EMILY MONTOSA NUNES

Propriedades biológicas e bioquímicas de variantes naturais de HPV-18

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Programa de Oncologia

Orientadora: Prof.ª Dr.ª Laura Cristina Sichero Vettorazzo

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Dados Internacionais de Catalogação na Publicação (CIP)

Preparada pela Biblioteca da
Faculdade de Medicina da Universidade de São Paulo

Nunes, Emily Montosa
Propriedades biológicas e bioquímicas de variantes naturais de HPV-18 / Emily Montosa Nunes. -- São Paulo, 2020.
Tese(doutorado)--Faculdade de Medicina da Universidade de São Paulo.
Programa de Oncologia.
Orientadora: Laura Cristina Sichero Vettorazzo.

Descritores: 1.Papilomavírus humano 18 2.Neoplasias do colo do útero 3.Proteínas oncogênicas virais 4.Transformação celular viral 5.Vírus oncogênicos 6.Transformação celular neoplásica

USP/FM/DBD-100/20

Responsável: Erinalva da Conceição Batista, CRB-8 6755

Aos que dedicaram e dedicam suas vidas às Ciências.

Aos que forneceram e fornecem seu corpo e mente a compreensão das patologias.

Aos acometidos por HPV e cânceres associados.

AGRADECIMENTOS

À Deus por todas as oportunidades concedidas ao longo da minha vida e que me possibilitaram chegar até aqui.

Aos meus pais que sempre acreditaram nos meus sonhos e lutaram com garra até o momento que puderam para que eu os realizasse.

À minha mãe (Solange Montosa Nunes), irmã (Gabriela Montosa Nunes) e namorado (Edoardo Asquini) pela compreensão, paciência, estimulo e também distrações necessárias durante os longos dias dedicados à pesquisa e principalmente à elaboração desta tese.

À Prof.ª Dr.ª Laura Sichero que, durante nossos quase oito anos juntos, foi mais do que orientadora, mas também mãe e amiga. Ela sempre acreditou em meu potencial, defendeu-me, estimulou-me e possibilitou que eu alcançasse novos horizontes na área da pesquisa.

À Prof.ª Dr.ª Luisa Villa por sempre acolher a todos como uma grande mãe nesta família que é o Grupo HPV.

Ao João Simão Sobrinho e Neide Ferreira por toda a parceria e colaboração. Obrigada pelas horas passadas no laboratório entre piadas, lanches, almoços e desabafos. Com certeza este trabalho não seria possível sem vocês.

Às minhas colegas de laboratório (Aline Lopes, Amanda Schiersner e Valeria Nunes) por toda ajuda, colaboração e compreensão. Obrigada por todas as horas de lazer, foi maravilhoso estar com vocês.

Ao Dr. Lawrence Banks e seu grupo de pesquisa *Tumour Virology* por me receberem e integrarem à família. Obrigada pela paciência perante minhas dificuldades, ajuda e conselhos. Com certeza parte deste trabalho não seria o mesmo sem vocês.

Ao Grupo HPV por todo o esclarecimento de dúvidas, conselhos e colaborações.

A todos os colegas do Instituto do Câncer do Estado de São Paulo por todos os conselhos e ajuda.

Às agências de fomentos Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) por depositarem confiança à mim e minha orientadora, possibilitando a realização deste trabalho e a divulgação dos resultados no país e exterior através dos seguintes financiamentos: Auxílio Regular (FAPESP Processo nº 2007/58590-7 à Laura Sichero), Bolsa de Produtividade em Pesquisa 2 (CNPq 303431/2018-0 à Laura Sichero), Bolsa de Doutorado (Processo nº 2015/26346-6 à mim) e Bolsa de Estágio no Exterior (Processo nº 2018/14697-7 à mim).

E por fim, aos integrantes desta banca que aceitaram avaliar este trabalho e dedicaram seu tempo nesta importante etapa da minha formação profissional.

Muito Obrigada!

“Já há muito tempo, atentei para o fato de que pessoas de grandes realizações raramente relaxam e deixam as coisas acontecerem para elas. Elas correm atrás e fazem acontecer”.

Leonardo da Vinci.

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Nunes EM. *Propriedades biológicas e bioquímicas de variantes naturais de HPV-18* [Tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2020.

