UNIVERSIDADE FEDERAL DO CEARÁ

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

NÚCLEO DE PESQUISA E DESENVOLVIMENTO DE MEDICAMENTOS

MECANISMOS EPIGENÉTICOS E VARIANTES GENÔMICAS ASSOCIADOS A INFECÇÃO POR HPV NO CÂNCER DE PÊNIS

RENAN DA SILVA SANTOS

FORTALEZA-CE

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Ceará como parte dos requisitos para obtenção do título de Doutor em Farmacologia.

Orientadora: Profª Drª Claudia do Ó Pessoa

Coorientadora: Profª Drª Cristiana Libardi Miranda Furtado

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RESUMO

O câncer de pênis (CPE) é um tumor raro, que acomete cerca de 2% da população masculina. O aumento da incidência da doença está relacionado às baixas condições socioeconômicas, sendo uma crescente preocupação dos órgãos de saúde pública. A infecção por papilomavírus humano (HPV) está relacionada com aproximadamente 50% dos casos de CPE. Múltiplos fatores de risco estão relacionados à doença e a falta de informação associado ao estigma social, diagnóstico tardio e terapia limitada reduzem as chances de cura. O objetivo deste trabalho é avaliar aspectos prognósticos do CPE, bem como identificar a relação da infecção por HPV de alto risco em 224 participantes. Também descrever o padrão das marcas de metilação e hidroximetilação global do DNA, 5-metilcitosina (5mC) e 5-hidroximetilcitosina (5hmC), respectivamente, identificar o padrão de metilação da região diferencialmente metilada, H19DMR, e apresentar dados preliminares de um painel de exoma. Observamos que a incidência de HPV foi de 53,2% para HPV de alto risco e 22,32% para o marcador p16INK4a. O HPV de alto risco não foi relacionado a metástase sistêmica ou linfonodal e recorrência locorregional, nem influenciou a taxa de sobrevivência. A expressão do marcador p16INK4a parece ser um fator para um desfecho positivo e que não afeta a metástase ou a recorrência tumoral. Metástases em linfonodo e sistêmica e recorrência locorregional aumentam o risco de morte. Nossos resultados indicam um aumento expressivo na marca de 5mC em CPE, independentemente da infecção pelo HPV. Porém, relatamos a redução de 5hmC para p16INK4a+ (P = 0,024). O aumento da relação 5mC/5hmC (> 1) foi observado em 94,2% dos CPE, independentemente da infecção pelo HPV. Apesar do aumento de 5mC, esse fato parece não afetar a taxa de sobrevivência (HR = 1,06; IC 95% 0,33–3,38). Observamos uma metilação média de 32,2%±11,6% no H19DMR do CPE, sem associação entre os marcadores p16INK4a+ (p = 0,59) e HPV+ de alto risco (p = 0,338) com o nível de metilação. Observamos correlação positiva entre infiltração de células polimorfonucleares e hipometilação no H19DMR (p = 0,035). Em uma análise preliminar de um painel de exoma, observamos genes com alta frequência mutacional não descritos anteriormente (*MUC5B*, *MUC16*, *OBSCN*, *MUC12*, *CMYA5*, *SVEP1*, *LEMAS5*, *SPTA1* e *FSIP2*). A caracterização molecular do CPE incentiva e favorece que novos estudos possam sugerir estratégias de tratamento específicas, como agentes hipometilantes para terapias direcionadas epigenéticas, estabelecer novos alvos com perfil de genes drogáveis e incentivar a busca de terapias menos invasivas.

Palavras-Chave: Câncer de Pênis, Prognóstico, HPV, Epigenética.

EPIGENETIC MECHANISMS AND GENOMIC VARIANTS ASSOCIATED WITH HPV INFECTION IN PENILE CANCER

Penile cancer (CPE) is a rare tumor, which affects approximately 2% of the male population. The incidence increase of the disease is mainly related to low socioeconomic conditions. Human papillomavirus (HPV) infection is related to approximately 50% of penile SCC cases. Multiple risk factors are related to the disease and the lack of information associated with social stigma, late diagnosis and limited therapy limits the chances of cure. The objective of this work is to evaluate epidemiological and prognostic aspects of CPE, as well as identify the relationship between high-risk HPV (hrHPV) infection in 224 participants. We also describe the pattern of the global DNA methylation and hydroxymethylation marks, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), respectively, identify the methylation pattern of the differentially methylated region, H19DMR, and present preliminary data from an exome panel. We observed that the incidence of HPV was 53.2% for high-risk HPV and 22.32% for the p16INK4a marker. hrHPV was not related to systemic or lymph node metastasis and locoregional recurrence, nor did it influence the survival rate. Expression of the p16INK4a marker appears to be a factor for a positive stage and does not affect metastasis or tumor recurrence. Lymph node and systemic metastases and locoregional recurrence increase the risk of death. Our results indicate a significant increase in the 5mC mark in CPE, regardless of HPV infection. However, we reported a reduction in 5hmC for p16INK4a+ ($P = 0.024$). An increase in the 5mC/5hmC ratio (> 1) was observed in 94.2% of SCC, regardless of HPV infection. Despite the 5mC increase, this fact does not seem to affect the survival rate (HR = 1.06; 95% CI 0.33–3.38). We observed an average methylation of 32.2%±11.6% in H19DMR of CPE, with no association between the markers p16INK4a+ ($p = 0.59$) and hrHPV+ ($p = 0.338$) with the methylation level. We observed a positive brightness between infiltration of polymorphonuclear cells and hypomethylation in H19DMR ($p = 0.035$). Preliminary analysis of an exome panel, we observed previously undescribed genes with high mutational frequency (*MUC5B, MUC16, OBSCN, MUC12, CMYA5, SVEP1, LEMAS5, SPTA1* and *FSIP2*). The molecular characterization of CPE encourages and favors new studies that can suggest specific treatment strategies, such as hypomethylating agents for epigenetic targeted therapies, establish new targets with druggable gene profiles and encourage the search for less invasive therapies.

Keywords: Penile Cancer, Prognosis, HPV, Epigenetics.

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1. CAPÍTULO I

FUNDAMENTAÇÃO TEÓRICA

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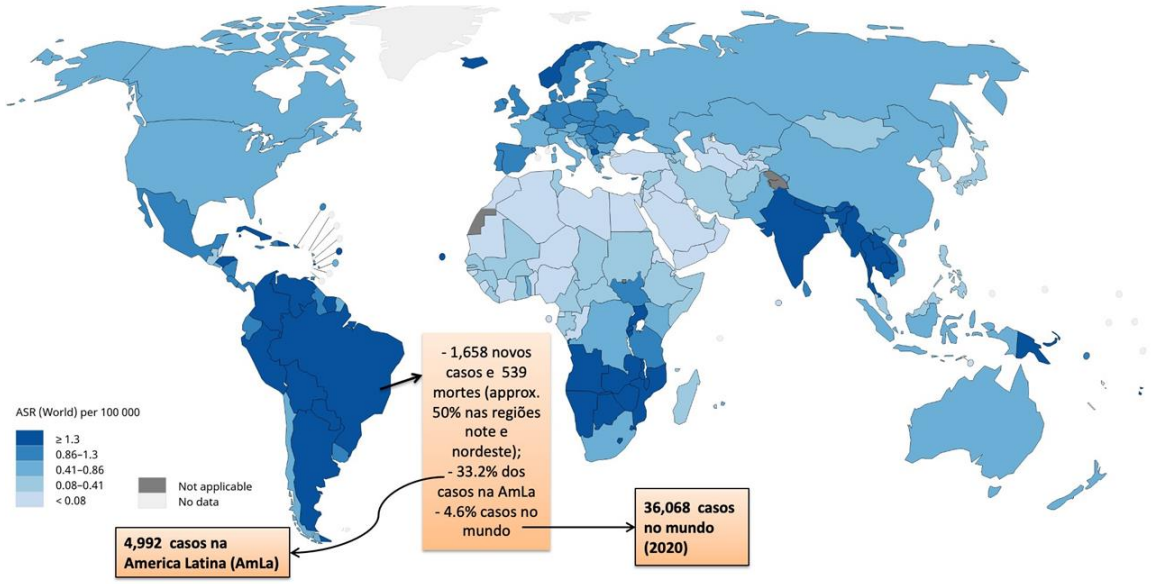
1.1. Fundamentação teórica.

1.2. Câncer de pênis.

O câncer é um problema de saúde pública mundial, sendo a primeira causa de morte em países desenvolvidos e a segunda maior causa em países em desenvolvimento. O crescente aumento no número de casos de câncer e mortalidade associada à doença é resultado de diversos fatores como crescimento e envelhecimento populacional e a associação de hábitos de vida não saudáveis como o tabagismo, falta de atividade física e dietas ocidentalizadas (*fast foods*) (Zhang et al., 2020). Dente as neoplasias que afetam a população masculina, a crescente incidência do câncer de pênis (CPE) tem preocupado os órgãos de saúde pública.

O CPE é um tumor raro, característico de regiões subdesenvolvidas, que acomete homens principalmente a partir dos 50 anos, embora possa atingir indivíduos mais jovens (Vieira et al., 2020). A incidência do CPE no mundo é de 0.8/100,000.0 e acredita-se que esteve valor aumente em 56% até 2040 (Bray et al., 2018). No Brasil essa estimativa é de 2,9 - 6.8/100.000, uma das maiores do mundo (Sung et al., 2021). Segundos dados disponíveis na plataforma Globocan (2021) o Brasil apresentou 1658 casos da doença em 2020 e 539 obitos da doença distribuídos entre as regiões norte e nordeste. Esses valores representam 33.2% dos casos da América Latina e 4.6% no mundo. Apesar da incidência relativamente baixa quando comparado aos demais tipos de tumores no Brasil, o CPE é uma doença extremamente agressiva, com prognóstico negativo, alta mortalidade, tempo médio de sobrevida de quatro anos e que afeta diretamente a autoestima e a vida sexual do paciente (Souza, de et al., 2018).

Figura 1. Regiões com maior incidência de carcinoma peniano mundialmente.



Fonte: adaptado de SUNG, H. et al. 2020.

O diagnóstico tardio reduz as chances de cura, pois as opções de tratamento como a quimioterapia e radioterapia são limitadas e na maioria das vezes usados como terapia paliativa ou neoadjuvante. Na maioria dos casos, a amputação parcial ou total do órgão genital é a principal estratégia utilizada como terapia, entretanto amputação não está associada a diminuição da morbidade/mortalidade dos portadores da doença (Silva Amancio et al., 2017). Atualmente não há consenso para uma terapia adequada para o CPE e poucas informações a acerca dos mecanismos envolvidos na doença e as alterações relacionadas dificultam o aprimoramento das medidas preventivas (Silva Amancio et al., 2017). Outro fator agravante é a escassez de dados epidemiológicos e a dificuldade na classificação dos vários subtipos do CPE.

O aumento da incidência do CPE é crescente no Brasil, principalmente nas regiões Norte e Nordeste, onde são registrados a maioria dos casos de ressecção cirúrgica da genitália (penectomia), representando 53.02% dos casos cirúrgicos no país (Favorito et al., 2008). Diferentes tipos histológicos podem estar associados ao CPE, como sarcoma, melanoma e carcinoma basocelular (Douglawi e Masterson, 2019), entretanto o carcinoma de células escamosas de pênis compreende 95% dos casos reportados e pode ser originado do epitélio escamoso que cobre a glande, sulco coronal e prepúcio (Christodoulidou et al., 2015).

Existem múltiplos fatores risco descritos associados ao CPE e em sua grande maioria se destacam aspectos relacionados principalmente ao estilo de vida ou hábito do paciente, como

baixas condições socioeconômicas, falta de higiene pessoal, fimose, número de parceiros sexuais e sexo com animais (zoofilia) estão entre os principais fatores de risco para o desenvolvimento da doença (Júnior et al., 2018; Vieira et al., 2020). A infecção pelo papilomavírus humano (HPV, do inglês, *Human papillomavirus*) pode estar relacionada a uma incidência de até 62% dos casos de CPE (Vieira et al., 2020), no entanto a relação da infecção viral com o pior prognóstico ou agravamento de CPE ainda não está bem esclarecida (Giuliano et al., 2011).

Como uma doença estreitamente ligada aos hábitos de vida, medidas preventivas simples poderiam evitar e/ou reduzir número de mortes por CPE. No entanto a distribuição irregular ao longo do Brasil, aliada à falta de documentação científica e clínico-cirúrgica, impede o estabelecimento de estratégias mais eficientes para diagnóstico e tratamento do CPE. Não obstante a imprecisa relação com o HPV e a pouca informação da etiopatologia molecular constitui um grande desafio na busca dos mecanismos envolvidos com o CPE (Ficarra et al., 2010).

Apesar da crescente busca dos mecanismos moleculares envolvidos no processo carcinogênico do CPE, as diversas vias envolvidas, alterações genéticas e epigenéticas relacionadas, bem como a atuação de diferentes medicamentos no combate ao tumor, ainda é um campo vasto a ser explorado. Devido a grande heterogeneidade genotípica e fenotípica dos diversos tipos de tumores, incluindo o CPE, uma maior compreensão dos mecanismos moleculares envolvidos pode ajudar no diagnóstico precoce e combate à doença (Koifman et al., 2015). As diferenças genéticas dos diferentes subtipos e sua relação com a infecção por HPV representam um crescente e novo campo na terapia alvo dirigida, que pode representar uma importante alternativa no tratamento e prognóstico dos portadores de CPE.

1.3. Papilomavírus humano (HPV) e Câncer.

O Ministério da Saúde (MS) instituiu em 2009 no âmbito do Sistema Único de Saúde (SUS) a Política Nacional de Atenção Integral à Saúde do Homem (PNAISH), visando promover estratégias para reforçar a atenção primária no cuidado à saúde dos homens e promover a prevenção de doenças à população jovem e adulta masculina (20 a 59 anos). A proposta de atenção à saúde do homem pretende evitar os agravos proporcionados por diversas comorbidades e implantar medidas de prevenção como vem fazendo com a população feminina (http://portalsaude.saude.gov.br/saude).

Diferentemente das mulheres, os homens geralmente entram no Sistema Único de Saúde

(SUS) através da atenção especializada e em estado avançado de algumas doenças, fatos que geram maiores custos para o SUS e um pior prognóstico para doenças que poderiam ser evitadas, aumentando a morbidade e mortalidade da população masculina. Esse contexto é observado em pacientes com CPE, onde uma grande parcela desses portadores busca a atenção hospitalar com uma lesão de maior risco de invasão tecidual. Essa busca tardia provavelmente está relacionada ao estigma da doença, medo do tratamento e falta de conhecimento sobre o diagnóstico (Korkes et al., 2020).

O aumento da prevalência de HPV na população brasileira tem sido objeto de crescente atenção dos órgãos de saúde pública. O HPV é uma das principais causas de infecção do trato reprodutivo masculino e feminino, ocorre na maioria dos casos via contato sexual. De acordo com o estudo publicado na revista *The Lancet*, Giuliano e colaboradores (2011) indicam um aumento da infecção por subtipos de HPV de alto risco no Brasil e que aproximadamente 50% da população masculina brasileira apresenta a infecção pelo vírus (Giuliano *et al.*, 2011).

O HPV é um vírus do tipo não envelopado que possui DNA dupla fita como material genético. O vírus HPV é um parasita intracelular obrigatório e que pela sua semelhança de estrutura genética encontra no genoma humano diferentes regiões de integração conhecidas como *hot spots* (Hu et al., 2015). Infecta principalmente células epiteliais, estando presente na pele e mucosas de vários tecidos. Cerca de 220 diferentes subtipos de HPV já foram identificados e sequenciados, dentre estes, aproximadamente 40 estão relacionados com infecção na mucosa anogenital (Leto et al., 2011; Mobini Kesheh et al., 2023). Além de estar relacionado com lesões benignas, um fator agravante da infecção por HPV é a estreita relação com lesões malignas, em que tem sido associado ao desenvolvimento tumores cutaneomucosos (Leto et al., 2011).

A classificação dos diferentes tipos de HPV está relacionada à predisposição ao desenvolvimento de neoplasias benignas ou malignas, sendo divididas basicamente em HPV de alto risco (hrHPV, do inglês, *high-risk HPV*) e baixo risco (lrHPV, do inglês, *low-risk HPV*). As lesões malignas estão relacionadas ao HPV de alto risco que pode induzir o surgimento de diversos tipos de tumores em indivíduos do sexo masculino e feminino (Bleeker *et al.*, 2009). Existem ao menos 14 genótipos descritos como HPV de alto risco (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 e 68) (Mobini Kesheh et al., 2023).

Embora a inserção do material genético do vírus possa levar a alterações que induzam eventos neoplásicos, a existência de DNA do vírus no DNA da célula hospedeira não é o

suficiente para induzir um tumor, pois decorrências genéticas e epigenéticas adicionais seriam necessárias (Wang, Huang e Zhang, 2018). Das oito proteínas expressas pelo genoma do vírus HPV, existem duas proteínas oncogênicas, E7 e E6, que estão associadas ao controle do ciclo célula e a apoptose da célula hospedeira (Graham, 2010). A proteína E7 apresenta capacidade de se ligar à forma não fosforilada da proteína pRb, competindo com fatores de transcrição do tipo E2F dentro do núcleo celular. A união entre a proteína E7-pRb perturba o complexo pRb-E2F, causando um favorecimento da expressão de genes que estimulam a síntese de DNA durante o ciclo celular (Tomita, Huibregtse e Matouschek, 2020).

Por sua vez, a oncoproteína E6 apresenta estrutura similar a dois domínios dedo de zinco e quatro motivos Cys-X-X-Cys, o que foi descrito como domínios de alto potencial de interatuar com diversas proteínas da célula hospedeira (Pal e Kundu, 2020). Uma desses alvos é a proteína supressora tumoral P53, que é degrada através de uma via de ubiquitinação estimulada pela proteína E6 (Wang, Huang e Zhang, 2018). As alterações geradas pelas oncoproteínas virais estão associadas ao estimulo de diferentes marcas de indução tumoral, *cancer hallmarks*, como desregulação da proliferação celular, resistência a morte celular, "imortalidade" celular através do controle da expressão da transcriptase reversa da telomerase humana, indução a angiogênese e ativação de mecanismos de invasão (Wang, Huang e Zhang, 2018).

Infecção por subtipos de HPV de alto risco, como HPV-16 e HPV-18, parece ser obrigatório para o desenvolvimento de câncer cervical. Embora seja amplamente relatado que os tumores de colo do útero sejam estimulados a partir da infecção por HPV, apenas uma pequena fração da população que desenvolve CPE são causados pelo HPV. Esta observação levou a suposição de que o tipo de células escamosas do pênis tem uma resistência aumentada à transformação maligna em comparação com o tecido cervical. A maioria das infecções por HPV não evoluem para lesões externas e permanecem assintomáticos, sendo eliminados dentro de um prazo médio de um ano (Dunne et al., 2006).

1.4. CPE e marcadores moleculares.

A característica complexa e multifatorial da carcinogenese, em que diferentes vias podem ser ativadas ou silenciadas, tanto para o desenvolvimento tumoral, quando em resposta a um tratamento impulsionam o interesse pelo estudo sistêmico, que leva em conta as complexas redes de interações e regulações existentes no contexto celular para explicar os fenômenos biológicos presentes em organismos vivos (Welch e Clegg, 2010). Atualmente ainda não há uma terapia

específica para o tratamento do CPE e a carência de informações a acerca dos mecanismos envolvidos na doença inviabilizam o desenvolvimento de estratégias de tratamento mais eficazes e menos agressivas para os pacientes (Koifman et al., 2015). O conhecimento das vias moleculares que envolvem o CPE se restringem principalmente a presença ou ausência do HPV.

Os mecanismos moleculares envolvidos no CPE associado ao HPV são caracterizados por instabilidade genômica secundária à superexpressão das oncoproteínas E6 e E7, o que impacta diretamente na ativação descontrolada do ciclo celular (Chaux et al., 2010). Em contraste, os mecanismos moleculares do CPE independente do HPV são menos compreendidos, inclusive o prognóstico de CPE HPV negativo é descritos como pior do que os casos HPV positivo (Kashofer et al., 2017). Variantes no gene *TP53* são relatadas neste subconjunto de pacientes que desenvolvem a doença e não apresentam infecção por HPV. Estudos sugeriram que as variantes no *TP53* podem estar associadas a uma alta frequência de metástases nodais, um fator conhecido por estar fortemente correlacionado com um pior prognóstico (Ficarra et al., 2010; Trias et al., 2023)

Biomarcadores com valor terapêutico têm sido descritos para CPE e diferentes estudos relatam a presença de variantes somáticas nos genes, *CDKN2A, PIK3CA, HRAS, KRAS e STK11*, previamente identificados em amostras de CPE, sugerindo um impacto importante da via fosfatidilinositol 3-quinase ou Ras (Wang et al., 2019). Outra via de interesse na compreensão das alterações moleculares do CPE é amplificação do gene *EGFR*. Entretanto, diferentes estudos identificaram que a superexpressão da proteína EGFR não está associada à amplificação genética ou ao ganho do número de cópias genéticas no CPE (Ali et al., 2016; McDaniel et al., 2015). Amplificações em *EGFR* são reportadas em aproximadamente 10% dos casos de CPE, com heterogeneidade significativa entre tumores primários pareados e metástases linfonodais (McDaniel et al., 2015).

