in a particular age group, the results were normalized. As expected with the progression of age it was observed a gradual increase in the number of patients harboring higher stages of SCCP (**figure 3**) in all groups.

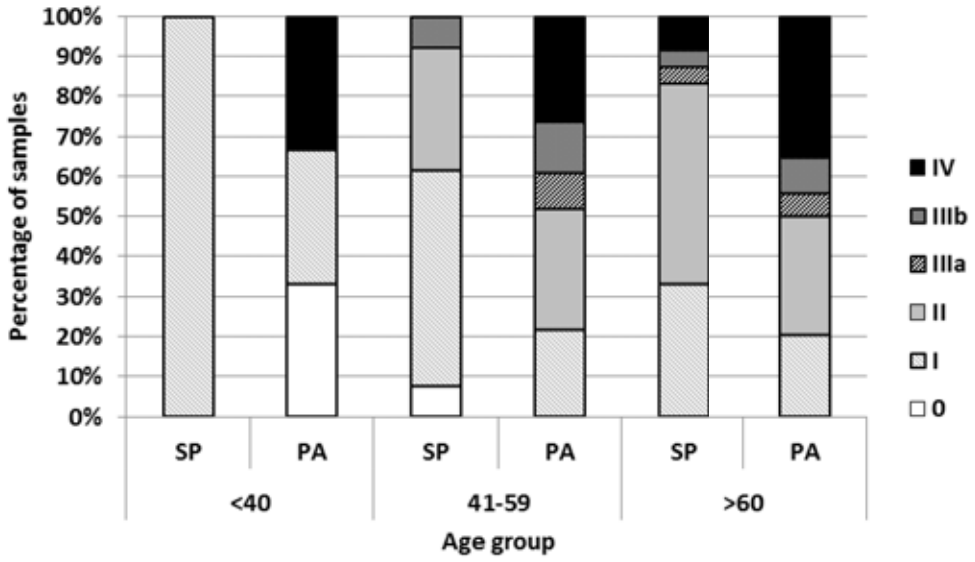

Figure 3. Differences in clinical staging in relation to age between the groups. SP, São Paulo group; PA, Pará group.

In the PA group there are an increased number of patients harboring higher clinical tumor stages (IIIa, IIIb and IV) compared with SP group, even in the age group of under 40 years.

8. DISCUSSION

Brazil is a large country, geopolitically divided in five regions, with striking contrasts in human development and which population has very different cultural patterns. Pará State is in North region and São Paulo state is in Southeast region. The North region has the worst human development indices while Southeast region is the most industrialized region of the country. These cultural diversity and variances in socioeconomic status reflects on lower scholarity, poor hygienic habits, and less access to medical care. These circumstances together, associated with variations in the prevalence of oncogenic HPV strains may contribute for the higher rates of prevalence of SCCP in Pará state.

In this work we found a higher prevalence of HPV in patients harboring SCCP from Pará compared to those from São Paulo. The number of patients infected with multiples HPV genotypes in the PA group (43.33%) is higher compared with SP group (16.67%) but this found is expected since other works already found multiple HPV genotypes infections in patients with cervical carcinoma in other regions of Brazil [34, 35]

The SCCP samples collected in Pará are mainly from men who live in villages on the shores of rivers. This population usually bathing in rivers, inherited habit from the native peoples. Probably the poor personal hygiene habits of the population in combination with little access to medical services are responsible for the higher rates of prevalence of both SCCP and HPV in this area.

Despite the natural history of HPV seems to be different between men and women, with high infection and low disease rates in men and low infection and high disease rates in

women [36], the data of the PA group leads to the presumption that there is a wide range of HPV genotypes circulating in the population from this State.

The findings in this work not permit to establish a causal link between this greater diversity and the higher prevalence of SCCP in the population of Pará, but are tempting to propose a direct link between them.