As infecções por HPV-16 e HPV-18 estão fortemente associadas ao risco de desenvolvimento de câncer cervical. Em todo o mundo, o HPV-16 é o tipo mais prevalente em carcinomas escamosos invasivos do colo do útero, seguido de HPV-18, o qual é similarmente prevalentes nos casos de adenocarcinoma. A variabilidade nucleotídica intra-típica de HPV-18 tem sido estudada resultando em importantes achados no que concerne à filogenia e evolução do vírus e a história natural das infecções. Embora os estudos acerca da relevância clínica da variabilidade genética de HPV-18 são limitados, sugeriu-se que variantes Ameríndias (As+AI) e Européias (E) representem isolados com um maior potencial oncogênico, quando comparadas às variantes Africanas (Af). Ademais, foi observado que variantes Af de HPV-18 são exclusivamente detectadas nas amostras de carcinoma escamoso invasivo do colo do útero, ao passo que variantes E e As+AI são mais prevalentes em adenocarcinoma e carcinoma adenoescamoso. No que concerne às diferenças biológicas entre as variantes de HPV-18, é fundamental ressaltar que há poucos estudos conduzidos até o momento e estes exploraram somente a variabilidade da oncoproteína viral E6, sendo que nenhum dos estudos foi realizado em modelos de células hospedeiras naturais de HPV-18, que são queratinócitos humanos primários (PHK). Pelo exposto, nós caracterizamos funcionalmente PHKs imortalizados por duas variantes distintas de HPV-18. Os PHKs foram transduzidos com E6/E7 das variantes As+AI e Af de HPV-18 (PHK18AA e PHK18Af, respectivamente) e subcultivados até p30, quando estes foram considerados imortalizados. Os PHK18AA atingiram à imortalização significativamente mais rápido do que os PHK18Af, ainda que uma “crise” não fosse observada em nenhum dos PHKs transduzidos. No entanto, não houve diferença nas taxas de proliferação entre os PHK18AA e PHK18Af em p30. Contudo, os PHK18AA imortalizados foram mais eficientes em formar colônias e esferoides em monocamada e cultura tridimensional, respectivamente, em experimentos diversos. Ademais, os PHK18AA imortalizados apresentaram uma maior eficiência em invadir através de uma matriz de colágeno, ainda que diferenças na migração não fossem observadas entre as variantes. Nós também observamos que células primárias de rim de ratos neonatos (BRK) co-transfectadas com *E7* Af e *RAS* geraram um menor número de colônias, o que pode estar associado em parte à incapacidade de E7 Af em formar um complexo com a ubiquitina ligase UBR4 e degradar a proteína supressora tumoral PTPN14. Por outro lado, os PHK18Af imortalizados apresentaram maior resistência à diferenciação em meio enriquecido com soro e cálcio. Finalmente, culturas organotípicas derivadas dos PHKs imortalizados por ambas as variantes de HPV-18 eram semelhantes na morfologia e apresentaram níveis equivalentes de proteínas associadas à diferenciação e proliferação celular. Em conjunto, nossos dados indicam que a variante As+AI de HPV-18 tem um maior potencial oncogênico em comparação à variante Af, além das variantes interagirem diferentemente com alguns alvos celulares. Este estudo é único em analisar as propriedades bioquímicas e biológicas das oncoproteínas E6/E7 de variantes naturais HPV-18 no contexto do hospedeiro natural, isto é, as células epiteliais.

Descritores: Papilomavírus humano 18; Neoplasias do colo do útero; Proteínas oncogênicas virais; Transformação celular viral; Vírus oncogênicos; Transformação celular neoplásica.

ABSTRACT

Nunes EM. *Biological and biochemical properties of natural variants of HPV-18* [Thesis]. São Paulo: “Faculdade de Medicina, Universidade de São Paulo”; 2020.