Os dados disponíveis na literatura ainda não são suficientemente determinantes para incluir o uso rotineiro de biomarcadores no diagnóstico e tratamento de CPE. A Tabela 1 apresenta potenciais marcadores moleculares e que foram descritos como associados com grau maior grau histológico do tumor, estadiamento alto, maior risco de metástase inguinal e de progressão da doença (SCCAg, p53, CRP, Ki-67, PCNA, Cyclin D1, E-cadherin, MMP-2, MMP-9, Fox-P3 e ARID1A). O marcador de infecção de HPV de alto risco, $p16^{ink4a}$, foi associado a menor invasão tumoral, menor risco de recorrência da doença e possivelmente melhor sobrevida (Zargar-

Shoshtari, Sharma e Spiess, 2018).

Tabela 1. Potenciais marcadores moleculares e seu valor prognóstico para o carcinoma de células escamosas de pênis.

Biomarcador	Função	Prognóstico
SCCAg	Glicoproteína associada a tumor	Pode ser usado para monitorar a progressão da doença e a resposta ao tratamento
p53	Gene supressor de tumor	A expressão indicou maior risco de metástase de LN, progressão da doença e pior sobrevivência específica da doença
CRP	Marcador pró-inflamatório	Níveis plasmáticos elevados encontrados com mais frequência em pacientes com tumor em estágio avançado, doença nodal positiva e pior sobrevivência específica da doença
Ki-67	Marcador para célula tumoral proliferação no ciclo celular	Rotulagem correlacionada com maior grau de tumor, estágio avançado de tumor local, maior risco de metástase nodal e progressão clínica da doença
PCNA	Marcador de proliferação celular essencial para replicação	A expressão foi associada à presença de metástase nodal
Cyclin D	Regula a progressão das células através da fase G1 da célula ciclo	Nenhum valor prognóstico claro; implicado na diferenciação tumoral
p16ink4a	Marcador substituto para alto risco Infecção por HPV	A positividade foi associada a menos invasão tumoral, menor risco de recorrência da doença e possivelmente melhor sobrevida
E-cadherin	Mantém a adesão celular e transdução de sinal	A imunorreatividade foi associada a um maior risco de metástase no LN
MMP-2 e MMP-9	Degrada o porão membrana de uma célula	A imunorreatividade foi associada a um maior risco de recorrência da doença
Fox-P3	Supervisiona o desenvolvimento e função das células T reguladoras	Níveis aumentados correlacionados com menor infiltrado inflamatório pior SG
ARID1A	Envolvido na cromatina remodelação	Maior expressão foi associada a um maior grau histológico

Fonte: adaptado de Zargar-Shoshtari, Sharma e Spiess (2018).

1.4.1. CPE e marcadores epigenéticos.

Devido a estreita relação com as alterações ambientais e a infecção viral com o desenvolvimento do CPE, além das alterações genéticas relacionadas, um componente epigenético tem sido relacionado com a doença. O acúmulo de variantes genéticas e epigenéticas têm sido apontados como a maior causa das neoplasias, as quais desempenham um importante papel na iniciação e progressão do tumor, e é uma importante ferramenta para a compreensão e

tratamento de doenças complexas como o câncer. O termo epigenética pode ser definido como qualquer alteração estável (a nível celular) na função gênica que não são explicadas por mudanças na sequência de DNA (Haig, 2004). Os mecanismos epigenéticos em geral alteram a estrutura da cromatina conferindo um programa diferencial de expressão gênica, dentre os quais encontram-se as modificações de histonas, os RNAs não codificadores (ncRNA) e a metilação do DNA.

Os mecanismos epigenéticos que controlam a expressão gênica (epigenótipo), juntamente com as alterações genéticas (genótipo), conferem as células tumorais um fenótipo peculiar, com alta capacidade proliferativa autônoma e monoclonal, anti-apoptótica, altamente invasiva e com a possibilidade de metástase favorecida pela angiogênese tumoral (formação de vasos sanguíneos para o suprimento de nutrientes e oxigênio) (Feinberg, Ohlsson e Henikoff, 2006). Estudos têm demonstrado um padrão de metilação do DNA aberrante que estão relacionados a um pior prognóstico, risco de metástase e prevalência de HPV no CPE (Kuasne et al., 2015; Kuasne et al., 2017). Dentre elas, a hipermetilação de genes supressores tumorais *CDO1, AR1, WT1, CD133, HOXA3, RSPO2* e *SOX17* (Kuasne et al., 2015).

Alterações epigenéticas são contribuintes críticos para a progressão do câncer, e a metilação do DNA é um dos biomarcadores de doenças mais extensivamente estudados. Marcadores de metilação no DNA já foram reportados com valor prognóstico, como $p16^{INK4A}$, p^{14ARF}, *TSP-1, RASSF1-A, DAPK, FHIT, MGMT, RASSF2* e *TSLC1* (Gu et al., 2021). Feber e colaboradores (2015) demonstraram 997 regiões hipermetiladas associadas a genes supressores de tumor. A instabilidade gerada pela expressão das oncoproteínas E6 e E7 não são os únicos eventos associados a um estímulo tumoral, a infecção por HPV também pode induzir a um cenário de expressão alterada de micro RNAs. Ayoubian e colaboradores (2021) descreveram o perfil de micro RNA em pacientes com CPE HPV positivos e indivíduas saudáveis, observando a diferença de expressão de 876 micro RNAs entre os grupos (Ayoubian et al., 2021).

O padrão de metilação do DNA em 38 CPE pareadas com 11 amostras de tecidos não tumoral vizinho circundante revelou um padrão de hipometilação em tumores não metástatico (Feber et al., 2015). O segundo estudo que descreve a metilação do DNA e comparou com resultados de transcriptoma de 25 amostras de CPE e 10 não tumoral vizinho circundante encontraram um painel de 54 genes (como *TWIST1, RSOP2, SOX3, SOX17, PROM1, OTX2, HOXA3* e *MEIS1*) com correlação inversa entre metilação do DNA e expressão gênica. Esses estudos apontaram que a metilação do DNA influenciam a regulação de vias associadas ao CPE,

incluindo ciclo celular, resposta imune e sinalização Wnt/β-catenina (Kuasne et al., 2015).

Muitos estudos sobre alterações epigenéticas no CPE avaliam os padrões de genes específicos, como regiões promotoras, genes e exons. No entanto, a avaliação de marcadores para esta doença seria favorecido por métodos que detectem alterações de metilação em nível genômico. Estudos em larga escala em CPE são muito importantes para compreender melhor o comportamento do tumor e conhecer seu microambiente e determinar os marcadores moleculares envolvidos nesta doença. Apesar de grandes esforços na busca de marcadores moleculares para melhor compreensão dos mecanismos envolvidos na patogênese do CPE, poucos estudos investigaram as alterações genéticas e epigenéticas relacionadas ao CPE na população brasileira, especialmente no nordeste do Brasil. Ainda, as diversas vias envolvidas, os genes que são ativados ou silenciados, bem como a atuação de diferentes medicamentos no combate a doença, ainda é um campo vasto a ser explorado. A associação da caracterização morfológica e os mecanismos moleculares envolvidos no CPE pode levar a um melhor entendimento do câncer como um sistema e propiciar novos alvos terapêuticos que possibilitem um melhor prognóstico e tratamento da doença.

1.5. Objetivos

1.5.1. Objetivo geral

Identificar marcadores epigenétcos e genômicos relacionados ao carcinoma de células escamosas de pênis e avaliar a possível relação destes com a infecção por HPV.

1.5.2. Objetivos específicos

- Avaliar os aspectos clínicos e patológicos da doença e sua relação com o desenvolvimento do CPE;
- Identificar a presença da infecção do HPV de alto risco em grupo amostral de 224 amostras de CPE em amostras de tecido parafinado;
- Avaliar a associação entre a presença de HPV de alto risco e dados epidemiológicos da doença;
- Avaliar a associação entre dados epidemiológicos e sobrevida livre da doença;
- Avaliar o padrão das marcas epigenéticas, 5-metilcitocisa (5mC) e 5-hidroximetilcitosina (5hmC), em amostras de tecido parafinado;
- Avaliar a associação entre as marcas epigenéticas e a infecção por HPV de alto risco;
- Avaliar o padrão de metilação da região diferencialmente metilada H19 em amostras de CPE (chr11:1,999,757- 2,000,060) em amostras de tecido congelados;
- Avaliar o padrão de metilação da região diferencialmente metilada H19 em amostras de CPE e sua relação com HPV de alto risco;
- Avaliar os genes com maior carga mutacional em amostras de tecido congelado de CPE através do sequenciamento de exons (Exoma);
- Avaliar a os genes com maior carga mutacional e sua relação com a presença da infecção por HPV de alto risco.

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2. CAPÍTULO II

HPV INFECTION AND 5MC/5HMC EPIGENETIC MARKERS IN PENILE SQUAMOUS CELL CARCINOMA: NEW INSIGHTS INTO PROGNOSTICS

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Clinical Epigenetics

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HPV infection and 5mC/5hmC epigenetic markers in penile squamous cell carcinoma: new insights into prognostics

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Abstract

Background: Penile cancer is one of the most aggressive male tumors. Although it is reventable, the main etiologic causes are lifestyle behaviors and viral infection, such as human apillomavirus (HPV). Long-term epigenetic changes due to environmental factors change cell fate and promote carcinogenesis, being an important marker of prognosis. We evaluated epidemiological aspects of penile squamous cell carcinoma (SCC) and the prevalence of HPV infection using high-risk HPV (hrHPV) and p16INK4A expression of 224 participants. Global DNA methylation was evaluated through 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC).

Results: The incidence of HPV was 53.2% for hrHPV and 22.32% for p16INK4a. hrHPV was not related to systemic or lymph node metastasis and locoregional recurrence, nor influenced the survival rate. $P16^{INK4a}$ seems to be a protective factor for death, which does not affect metastasis or tumor recurrence. Lymph node and systemic metastases and locoregional recurrence increase the risk of death. An increased 5mC mark was observed in penile SCC regardless of HPV infection. However, there is a reduction of the 5hmC mark for p16INK4a + ($P = 0.024$). Increased 5mC/5hmC ratio (> 1) was observed in 94.2% of penile SCC, irrespective of HPV infection. Despite the increase in 5mC, it seems not to affect the survival rate (HR = 1.06; 95% CI 0.33–3.38).

Conclusions: P16INK4a seems to be a good prognosis marker for penile SCC and the increase in 5mC, an epigenetic mark of genomic stability, may support tumor progression leading to poor prognosis.

Keywords: Penile cancer, Human papillomavirus, $p16^{INK4a}$, High-risk HPV, Global DNA methylation, Global DNA Hydroxymethylation

2.1. Background

Penile cancer is a rare tumor type with an increased worldwide incidence, having 36,068 new cases registered in 2020. The majority of cases occur in regions with a low human development index, where India (10,677), China (4,628) and Brazil (1,698) are the most affected countries [1]. Despite the low incidence compared to other types of male malignancies, penile cancer has a poor prognosis and is associated with high mortality [2] and morbidity [3]. Therapeutic strategies are very limited and, therefore, the main related therapy is partial or total penectomy, which directly affects the emotional and social life of patients. Given narrow options for early diagnosis and non-surgery treatments, restricted funding for medical care, and mutilating treatments resulting in negative effects on well-being, penile cancer can be regarded as a neglected disease [4–6].

Different histological types are associated with penile cancer, such as sarcoma, melanoma and basal cell carcinoma [7, 8], nevertheless, penile squamous cell carcinoma (SCC) is reported in 95% of cases worldwide [9, 10]. Multiple risk factors are described, mostly related to lifestyle behaviors, such as promiscuous sexual behavior [11], history of zoophilia [12], poor hygiene [13], psoralen UV-A phototherapy [14], smoking [15] and obesity [16]. Non-circumcision (phimosis) leads to chronic inflammation conditions like posthitis, lichen sclerosus and balanitis xerotic obliterans [9] increasing the risk of developing penile cancer by 22-fold [17]. However, human papillomavirus (HPV) infection in penile cancer is one of the main etiologic causes, especially for squamous cell carcinomas [18].

The prevalence of HPV in penile neoplasia can vary widely depending on the literature and among different regions of the world, ranging from 11 to 87% [19, 20]. Associations of HPV infection and death risk are still unclear, as the results are controversial [21–23]. Positive survival prognosis for high-risk HPV (hrHPV) in penile cancer has already been highlighted [24] and Wang and collaborators (2020) [25] have recently suggested a regional lymph node infiltration staging based on the presence of hrHPV. Subtypes of hrHPV are linked to malignant lesions due to the degradation of the cell cycle control proteins P53 and Rb, and the expression of the viral oncoproteins, HPV E6 and HPV E7, which causes evasion to cell death and DNA damage repair, respectively [26]. These viral oncoproteins' expression promotes chromosomal rearrangements, multiple centromeres and aneuploidy [27]. Viral infection, as HPV is often related to an increase in genomic instability by aberrantly reprogramming the epigenome [28]. In different squamous

cell carcinomas hrHPV directly modulates enzymes that maintain the conformation of nucleosomes [28, 29] and enzymes responsible for maintaining DNA methylation [30, 31].

Environmentally induced epigenetic changes have been recently added as hallmarks of cancer, which contribute to tumor initiation and progression [32]. Global DNA methylation is characterized by the addition of a methyl at position 5 of cytosine in a dinucleotide CpG (cytosine-phosphate-guanine) resulting in a 5-methylcytosine (5mC) [33], which affects gene expression, chromatin remodeling and genomic stability. DNA methyltransferases (DNMTs) mediate the transfer of the methyl group to DNA, and loss of the 5mC marker can occur either passively by DNA replication or actively by the enzymes known as Ten-Eleven-Translocation (TETs) [34]. These enzymes catalyze the conversion of 5mC into 5-hydroxymethylcytosine (5hmC). Imbalances between 5mC and 5hmC marks cause transcriptional dysregulation of promoters and enhancers, changing the cell fate [35]. The imbalance of 5mc/5hmc dynamics has been described in many types of cancers, but not for penile squamous cell carcinomas.

Since HPV infection may drive epigenetic changes, the characterization of the global DNA methylation and its association with hrHPV DNA and p16INK4a expression will provide better knowledge about the molecular mechanisms related to penile SCC. Therefore, evaluate the prevalence of HPV infection and 5mC and 5hmC epigenetic marks in penile SCC and its association with clinicopathological alterations. As an important mechanism of genomic stability, aberrant epigenetic reprogramming related to penile SCC pathogenesis and viral infection may be used as a biomarker for prognosis and targeted therapies. Determining the prevalence of HPV, one of the most common infections of the reproductive tract, is important for the development of public health strategies for the prevention and treatment of related diseases.

2.2. Methods

2.2.1. Participants and study design

This is a retrospective study that included 224 participants with penile SCC who underwent partial or total penectomy, and advantage stage with enlarged prostatectomy and emasculation, without any prior history of chemotherapy or radiotherapy. Penile SCC samples were obtained at Hospital Haroldo Juaçaba, Ceará, Northeast Brazil, and detailed clinicopathological and follow-up data were assessed from 2000 to 2018. The study was approved by our Institutional Review Board (process number 2.427.846).

Available epidemiological data for penile SCC were obtained. The anatomopathological evaluation was performed by two pathologists who were blinded to the clinical data, and the regions corresponding to the neoplasm were marked on the histological slides. Pathological staging was performed according to the eighth edition of the American Joint Committee on Cancer (AJCC) (2017) [36]. Local recurrence in the amputation stump, as well as lymph node and systemic metastasis, was evaluated. The mean follow-up was 29.69 (37.3) months from surgery to the last visit or death. As we had different types of block quality and the amount of material had to be fractionated for pathological analysis and p16INK4a, hrHPV, 5mC, and 5hmC markings, the number of samples available for analysis varied (Table 1 and Additional file 1: Supplementary Fig. 1).

2.2.2. Tissue microarray construction

The Tissue MicroArray (TMA) blocks were composed of representative 2.0 mm cores, in duplicate, from the 224 samples from the epidemiological study cases. Blocks were sectioned to 4 μm thick and mounted on glass slides with an organosilane-based adhesive (3-aminopropyltriethoxy-silane; Sigma Chemical Co®, St Louis, MO, USA). TMA sections were then used for staining in HPV detection assays and analyses of 5mC/5hmC epigenetic markers. All the experiments were analyzed by a blinded observer.

2.2.3. p16INK4a immunoexpression and HPV in situ hybridization

The p16INK4a immunoexpression was carried out using an anti-p16 antibody clone E6H4 (Roche, USA). p16INK4a expression must be ≥ 75% in neoplastic cells to be considered positive in immunohistochemistry (IHC) staining, with continuous and complete cytoplasmic and nuclear staining [37]. Chromogenic in situ hybridization (CISH) was used to distinguish hrHPV and low-risk HPV (lrHPV) forms. For that, Ventana Inform HPV III Family 16 Probe (Ventana Medical Systems, Tucson, AZ) diagnostic kit was used to identify hrHPV (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66) and Ventana Inform HPV II Family 6 (Ventana Medical Systems, Tucson, AZ) for lrHPV identification (genotypes 6 and 11). High-grade cervical intraepithelial neoplasia was used as a positive control for hrHPV and condyloma acuminate for lrHPV. Skeletal striated muscle was used as a negative control for both assays. Representative images of the positive and negative markings of the tumor area for each assay used in this work are available in Additional file 1: Supplementary Fig. 2.

2.2.4. Global DNA methylation and hydroxymethylation assays

The TMA sections were stained with anti-5-methylcytosine (anti-5mC) and anti-5-hydroxymethylcytosine (anti- 5hmC) antibodies using streptoavidin-biotin IHC. Briefly, after deparaffinization and rehydration, TMA slides were submitted to antigenic recovery in citrate pH 6.0 0.01 M. The slides were, then, blocked by endogenous peroxidase in a 3% hydrogen peroxide solution for 30 min, to block unspecified staining. Subsequently, slides were incubated with primary antibodies (anti-5mC: Abcam–Ab214727ug50; anti-5hmC: Abcam–Ab214728ug50) in a humid and dark chamber at 4 °C overnight. Secondary incubation took place with the DAKO EnVision™ kit (DAKO®, Carpentaria, CA, USA) for 30 min at room temperature and counterstained with Mayer's hematoxylin. The analysis of 5mC and 5hmC was performed by evaluating the percentage of positivity of 100 cells in the tumor area of each TMA sample, in duplicate, for each target. Counting was made by two independent observers and the percent positivity rate was used to construct the score (% score). As each sample had 5mC and 5hmC scores, the 5mC/5hmC ratio was calculated for all participants. We used brain samples as positive control and the absence of the primary antibody during the incubation as the negative control.

2.2.5. Statistical analyses

Qualitative variables were summarized considering absolute and relative frequencies. The Fisher's exact test was applied to verify which qualitative variables are associated with p16INK4a and hrHPV and the Wilcoxon nonparametric test for independent variables was applied to verify the relation between quantitative variables and p16INK4a and hrHPV. A multivariate regression analysis of survival data was based on the Cox proportional hazards model using PHREG procedure. The characteristics with reduced sample size as staging (pT4) were not included in the model. The Student's t-test was used to compare 5mC and 5hmC marks and HPV infection. All statistical analyses were performed using SAS® 9.3 software (SAS Institute Inc, University of North Carolina, North Carolina), and $P < 0.05$ was considered significant. The Kaplan–Meier curves were predicted by log-rank (Mantel-Cox) test performed in GraphPad Prism version 8.4.2. For survival curves, the sample size evaluated to time to death is presented in Table 1.

2.2.6. Supplementary Information

The online version contains supplementary material available at https:// doi.org/ 10.1186/ s13148-022-01360-1.

2.3. Results

2.3.1. Clinicopathological characteristics and HPV infection

The participants' age, HPV infection, pathological classification, and treatments of penile SCC are resented in Table 1. Participants' mean age was 63.8 (± 15.86) years old, and the incidence of SCC was higher in men over 60 years old (59.2%; 133/224), even though the manifestation of the disease was also detected before the age of 60 (33.2%; 74/224) and 40 (7.6%; 17/224). The frequency of HPV infection was 22.3% when evaluated with p16INK4a immunodepression and reached 53.2% when tested by hrHPV hybridization. Low-risk HPV infection was not identified. Tumor staging based on the primary tumor was mainly corpus spongiosum invasion (39.3%, pT2) followed by the first stages including carcinoma in situ (pTis), noninvasive carcinoma (pTa), subepithelial invasion without lymphovascular invasion (pT1a) and with lymphovascular invasion (pT1b) (30.1%).

Table 1: Clinical and pathological aspects. staging and treatment of penile squamous cell carcinoma patients.