Although regional variations in genotypes that are circulating in the population, HPV16 is the most prevalent HPV genotype worldwide and is responsible for more than a half of all cases of cervical carcinoma worldwide [37]. The prevalence of HPV16 in São Paulo found in this work is similar to the prevalence found in other works [38-40]. The prevalence rates found in Pará state is very higher than the observed in two previous works [41, 42], but this can be attributable to differences in the approach like population studied, sampling and HPV detection methodology.

When the groups are analyzed in regard of age and clinical stage of the tumor, it was observed that the PA group had a greater number of patients with tumors in higher stages even in early ages. These higher stages of SCCP are associated to poor prognostic and lower survival rate of the patients.

The simplest explanation would be the lower access medical care in the Pará state. However this does not explain the higher stages (mainly IV) in the early age group (under 40 years) in this state. Another cause may be the multiple infections with different HR HPV genotypes. Infections with multiple HPV genotypes are common in men [43]. In women persistent infections with different HR genotypes can act synergistically to promote a cervical tumor with a faster growth or more aggressive [44, 45]. The same mechanism can occur in the SCCP.

Many issues remain in Brazil about differences in prevalence and HPV genotype distribution, mainly in men. A large multinational study, the HPV Infection in Men (HIM)

study [36, 46, 47] is currently ongoing, whose purpose is to understand the natural history of HPV infection in men. Despite studies are needed that include larger number of samples and from other regions of the country, the data on prevalence and genotypic distribution of HPV provided by HIM and other studies may prove useful in development of guidelines for the implementation of more efficient and less expensive local vaccination programs.

9. ACKNOWLEDGES

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DISCUSSÃO

A baixa incidência de CEP em países desenvolvidos acaba por traduzir-se em poucos estudos sobre os mecanismos moleculares deste câncer quando comparado a outros tumores semelhantes como o cervical. Ainda não há consenso sobre o papel exercido pela infecção por HPV no desenvolvimento do CEP. O presente estudo teve por objetivo esclarecer alguns aspectos moleculares do CEP e verificar o papel do HPV nesta doença.

Este trabalho teve dois objetivos gerais: encontrar genes diferencialmente expressos entre tecidos tumorais e normais e verificar a presença de HPV nestes tecidos, identificar seus genótipos e correlacionar com os parâmetros clínicos dos pacientes portadores de CEP nestes tecidos e correlacionar com os dados clínicos dos pacientes portadores de CEP.

Pela metodologia de RaSH foi possível selecionar 57 sequências diferencialmente expressas entre tecidos normais e tumorais. Após análise por ferramentas de bioinformática foram evidenciados 5 sequências correspondentes a genes com possíveis papéis na carcinogênese. Estes genes (*CDKN2A*, *KIAA1033*, *NAMPT*, *RPL6* e *ANXA1*) tiveram sua expressão diferencial entre os tecidos normais e tumorais validada por PCR em tempo real e as informações gerais sobre estes genes foram obtidas das páginas NCBI/Gene (http://www.ncbi.nlm.nih.gov/gene) e Kyoto Encyclopedia of Genes and Genomes - KEGG (http://www.genome.jp/kegg).

O gene *KIAA1033* (também conhecido como *SWIP* ou *WASH7*), localizado na região 12q24.11, codifica a subunidade 7 do complexo WASH. Este complexo está presente na superfície de endossomos que recruta e ativa o complexo Arp2/3 que por sua vez induz a polimerização da actina (Duleh e Welch, 2012). O complexo WASH tem um importante papel na fissão de túbulos que servem de intermediários no direcionamento de endossomos (Ropers, Derivery, Hu *et al.*, 2011; Derivery, Helfer, Henriot *et al.*, 2012).