HPV-16 and HPV-18 infections are strongly associated with the risk of developing cervical cancer. Worldwide, HPV-16 type is the most prevalent type in invasive squamous cell carcinomas of the cervix followed by HPV-18, whereas both viral types are similarly prevalent in adenocarcinoma cases. HPV-18 intratypic nucleotide variability has been studied resulting in important findings concerning the evolution and phylogeny of the virus and the natural history of infections. Although the studies about the clinical relevance of HPV-18 genetic variability are very limited, overall, it is suggested that Amerindian (As+AI) and European (E) variants represent isolates with a higher oncogenic potential compared to the African (Af) variants. Furthermore, it was observed that HPV-18 Af variants are exclusively detected in samples of invasive squamous cell carcinoma of the cervix, whereas As+AI and E variants are more prevalent in adenocarcinoma and adenosquamous cell carcinoma. With regard to biological differences among HPV-18 variants, it is crucial to emphasize that the few studies conducted so far explored only the variability of the viral E6 oncoprotein, and most importantly, none of the studies was performed in HPV-18 natural host cell models, which are primary human keratinocytes (PHK). For these reasons, we characterized functionally PHKs immortalized by two different variants of HPV-18. PHKs were transduced with E6/E7 of HPV-18 As+AI and Af variants (PHK18AA and PHK18Af, respectively), and subcultured until p30, when these were considered immortalized. PHK18AA reached immortalization significantly faster than PHK18Af, even though a “crisis” was not observed in any of the transduced PHKs. Nevertheless, there were not proliferation rates difference among PHK18AA e PHK18Af at p30. However, immortalized PHK18AA were more efficient in forming colonies and spheroids in monolayer and three-dimensional cultures, respectively, using different assays. In addition, immortalized PHK18AA showed greater efficiency in invading through a collagen matrix, although no differences in migration were observed among variants. We also observed that baby rat kidney cells (BRK) co-transfected with E7 Af and RAS generated a smaller number of colonies, which may be partly associated with the inability of E7 Af to form a complex with the ubiquitin ligase UBR4, and further degrade the tumor suppressor protein PTPN14. On the other hand, immortalized PHK18Af showed greater resistance to differentiation in a medium enriched with serum and calcium. Finally, raft cultures derived from PHKs immortalized by both HPV-18 variants were morphologically similar and showed equivalent levels of proteins associated with cell differentiation and proliferation. Taken together, our data indicates that the HPV-18 As+AI variant has a greater oncogenic potential compared to the Af counterpart, in addition to interacting differently with some cellular targets. This study is unique in analyzing the biological and biochemical of E6/E7 oncoproteins of natural variants of HPV-18 in the context of the natural host, that is, epithelial cells.

Descriptors: Papillomavirus humano 18; Uterine cervical neoplasms; Oncogene proteins, viral; Cell transformation, viral; Oncogenic viruses; Cell transformation, neoplastic.

1 INTRODUÇÃO

O câncer do colo do útero é o quarto tipo de neoplasia mais incidente entre as mulheres no mundo (IARC, 2018) e o terceiro no Brasil (INCA, 2020). A infecção persistente por papilomavírus humano (HPV) de alto risco oncogênico é o principal fator etiológico para o desenvolvimento de neoplasias na região anogenital, principalmente no colo do útero (revisado por Wheeler, 2013; Walboomers *et al.*, 1999).

Os HPVs, vírus pertencentes à família *Papillomaviridae* (revisado por Bernard, 2005), possuem um capsídeo não-envelopado de 50 nm de diâmetro que engloba uma molécula de DNA dupla fita circular de aproximadamente 8 kb dividida em três regiões: precoce, tardia e LCR. A região precoce (*early*, E) tem como função regular a transcrição e replicação do DNA (E1, E2), assim como a transformação celular (E5, E6 e E7). Os genes *L1* e *L2*, da região tardia (*late*, L), transcrevem as proteínas principal e secundária do capsídeo, respectivamente (revisado por IARC, 2012). A região não codificante, encontrada entre os genes *L1* e *E6*, é denominada de região longa de controle (LCR) e compreende aproximadamente 10% do genoma viral (figura 1A). A regulação da expressão viral é complexa e controlada por fatores de transcrição virais e celulares que se ligam a sequências específicas da LCR regulando a transcrição dos genes precoces (revisado por Bernard, 2002; Chan *et al.*, 1989).

O ciclo de vida do HPV está intimamente relacionado ao programa de diferenciação do epitélio estratificado, sendo que as células epiteliais em proliferação, encontradas na camada basal, contêm o genoma viral na forma latente e episssomal. Nas camadas suprabasais ocorre a replicação vegetativa do DNA viral e tradução das proteínas virais. Por fim, nas células mais superficiais do epitélio, há a formação dos virions (figura 1B).