	N (%)
Age (years)	
Mean (range)	63.8 years (18-103)
< 40	17/224 (7.6)
40 – 60	74/224 (33.2)
> 60	133/224 (59.2)
p16^{INK4a}	
No	174/224 (77.7)
Yes	50/224 (22.3)
hrHPV	
No	88/188 (46.8)
Yes	100/188 (53.2)
Staging	
pTis + pTa + pT1(a.b)	66/219 (30.1)
pT2	86/219 (39.3)
pT3	62/219 (28.3)
pT4	5/219 (2.3)
Sistemic metastasis	
No	157/188 (83.5)
Yes	31/188 (16.5)
Lymph node metastasis	
No	124/207 (59.9)

Yes	83/207 (40.1)
Locoregional recurrence	
No	139/188 (73.9)
Yes	49/188 (26.1)
PLI	
Mild and Moderate	130/217 (59.9)
Intense	87/217 (40.1)
PPI	
Mild and Moderate	205/217 (94.5)
Intense	12/217 (5.5)
ILI	
Mild and Moderate	215/217 (99.1)
Intense	2/217 (0.9)
IPI	
Mild and Moderate	127/217 (58.5)
Intense	90/217 (41.5)
Surgical procedure	
Partial amputation	164/224 (73.2)
Total amputation	33/224 (14.7)
Others	27/224 (12.1)
Chemotherapy	
No	144/194 (74.2)
Yes	50/194 (25.8)
Radiotherapy	
No	165/192 (85.9)
Yes	27/192 (14.1)

pTis: carcinoma in situ; pTa: noninvasive carcinoma; pT1a: subepithelial invasion without lymphovascular invasion, perineural invasion or grade 3; pT1b: subepithelial invasion with lymphovascular invasion, perineural invasion or grade 3; pT2: invasion of corpus spongiosum; pT3: invasion of corpus cavernosum; pT4: invasion of adjacent structures including scrotum, prostate and pubic bone; PLI: peritumoral lymphocyte infiltrate; PPI: peritumoral polymorphonuclear infiltrate; ILI: intratumoral lymphocyte infiltrate; IPI: intratumoral polymorphonuclear infiltrate.

Systemic metastasis was observed in 16.5% of participants diagnosed with penile SCC, while lymph node metastasis was presented in 40.1% and locoregional recurrence in 26.1%. Intense peritumoral lymphocyte infiltrate (PLI) and intratumoral polymorphonuclear infiltrate (IPI) reached 40.1% and 41.5% of penile SCC, respectively. The main therapeutic strategies were partial amputation 73.2% and total amputation 14.7%, followed by other associated strategies

(12.1%) such as prostatectomy, emasculation, and exercise injury. Chemotherapy was the treatment option for 25.8% of men, which consisted of cisplatin associated with 5-Fluorouracil or cisplatin associated with taxol or paclitaxel, as a palliative or neoadjuvant therapy before and after lymphadenectomy. Radiotherapy was used only as a palliative alternative for pain in cases of metastasis (14.1%) (Table 1). Despite the differences in HPV infection diagnosis using p16INK4a or hrHPV, a positive correlation was observed between these markers (P = 0.003, Table 2). Radiotherapy was associated with p16INK4a positive cases (P = 0.0136). All other clinicopathological variables were not associated with HPV markers p16INK4a and hrHPV.

Table 2. Clinical-pathological aspects, staging and therapy of penile SCC patients and $p16^{INK4a}$ and hrHPV correlation.

	$p16^{INK4a}$ *			**hrHPV**		
	Negative (%)	Positive (%)	*p*-value	Negative (%)	Positive (%)	*p*-value
hrHPV						
Negative	76 (52.8)	12 (27.3)	***0.003***	-	-	
Positive	68 (47.2)	32 (72.7)				
Staging						
pTis + pTa + pT1(a.b)	50 (29.4)	16 (32.6)	0.7138	25 (29.1)	26 (26.5)	0.4003
pT2	69 (40.6)	17 (34.7)		38 (44.2)	35 (35.7)	
pT3	48 (28.3)	14 (28.6)		22 (25.6)	34 (34.7)	
pT4	3 (1.7)	2 (4.1)		1 (1.1)	3 (3.1)	
Lymph Node Metastasis						
Negative	101 (62.3)	23 (51.1)	0.1737	55 (66.3)	49 (52.1)	0.0566
Positive	61 (37.7)	22 (48.9)		28 (33.7)	45 (47.9)	
Locoregional Recurrence						
Negative	106 (72.6)	33 (78.6)	0.4374	57 (76)	60 (69.8)	0.3761
Positive	40 (27.4)	9 (21.4)		18 (24)	26 (30.22)	
Metastasis						
Negative	123 (84.2)	34 (80.9)	0.6122	62 (82.7)	72 (83.7)	0.8582
Positive	23 (15.8)	8 (19.1)		13 (17.3)	14 (16.3)	
PLI						
Mild and Moderate	99 (58.6)	31 (64.6)	0.4539	49 (58.3)	58 (59.8)	0.842
Intense	70 (41.4)	17 (35.4)		35 (41.7)	39 (40.2)	

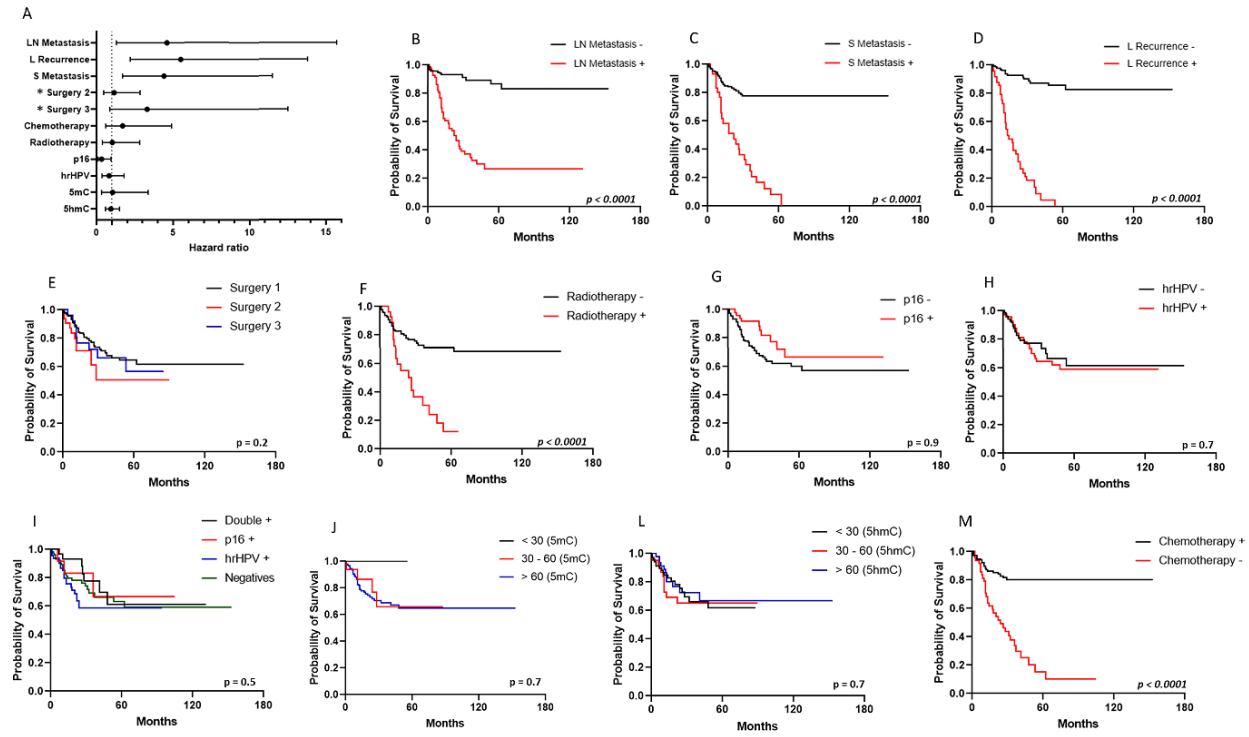
PPI						
Mild and Moderate	157 (92.9)	48 (100)	0.0575	82 (97.6)	90 (92.8)	0.1356
Intense	12 (7.1)	0 (0)		2 (2.4)	7 (7.2)	
ILI						
Mild and Moderate	167 (98.8)	48 (100)	0.4489	83 (98.8)	96 (99)	0.9184
Intense	2 (1.2)	0 (0)		1 (1.2)	1 (1)	
IPI						
Mild and Moderate	99 (58.6)	28 (58.3)	0.9756	46 (54.8)	56 (57.7)	0.6878
Intense	70 (41.4)	20 (41.7)		38 (45.2)	41 (42.3)	
Surgical procedure						
Partial amputation	127 (73)	37 (74)	0.1441	64 (72.7)	75 (75)	0.5434
Total amputation	29 (16.7)	4 (8)		16 (18.2)	13 (13)	
Others	18 (10.3)	9 (18)		8 (9.1)	12 (12)	
Chemotherapy						
Negative	115 (76.7)	29 (65.9)	0.1514	58 (76.3)	65 (72.2)	0.5486
Positive	35 (23.3)	15 (34.1)		18 (23.7)	25 (27.8)	
Radiotherapy						
Negative	133 (89.3)	32 (74.4)	***0.0136***	65 (85.5)	76 (85.4)	0.9807
Positive	16 (10.7)	11 (25.6)		11 (14.5)	13 (14.6)	
5mC						
< 30%	2 (1.5)	0 (0)	0.528	1 (1.5)	0 (0)	0.4534
> 30% - < 60%	11 (8.5)	5 (13.2)		6 (9)	10 (12)	
> 60%	116 (90)	33 (86.8)		60 (89.5)	73 (88)	
5hmC						
0%	18 (14.2)	8 (21.6)	0.1236	14 (21.2)	11 (13.5)	0.5893
< 30%	34 (26.7)	11 (29.7)		17 (25.8)	26 (31.7)	
> 30% - < 60%	29 (22.8)	12 (32.5)		17 (25.8)	20 (24.4)	
> 60%	46 (36.2)	6 (16.2)		18 (27.2)	25 (30.4)	

*Each column adds up to 100%. pTis: carcinoma in situ; pTa: noninvasive carcinoma; pT1a: subepithelial invasion without lymphovascular invasion, perineural invasion or grade 3; pT1b: subepithelial invasion with lymphovascular invasion, perineural invasion or grade 3; pT2: invasion of corpus spongiosum; pT3: invasion of corpus cavernosum; pT4: invasion of adjacent structures including scrotum, prostate and pubic bon PLI: Peritumoral Lymphocytic Infiltrate; PPI: Peritumoral Polymorphonuclear Infiltrate; ILI: Intratumoral Lymphocyte Infiltrate; IPI: Intratumoral Polymorphonuclear Infiltrate; 5mC: 5-methylcytosine; 5hmC: 5-hydroxymethylcitosine.

The survival rate was measured by a multivariate-adjusted Cox hazards regression model (Fig. 1A, Additional file 1: Supplementary Table 1). Locoregional recurrence (Hazard Ratio, HR = 5.51, 95% CI 2.20–13.84; P < 0.001), systemic metastasis (HR = 4.40, 95% CI 1.68– 11.49, P = 0.003) and lymph node metastasis (HR = 4.65, 95% CI 1.3–15.7, P = 0.013) decreased the

survival rate in penile SCC. On the other hand, p16INK4a expression seems to be a protective factor for death (HR = 0.34, 95% CI 0.531–2.05; P = 0.04) and hrHPV (HR = 0.82, 95% CI 0.36–1.86; P = 0.637) did not affect survival even with a HR < 1. The remaining parameters evaluated did not affect the survival rate. However, on the nonadjusted Kaplan–Meier curves metastasis, recurrence, chemotherapy, radiotherapy (Fig. 1B–D, F, M) and staging (Additional file 1: Supplementary Fig. 3E) seems to decrease the time to death (P < 0.05).

Figure 1. Survival analyses. Multivariate-adjusted Cox hazards regression model (A) and Kaplan–Meier survival curves: B lymph node metastasis; C systemic metastasis; D locoregional recurrence; E surgery; F radiotherapy; G p16 INK4a; H hrHPV; I double positive (+), p16INK4a (+), hrHPV (+) and negative (−); J 5-mehtylcitosine, 5mC; L 5-hydroximetilcitosine, 5hmC and M chemotherapy. Surgery 1, partial amputation; Surgery 2, total amputation; Surgery 3, others. *Adjusted with surgery 1

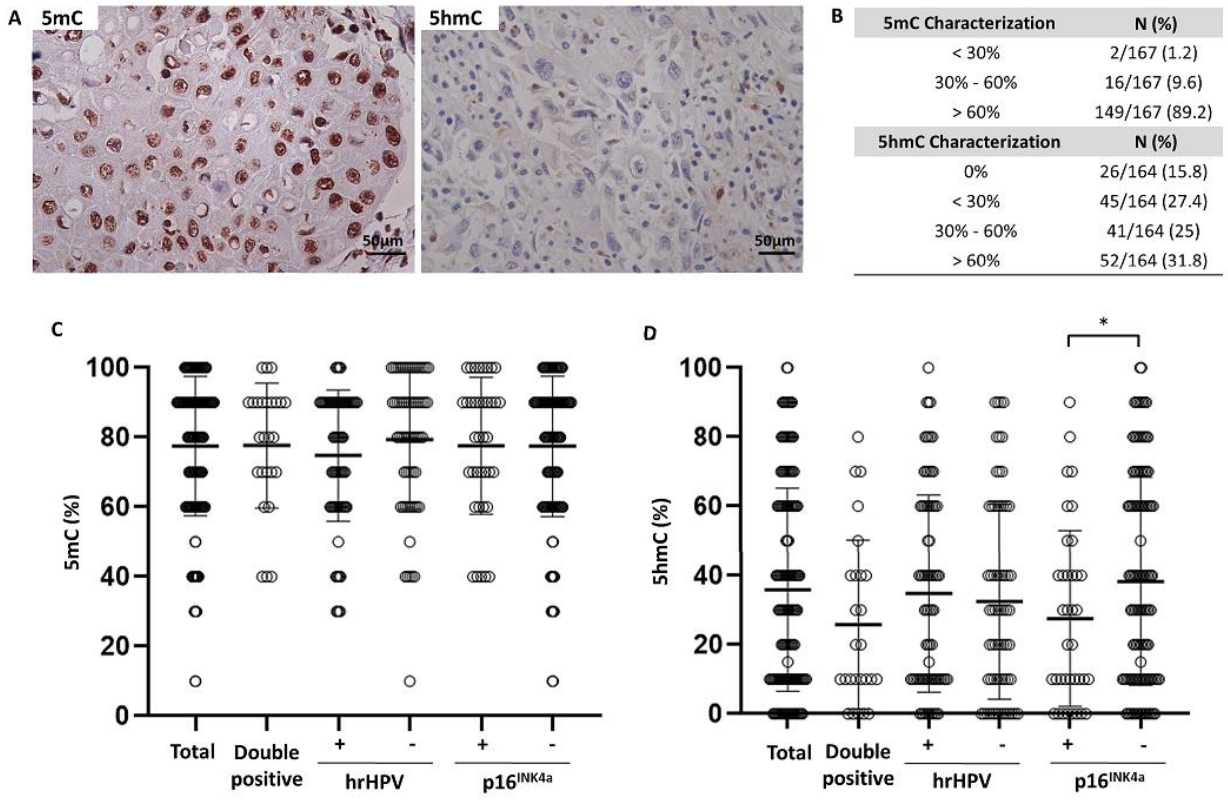


2.3.2. 5mC and 5hmC marks in penile SCC

Increased global 5mC (> 60%) mark was observed in 89.2% of penile SCC cases, regardless of HPV infection (Fig. 2A , B). The average of global 5hmC levels varied from lower (< 30%), intermediate (30–60%), and increased (> 60%) hydroxymethylation levels (Fig. 2B).

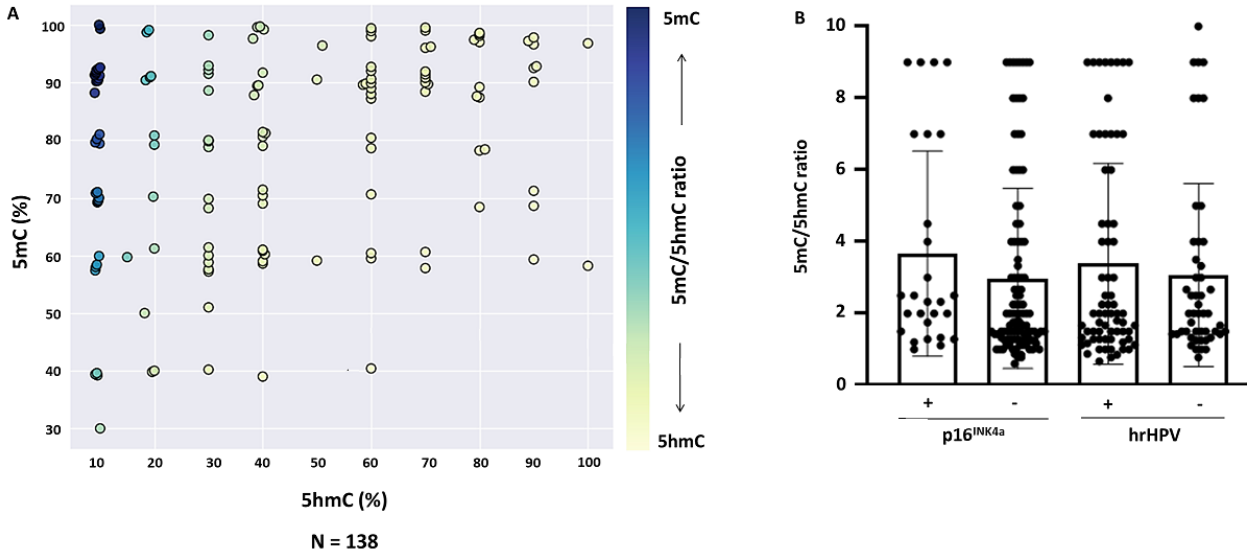
The total cases of penile cancer evaluated for the 5mC mark (Fig. 2C), corresponding to 77.5% ± 19.9, have a higher percentage value than the 5hmC mark (35.9% ± 29.3, Fig. 2D). No association was observed between global DNA methylation marks and HPV infection (Table 2). Also, no differences were observed in the distribution of the 5mC mark for both hrHPV + and p16INK4a + (Fig. 2C), but an increase in the 5hmC mark for p16INK4a negative (P = 0.024) was observed (Fig. 2D). 5mC in double positive HPV was similar to hrHPV and p16INK4a (Fig. 2C) and 5hmC double positive HPV was similar to p16INK4a + (Fig. 2D). The 5mC/5hmC ratio represents the proportion between the marks for each sample (Fig. 3A). The majority of penile SCC showed 5mC/5hmC ratio greater or equal to 1 (94.2%), which was not associated with viral infection (Fig. 3B). Despite increased 5mC mark, methylation level was not associated with survival rate (HR = 1.05, 95% CI 0.33–3.38; P = 0.92), as well as 5hmC (HR = 0.95, 95% CI 0.60–1.50; P = 0.82).

Figure 2. The pattern of 5mC and 5hmC epigenetic marks on penile SCC. A 5mC and 5hmC marks staining in penile squamous cell carcinoma. B Stratification of 5mC and 5hmC mark levels. Distribution of 5mC C and 5hmC D levels in a total number of samples and separated by hrHPV and p16INK4a groups. Data are presented as mean and standard deviation. *P = 0.024



5mC Characterization	N (%)
< 30%	2/167 (1.2)
30% - 60%	16/167 (9.6)
> 60%	149/167 (89.2)
5hmC Characterization	**N (%)**
0%	26/164 (15.8)
< 30%	45/164 (27.4)
30% - 60%	41/164 (25)
> 60%	52/164 (31.8)

Figure 3. 5mC/5hmC ratio distribution. A 5mC/5hmC ratio dispersion by 5mC and 5hmC percentage, y and x-axis, respectively. The higher the color tone, the higher the ratio value. B 5mC/5hmC ratio distribution values by hrHPV and p16INK4a groups



2.4. Discussion

Penile cancer is one of the most aggressive male malignancies, although it is one of the easiest to prevent, as the main etiologic causes are lifestyle conditions and viral infection [38]. We observed a high incidence of HPV infection considering hrHPV detection (53.2%) while the incidence reduces using p16INK4a (22.3%). Despite the elevated incidence, HPV infection was not related to systemic or lymph node metastasis and locoregional recurrence and, therefore, does not influence the survival rate. Long-term changes in DNA methylation are characteristic of environmentally induced carcinogenesis as penile cancer [13]. Increased 5mC was observed in penile SCC, which seems to be a stable epigenetic marker, while the 5hmC was lower and more variable. The HPV infection was not related to the overall 5mC/5hmC ratio. Interestingly, 5mC may be a risk factor for poor prognosis and survival rate.