O gene *NAMPT* (também conhecido como *VF*; *PBEF*; *PBEF1*; *VISFATIN* ou *1110035O14Rik*), localizado na região 7q22.2, codifica a enzima nicotinamida

fosforibosiltransferase (NAmPRTase ou NAMPT), da família das glicosiltransferases, que catalisa a reação de nicotinamida com 5-fosforibosil-1-pirofosfato para gerar nicotinamida mononucleotídeo que é o passo limitante na taxa de biossíntese da NAD coenzima (*nicotinamide adenine dinucleotide*, dinucleotídeo de nicotinamida e adenina). É uma adipocina que tem várias funções incluindo a maturação de miócitos vasculares, maturação de linfócitos B e inibição de apoptose de neutrófilos (Wieser, Moschen e Tilg, 2012). Ela também é capaz de se ligar aos receptores de insulina diminuindo a glicemia e aumentando a sensibilidade a insulina (Jacques, Holzenberger, Mladenovic *et al.*, 2012).

O gene *RPL6* (também conhecido como *L6*; *TXREB1*; *SHUJUN-2* ou *TAXREB107*), localizado na região 12q24.1 codifica uma proteína da fração 60S do ribossomo, pertencente a família L6E de proteínas ribossomais e portanto tem funções ligadas à regulação da transcrição e da tradução. Esta proteína se liga ao domínio C do elemento potencializador de resposta à proteína Tax do HTLV 1 e pode estar também relacionada a transativação e transcrição mediadas por esta proteína viral (Yang, Wang e Han, 2002).

Outro gene selecionado pela metodologia de RaSH foi o gene *CDKN2A* (também conhecido como *ARF*; *MLM*; *P14*; *P16; P19*; *CMM2*; *INK4*; *MTS1*; *TP16*; *CDK4I*; *CDKN2*; *INK4A*; *MTS-1*; $P14^{ARF}$; $P19^{ARF}$ ou $P16^{INK4}$) está localizado na região 9p21. Este gene gera diversos transcritos que diferem nos primeiros exons. Pelo menos três produtos obtidos pelo processamento alternativo já foram descritos. Dois são isoformas do inibidor p16 de quinase ciclina-dependente que inibem a CDK4 (*cyclin-dependent kinase*, ciclina dependente de quinase 4) e o outro é a proteína supressora de tumor $P14^{ARF}$ que funciona como estabilizador da proteína supressora de tumor p53, através do sequestro de MDM2, que é capaz de degradar p53 (Saegusa, Hashimura, Suzuki *et al.*, 2012).

A p16 pertence à família 4 de inibidores de CDK (*inhibitors of CDK*, INK) conhecida como INK4a. Inibe a interação entre ciclina D a as CDK4 e 6, um complexo que estimula a

proliferação celular (Nam e Kim, 2008). O fator de transcrição E2F normalmente encontra-se em forma inativa complexado com pRb (Fera, Schultz, Hodawadekar *et al.*, 2012). Quando a pRb é fosforilada por CDK há a liberação deste fator de transcrição resultando na transição da fase S para G1. A p16 é capaz de inibir a fosforilação de pRb induzida por CDK (Fahraeus, Paramio, Ball *et al.*, 1996).

O HPV é capaz de causar a liberação e E2F através da ligação da proteína viral E7 à pRb. A inativação de pRb por E7 causa a superexpressão de p16 (Nam, Kim, Kim *et al.*, 2007; Myklebust, Bruland, Fluge *et al.*, 2011).

Correlação entre o aumento da expressão de p16 e presença de infecção por HR-HPV já foi relatada em diversos tumores como cervical (Nam e Kim, 2008), cabeça e pescoço (Begum, Gillison, Nicol *et al.*, 2007), oral (Fregonesi, Teresa, Duarte *et al.*, 2003), anorretal (Lu, El-Mofty e Wang, 2003) e inclusive CEP (Prowse, Ktori, Chandrasekaran *et al.*, 2008). O aumento da expressão de p16 é considerado um indicador confiável de HR HPV em CEP (Cubilla, Lloveras, Alejo *et al.*, 2011).