The incidence of penile cancer directly impacts public health, as the specialized medical care, psychosocial impact and the risk of cervical cancer in their female partners increase the cost of treatment [66]. Despite significant reductions in hospital admissions in Brazil over the past two decades, the incidence of penile carcinoma is still high and quite unequal across the five regions of the country, whereby the Northwest stands out with the highest incidence and the lowest per capita income in the country [39]. Socioeconomic status and low education levels reflect on penile SCC staging at diagnosis [40, 41] and, a recent study conducted in the states of Maranhão and Rio de Janeiro, Brazil, report that 69.9% of patients were diagnosed with pT2 and pT3, and 87.9% of them underwent surgical removal of the organ. A contrasting scenario is observed in developed countries, such as the USA, where 54.1% of penile SCC staging are pT1 and there were 75.8% of amputated cases [42]. Penile SCC was observed in young men, ranging from 18 to 103 years old, different from other cities in Brazil as the age varied between 23 to 98 years [13, 41], and even more discrepant in developed countries, where the disease is rare before the third decade of life [43, 44].

Despite the correlation between hrHPV and p16INK4a, the incidence of HPV infection was twofold higher using hrHPV than p16INK4a, and only 17% of all cases were double positive for both markers. Considering that the tumor suppressor protein p16INK4a is upregulated by the HPV oncogene [45, 46], HPV DNA detection may be a primary event that is followed by increased expression of this protein. Also, negative testing for p16INK4a expression may be linked to gene expression loss, caused by promoter methylation events and loss of heterozygosity

[45, 46], implying a false negative result [49]. A recent systematic review and meta-analysis showed 49% (95% CI 43.1–54.9) of HPV prevalence using qPCR or hybridization analyses (n = 3772 cases in 47 studies) and 42.1% (95% CI 36.4–47.8) using p16INK4a (n = 1296 cases in 23 studies) in SCC [47]. Both techniques are widely used for HPV detection, however, the values may show discrepancies. Therefore, double positive HPV DNA and p16INK4a must be considered for some types of cancer, such as head and neck SCC [74].

Although previously mentioned as a risk factor for penile SCC [10, 23], our present analysis indicates that hrHPV and p16INK4a were not related to disease staging, metastasis, or infiltration of the immune system cells. On the other hand, p16INK4a seems to be a protective factor for death, as well as hrHPV (HR < 1), although it was not significant, an increase in sample size would answer this question. In addition, positive cases for p16INK4a seem to be associated with radiotherapy. Although previous studies associate p16INK4a expression and radiotherapy [75, 76], this might be a random correlation. Increased p16INK4a was correlated with a good prognosis, as lack of nodal metastasis in head and neck SCC [67, 77] and cervical cancer [78]. In penile SCC, Martins and collaborators showed that p16INK4a was not associated with prognosis parameters or survival rate [46].

The presence of lymph node metastasis in the inguinal and iliac regions increased the risk of death, which is the most common metastatic event [50] and the main prognostic marker [51]. Systemic metastases are rarely reported [41, 50] and locoregional recurrences are also less frequent [52], but these two parameters were associated with an increased risk of death by 4.3 and 5.5-fold, respectively. Penectomy is still a widely used treatment for extensive lesions or tumors involving the base and the bulbar urethral part of the penis [13, 41, 53], but our results indicate that this type of surgery was not linked to survival rate. However, morbidity and psychological traumas are detrimental to the quality of life [53]. The absence of minimally invasive therapies and predictive characteristics of penile cancer is an overwhelming burden for medical practice and scientists, who are challenged to search for molecular targets of the disease [54].

Epigenetic mechanisms, like DNA methylation, present great plasticity as the epigenome can be reprogrammed through environmental factors [68, 69]. Aberrant epigenetic reprogramming changes cell landscape given the malignant phenotype, as increased proliferation, resistance to apoptosis, invasion, and metastasis, triggering tumorigenesis [32]. Several studies

have been dedicated to the characterization of epimutations in penile SCC [19, 70]. In this study, an increased level of the global 5mC mark was observed regardless of HPV infection. Genomic instability and mutations are important molecular alterations that trigger carcinogenesis. Increased global 5mC (hypermethylation) contributes to genomic stability in tumor progression and, therefore, may be related to tumor invasion and chemotherapy resistance [58, 73]. Unlike our findings, reduced global DNA methylation was reported in other types of the tumors such as colorectal [55] and prostate [56]. As a repressive epigenetic marker, it changes gene expression of specific targets and large chromosomal regions, as repetitive DNA elements [58].

The increased 5mC marker was accompanied by a reduced level of the 5hmc mark in the majority of penile SCC, which was associated with a p16INK4a negative diagnosis. Decreased level of 5hmC mark was observed in head and neck cancers positive for HPV, and virus presence might influence the oxidation process from 5mC to 5hmC in genes of cell junction pathways [65]. 5hmC is an epigenetic marker for active DNA demethylation [57], which depends on TET proteins that mediate this process [71]. Aberrant active DNA demethylation was also observed in non-small cell lung cancer [72]. Furthermore, we observed a complete absence of 5hmC staining in several SCC samples, similar to previous reports about global loss of the 5hmC mark in large subsets of oral squamous cell carcinoma [59] and cervical cancer [60].

Given that 5mC is a protective marker against DNA damage and the hydroxymethylation of 5mC is an active demethylation process, the balance between 5mC and 5hmC marks is directly linked to genomic stability, contributing to cancer development and progression [34]. We observed that a higher 5mC may be a risk factor for death in penile SCC, irrespective of HPV infection, and an increase in sample size may confirm these findings. The 5mC/5hmC mark is an important prognostic and predictive parameter, and we have recently demonstrated the 5mC/5hmC imbalance related to hypercellular bone marrow, dyserythropoiesis and cases of high-risk myelodysplastic syndromes [61]. Moreover, the clinical relevance of 5mC and 5hmC levels was reported as a critical marker for prognosis in colorectal cancer, as increased 5mC was associated with lymph node metastasis [55]. Global epigenetic marks should be deciphered as they indicate characteristics of advanced tumor staging [60] and tumor subtypes biomarker [62], and targeted therapy using DNMT inhibitors [63, 64].

Despite the important findings regarding the HPV incidence and the association with the 5mC/5hmC mark with penile SCC prognosis, other studies should address the epidemiological

characteristics of penile cancer and epigenetic reprogramming through lifestyle changes. Our study was limited by the lack of information regarding the participant's background, such as sexual behavior, sociodemographic profile, HPV vaccination, and the identification of other related histological subtypes and tumor topography. Even though we evaluated hrHPV and lrHPV, there is a diversity of viral genotypes that have not been identified individually and should give better knowledge regarding the infection and disease prognosis, as well as the time of exposure to infection that was not tracked. Although partial and total penectomy represents better survival compared to even more invasive ones, these techniques have drastic implications for the individual's personal life, which calls for less invasive therapies. The epidemiologic data, for staging and surgical excision, suggest that medical care is just sought late raising costs in specialized treatment. The elevated incidence of HPV highlights the importance of policies to encourage vaccination to control viral infection in the male population.

2.5. Conclusions

We reported an incidence of 53.2% of hrHPV infection in men with penile SCC in the State of Ceará, Northeast Brazil, a region with the lowest per capita income in the country. Despite the increased incidence, HPV infection was not associated with poor prognosis, such as systemic or lymph node metastasis, locoregional recurrence, but it seems to increase the survival rate. The current research also indicates, for the first time, that increased global DNA 5mC and reduced 5hmC marks are characteristics of penile SCC. Despite no statistical difference, increased 5mC may contribute to poor prognosis as the HR was 1.06 in SCC. Although viral infection contributes to the loss of aberrant DNA reprogramming, the 5mC/5hmC ratio was not related to HPV infection, and 5hmC levels were increased in p16INK4a negative samples. This information should be further explored as this data may predict potential clinical relevance for penile cancer prognosis and may suggest new treatment strategies, as hypomethylating agents for epigenetic targeted therapies (epidrugs). The poor prognosis of penile cancer and its relationship with environmentally induced changes reinforces the benefits of primary health care and the elevated incidence of hrHPV highlights the importance of vaccination and continued educational strategies for both prevention and treatment of malignant lesions.

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3. CAPÍTULO III

HYPOMETHYLATION AT H19DMR IN PENILE SQUAMOUS CELL CARCINOMA IS NOT RELATED TO HPV INFECTION

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Hypomethylation at H19DMR in penile squamous cell carcinoma is not related to HPV infection

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Abstract

Penile squamous cell carcinoma (SCC) is a rare and aggressive tumor mainly related to lifestyle behavior and human papillomavirus (HPV) infection. Environmentally induced loss of imprinting (LOI) at the H19 differentially methylated region (H19DMR) is associated with many cancers in the early events of tumorigenesis and may be involved in the pathogenesis of penile cancer. We sought to evaluate the DNA methylation pattern at H19DMR and its association with HPV infection in men with penile SCC by bisulfite sequencing (bis-seq). We observed an average methylation of 32.2%±11.6% at the H19DMR of penile SCC and did not observe an association between the p16INK4a+ ($p = 0.59$) and high-risk HPV+ ($p = 0.338$) markers with methylation level. The average methylation did not change according to HPV positive for p16INK4a+ or hrHPV+ (35.4%±10%) and negative for both markers (32.4%±10.1%) groups. As the region analyzed has a binding site for the CTCF protein, the hypomethylation at the surrounding CpG sites might alter its insulator function. In addition, there was a positive correlation between intense polymorphonuclear cell infiltration and hypomethylation at H19DMR ($p = 0.035$). Here, we report that hypomethylation at H19DMR in penile SCC might contribute to tumor progression and aggressiveness regardless of HPV infection.

Keywords: Penile squamous cell carcinoma; Human Papillomavirus; H19DMR; Hypomethylation; Genomic Imprinting; Single Nucleotide Polymorphism.

3.1. Introduction

Penile squamous cell carcinoma (SCC) is a rare and aggressive neoplasia with increasing incidence in developing countries. Lifestyle conditions, such as poor hygiene, promiscuous sexual behavior, and the presence of phimosis are the main risk factors associated with penile cancer [1]. Human papillomavirus (HPV) infection is often related to penile SCC with a variable incidence in the male population from 11% to 87% which is slightly related to the diagnostic method [2]. We recently reported that the incidence of HPV infection in the penile SCC population was 53.2% using a hybridization assay to capture high-risk HPV (hrHPV) and 22.3% using the p16INK4a marker, which does not affect the prognosis and survival rate [3].

Owing to the sporadic etiology of penile cancer, with an important environmental contribution, epigenetic alterations in gene expression control may trigger tumor development and tighter with genetic mutations propagate carcinogenesis and contribute to its aggressiveness [4]. The biallelic expression of imprinted genes or loss of imprinting (LOI) is one of the most affected epigenetic processes that appear during early tumor development [5]. The imprinting control regions (ICRs) are differentially methylated regions (DMRs) in a parent-of-origin manner leading to a monoallelic expression of the clustered imprinted genes [6]. Since imprinted genes regulate cell differentiation [7], metabolism [8], proliferation [9], and other biological processes, epigenetic alterations at the ICRs change the cell landscape and trigger carcinogenesis, being reported in many cancers such as lung [10], colorectal [11], glioblastoma [12], and acute myeloid leukemia [13].

One of the well-recognized ICRs involved in carcinogenic transformation is the H19DMR (ICR1) [14], mapped to human chromosome 11p15.5 region that controls the monoallelic expression of the clustered H19 and IGF2 genes [15]. This region is localized at 2 kilobases upstream of the H19, a long non-coding RNA (lncRNA) with a controversial role during carcinogenesis, acting either as a tumor suppressor or oncogene [16,17]. The H19DMR contains multiple CTCF-binding domains (CCCTC-binding factor) with insulator activity and represents an enhancer competition model for gene regulation [18]. The H19DMR is unmethylated at the maternal allele allowing the CTCF protein to bind to its DNA domains and H19 activation through enhancers located upstream of this gene. In the paternal allele, methylation promotes conformational changes near to H19 promoter region, which is silenced, while IFG2 is transcriptionally activated by the shared enhancers [19].

In addition to its complex structural region, the H19DMR has two non-CpG single nucleotide polymorphisms (SNPs), the rs2107425:C>T and rs2071094:G>T, that are associated with parental allele-specific DNA methylation (ASM) status, thereby altering gene expression and chromatin remodeling [20, 21]. However, ASM can be altered through environmental exposure and a high confluence of ASM regions has been reported, possibly due to a genetic variation at a regulatory SNP locus [22]. The genomic variation in DMRs can be valuable as prognostic markers and may contribute to changes in DNA methylation pattern during carcinogenesis [22]. Analysis based on DNA methylation and SNPs on conjoint has guided the stratification into high and low-risk groups for breast cancer, supporting the relevant prognostic value of genomic and epigenomic combined analysis for cancer biomarkers [23].

Considering the importance of the H19DMR in growth-related pathways and cell differentiation [24], LOI or biallelic expression of imprinted genes has been associated with carcinogenic transformation [25], tumor progression [26], metastasis [26], and resistance to treatment [27]. We previously reported an increase in 5-methylcytosine (5mC) and a decrease in 5-hydroxymethylcytosine (5hmC), markers of global methylation and demethylation respectively, were previously observed in our penile SCC cohort. However, HPV infection seems not to affect 5mC or 5hmC epigenetic markers [3]. These global changes may affect the methylation pattern at ICRs in penile SCC. Thus, we sought to evaluate the DNA methylation at H19DMR and its association with clinical aspects of penile SCC and HPV infection.

3.2. Materials and methods

3.2.1. Study design and data collection

This is a retrospective study that included 30 penile SCC samples from patients who underwent partial or total penectomy, enlarged prostatectomy, or emasculation due to penile SCC, without any prior history of chemotherapy or radiotherapy and with detailed clinicopathological and follow-up data available, from 2015 to 2018 at Hospital Haroldo Juaçaba, Ceará, Brazil. The samples used came from the Hospital tumor tissue sample bank, stored at -80 °C. The study was approved by the ethics committee of the Federal University of Ceará and Haroldo Juaçaba Hospital, according to process number 2.427.846. Data from the anatomopathological reports and medical records were collected for epidemiological evaluation. Pathological staging was performed according to the eighth edition of the American Joint Committee on Cancer (AJCC).

3.2.2. p16INK4a expression and high-risk HPV identification assays

The immunohistochemistry (IHC) assay was performed to $p16^{INK4a}$ protein expression using the anti-p16 antibody clone E6H4 (Roche CINtec® Histology) according to the manufacturer's instructions. The $p16^{INK4a}$ expression must be ≥ 75% to be considered positive, with continuous and complete cytoplasmic and nuclear staining [23]. Chromogenic *in situ* hybridization (CISH) was used to identify high-risk HPV (hrHPV) using the Ventana Inform HPV III Family 16 Probe diagnostic kit (Ventana Medical Systems, Tucson, AZ) (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66). High-grade cervical intraepithelial neoplasia was used as a positive control for hrHPV. Skeletal striated muscle was used as a negative control for both assays. All the experiments were analyzed by a blinded observer.

3.2.3. Isolation of genomic DNA

Genomic DNA from penile SCC samples was isolated using the salting-out protocol. Briefly, samples were lysed (80 mL proteinase-K buffer [0.375 M NaCl, 0.12 M EDTA, pH 8.0], 8 mL proteinase K [25 mg/mL], 10 mL 20% sodium dodecyl sulfate, and 280 mL H_2O) overnight at 55 °C with shaking. The samples were cooled down and 120 mL of 5M NaCl was added. The samples were then shaken vigorously for 8 seconds and centrifuged (13,000 g for 5 minutes at 4 °C). Next, 400 mL of the supernatant was mixed with 1 mL of 99% cold ethyl alcohol, inverted a few times, and kept at 20 °C overnight. The precipitated DNA was washed twice with 70% cold ethyl alcohol and centrifuged (13,000 g for 5 minutes at 4 °C). The concentration and quality of the isolated DNA were evaluated using a spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific, USA). The genomic DNA was stored at -20 °C until they were used.

3.2.4. Bisulfite conversion and PCR amplification

Genomic DNA was used for DNA bisulfite conversion using the EZ DNA Methylation-Lightning kit (Zymo Research, USA) according to the manufacturer's instructions. The amplification of the H19DMR was performed using Platinum Taq DNA polymerase (Invitrogen, USA) with primers containing Nextera (Illumina, USA) adapters (Supplementary Table 1) as previously described elsewhere [24]. The PCR conditions to amplify the H19DMR were 95 °C for 5 minutes, 50 cycles of denaturation at 94 °C for 45 seconds, annealing at 59 °C for 45 seconds, and extension at 72 °C for 45 seconds, and a final extension step at 72 °C for 10 minutes. The amplification of H19DMR was confirmed by electrophoresis on 1.5% agarose gel.

Supplemental Table 1. Primers sequences, genomic position, and size of the analyzed sequence.

Sequences in black are the adaptors for genomic sequencing. BS, Bisulfite sequencing; bp, base pairs.

*Chromosome position according to GenBank genome browser (https://www.ncbi.nlm.nih.gov/genbank/) and genome reference GRCh38/hg38.

Primers BS	**Sequence**	**Chromosome Position***	**Length (bp)**
H19DMR	Fw 5' - **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TATGGGTATTTTTGGAGGTTTTTT - 3' Fw 5' - **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**AATCCCAAACCATAACACTAAAAC - 3'	2000040-1999780	330

3.2.5. Next-generation sequencing

Targeted bisulfite sequencing was performed using the MiSeq platform (Illumina, San Diego, CA, USA), covering 23 CpG sites at H19DMR (chr11:1,999,757- 2,000,060). PCR products, containing the adapters, were barcoded using the Illumina Nextera XT library preparation kit (Illumina, USA) and the sequencing was performed using the 600bp V3 reagents kit according to the manufacturer's instructions. The FASTQ files for individual samples were generated using Illumina's pipeline (bcl2fastq2-v2-20). The adapter and indexes were removed from the sequence using Trimmomatic v0.38.1 [25]. The paired read sequences were merged using the default settings of FLASH v1.2.11.4 [26] and aligned to the bisulfite converted genome using Bismark v0.18.2 with the following settings: –ambig_bam [27], which was also used to count the reads with different methylation patterns. Reads were considered methylated with ≥ 61% methylated CpG sites, partially methylated between 31% - 60% methylated CpG sites, and unmethylated with ≤ 30% methylated CpG sites. Visualization of the methylated CpGs in the regions of interest was performed based on Tabsat v1.0.2 [28]. The two single nucleotide polymorphism (SNP) genotyping, rs2107425 (C>T; Chr11:1999845) and rs2071094 (G>T; Chr11:1999934), were visualized with Integrative Genomics Viewer (IGV). For methylation

analyses, positive (+) samples for either $p16^{INK4a}$+ or hrHPV+ were mentioned as HPV+. Negative samples (-) for both marks were considered HPV-.

3.2.6. CTCF consensus binding sites search and in silico analysis of gene expression

CTCF binding sites in H19DMR were detected using the scan settings of the CTCFBSDB 2.0 database (https://insulatordb.uthsc.edu/) [29], a comprehensive collection of experimentally determined and computationally predicted CTCF binding sites from the literature. The database uses sex position weight matrices (PWM) to report the single best hit in the query sequence. Usually, a short sequence with a PWM score >3.0 is a suggestive match. Query sequence and database output result are available in Supplementary Table 2. In silico analyses of CTCF, H19, and IGF2 expression were performed in squamous cell carcinoma as per available data of RNA-seq from the OncoDB database (https://oncodb.org/) [63]. Expression of three tumor types and their respective non-tumor tissues were accessed (Supplementary Table 3): head and neck squamous cell carcinoma (HNSC) (520 tumor samples and 44 non-tumor samples), cervical squamous cell carcinoma (CESC) (304 tumor samples and 22 non-tumor samples), and lung squamous cell carcinoma (LUSC) (503 tumor samples and 51 non-tumor samples). Expression data were normalized through transcripts per million (TPM).

Supplementary Table 2. CTCF binding site search result within the H19/IGF2 DMR sequenced region using the CTCFBSDB 2.0 database.

Motif PWM	Motif Sequence	Input Sequence Name	Motif Start Location	Motif Length	Motif Orientation	Score
EMBL_M1	TGCCGCCGCGCGGC	IGF2/H19DMR	186	14	+	-0.717394
EMBL_M2	*GGCAGTGCA*	*IGF2/H19DMR*	*181*	*9*	-	*11.0207*
REN_20	GTGGCCGCGCGGCGGCAGTG	IGF2/H19DMR	183	20	-	-3.2606
MIT_LM2	CACCGCCTGGATGGCACGG	IGF2/H19DMR	229	19	-	-9.44131
MIT_LM7	AGATCTTCAGGTCGGGCATT	IGF2/H19DMR	31	20	-	-4.72565
MIT_LM23	AGATCTTCAGGTCGGGCATT	IGF2/H19DMR	31	20	-	-1.56947

Region with the greatest potential for interaction.

FASTA sequence used in CTCFBSDB 2.0:

>ref|NC_000011.10|:1999783-2000036 Homo sapiens chromosome 11, GRCh38.p14 Primary Assembly

GAGTGTGACCCGGGGCCACGGGGCTGTGGATAATGCCCGACCTGAAGATCTGGTGCGGCTCCCATGAGTGTCCTA
TTCCCAGATGACCCCCGTGAACCCTGCGACGCGTGGCTTGGGTGACCCGGGACGTTTCCACGGGCGAACCCCAGT

TGGGGCGGGCTCGGGCTGTGATGTGTGAGCCTGCACTGCCGCCGCGCGGCCACTTCCGATTCCACAACTACAACC
AATTCCGTGCCATCCAGGCGGTGAGACCG

3.2.7. Statistical Analysis

Student's t-test was used to compare the average number of methylated, unmethylated, and partially methylated reads between HPV positive and negative groups and in silico gene expression. Fisher's exact test was applied to verify the association of clinical variables and the SNPs rs2107425 and rs2071094 with methylation pattern at H19DMR. The Kaplan–Meier curves were predicted by the log-rank (Mantel-Cox) test and linear regression was used to correlate H19DMR methylation levels and age. Statistical analyses were carried out using GraphPad Prism 8.4.2 (Intuitive Software for Science, San Diego, California, USA). $p < 0.05$ was considered statistically significant.

3.3. Results

The clinical characteristics, pathological classification, and HPV infection of penile SCC participants are presented in Table 1. The average age of patients was 63.8 (± 18.8) years old and the most frequent primary tumor staging grades were pT2 (40%; 12/30) and pT3 (36.7%; 11/30). Lymph node metastasis was observed in 51.9% (14/27) of the participants, followed by locoregional recurrence (29.7%; 7/27) and systemic metastasis (15.4%; 4/26). Immune cell infiltration was mostly mild and moderate for peritumoral lymphocyte infiltrate (PLI) (60%; 18/30) and intratumoral polymorphonuclear infiltrate (IPI) (53.3%; 16/30). HPV infection was observed in 40% (12/30) of the participants using the p16INK4a+ marker and 53.2% (16/30) using hrHPV+ and 63.3% (19/30) were positive for at least one of the tests.

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Table 1. Clinical and pathological aspects of patients with penile squamous cell carcinoma.

	N (%)
Age (years)	

Mean (range)	63.8 years (23-94)
< 40	4/30 (13.3)
40 – 60	9/30 (30)
> 60	17/30 (56.7)
p16^{INK4a}	
No	18/30 (60)
Yes	12/30 (40)
hrHPV	
No	14/30 (46.6)
Yes	16/30 (53.2)
Staging	
pTis + pTa + pT1(a.b)	7/30 (23.3)
pT2	12/30 (40)
pT3	11/30 (36.7)
Sistemic metastasis	
No	22/26 (84.6)
Yes	4/26 (15.4)
Lymph node metastasis	
No	13/27 (48.1)
Yes	14/27 (51.9)
Locoregional recurrence	
No	19/27 (70.3)
Yes	7/27 (29.7)
PLI	
Mild and Moderate	18/30 (60)
Intense	12/30 (40)
IPI	
Mild and Moderate	16/30 (53.3)
Intense	14/30 (46.7)

pTis: carcinoma in situ; pTa: noninvasive carcinoma; pT1a: subepithelial invasion without lymphovascular invasion, perineural invasion or grade 3; pT1b: subepithelial invasion with lymphovascular invasion, perineural invasion or grade 3; pT2: invasion of corpus spongiosum; pT3: invasion of corpus cavernosum; PLI: peritumoral lymphocyte infiltrate; IPI: intratumoral polymorphonuclear infiltrate.

The average methylation was 32.2%±11.6% in all samples, however, 43.3% of penile SCC showed methylation levels lower than 30%. The mean number of unmethylated reads was higher, representing 63.3%±10.6% of all reads, and the mean number of partially methylated reads was

4.5%±5.8 (Figure 1). Methylation level at H19DMR was not associated with HPV infection using both p16INK4a + ($p = 0.59$) and hrHPV ($p = 0.338$) markers. However, reduced H19DMR methylation level was positively correlated with intense intratumoral polymorphonuclear infiltrate (IPI) ($p = 0.035$), but with no other clinical and pathological variables (Table 2). When the samples were stratified into those below and above 60 years of age, no correlation was observed between the methylation levels of 30% and 60% (Table 2). Considering all ages, a negative correlation between DNA methylation and age was observed (Supplementary Figure 1). The average distribution of methylated ($p = 0.15$), unmethylated ($p = 0.38$), and partially methylated reads ($p = 0.06$) was not different between HPV+ (p16INK4a+ or hrHPV+) and HPV- samples (Figure 2) and the methylation average did not change according to HPV positive (35.4%±10%) and negative (32.4%±10.1%) groups (Figure 3).

Table 2. Correlation between clinical data and methylation level at H19DMR.

Variables (n)	Methylation level (%)		
	0 - 30	**31 - 60**	***P*-value**
Age (23-94 years)			
≤ 60 (12/30)	7	5	0.164
>60 (18/30)	6	12	
p16^{INK4a}			
Positive (12/30)	8	10	0.590
Negative (18/30)	5	7	
hrHPV			
Positive (16/30)	5	9	0.338
Negative (14/30)	8	8	
LNM			
Positive (14/27)	6	7	0.584
Negative (13/27)	6	8	
LR			
Positive (7/26)	8	11	0.655
Negative (17/26)	3	4	
SM			
Positive (4/26)	8	14	0.187
Negative (22/26)	3	1	
PLI			
Mild/Moderate (18/30)	7	11	0.409
Intense (12/30)	6	6	
IPI			
Mild/Moderate (16/30)	4	12	***0.035***
Intense (14/30)	9	5	
Staging			
pTis + pTa + pT1(a.b)	3	4	0.652
pT2	4	8	

pT3	6	5	

P-value for Fisher Exact Probability Test. In bold and italics, $p < 0.05$. LNM: Lymph Node Metastasis; LR: Locoregional Recurrence; SM: Sistemic Metastasis; PLI: Peritumoral Lymphocytic Infiltrate; IPI: Intratumoral Polymorphonuclear Infiltrate. pTis carcinoma in situ, pTa noninvasive carcinoma, pT1a subepithelial invasion without lymphovascular invasion, perineural invasion or grade 3, pT1b subepithelial invasion with lymphovascular invasion, perineural invasion or grade 3, pT2 invasion of corpus spongiosum, pT3 invasion of corpus cavernosum.

Figure 1. Bisulfite sequencing of H19DMR in penile squamous cell carcinoma. Each row represents a sample (S) and its HPV status (positive samples were considered as $p16^{INK4a}$+ or hrHPV+). The number of reads is specified for each sample. The SNPs position are specified by the red arrow.

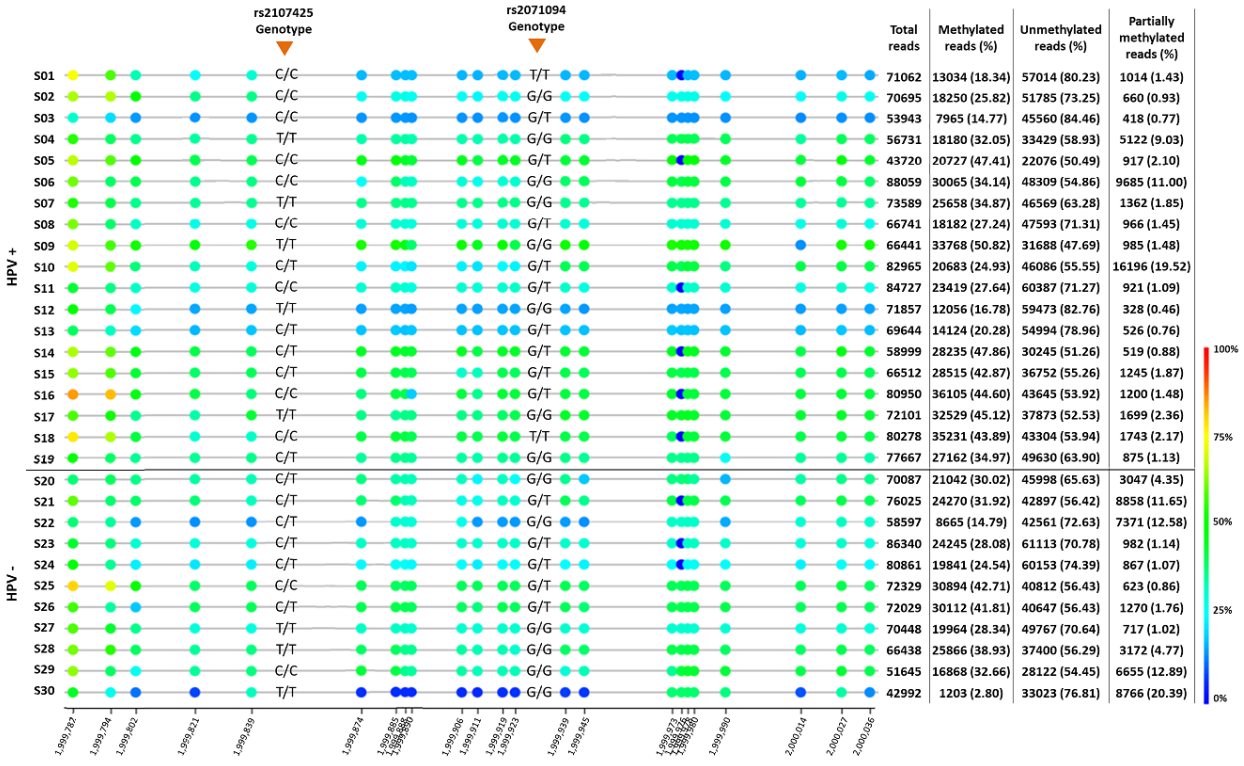


Figure 2. Average of methylated, unmethylated and partially methylated reads in HPV positive (HPV+) and negative (HPV-) of penile squamous cell carcinoma. Positive samples were considered as $p16^{INK4a}$+ or hrHPV+.

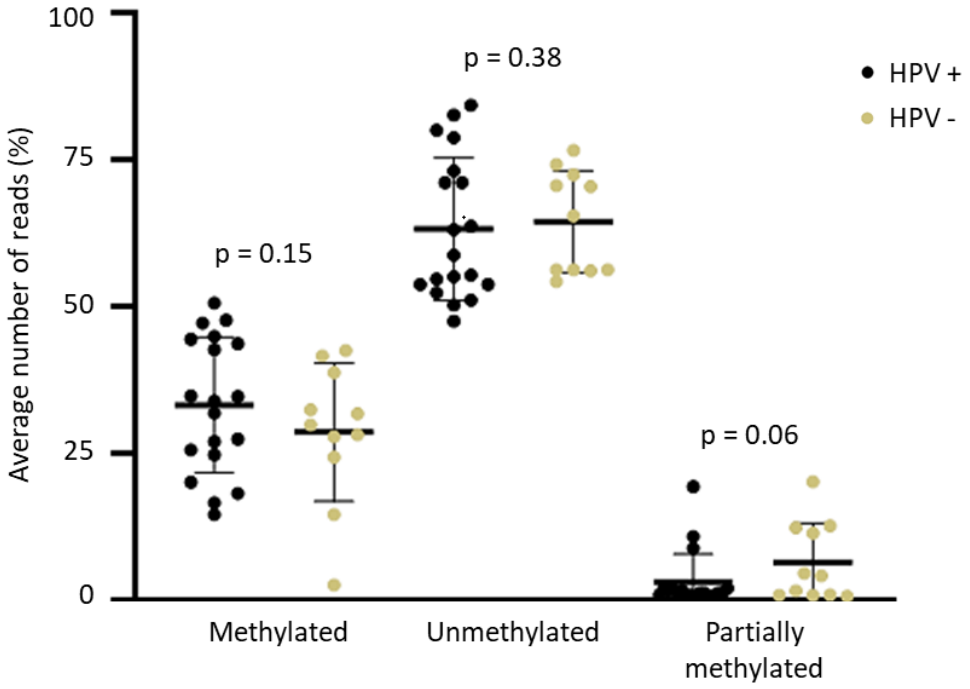
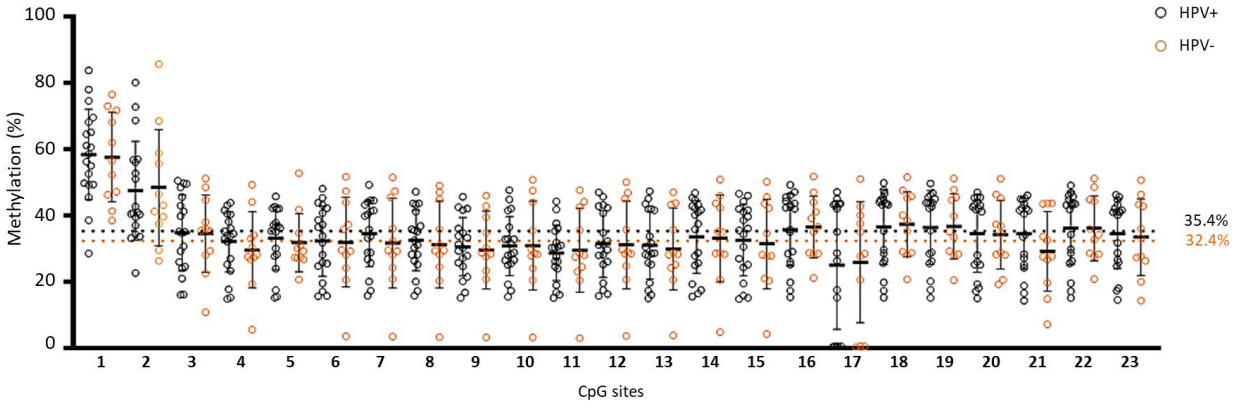
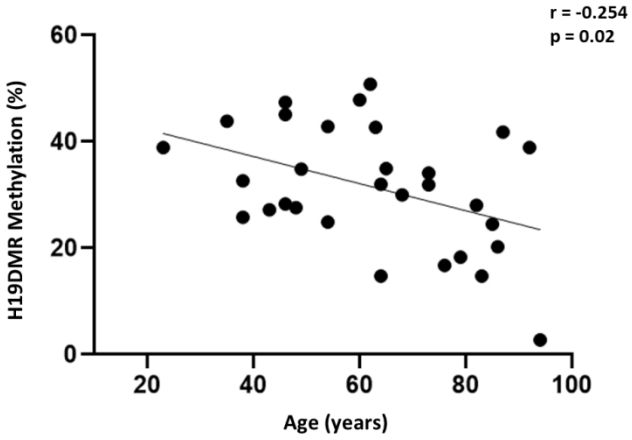


Figure 3. Percentage of methylation for each CpG site in HPV positive (HPV+) and negative (HPV-) of penile squamous cell carcinoma. The CTCF binding site is indicated by the gray arrow. Positive samples were considered as $p16^{INK4a}$+ or hrHPV+.



Supplementary Figure 1. Linear regression used to correlate H19DMR methylation levels (%) and age (years).

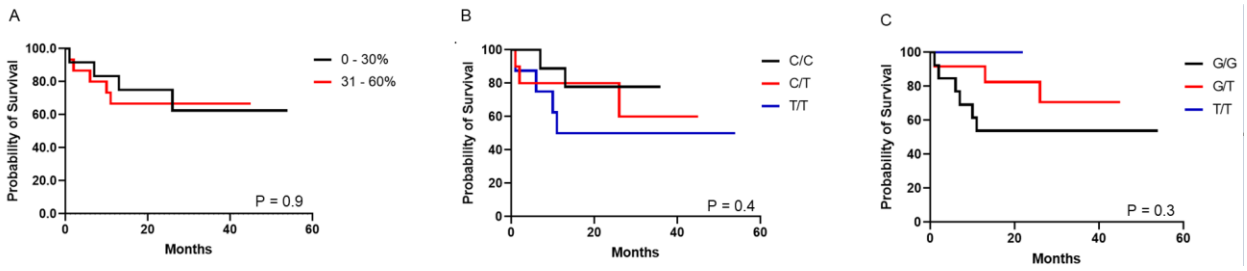


Reduced methylation level (hypomethylation) was observed for most CpG sites in the H19DMR, however, the CpG1 (Chr11: 1,999,782), CpG2 (Chr11: 1,999,794), and CpG17 (Chr11: 1,999,976) showed a different pattern in both HPV+ (CpG1 58.4%±13.6%, CpG2 47.4%±14.9%, and CpG17 25.1%±19.3%), and HPV- (CpG1 57.6%±13.4%, CpG2 48.4%±17.5%, and CpG17 25.9%±18.2%) (Figure 3). Interestingly, the sequenced region presents a potential binding site for the CTCF factor (Chr11: 1,999,964 - 1,999,973), which comprises exactly the CpG16 site (Chr11: 1,999,973) (Figure 3; Supplementary Table 2). Additionally, the genotypic frequency of both rs2107425 (C>T) and rs2071094 (G>T) SNPs was not related to changes in the methylation pattern

at H19DMR (Table 3). Hypomethylation at H19DMR and SNPs genotype did not affect the survival rate in penile SCC (Figure 4).

In silico analysis of gene expression in three different squamous cell carcinomas showed an increased expression of CTCF in HNSC (p = 1.7e-10) and LUSC (p = 1.4e-08) in comparison to non-tumor samples. H19 showed reduced expression in HNSC (p = 3.5e-02) and increased expression in LUSC (p = 8.5e-06) when compared to their respective non-tumor samples. IGF2 had lower expression in CESC (p = 1.2e-05) and increased expression in LUSC (p = 1.1e-02) when compared to non-tumor samples (Supplementary Table 3).

Figure 4. Kaplan–Meier curve for survival probability (n = 27). (A) methylation levels; (B) rs2107425 genotype; (C) s2071094 genotype.



Supplementary Table 3. *CTCF*, *H19* and *IGF2* RNA-Seq expression data in tumor and not tumor squamous cell carcinomas from OncoDB database. Data is normalized using Transcripts Per Million (TPM).

	CTCF	**H19**	**IGF2**
Cancer type	Head and neck squamous cell carcinoma (HNSC)		
Tumor Expression (TPM)	43.9	200.5	0.9
Not Tumor Expression (TPM)	31.8	380.9	0.8
p-value*	*1.7e-10*	*3.5e-02*	6.1e-01
Cancer type	Cervical squamous cell carcinoma (CESC)		
Tumor Expression (TPM)	50.2	153.9	0.5
Not Tumor Expression (TPM)	47.2	113.5	78.6
p-value*	2.5e-01	2.6e-01	*1.2e-05*
Cancer type	Lung squamous cell carcinoma (LUSC)		
Tumor Expression (TPM)	51.0	168.4	3.9
Not Tumor Expression (TPM)	41.3	16.6	0.5
p-value*	*1.4e-08*	*8.5e-06*	*1.1e-02*

*student's t-test

3.4. Discussion

LOI is associated with the early events of tumorigenesis due to the important role of imprinted genes in cell differentiation and growth. We reported a hypomethylated pattern at H19DMR in penile SCC, that was not related to HPV infection considering both p16INK4a and hrHPV markers. The hypomethylation was correlated to IPI but with no other clinical characteristics or survival rate. Additionally, the average methylation between the CpG sites was lower and similar, however, three specific sites showed a different pattern (CpG1, CpG2, and CpG17). The genotypic frequency of the SNPs rs2107425 (C>T) and rs2071094 (G>T) was not related to the methylation level at H19/IGF2 DMR.

The H19DMR gene cluster is related to the maintenance of cellular processes such as cell growth and proliferative activity [32]. Changes in the methylation levels at this locus or LOI affect the monoallelic expression of both H19 and IGF2 genes [33]. The impact of LOI at H19DMR has been previously linked to an increased IGF2 gene expression in prostate, colorectal, and rectum cancer [34,35]. In colorectal cancer, the increase in IGF2 protein expression via LOI is related to an increase in the carcinogenic pathways AKT1 (AKT serine/threonine kinase 1), IR-A (insulin receptor A), and WNT/beta-catenin. Given the modulatory role in such pathways, the IGF2 gene has been seen as a potential anticancer target. Xenographic models with increased IGF2 expression showed high inhibition of tumor growth and tumor regression when treated with new anti-IGF2 targets, such as BI 885578, MEDI-573, and anti-VEGF therapy [36,37].

Loss of methylation at H19DMR has also been observed in bladder tumors. Byun et al (2007) showed 20% of hypomethylation at the paternal allele when compared to normal mucosa in matched cases of bladder tumors [14]. Biallelic expression of the lncRNA H19 or the inactivation of its maternally active copy due to LOI at H19DMR is the main alteration behind the preneoplastic Beckwith–Wiedemann syndrome [38] and the pediatric Wilms' tumor and rhabdomyosarcoma [39]. The lncRNA H19 is a known oncofetal gene, with an intricate role during tumorigenesis, acting as a tumor suppressor in cancer initiation [16] and as an oncogene during malignant progression [40]. The biallelic expression of the lncRNA H19 due to LOI increases the risk of colorectal [41], bladder [42], breast and oral squamous cell carcinoma (REF?), and acute myeloid leukemia (LMA), among others [43]. Recently, we have shown that the knockdown of

the lncRNA H19 gene and its reduced expression increases cell proliferation and metaphase translocation events [32].