O gene p16 é um conhecido gene supressor de tumor, cuja expressão encontra-se alterada em diversos tumores incluindo o CEP (Poetsch, Hemmerich, Kakies *et al.*, 2011; Witkiewicz, Knudsen, Dicker *et al.*, 2011). Mutações em p16 aumentam o risco de desenvolvimento de diversos tipos de câncer visto que as proteínas codificadas por este gene têm importante papel na regulação do ciclo celular. Normalmente, a expressão aumentada de p16, previne a fosforilação dos membros da família pRb, parando o ciclo celular e favorecendo a senescência celular, funções que em última análise, auxiliam na inibição da formação de tumores(Myklebust, Bruland, Fluge *et al.*, 2011). A oncoproteína E7, possibilita o HPV superar este problema. Esta oncoproteína tem a capacidade de degradar membros da família pRb, o que resulta na liberação e ativação do fator de transcrição E2F que direciona a expressão de genes da fase S mesmo com altas concentrações de p16. Assim tem sido

sugerido que o aumento de expressão de p16 pode ser útil como um marcador para atividade patológica de HPV (Narisawa-Saito e Kiyono, 2007; Mclaughlin-Drubin, Meyers e Munger, 2012).

Observou-se neste trabalho que há expressão significantemente maior de mRNA de p16 em amostras de CEP com HR-HPV quando comparadas com amostras HPV negativas. A imunohistoquímica mostrou que o aumento de mRNA levou ao aumento da proteína p16. Outros estudos também mostraram aumento de p16 em CEP em relação com HPV como, por exemplo, os estudos de Ferreux e colaboradores e de Prowse e colaboradores que encontraram respectivamente aumento de p16 em 29% e 46% e das amostras de CEP, ambos com relação a presença de HPV (Ferreux, Lont, Horenblas *et al.*, 2003; Prowse, Ktori, Chandrasekaran *et al.*, 2008). Por outro lado Senba e colaboradores não encontraram diferenças na expressão de p16 entre amostras positivas ou negativas para HPV (Senba, Buziba, Mori *et al.*, 2009). Estas diferenças podem ser devidas a variações na incidência de HPV nas diferentes áreas estudadas, ao uso de métodos diversos de detecção de HPV e aos subtipos histológicos de CEP empregados.

Os dados deste trabalho sugerem que p16 pode ser um marcador para CEP, confirmando o diagnóstico de lesões penianas malignas com HR-HPV. Os dados da literatura corroboram os dados deste trabalho (Prowse, Ktori, Chandrasekaran *et al.*, 2008; Cubilla, Lloveras, Alejo *et al.*, 2011)

O quinto gene selecionado pela metodologia de RaSH foi o gene *ANXA1* (também conhecido por *ANX1* e *LPC1*). Este gene está localizado na região 9q12-q21.2, codifica a proteína anexina A1, também conhecida como lipocortina I ou calpactina II. A anexina A1 é uma proteína de 37kDa, sendo o primeiro membro descrito da superfamília das anexinas. Os 13 membros desta superfamília têm similaridade estrutural e funcional entre 40 a 60%. As anexinas apresentam uma região C-terminal altamente conservada e uma região N-terminal

única. Acredita-se que esta sequência única seja responsável pelas funções específicas de cada anexina (Fatimathas e Moss, 2010).

A principal propriedade bioquímica das anexinas é a capacidade de se ligar a membranas fosfolipídicas de forma cálcio-dependente. Encontrada normalmente na face citoplasmática da membrana, regula a fosfolipase A2 e, portanto, tem atividade anti-inflamatória. Promove a fusão de membranas e está envolvida na exocitose. Tem capacidade induzir apoptose por inibir a via de transdução de sinal de NF-κB através da ligação a p65. Esta via é comumente ativada em diversos tumores. Todas as anexinas possuem quatro regiões conservadas onde se ligam o cálcio e os fosfolipídeos. (Lim, Hamid, Lau *et al.*, 2007). Sua estrutura é descrita na **figura 8**.

Figura 8. Representação esquemática da anexina A1, mostrando seus principais domínios.

Este gene apresentou expressão elevada em 80% das amostras testadas por PCR em tempo real quando comparado com tecido normal, podendo assim vir a se constituir num possível marcador tumoral.

Foi verificado também se o aumento de mRNA de *ANXA1*, indicado pela metodologia de RaSH levaria a aumento da proteína nos tecidos. Para isso amostras de tecidos foram testadas por imunohistoquímica com anticorpo contra anexina A1. A expressão da proteína anexina A1 em amostras de CEP com HPV de alto risco mostrou-se aumentada em relação com amostras de CEP com HPV de baixo risco ou negativas para HPV.

Normalmente a anexina A1 é expressa em células do sistema imune e alguns tecidos epiteliais. Esta proteína tem sido muito estudada por sua ação moduladora do sistema imunológico (D'acquisto, Perretti e Flower, 2008), entretanto ela participa de diversos processos celulares no organismo como: organização do citoesqueleto, transporte transmembrânico de substâncias e sinais, fusão de membranas celulares, diferenciação e

migração celular, onde participa da maturação de queratinócitos (Sakaguchi, Murata, Sonegawa *et al.*, 2007) e regula mecanismos de apoptose.

Considerando estes processos biológicos era razoável se esperar alterações desta proteína em tumores. Diversos estudos mostraram alterações da expressão de anexina A1 em diversos tumores, entretanto sem consenso sobre seu papel na gênese e progressão tumoral. Em alguns tumores a expressão de anexina A1 encontra-se aumentada (por exemplo, carcinoma de pâncreas e carcinoma hepatocelular, leucemia, câncer de pele e mama), e em outros tumores encontra-se diminuída (por exemplo, próstata, esôfago, estômago, cabeça e pescoço e laringe) (Lim, Hamid, Lau *et al.*, 2007).

Observa-se também que a expressão de anexina A1 pode alterar a severidade da doença (Biaoxue, Xiling, Shuanying *et al.*, 2012; Cheng, Wu, Lin *et al.*, 2012; Kang, Ko e Jang, 2012). Por exemplo, camundongos selvagens inoculados com células de carcinoma pulmonar de Lewis desenvolvem tumores cerca de cinco vezes maiores e muito mais agressivos do que em camundongos nocaute para ANXA1. Aparentemente a anexina A1 é induzida no endotélio tumoral e a ausência desta proteína nos camundongos nocaute dificulta a angiogênese induzida pelo tumor, reduzindo o suprimento de sangue para o tumor e retardando o aparecimento de metástases (Yi e Schnitzer, 2009).

Os dados deste trabalho mostram que a anexina tem um importante papel no CEP. A maior expressão desta proteína correlaciona-se com a maior severidade do tumor (estágios patológicos T3 e T4). É provavelmente que a expressão de anexina A1 induza angiogênese tumoral, disponibilizando maior quantidade de sangue e nutrientes para o tumor e permitindo seu crescimento mais rápido (Pin, Houle, Fournier *et al.*, 2012). Estes dados são corroborados por estudo de Protzel e colaboradores que verificaram que a alta expressão de anexina A1 em células da frente de invasão tumoral correlaciona-se com maior ocorrência de metástases

linfonodais em CEP, provavelmente devido ao papel regulatório da anexina A1 na invasão e migração celular (Protzel, Richter, Poetsch *et al.*, 2011).

Os dados obtidos neste trabalho também revelaram uma correlação entre a infecção por HPV e o aumento da expressão de anexina A1. Apesar dos mecanismos regulatórios da anexina ainda não serem inteiramente conhecidos, sabe-se que o HPV tem papel na modulação desta proteína.