As the region analyzed has a binding site for the CTCF protein, the hypomethylation at the surrounding CpG sites might alter its insulator function [18]. This alteration may promote the biallelic expression of the lncRNA H19 and the silencing of the IGF2 in penile SCC. Furthermore, the hypomethylation observed in our current study seems to be independent of HPV infection, given the similar methylation levels between the HPV+ and HPV- samples considering either p16INK4a and hrHPV markers. Similarly, H19DMR hypomethylation was not associated with HPV infection in invasive cervical cancer (ICC) [44] and cervical intraepithelial neoplasia (CIN) [45]. We previously showed that the 5mC Global DNA methylation mark is increased in penile SCC and is not influenced by HPV infection [3]. Although the genome-wide loss of CpG DNA methylation is an age-related event, our results did not show an association between age < 60 and > 60 years old with methylation levels of 0% - 30% and 31% - 60%, but a negative correlation was observed between age and DNA methylation at H19DMR in penile SCC. Horvath (2013) reported that DNA methylation per tissue may differ from chronological age, and for squamous cell carcinomas, a lower time-dependent acceleration of methylation loss was observed when compared to other cancers [46].

The H19DMR hypomethylation may influence the tumor microenvironment, as increased IPI was correlated with reduced methylation at this region. Likewise, immune cell infiltration in the tumor microenvironment was associated with DNA and RNA methylation modifications in colorectal cancer [47] and gastric cancer [48]. As this region controls the H19 and IFG2 imprinting cluster, LOI is related to altered glucose metabolism and diabetes [49]. Intrauterine hyperinsulinemia changes H19DMR methylation in the fetuses which exhibited impaired glucose tolerance and insulin resistance [50]. Obesity is a risk factor for cancer in general and is associated with an increased risk of invasive penile cancer, inducing chronic inflammation and insulin resistance [51]. Overexpression of IGF1R was reported by Ball and colleagues (2016) in 62% of a cohort of 53 men diagnosed with penile cancer and was associated with inferior progression-free survival (PFS) [52]. A comparison between patients without IGF1R overexpression and those with overexpression revealed a significant difference in 5-year PFS rates, with 94.1% versus 45.8%, respectively. In a subsequent study by the same research group, IGF1R overexpression was observed in approximately two-thirds of penile squamous cell carcinoma (SCC) cases among 112

patients. The findings indicated a noteworthy association with histologic subtype and grade, suggesting a worse prognosis for tumors exhibiting IGF1R overexpression [53].

The HPV virus sequence can be integrated into the genome of the host cell, leading to mutational events, disrupted gene expression, and genomic instability [54]. Furthermore, we also analyzed two non-CpG SNPs (rs2107425 and rs2071094) at H19DMR previously reported as biomarkers for allele-specific DNA methylation (ASM) pattern [55]. However, these SNPs were not related to ASM in penile SCC, as the genotype frequency was not different between HPV+ and HPV-, and it was not related to the methylated or unmethylated stretch. Canto et al (2022) showed HPV-related mutations in penile cancer, and these alterations were localized to HPV integration sites (HPVis) and miRNA regions [56]. A recent study showed that a high somatic tumor mutation burden (TMB) is associated with HPV-positive penile SCC, and these data show that the molecular scenario for this disease may depend on viral infection [57]. The SNP rs1042522 (pArg72Pro) in the TP53 gene has been associated with cancer risk susceptibility, as the variant is more likely to be degraded via ubiquitinylation by the hrHPV E6 oncoprotein in various cancers [58], but this association was not confirmed for penile SCC [59].

The accumulation of myeloid-derived suppressor cells (MDSCs), such as polymorphonuclear neutrophils, is a hallmark of cancer, and changes in the tumor microenvironment associated with MDSC reduction have been reported in an in vitro model of penile SCC [60, 61]. This marker has potent immunosuppressive effects, although the mechanisms that cause MDSC growth in the tumor microenvironment remain unknown. It has been reported that MDSCs respond to DNA methylation modulation, as the reduction of MDSC growth and accelerated activation of antigen-specific cytotoxic T cells are altered by the use of DNA methyltransferase inhibitors such as decitabine [62]. Penile SCC DNA methylation signature should be further explored since the hypomethylation pattern at H19DMR in different types of tumors is associated with tumor progression, aggressiveness, and tumor microenvironment.

The identification of epigenetic reprogramming and molecular alterations in penile SCC, an extremely aggressive tumor with increased incidence in developing countries, whose therapies are limited and mostly based on surgical excision, can assist in disease diagnosis, prognosis, and treatment. To our knowledge, this is the first study that has evaluated the methylation pattern at H19DMR in penile SCC. However, some limitations need to be mentioned. First, we do not have the participant's background information such as sociodemographic profile, sexual behavior, HPV

vaccination, and the identification of other related histological subtypes and tumor topography. Despite the high incidence of hrHPV in our cohort, two other viral genotypes, hrHPV 59 and 68, were not evaluated. Since we did not evaluate the expression of these imprinted genes in the samples (in vitro), we performed an in silico analysis in squamous cell carcinomas, similar to penile cancer, using the OncoDB database. H19 and IGF2 expression varied according to tumor origin as HNSC, LUSC, and CESC, while the insulator gene CTCF showed increased expression in both HNSC and LUSC. We could not find gene expression results in OncoDB in penile SCC, highlighting the importance of studies related to the molecular mechanisms of this tumor type. Despite the monoallelic pattern in most cell types, the expression of imprinted genes varies depending on the stage of development [6]. The hypomethylation observed in this study may favor the expression of the H19 gene, an oncofetal lncRNA that is frequently overexpressed in many types of cancer, favoring carcinogenesis and tumor aggressiveness [37, 38]. Given its relatively low incidence worldwide, but increased in underdeveloped countries, little is known about the genetic and epigenetic epidemiology of penile SCC, and future studies are needed to better understand the molecular profile of this tumor..

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4. CAPÍTULO IV

DESCRIÇÃO DE VARIANTES SOMÁTICAS DE EXOMA EM CARCINOMA DE CÉLULAS ESCAMOSAS DE PÊNIS

RENAN DA SILVA SANTOS

4.1. Introdução.

O carcinoma de células escamosas de pênis é uma neoplasia rara, porém agressiva e representando um desafio significativo no campo da oncologia devido à sua alta taxa de morbidade. Embora menos comum em países desenvolvidos, a incidência deste tipo tumoral é maior em regiões em desenvolvimento, refletindo a importância de fatores socioeconômicos e de saúde pública na sua etiologia (McDaniel *et al*., 2015) (Júnior *et al.*, 2018). A compreensão das bases moleculares e genéticas do CPE é essencial para o desenvolvimento de estratégias terapêuticas e prognósticas mais eficazes. A pesquisa em câncer tem se voltado cada vez mais específicas para a identificação de biomarcadores genéticos que possam servir como ferramentas diagnósticas, prognósticas e terapêuticas.

No avanço do estudo do CPE, as análises genéticas que compreendem as regiões codificantes do genoma, tem revelado um panorama complexo de variantes somáticas que poderiam contribuir para o desenvolvimento da carcinogênese. Diferentes estudos tem apresentado a incidência de variantes somáticas associadas aos genes *TP53, CDKN2A, FAT1*, *NOTCH-1* e *PIK3CA*. As mutações de variação de número de cópias (do inglês, *Copy Number Variation* - CNV), ou seja, o aumento ou a diminuição do número de cópias de um determinado gene também já foram reportadas para o CPE. O ganho de cópias nos genes *MYC* e *EGFR* são os mais reportadas. Genes como *TP53, CDKN2A, PIK3CA* e *CCND1*, foram descritos com a presença de variantes somáticas e também alterações no número de cópias. (Chahoud *et al.*, 2021) (Ribera-Cortada *et al.*, 2022).

Variantes em genes da via NOTCH, particularmente o gene *NOTCH1*, estão entre os genes mais frequentemente mutados no CPE. A via NOTCH é crucial para a regulação do desenvolvimento celular e sua disfunção pode levar a uma proliferação celular descontrolada. Variantes em *NOTCH1* podem desempenhar um papel dual, atuando tanto como oncogene quanto como supressor tumoral, dependendo do estímulo microambiental (Parmigiani, Taylor e Giachino, 2020). Outro gene destacado em ensaios moleculares de interesse é o *FAT1* (Canto *et al.*, 2022), onde sua proteína envolvida em mecanismos de sinalização celular e adesão. Esse gene é regulado negativamente em muitos tipos de câncer e seu desempenho no papel do controle da proliferação e migração celular são prejudicados, sendo considerado um supressor tumoral. Variantes em *FAT1* têm sido associadas ao potencial metástase no CPE (Ribera-Cortada *et al.*, 2022).

A integração de dados genômicos associados a parâmetros clínicos e moleculares pode melhorar significativamente a compreensão das bases moleculares do CPE. Biomarcadores

genéticos não só auxiliam na estratificação de um grau de prognóstico do paciente, também podem orientar decisões terapêuticas, promovendo uma abordagem mais personalizada ao tratamento. Entretanto existe uma carência de compreensão na interação do vírus HPV e a célula hospedeira tumoral no CPE. A prevalência do HPV no CPE pode variar amplamente dependendo da literatura e entre diferentes regiões do mundo, podendo variar de 11 a 87% (Yanagawa *et al.*, 2003; Ferreux *et al.*, 2003). A associação entre infecção por HPV e risco de desenvolvimento desse tipo tumoral ainda não é clara, pois os resultados podem ser controversos (Gu *et al.*, 2020; Takamoto *et al.*, 2018).

O potencial carcinogênico dos subtipos de alto risco do vírus HPV vem em parte pela expressão de proteínas oncovirais na célula hospedeira. Embora apresente um genoma pequeno, de aproximadamente 8 kb, dois dos oito genes do vírus podem impactar vias de controle do ciclo celular da célula (Doorbar *et al.*, 2015). Os oncogenes virais E6 e E7, que são ativamente transcritos nas células infectadas, são essenciais para a transformação maligna induzida por vírus. Esses genes interrompem a síntese do centrossomo, um componente vital da divisão celular, com o desenvolvimento de divisões celulares multipolares. Além disso, a proteína E6 tem como alvo a proteína supressora de tumor p53, enquanto a proteína E7 tem como alvo a proteína supressora de tumor retinoblastoma-1, dois reguladores negativos da proliferação celular, cuja inativação induz à proliferação celular descontrolada (Pal e Kundu, 2020; Scarth *et al.*, 2021).

Além do bloqueio de vias de controle proliferativo, o genoma de dupla hélice do HPV encontra alto potencial de inserção do seu material genético com o genoma hospedeiro. Essa interação promove instabilidade genômica na célula não tumoral. Recentemente Labarge e colaboradores (2022) mostraram que tumores de células escamosas de cabeça e pescoço nos quais o HPV foi integrado ao DNA da célula hospedeira continham um número significativamente maior de CNVs de diferentes tipos, como deleções, inserções, translocações e inversões, e um aumento nas variantes de nucleotídeo único e de alteração do quadro de leitura em relação às encontradas em tumores com apenas HPV extracromossômico. Os tumores sem HPV integrado não continham essencialmente CNVs e relativamente poucas mutações pontuais (Labarge *et al.*, 2022).

4.2. Materiais e métodos

4.2.1. Participantes e desenho do estudo.

Este é um estudo retrospectivo que incluiu 24 participantes com CPE que foram submetidos à penectomia parcial, total ou emasculação, sem qualquer história prévia de outro tipo tumoral e

aplicação de quimioterapia ou radioterapia. As amostras utilizadas foram provenientes do banco de amostras de tecidos tumorais do Hospital Haroldo Juaçaba, Ceará, armazenadas a -80°C entre os anos de 2015 a 2018. O estudo foi aprovado pelo comitê de ética da Universidade Federal e do Hospital Haroldo Juaçaba, conforme processo número 2.427.846.

4.2.2. Expressão da proteína $p16^{INK4a}$ e identificação de HPV de alto risco.

O ensaio de Imunohistoquímica foi realizado para identificar a expressão da proteína $p16^{INK4a}$ utilizando o anticorpo anti-p16 clone *E6H4 (Roche CINtec® Histology)* de acordo com as instruções do fabricante. A expressão $p16^{INK4a}$ deve ser ≥ 75% para ser considerada positiva, com continuidade e completa coloração citoplasmática e nuclear (Cubilla *et al.*, 2011). A hibridização cromogênica *in situ* (CISH) foi usada para identificar os genótipos de hrHPV através do Kit de diagnóstico *Ventana Inform HPV III Family 16 Probe (Ventana Medical Systems, Tucson, AZ) (genótipos 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 e 66)*. Neoplasia intraepitelial cervical de alto grau foi utilizada como controle positivo para hrHPV. Músculo estriado esquelético foi utilizado como controle negativo para ambos os ensaios. Todos os experimentos foram analisados por observadores cegos.

4.2.3. Sequenciamento de exons

Para processamento de amostras para Sequenciamento de Nova Geração (NGS) foram utilizados um total de 32 amostras distribuídas nos seguintes grupos: 24 amostras de CPE (HPV+ = 12, HPV- = 12) e 8 amostras de tecido não tumoral pareadas com amostras tumorais (HPV+ = 4, HPV- = 4). Para extração do DNA genômico dos tecidos utilizamos o kit *DNeasy Blood and Tissue (Qiagen)* seguindo as recomendações do fabricante. A concentração de DNA foi avaliada através de ensaio de fluorometria utilizando o *Qubit® 4.0 (Thermo Fisher Scientific, Inc).* Além da quantificação do rendimento, a qualidade do DNA foi avaliada em eletroforese em gel de agarose 1%.

4.2.3.1. Preparo de bibliotecas para sequenciamento.

O preparo de bibliotecas e o sequenciamento foram realizados em colaboração com o Institudo Nacional do Câncer (INCA) utilizando a Plataforma Multiusuário de Genômica vinculada ao Programa de Genética. O sequenciamento de exons foi realizado utilizando a plataforma Illumina, HiSeq 2500, cujas três etapas de execução foram: preparo das bibliotecas de DNA, construções de clusters por amplificação clonal e sequenciamento por síntese. Para o

preparo de bibliotecas foi utilizado o kit *Illumina Nextera DNA Flex LPK - Small (16 Spl), Nextera DNA Flex LPK Enrich (16 Spl)* e *Nextera Compatible Unique Dual Id*, conforme indicações do fabricante. Brevemente, a biblioteca é preparada por fragmentação da amostra de DNA, seguida pela adição de adaptadores que marcam as extremidades 5′ e 3′ das sequencias fragmentadas (Illumina Inc., 2017). A análise de qualidade de biblioteca ainda foi avaliada em eletroforese capilar pelo equipamento *Bioanalyzer 2100 (Agilent)*, onde a maioria dos fragmentos gerados apresentou tamanho médio de 250 pares de base (pb).

4.2.3.2. Geração de clusters e sequenciamento global de exons (Exoma).

A geração de *cluster* (grupos) consiste na etapa seguinte ao preparo de biblioteca das amostras. As amostras são então carregadas em uma célula de fluxo, compartimento onde ocorre a amplificação clonal dos fragmentos gerados (*clusters*) e o sequenciamento. Cada fragmento ligado aos adaptadores se ligam em uma região específica na célula de fluxo, seguindo as especificações para a plataforma *HiSeq 2500* onde utilizamos o *kit HiSeq® Rapid PE Cluster Kit v2* para geração de clusters. O painel de sondas de sequenciamento global de exons utilizado foi o *Twist Human Core Exome, 2 Reactions (Twist Bioscience)* gerando um total de 97 gigabases.

4.2.4. Workflow para análise de sequenciamento.

Após o sequenciamento, para validação da qualidade das amostras sequenciadas e para a retirada de sequências contaminantes como indexes e adaptadores serão utilizadas as ferramentas FastQC e Trimmomatic, respectivamente. O mapeamento ao genoma referência (genoma humano–hg19) será realizado utilizando-se o algoritmo “bowtie2” (genoma humano - hg19). Para a conversão dos arquivos em formato “SAM” e “BAM”, será utilizado o software SAMtools. Após o mapeamento os arquivos serão submetidos à análise de duplicação e calibração usando o pacote Genome Analysis Toolkit (GATK) e a ferramenta Picard. A chamada de variantes somáticas, que incluem variantes de nucleotídeo único (SNV) e de inserção e deleção (indel), serão geradas através da ferramenta VarScan2. As variantes encontradas serão anotadas pela comparação com genoma de referência usando a ferramenta Annovar.

4.2.5. Análise de enriquecimento de vias.

Para a análise de enriquecimento de vias, método computacional usado para inferir a relação sobre um conjunto de genes, comparando os genes alvos com um conjuntos de genes

anotados e disponíveis para acesso em bancos de dados, utilizamos de duas ferramentas, *Enrichr* e *WebGestalt* (*WEB-based GEne SeT AnaLysis Toolkit*). *Enrichr* é um servidor robusto que contém muitos tipos de conjuntos de dados (https://maayanlab.cloud/Enrichr/) (Kuleshov *et al.*, 2016), entre os quais usamos os bancos de dados KEGG 2021 Human, ClinVar 2019, GO Molecular Function 2021, Jensen DISEASES, DisGeNET, Kinase Perturbations from GEO up, PheWeb 2019, PPI Hub Proteins, GO Molecular Function 2021, Reactome 2016, BioPlanet 2019, MSigDB Hallmark 2020 e DisGeNET. Para análise de interações de vias baseada em topologia utilizamos a ferramenta *WebGestalt* também disponível em domínio público (https://www.webgestalt.org/) (Zhang, Kirov e Snoddy, 2005).

4.2.6. Análise estatística.

Para os resultados de comparação da frequência das médias de genes por grupo HPV+ e HPV- utilizamos de Test-t de Studant. Para os resultados de significância de vias de enriquecimento utilizamos Teste exato de Fisher. A significância estatística foi fixada em $p < 0.05$.

4.3. Resultados.

4.3.1. Painel de variantes somáticas de CPE.

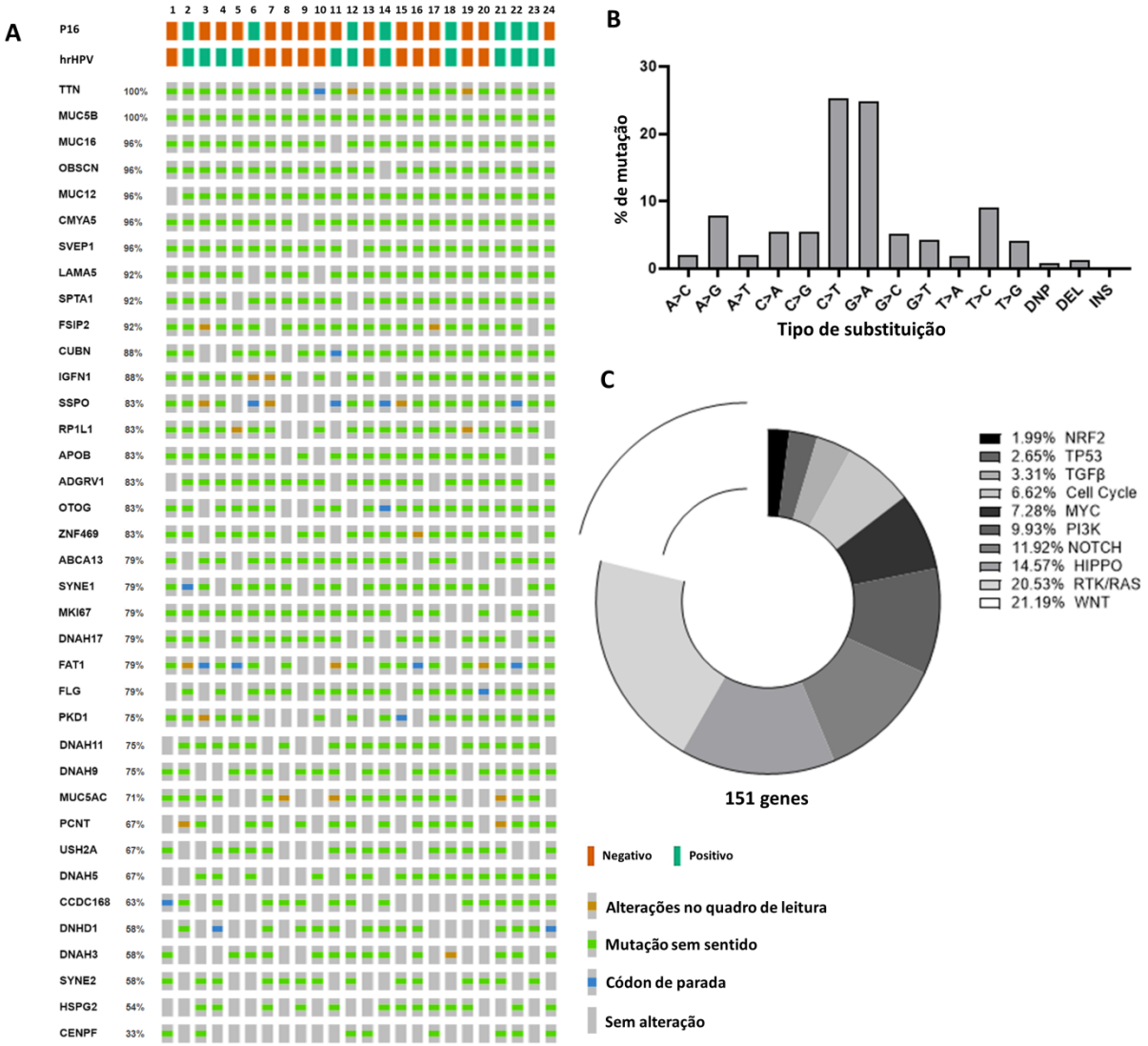
A figura 1 apresenta um sumário dos principais achados do sequenciamento global de exons em 24 amostras de CPE. O painel de variantes somáticas apresenta uma lista com 37 genes com uma ou mais variantes identificadas em sua sequencia nucleotídica. De acordo com o painel, os dois primeiros genes, *TTN* e *MUC5B*, reportam variantes em todas as amostras tumorais (24/24; 100%) sequenciadas. Os genes *MUC16*, *OBSCN*, *MUC12*, *CMYA5* e *SVEP1* também apresentam variantes e se distribuem em 23 (23/24; 96%) amostras. Variantes nos genes *LEMAS5*, *SPTA1* e *FSIP2* estão presentes em 22 (22/24; 92%) amostras.