A expressão de anexina A1 é controlada pela proteína celular E6AP (*E6 associated protein*, proteína associada a E6) codificada pelo locus *Ube3A*. A proteína E6AP tem este nome devido ao fato de ter sido inicialmente identificada quando recrutada pela E6 viral para degradação de p53 (Wolyniec, Levav-Cohen, Jiang *et al.*, 2012). Esta proteína é a uma E3 ubiquitinase e é o protótipo dos subtipos HECT (*homologous to E6-AP carboxyl terminus*) de ligases E3 (Bernassola, Karin, Ciechanover *et al.*, 2008). Nestes sistemas há uma cascata de enzimas, genericamente chamadas E1, E2 e E3 que transferem a molécula de ubiquitina para o alvo celular. A enzima E3 é a enzima responsável pela especificidade do sistema, reconhecendo o alvo celular e marcando-o com uma cadeia de poliubiquitina (Bernassola, Karin, Ciechanover *et al.*, 2008).

Já foi observado que a relação entre a interação da E6 viral e a E6AP celular tem papel importante na carcinogênese de pele em modelo animal (Song, Pitot e Lambert, 1999; Nguyen, Song, Liem *et al.*, 2002).

O alvo celular da E6AP era desconhecido até pouco tempo. Em 2009 Shimoji e cols. (Shimoji, Murakami, Sugiyama *et al.*, 2009) descobriram que o alvo celular desta ubiquitinase é a anexina A1. Normalmente a E6AP se liga a anexina marcando-a com uma cadeia de poliubiquitina e causando sua degradação via proteassomo. Este mecanismo pode ser interrompido por uma infecção por HPV de alto risco. Quando um HPV estabelece uma infecção, a célula passa a expressar E6. Esta proteína viral liga-se a E6AP causando mudanças

conformacionais na sua estrutura. A alteração leva a mudança de alvo da E6AP de anexina A1 para p53. Isto permite a estabilização de anexina A1 e a degradação proteassomal de P53. Assim a expressão de anexina aumenta e a expressão de p53 diminui, contribuindo para a patogênese viral. O mecanismo é descrito na **figura 9**.

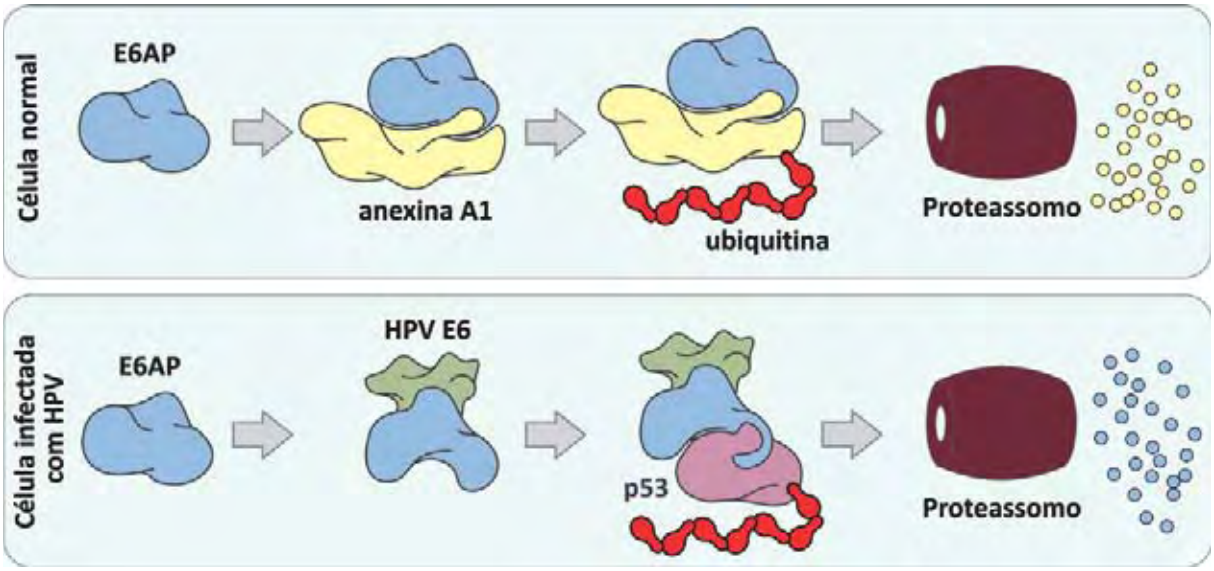

Figura 9. O mecanismo de regulação da expressão de anexina por E6AP e como a expressão de E6 viral interfere neste processo.