Mutações sem sentido (*missense mutation*, do inglês) foram as variantes mais abundantes encontradas nos genes descritos do painel, onde uma substituição de um nucleotídeo passa a traduzir um aminoácido incorreto. Alterações no quadro de leitura (*in-frame mutation*, do inglês) e alteração de nucleotídeos que antecipem códons de parada (*truncating* ou *nonsense mutation*, do inglês) aparecem como variantes menos frequentes identificadas entre os genes do painel. Dentre as substituições nucleotídicas mais frequentes (Figura 1B), observamos que as bases citosinas e

guaninas foram substituídas por timinas e adeninas, respectivamente, representando juntas 50% das trocas de nucleotídeos.

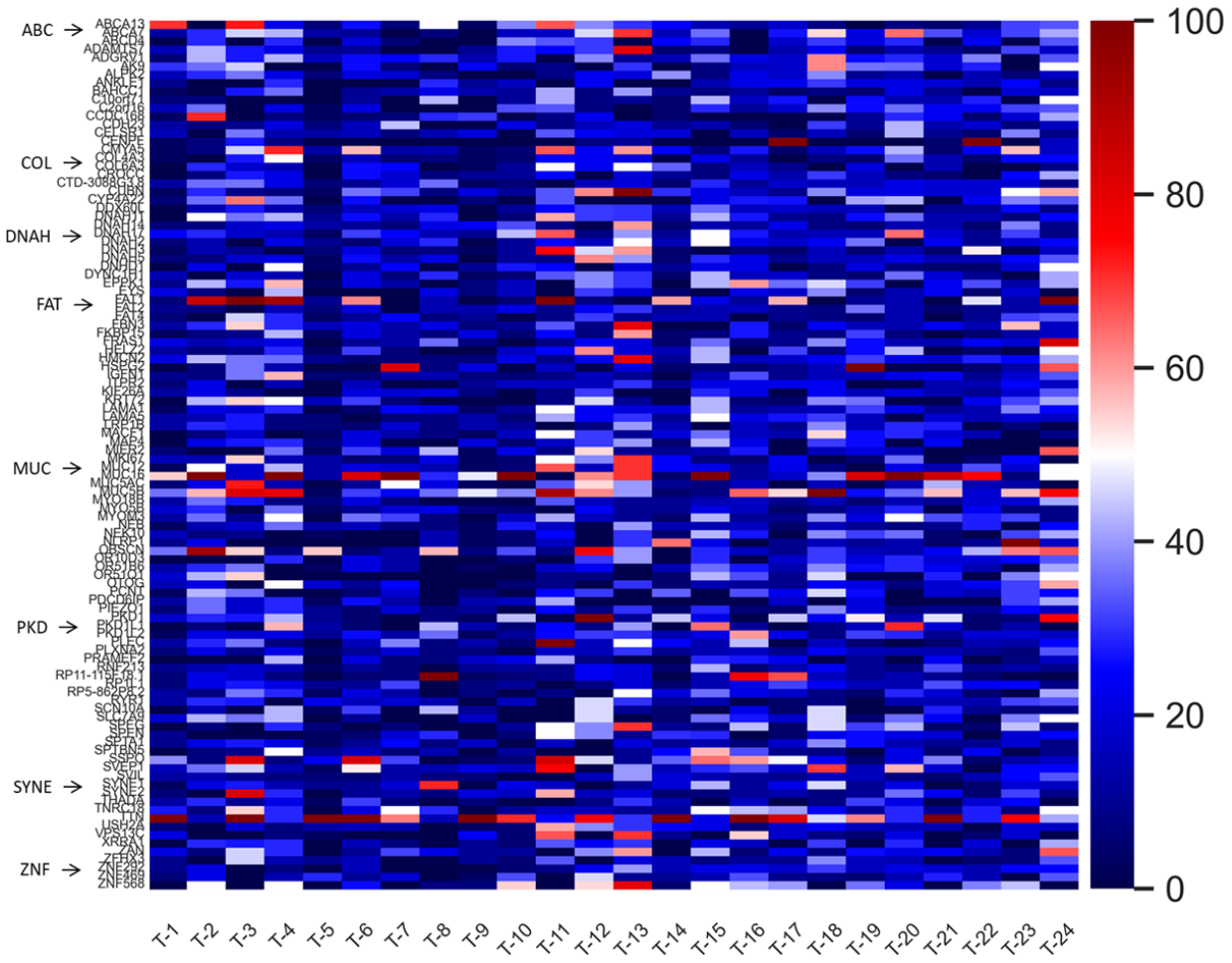
Do total de 14383 genes reportados com pelo menos uma variante em pelo menos uma amostra, observamos que 151 (1%) genes estão inseridos dentro de algumas de dez vias tumorais clássicas: ciclo celular, Hippo, Myc, Notch, NRF2, PI3-Kinase/Akt, RTK/RAS, TGFβ, P53 e β-catenin/WNT (Figura 1C).

Figura 1. Painel de variantes somáticas. (A) Genes com maior frequência de variantes por amostra, tipos de variantes e infecção por HPV. (B) Frequência de alteração de nucleotídeos. (C) Distribuição de variantes por vias tumorais.



A Figura 2 apresenta um painel mais abrangente, mostrando os 100 genes com maior carga mutacional e sua distribuição entre as amostras. É interessante ressaltar que encontrar mais de um gene que compõem uma família gênica, ou seja, conjunto de genes semelhantes que compartilham sequências de nucleotídeos ou proteínas. As famílias de genes em destaque foram ABC (*ATP-binding cassette*, do inglês; *ABCA13, ABCA7* e *ABCD4)*; COL (*collagen type*, do inglês; C*OL4A3* e *COL6A3)*; DNAH (*dynein axonemal heavy chain*, do inglês; *DNAH11*, *DNAH14, DNAH17, DNAH2*, *DNAH3* e *DNAH5)*; FAT (*atypical cadherin*, do inglês; *FAT1*, *FAT2* e *FAT4)*; MUC (*mucin*, do inglês; *MUC12, MUC16, MUC5AC* e *MUC5B)*; PKD1L1 (*polycystin 1 like*, do inglês; *PKD1L1* e *PKD1L2)*; SYNE (*spectrin repeat containing nuclear envelope protein*, do inglês; *SYNE1* e *SYNE2)*; ZNF (*zinc finger protein*, do inglês; *ZNF292, ZNF469* e *ZNF568)*.

Figura 2. Representação em *heatmap* dos 100 genes com maior frequencia de variantes em amostras de CPE. A sequência de genes obedece à ordem alfabética e é independente da presença de HPV. Setas destacam as famílias de genes.



4.3.2. Enriquecimento de famílias de amostras de CPE.

A Tabela 1 apresenta o resultado de enriquecimento de vias obtidas das oito famílias de genes destacadas no resultado da Figura 2. O resultado do enriquecimento de vias indica que muitas das famílias de genes avaliados estão associadas com eventos de manutenção metabólicos e proteção contra eventos de promoção tumoral. As principais alterações estão associadas à atividade de receptores ABC e receptores ativados pela via Wnt, atividade motora de microtúbulos, migração de células epiteliais e adesão celular, sinalização de vias carcinogênicas (Hippo, PI3K-Akt e ciclo celular) e alteração de expressão de proteínas quinase (MAPK14, MAPK13, MAPK1, MELK e JAK2). O enriquecimento de alvos destaca que os genes para expressão da proteína caderina *(FAT1, FAT2* e *FAT4*) são alvos relacionados à manifestação de eventos carcinogênicos para carcinomas de células escamosas.

Tabela 1. Enriquecimento de 25 genes distribuídos em oito famílias gênicas através da ferramenta de enriquecimento de vias gênicas *EnrichR*.

Família de genes	Gene	Descrição	Análise de enriquecimento (vias e ontologia)	Banco de dados	*p*-value*	OR**
ABC	*ABAC13*, *ABAC7* e *ABCD4*	Subfamília de cassetes de ligação ATP	Transportadores ABC	KEGG 2021 Human	1.1E-08	1.5E+01
DNAH	*DNAH2*, *DNAH3*, *DNAH5*, *DNAH11*,*DNAH14* e *DNAH17*	Cadeia pesada axonemal	Doença de Huntington	KEGG 2021 Human	1.2E-11	1.2E+05
			Atividade motora dos microtúbulos	GO Molecular Function 2021	4.1E-07	3.8E+02
FAT	*FAT1*, *FAT2* e *FAT4*	Caderina atípica FAT	Via de sinalização (Hippo)	KEGG 2021 Human	2.4E-02	6.1E+01
			Adesão célula-célula via adesão à membrana plasmática	GO Biological Process 2021	2.1E-04	2.4E+02
			Via de sinalização (Hippo)	GO Biological Process 2021	3.9E-03	4.0E+02
			Migração de células epiteliais	GO Biological Process 2021	6.1E-03	2.5E+02
			Carcinoma de células escamosas de cabeça e pescoço	Jensen DISEASES	9.0E-04	2.0E+03
			Carcinoma de células escamosas de esôfago	DisGeNET	1.3E-04	5.7E+04
MUC	*MUC12*, *MUC16*, *MUC5AC*, *MUC5B*	Mucina (proteínas glicosiladas produzidas por tecidos epiteliais)	Via de sinalização (IL-17)	KEGG 2021 Human	4.5E-04	8.7E+01
ZNF	*ZNF292*, *ZNF469* e *ZNF568*	Proteína de dedo de zinco	MAPK14 *knockdown*	Kinase Perturbations from GEO up	4.4E-02	3.3E+01
			MAPK1 *knockdown*	Kinase Perturbations from GEO up	4.4E-02	3.3E+01
			MELK *knockdown*	Kinase Perturbations from GEO up	4.4E-02	3.3E+01
			ligação de ácido nucleico da região reguladora da transcrição	GO Molecular Function 2021	3.1E-02	4.7E+01
			Displasia dos órgãos genitais femininos	PheWeb 2019	3.0E-03	5.3E+02
COL	*COL4A3* e *COL6A3*	Tipo de colágeno	Interação ECM-receptor	KEGG 2021 Human	1.9E-05	4.0E+04
			Infecção por papillomavirus human	KEGG 2021 Human	2.7E-04	3.9E+04
			Via de sinalização (PI3K-Akt)	KEGG 2021 Human	3.1E-04	3.9E+04
			Câncer de Colorre PheWeb 2019	2.4E-03	8.7E+02	

PKD	*PKD1, PKD1L1* e *PKD1L2*	Policistina	Via de sinalização (CDH1)	PPI Hub Proteins	1.9E-02	7.9E+01
			Via de sinalização (MAPK13)	PPI Hub Proteins	2.1E-02	7.2E+01
			Via de sinalização (JAK2)	PPI Hub Proteins	2.4E-02	6.3E+01
			Atividade do receptor ativado por Wnt	GO Molecular Function 2021	2.2E-03	7.1E+02
			Câncer do endométrio	Jensen DISEASES	4.3E-02	3.4E+01
SYNE	*SYNE1 e SYNE2*	Repetição de espectrina contendo proteína do envelope nuclear	Via de sinalização (Ciclo celular)	Reactome 2016	8.0E-04	3.9E+04
			Câncer de mama	Jensen DISEASES	4.7E-04	3.9E+04
			Câncer de fígado	Jensen DISEASES	8.9E-04	3.9E+04

*Teste exato de Fisher

****** Odds ratio

4.3.3. Enriquecimento de vias secundárias.

O resultado de enriquecimento das famílias gênicas (Tabela 1) apresenta eventos potencialmente alterados relacionados aos genes mutados apresentados no painel da Figura 2. A Figura 3 destaca as pricipais interações dos 100 genes ranqueados com maior frequencia de variantes e suas principais interações secundarias. O mapa de interações (Figura 3) mostra que as vias secundárias mais influenciadas pelo painel da Figura 2 são os genes *GAN, SRPK2, EGFR, UBC, CLK1, ARRB1, TRIM25, TNF, IRS4, HYPK, HSPB1, NXF1, HNRNPL, BRCA1, CUL1* e *MTNR1A*.

Os 16 genes mencionados estão inseridas em duas rotas tumorais, sinalização da via NOTCH e RTK/RAS. A análise de enriquecimento desses 16 genes mostra que três bancos de dados indicam associação com o funcionamento da via de sinalização NOTCH (*BioPlanet, MSigDB Hallmark* e *Reactome*) e quatro bancos de dados (*BioPlanet, KEGG, Reactome* e GO *Molecular Function*) indicam que diferentes receptores e enzimas quinases implicariam em alteração na via RTK/RAS (ERBB4, EGFR, MAPK e atividade em serina/threonina quinases) (Tabela 2).

Figura 3. Rede de interações secundarias dos 100 genes com maior carga de variantes somáticas construída pela ferramenta *WebGestalt*. A rede representa os 16 alvos de interações secundaria com maior relevância ($p<0.05$ ajustado).

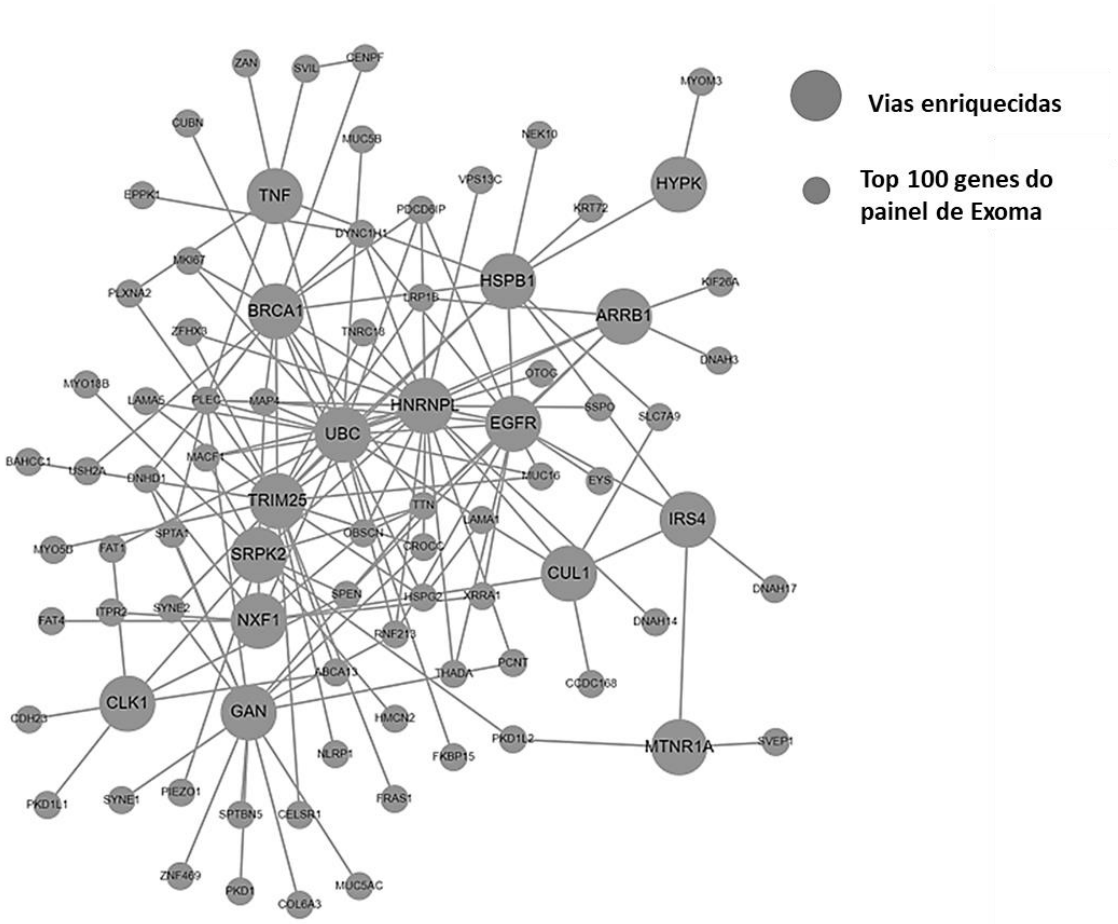


Tabela 2. Enriquecimento dos 16 alvos secundários e banco de dados públicos através da ferramenta de enriquecimento de vias gênicas *EnrichR*.

Vias	Enriquecimento (vias, ontologia e doenças)	Banco de dados	*p*-value*	OR**
GAN, SRPK2, EGFR, UBC, CLK1, ARRB1, TRIM25, TNF, IRS4, HYPK, HSPB1, NXF1, HNRNPL, BRCA1, CUL1 e *MTNR1A*	Sinalização por via NOTCH1	BioPlanet 2019	8.2E-03	5.0E+01
	Sinalização por via ERBB4	BioPlanet 2019	8.5E-03	3.9E+01
	Sinalização por via EGFR	BioPlanet 2019	9.4E-03	1.3E+02
	Sinalização por via NOTCH	BioPlanet 2019	9.4E-03	3.0E+01
	Sinalização por via MAPK	BioPlanet 2019	9.4E-03	1.6E+01
	Regulação do receptor de células T de apoptose	BioPlanet 2019	9.4E-03	1.1E+01
	EGFR downregulation	BioPlanet 2019	1.1E-02	8.9E+01
	Sinalização por via MAPK	KEGG 2021 Human	1.2E-02	1.7E+01
	Proteólise mediada por ubiquitina	KEGG 2021 Human	1.6E-02	2.6E+01
	MicroRNAs em câncer	KEGG 2021 Human	4.6E-02	1.1E+01

Sinalização por via Notch	MSigDB Hallmark 2020	5.4E-03	7.4E+01
Apoptose	MSigDB Hallmark 2021	5.4E-03	2.2E+01
Sinalização por EGFR em Câncer (Homo sapiens R-HSA-1643713)	Reactome 2016	6.2E-03	1.3E+02
Sinalização por NOTCH1 (Homo sapiens R-HSA-1980143)	Reactome 2016	6.2E-03	5.1E+01
Cascatas de sinalização da família MAPK (Homo sapiens R-HSA-5683057)	Reactome 2016	6.2E-03	1.8E+01
ligação à proteína ligase semelhante à ubiquitina (GO:0044389)	GO Molecular Function 2021	2.2E-04	2.4E+01
atividade da proteína tirosina quinase (GO:0004713)	GO Molecular Function 2022	3.3E-02	2.1E+01
atividade da proteína serina/treonina quinase (GO:0004674)	GO Molecular Function 2023	3.3E-02	1.0E+01
Câncer de pulmão	ClinVar 2019	2.8E-02	8.1E+01
Câncer de mama	ClinVar 2019	3.0E-02	4.0E+01
Carcinoma espinocelular de cabeça e pescoço	DisGeNET	3.5E-03	1.1E+01

*Teste exato de Fisher

****** Odds ratio

4.3.4. Perfil de variantes e infecção por HPV.

Dentre do total de 14383 genes reportados com pelo menos uma variante somática em alguma amostra sequenciada, observamos que 574 (3.99%) genes apresentam frequencia de variantes diferentes de acordo com a infecção por HPV ($p<0.05$; Figura 4). Através da busca bibliográfica realizada encontramos o trabalho "*Genome-wide methylation and transcriptome analysis in penile carcinoma: uncovering new molecular markers*" publicado na revista *Clinical Epigenetics* em 2015, que analisa a expressão gênica de 35 amostras de CPE separadas por grupos HPV+ (n = 13) e HPV- (n = 22) através de análises de transcrptoma. De acordo com o acesso dos resultados depositados no Gene Expression Omnibus (GEO; GSE57955), podemos ter acessar os dados de expressão diferenciada de 637 alvos. O curuzamento de dados entre os 574 genes reportados com variantes somáticas diferentemente observadas nos grupos de HPV+ e HPV- e o

dados de expressão diferencial dos 637 genes depositados em banco de dados mostram 15 alvos em comum, *ARHGAP35*, *CDH1*, *CEACAM5*, *CEACAM8*, *FAM83B*, *LAD1*, *LIG4*, *NBEAL1*, *NEK3*, *PHLPP2*, *RBBP6*, *RBSN*, *TMPRSS5*, *UGGT2* e *XPO4* (Figura 5A). O *heatmap* indica a carga mutacional de cada um dos 15 alvos comuns entre Exoma e expressão via transcriptoma por grupo de HPV (Figura 5B) e a alteração da expressão dos mesmos através da comparação do grupo HPV+ em relação ao HPV- de acordo com dados de expressão via transcriptoma (Figura 5C). O enriquecimento funcional de 9 dos 15 genes correlacionados apontam dez alvos (*CEACAM1*, *CEACAM6*, *ZPLD1*, *APP*, *HNRNPL*, *EWSR1*, *CD209*, *UQCC2*, *CTNNB1* e *HNRNPM)* potencilmente desregulados em amostras HPV+.

Figura 4. Representação em *heatmap* dos 574 genes sequenciados e separados em grupos HPV+ e HPV-.

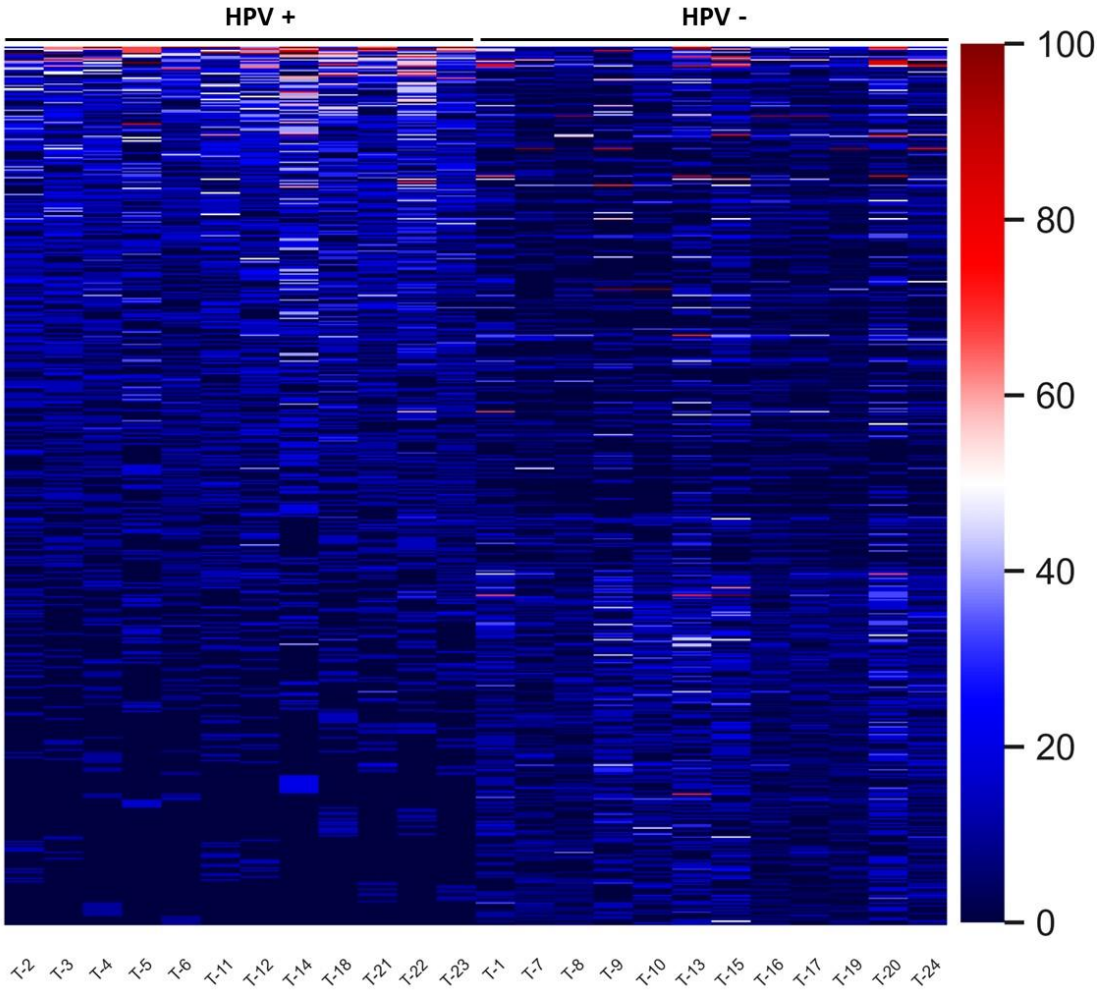
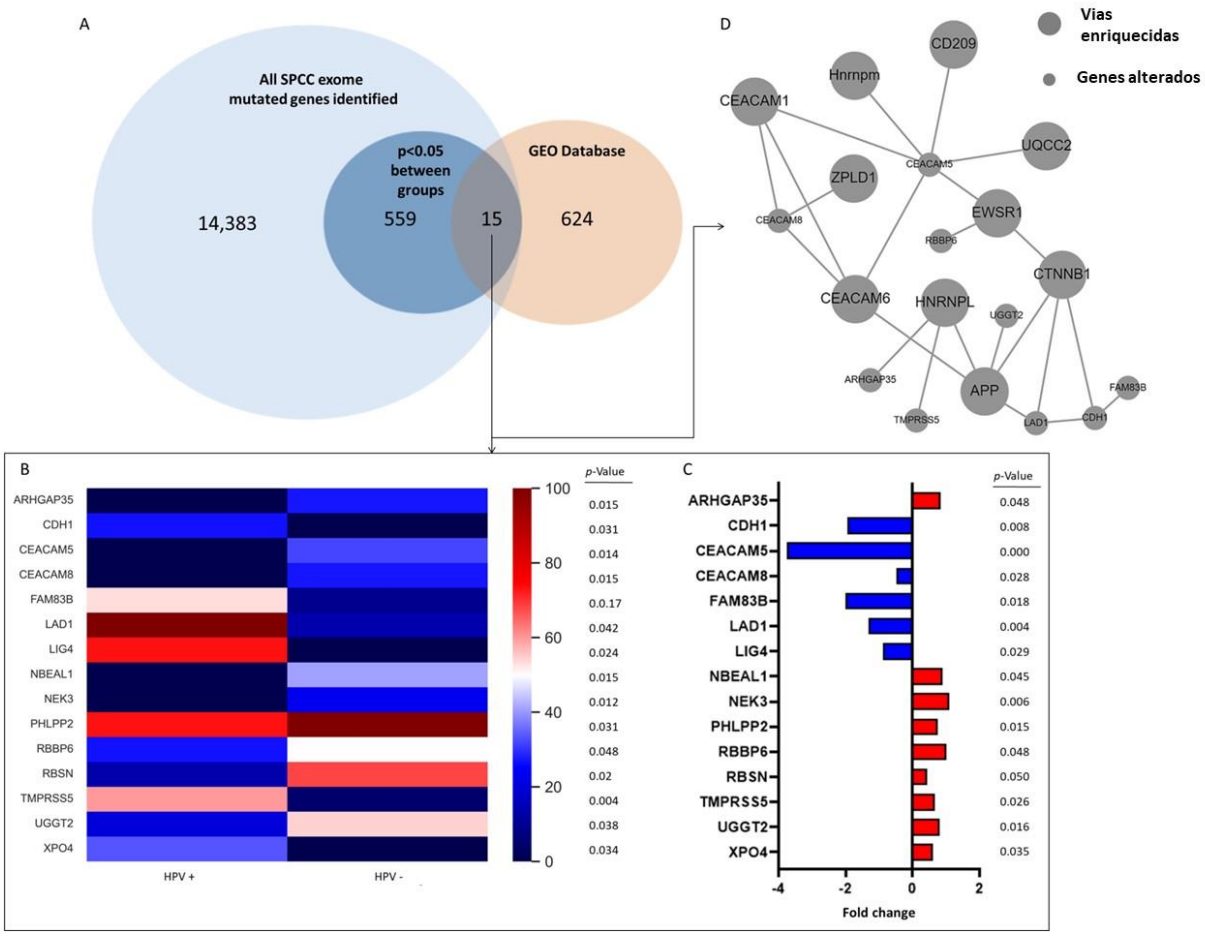


Figura 5. Comparação entre carga mutacional (Exoma) e expressão gênica via transcriptoma (GEO; GSE57955) entre grupos HPV+ e HPV- (A). Representação dos 15 alvos comuns entre Exoma e expressão via transcriptoma e sua frequência mutacional entre grupos HPV+ e HPV- (B). Alteração da expressão dos 15 alvos comuns de acordo com dados de expressão via transcriptoma entre grupos HPV+ e HPV- (C). O enriquecimento funcional de 9 dos 15 genes correlacionados construída pela ferramenta *WebGestalt*. A rede representa os 10 alvos de interações secundaria com maior relevância (p<0.05 ajustado) (D).



4.4. Discussão.

Os resultados do sequenciamento de exons mostram uma grande quantidade de genes com pelo menos uma alteração somática. Uma grande quantidade de variantes era esperado dado o grande potencial mutagênico de uma célula tumoral. Estima-se que pelo menos três mil variantes somáticas poderiam ser esperadas em regiões de exons em CPE (Canto et al., 2022). Embora nossos resultados indicam um número muito superior, observamos que uma parte dos nomes apresentados no painel de genes com maior frequência de variantes (Figura 1A) é semelhante a diferentes painéis de exoma já reportados anteriormente. Entretanto novos nomes também foram observados.

O gene *TTN* é responsável pela expressão da proteína titina, a maior proteína encontrada em células eucariotas, variando entre 2970 e 3700 kD, dependendo da isoforma. É codificada por um único gene, localizado no braço longo do cromossoma 2 no genoma humano (LeWinter et al., 2007). O gene *TTN* provavelmente está entre os genes com mais variantes descritos em diferentes trabalhos de sequenciamento de exons em CPE (Wang et al., 2019; Canto et al., 2022). Entretanto, embora o gene seja o primeiro da lista de genes com maior quantidade de variantes, seu nome é mais associado a cardiopatias que processos carcinogênicos (Tharp et al., 2019). Provavelmente a frequência de variantes encontradas esteja majoritariamente associada ao seu tamanho, que está composto por mais de 300 exons. Além disso, existe a possibilidade de que a grande maioria dessas variantes sejam do tipo nao patogenicas, com descrita por Campuzano e colaboradores (2015).

Um segundo gene de destaque em outros painéis e que também foi uma mutação observada na população estudada neste trabalho é o gene *FAT1 (*Wang et al., 2019; Chahoud et al., 2021*)*. A mutação em *FAT1* foi relatada em outros tipos de tumores, incluindo carcinoma de célula escamosa de cabeça e pescoço, câncer de cólon e glioblastoma (Morris et al., 2013), tanto em eventos de mutação e deleção. A mutação em *FAT1* promove tumorgenicidade através da desregulação da cascata de sinalização Wnt, promovendo a proliferação celular e o crescimento tumoral (Morris et al., 2013). Nossos resultados apresentam variantes somáticas que também foram encontradas em CPE em outras populações, como o gene *USH2A* (Canto et al., 2022), *FLG (*Wang et al., 2019*), DNAH6*, *PKD1* e *FSIP2 (Chahoud 2021)*. Entre o ranque dos dez primeiros genes mais frequentemente alterados, os genes *MUC5B*, *MUC16, OBSCN*, *MUC12*, *CMYA5*, *SVEP1, LEMAS5*, *SPTA1* e *FSIP2* não foram descritos anteriormente em peines de exoma.

Observamos que o padrão de variantes mais abundante foi de substituições nucleotídica de citosinas por timinas (C>T) e guaninas por adeninas (G>A). Curiosamente o enriquecimento de variantes C>T também foi o mais observado por Chahoud e colaboradores (2021). A substituição nucleotídica C > T é a alteração de base mais prevalente na maioria dos cânceres e é considerada ainda mais proeminente entre as substituições associadas a alterações de aminoácidos, sendo a mutação mais comum em cânceres de pulmão e neuroblastoma (Niroula e Vihinen, 2015). As substituições G>A também foi predominante em 40% de todas as substituições em amostras de câncer de pênis em um painel com 30 amostras de DNA sequenciadas de material parafinado (Wang et al., 2019) .

Dez vias de sinalização tradicionalmente ligadas ao ambiente carcinogênico, ciclo celular, Hippo, Myc, Notch, Nrf2, PI-3-Kinase/Akt, RTK-RAS, TGFβ, p53 e β-catenina/Wnt apresentaram alterações em frequências variadas nos resultados apresentados. Duas das vias com maior quantidade de variantes em gene observadas, RTK-RAS e HIPPO, foram descritas como vias de sinalização celular alteradas em CPE. Estima-se que elas possam representar as rotas de sinalização que mais contribuem para o processo de iniciação tumoral (Wang et al., 2019). A via RTK-RAS é provavelmente a via de transdução de sinal mais bem caracterizada na biologia celular. A sua função é transduzir sinais do meio extracelular para o núcleo celular, onde genes específicos são ativados para o crescimento, divisão e diferenciação celular (Molina e Adjei, 2006). A via Ras/Raf/MAPK também está envolvida na regulação do ciclo celular, cicatrização de feridas e reparação de tecidos, sinalização de integrina e migração celular (Stacey, 2003). Por sua vez, a regulação aberrante da via HIPPO confere às células-tronco cancerígenas propriedades como transformação epitelial-mesenquimal (EMT), resistência a medicamentos, auto-renovação e metástase (Lv e Zhou, 2023). Compreender esse contexto é importante dado que estudos indicam que genes de ambas as vias são alvos sensíveis a inibidores de EGFR (McFall e Stites, 2021; Alencar e Sonpavde, 2022)

Utilizamos de análises de enriquecimento funcional como uma ferramenta para descrever a anotação biológica das famílias de genes homólogos destacados no painel de ranqueamento dos 100 genes mais frequentemente alterados. Destacamos a correlação entre os genes *FAT1, FAT2* e *FAT3* a via HIPPO, onde a principal modulação da via se dá através da fosforilação das proteínas kinase LATS1 e LATS2 pelos genes FAT (Lv e Zhou, 2023). Embora o gene dos fatores de transcrição proteínas dedo de zinco não tenham sido destacados como genes mutados no CPE em

trabalhos anteriormente, nosso resultados mostram a presença de três genes pertencentes a família *ZNF* entre os mais alterados. Entretanto, em tumores gástricos (Li et al., 2014) e câncer (Sun et al., 2020) de esôfago a expressão dos genes *ZNF139* e *ZNF471* já foi associada a alta expressão de proteínas kinases MAPK10.

A interação entre esses 100 genes ranqueados mostra que outros 14 genes poderiam ser modulados de modo indireto em uma célula tumoral em CPE. Entre oncogênese (*NXF1, SRPK2, TRIM25, UBC, EGFR, CUL1* e *IRS4*) e supressores tumorais (*BRCA1, MTNR1A, ARRB1* e *HYPK*) que se destacaram no mapa de interação, observamos que a via de sinalização MAPK é novamente indicada como potencial rota perturbada no contexto tumoral estudado. As vias *NOTCH1* e *EGRF* também são apontadas em mais de um banco de dado, ressaltando a potencial influencia desses genes. O impacto que a atividade do supressor tumoral *NOTCH1* pode exercer sobre a carcinogênese em CPE foi apresentado recentemente por Necchi e colaboradores (2023), onde de um total de 397 amostras analisadas, 17.4% delas apresentavam mutação em *NOTCH1*. Para *EGRF*, 11.5% das amostras apresentaram alguma mutacao nesse gene (Necchi et al., 2023).

Muito embora a infecção por HPV tenha sido reportada como elemento de pouco o praticamente nula significância prognóstica em CPE (Santos et al., 2022; Hrudka et al., 2023) , sabe- se que o HPV é um vírus que pode comprometer a estabilidade genômica das células infectadas e além disso atualmente serve como um critério importante na classificação dos tumores de pênis pela Organização Mundial da Saúde (OMS), em subclassificações de lesões precursoras e tumores associados ao HPV ou não associados ao vírus. Nossos resultados mostraram que 574 genes apresentam diferença em carga mutacional entre grupos HPV+ e HPV. Entretanto, em uma perspectiva exploratória, a comparação entre grupos de HPV seja importante para a busca de marcadores moleculares associados a infecção, a carga mutacional tumoral, ou seja, a quantidade de variantes, em CPE provavelmente seja independente do estatus de HPV (Hrudka et al., 2024).

A comparação entre os resultados de exoma subdivididos por grupo de HPV+ e HPV- e de resultados de expressao de transcriptoma descrito previamente por Kuasne e colaboradores (2015), mostram a combinacao de 15 genes em comum, com perfil diferencialmente expressoes. O gene *CEACAM5* aparece destro dessa lista e o resultado de expressao previamente publicado mostrou o aumento da sua expressao em CPE. Esse dado é interessante visto que a expressao deste gene também foi maior em amostras HPV+ de carcinoma de células escamosas de cabeca e pescoco que em amostras HPV- (Wang et al., 2022). Curiosamente, insercao de fragmentos do material

genetico do subtipo viral HPV-16 foi vista em amostras de lesoes pré-cancerosas de colo de útero (Matovina et al., 2009).

Um segundo elemento de destaque que foi visto anteriormente como alvo de modulacao do vírus HPV é o gene *RBBP6*. Esse gene interage com as proteínas supressoras de tumor p53 e Rb, sugerindo o seu papel no controle da apoptose, entretanto sua expressao poderia estar regulada nagativamente pela preseca de HPV, como foi descrito para amostras de carcinoma de colo de útero (Dlamini et al., 2019). Em uma análise exploratória com os 15 genes em comum entre o painel de exoma e resultados de expressao em CPE, nove genes foram integrado pela análise de enriquecimento de vias. Essa útima análise mostrou o cruzamento com outros quetro genes, um gene supressor tumoral (*CEACMA1*) e três oncogenes (*CEACAM6, EWSR1* e *CTNNB1*).

A descrição dos resultados do painel de exoma apresentado aqui reafirma a presença de alvos já descritos anteriormente para o tumor CPE (TTN, FAT1, FLG e PKD1) e apresenta genes não reportados anteriormente (MUC5B, MUC16, OBSCN, MUC12, CMYA5, SVEP1, LEMAS5, SPTA1 e FSIP2). As análises de enriquecimento de vias apresentadas também apontam rotas tumorais (WNT, RTK/RAS, HIPPO e NOTCH) de interesse terapêutico e prognostico. Entretanto esses resultados devem ser revisados dado o alto número de variantes encontradas, o que poderia ser um número excessivo comparados com tumores de etiologia semelhante. Além disso, novas variantes descritas deveriam ser validades in vitro para confirmação do seu impacto na função gênica. Além disso, em uma nova revisao, devem passar por uma workflow de anotacao funcional de variantes somáticas (patogênica, provavelmente patogênica, significado incerto, provavelmente benigna e benigna) para que possamos entender a funcionalidade dessas variantes no contexto tumoral.

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5. CONCLUSÃO E CONSIDERAÇÕES FINAIS

Neste trabalho relatamos uma incidência de 53,2% de infecção por HPV de alto risco em homens com CPE no Estado do Ceará, Nordeste do Brasil, região com a baixa renda per capita no país e alta incidência da doença. Apesar do aumento da incidência, a infecção por HPV não foi associada a mau prognóstico, tal como metástase sistêmica ou linfonodal, locorregional recorrência, e sim poderia se associar ao aumento da taxa de sobrevivência. Dentr os aspectos correlacionados, metástases e recidivas foram as características que mais impactaram negativamente a sobrevida dos pacientes. Também observamos, pela primeira vez, que o aumento da marca epigenética 5mC e redução de 5hmC são características da doença. Apesar da ausência de significância estatística, o auto padrao de 5mC (aproximadamente 80%) pode contribuir para prognóstico ruim (HR = 1,06). Embora a infecção viral contribua para a perda da reprogramação de DNA, a relação das marcas 5mC/5hmC não foi relacionada à infecção por HPV, e os níveis de 5hmC foram aumentados em amostras p16INK4a negativas.

Observamos um padrao de hipometilacao na regiao controladora de imprinting H19DMR, dado a perda de metilacao esperada em comparacao com o padrao teórico descrito na literatura para uma célula nao tumoral. A hipometilacao descrita nao está relacionada a infecao viral por HPV. Em uma análise preliminar de um painel de sequenciamento de exons, observamos na nossa população de estudo genes com alta frequencia mutacional não descritos anteriormente (*MUC5B*, *MUC16*, *OBSCN*, *MUC12*, *CMYA5*, *SVEP1*, *LEMAS5*, *SPTA1* e *FSIP2*). Esses dados ainda devem ser melhorados através de análises cofirmatorias e análises *in vitro*, entretando ressaltamos que essa análise poucas vezes foi aplicada em CPE em amostras de indivíduos barsileiros.

A identificação de marcadores genéticos, epigenéticos e alterações moleculares no CPE, tumor extremamente agressivo e com maior incidência em países em desenvolvimento, cujas terapias atuais são limitadas e baseadas principalmente na excisão cirúrgica, pode auxiliar no

diagnóstico, prognóstico e tratamento da doença. A caracterização molecular e do padrão de metilação no CPE incentiva e favorece que novos estudos possam sugerir estratégias de tratamento específicas, como agentes hipometilantes para terapias direcionadas epigenéticas (epidfármacos), estabelecer novos alvos com perfil *druggable genes* e incentivar a busca de terapias menos invasivas.