A P53 é uma importante proteína supressora de tumores (Gunia, Kakies, Erbersdobler *et al.*, 2012). É uma das proteínas regulatórias do ciclo celular, controlando a transição da fase S para G1. Na presença de DNA danificado, ativa vários genes pró-apoptóticos e inibidores de crescimento. Alterações na sua expressão ou danos a este gene são relatadas em diversos tumores (Lehmann e Pietenpol, 2012; Sperka, Wang e Rudolph, 2012).

A expressão de anexina A1 está significantemente aumentada em células de CEP infectadas com HR-HPV quando comparadas com amostras de CEP negativas para HPV. O mecanismo molecular de controle da anexina A1 e p53 pela interação E6AP/E6, proposto por Shimoji e colaboradores fornece uma explicação aos dados obtidos neste trabalho e permite afirmar que há uma correlação entre a expressão aumentada de anexina A1 e a presença de HR-HPV (HPV16) e que este aumento tem importante papel oncogênico no CEP em pacientes portadores de HR-HPV.

O outro objetivo deste trabalho era verificar os genótipos de HPV mais prevalentes nas amostras de CEP. Com este fim amostras de CEP foram testadas por PCR com o par de oligonucleotídeos iniciadores gerais GP5+/GP6+ que reconhecem uma grande quantidade de genótipos de HPVs, que foram em seguida identificados com o kit INNO-LiPA HPV Genotyping Extra (Innogenetics) disponível comercialmente.

A incidência de HPV nos homens não é tão bem conhecida como nas mulheres (Hartwig, Syrjanen, Dominiak-Felden *et al.*, 2012). Há grande variação nas incidências de HPV encontrados em CEP, variando de 15% a 77,5%. Esta variação depende principalmente da utilização de subtipos histológicos diferentes, da metodologia empregada na detecção de HPV, da população estudada e da conservação das amostras (Stankiewicz, Prowse, Ktori *et al.*, 2011).

Os 99 pacientes testados neste trabalho foram agrupados em dois grupos de acordo com a região de origem: Pará (grupo PA, 60 amostras) e São Paulo (grupo SP, 39 amostras).

A prevalência geral foi de 74 (74,75%) para algum HPV. Os índices de prevalência encontrados neste trabalho são maiores do que os índices de outros recentes (Prowse, Ktori, Chandrasekaran *et al.*, 2008; Stankiewicz, Prowse, Ktori *et al.*, 2011) e de uma revisão recente (Backes, Kurman, Pimenta *et al.*, 2009) que encontrou 48% de 1266 casos de 30 estudos de câncer de pênis invasivo. A alta prevalência de HPV encontrada neste trabalho pode ser devido à sensibilidade do método empregado. Neste estudo optou-se por ensaio de PCR em dois rounds com o objetivo de compensar a degradação do DNA causada pela parafinização dos tecidos.

Neste trabalho foi observado maior prevalência de HPV em pacientes provenientes do Pará (81,7%) quando comparados a pacientes de São Paulo (64,1%) além da maior diversidade de genótipos nestes pacientes. Também foi observado que infecções múltiplas com diferentes genótipos de HPV são mais prevalentes no grupo PA (43,33%) do que no

grupo SP (16,67%) e correlacionam-se com maior severidade dos tumores no grupo PA (estágios III e IV). Infecções múltiplas em carcinoma cervical já foram relatadas em outras regiões do Brasil (Tozetti, Scapulatempo, Kawski *et al.*, 2006; Ribeiro, Figueiredo Alves, Costa *et al.*, 2011), apesar de a história natural do HPV aparentemente diferir entre homens e mulheres. Nas mulheres há menos infecções e mais doenças e no homem há mais infecções e menos doenças (Giuliano, Lee, Fulp *et al.*, 2011).

Os dados obtidos pelo presente trabalho não permitem estabelecer de forma conclusiva um elo causal entre a maior diversidade de genótipos e o maior número de casos de CEP no grupo PA, mas é possível que esta relação causal exista. Também é possível que múltiplas infecções com genótipos de alto risco atuem de forma sinérgica aumentando a probabilidade de gênese tumoral bem como acelerando sua progressão. A baixa idade de alguns pacientes portadores de CEP em estágio IV no grupo PA suporta esta hipótese. Entretanto, diferenças sócio-econômico-culturais (*status* imunitário precário, fumo, uso de bebidas alcoólicas, sexo sem preservativo, má higiene íntima) devem ter também importante contribuição para maior incidência de infecções simples ou múltiplas no Pará e para a progressão tumoral. Estes dados permitem pressupor que deve haver maior diversidade de genótipos circulando na população masculina paraense.

Durante a realização deste trabalho foram identificados dois pacientes portadores de CEP que desenvolveram tumores primários (carcinomas de células escamosas) em outros órgãos, anos após a penectomia. Um paciente desenvolveu tumor de laringe e outro tumor de esôfago, mas ambos vieram a óbito antes da possibilidade de tratamento destes tumores. Estes tumores têm grande incidência mundialmente e estão associados a infecção por HPV de alto risco (Duray, Descamps, Arafa *et al.*, 2011; Gupta, Barwad, Rajwanshi *et al.*, 2012). Nas amostras de tumores de ambos os tumores do mesmo paciente foram encontrados HPV do

mesmo genótipo (HPV16). Este trabalho é o primeiro relato sobre tumores primários consecutivos associados ao HPV em diferentes órgãos no mesmo paciente.

Esta observação mostra que, talvez seja possível o desenvolvimento de tumores primários consecutivos em diferentes órgãos no mesmo paciente, levando a necessidade de seguimento mais cuidadoso do paciente submetido a penectomia para tratamento de CEP.

Os dados deste trabalho juntamente com a extensão do território nacional e as grandes diferenças culturais entre as regiões do país apontam para a necessidade de mais estudos epidemiológicos regionais.

A metodologia de RaSH e subsequente validação por PCR em tempo real mostraram ser ferramentas eficientes e sensíveis para análise das alterações moleculares no desenvolvimento e progressão do CEP.

Os dados deste trabalho trazem contribuições importantes no entendimento dos mecanismos moleculares envolvidos na gênese e desenvolvimento do CEP e do papel do HPV nesta patologia. Compreender melhor os mecanismos moleculares desta doença pode auxiliar em diagnósticos e prognósticos mais precisos, além de resultar em abordagens clínicas e terapêuticas mais adequadas.

Estudos posteriores no sentido de verificar o *status* físico do HPV e compreender melhor seu papel nestes carcinomas deverão ser realizados no futuro.

CONCLUSÕES

Os dados obtidos neste trabalho permitem concluir que:

- O aumento de p16 e anexina A1 observados nos tecidos penianos de portadores de CEP estão correlacionados a infecção por HR-HPV;
- Existe a possibilidade de desenvolvimento de tumores primários consecutivos associados à infecção por HR-HPV em diferentes órgãos no mesmo paciente;
- Há alta prevalência de HPV, principalmente de alto risco na população masculina no estado de São Paulo;
- Há diferenças significativas entre os genótipos de HPV circulantes no estado de São Paulo e no estado do Pará, ao menos na população masculina.

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