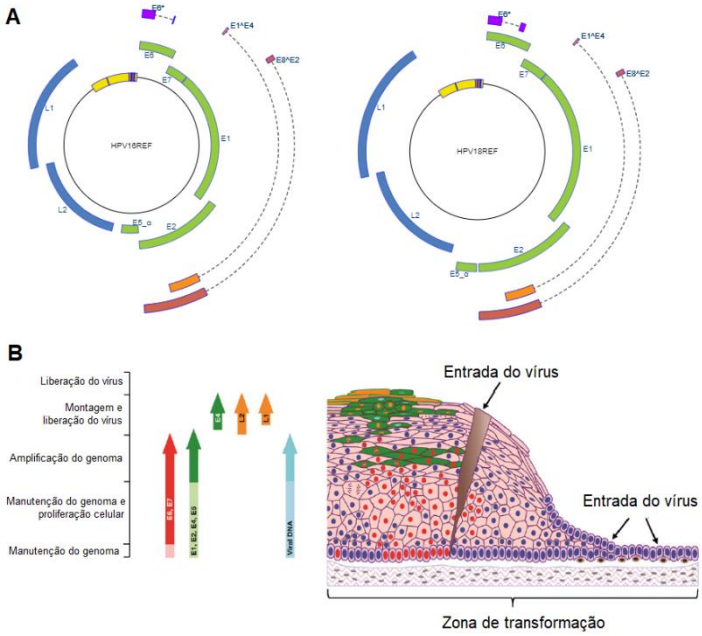

Figura 1 – Papilomavírus humano (HPV) e o ciclo viral. (A) Representação do genoma circular viral dos principais tipos de alto risco oncogênico, HPV-16 e HPV-18. Estão representados os genes (*E1-E8*, *L1*, *L2*), os produtos de processamento alternativo (*splicing)* (E6*, E1^E4, E8^E2), além da região longa de controle (LCR) em amarelo (Fonte: Papillomavirus episteme, PaVE,2020). (B) Representação do ciclo de vida do HPV intimamente relacionado ao programa de diferenciação do epitélio estratificado. A imagem apresenta a zona de transformação do epitélio do colo do útero e as principais regiões de entrada do vírus. Cada grupo de genes, além do genoma viral, estão ilustrado por cores (à esquerda) cujos produtos de expressão podem ser verificados na imagem do epitélio estratificado (à direita) (Fonte: adaptado da revisão Doorbar et al., 2012).

Até o momento mais de 200 tipos de HPV já foram descritos dos quais aproximadamente 40 infectam o trato anogenital (revisado por de IARC, 2012). A maioria dos HPV de alto risco oncogênico, àqueles associados com ao desenvolvimento do câncer de colo de útero, estão filogeneticamente agrupados nas espécies *Alphapapillomavirus* 9 (relacionados com o HPV-16) ou *Alphapapillomavirus* 7 (relacionados com o HPV-18). Tipos de HPV destas duas espécies são responsáveis por aproximadamente 75% e 15% de todos os tumores de colo de útero ao redor do mundo, respectivamente (Bouvard *et al.*,

2009). O HPV-16 é o tipo mais prevalente, tanto em neoplasias pré-invasivas intra-epiteliais quanto em carcinomas escamosos invasivos do colo do útero, seguido pelo HPV-18 (Walboomers *et al.*, 1999). Entretanto, os HPV-16 e HPV-18 são detectados com prevalência semelhante em amostras de adenocarcinoma (Fujiwara *et al.*, 1995). Foi sugerido que a infecção por HPV-18 possa estar associada com uma forma mais agressiva da neoplasia intra-epitelial cervical, adenocarcinoma e carcinoma adenoescamoso, além de maior taxa de integração do genoma, e maior probabilidade de recorrência do tumor e metástase no linfonodo (Arends *et al.*, 1993; Sellors e Sankaranarayanan, 2003; revisado por IARC, 2012). Entretanto, estes dados ainda são motivo de debate.

1. Potencial oncogênico de HPV

Atualmente se sabe que em lesões malignas (por exemplo, neoplasias intra-epiteliais do colo do útero de grau III, carcinoma *in situ* e câncer invasivo), o DNA de HPV está geralmente integrado ao genoma da célula (Cullen *et al.*, 1991), sendo que a integração parece acontecer ao acaso (revisado por Wentzensen *et al.*, 2004). Durante a integração, geralmente ocorre uma ruptura dos genes virais *E1* ou *E2* levando à interrupção do processo de regulação transcricional viral exercido por *E2*. Uma vez que esta proteína se liga à LCR, inibindo a transcrição a partir do promotor precoce principal P97 (revisado por Bernard, 2002), ocorre síntese contínua das oncoproteínas virais E6/E7 que propiciam a imortalização celular em cooperação (figura 1B).

Estudos funcionais sobre as propriedades transformantes dos HPVs de alto risco oncogênico foram principalmente conduzidos com HPV-16 e HPV-18 devido à forte associação aos cânceres já explanada. Atualmente, já se tem o

conhecimento de que ambos os tipos virais são capazes de induzir a imortalização de queratinócitos humanos primários (PHK, *primary human keratinocytes*), uma vez que estas células infectadas são capazes de ultrapassar duas barreiras proliferativas sucessivas: senescência e crise (Steenbergen *et al.*, 2005). A resistência à senescência se deve principalmente à expressão das oncoproteínas virais E6 e E7 que agem sinergicamente para desregular a apoptose e o ciclo celular da célula infectada, respectivamente. Após contornar a senescência, as células infectadas têm um tempo de vida estendido, embora ainda limitado, sendo que a subsequente imortalização das células é geralmente precedida por um segundo período de barreira proliferativa denominado de crise (Shay *et al.*, 2005). Na maior parte dos casos, a imortalização celular é caracterizada pelo aumento da atividade da enzima telomerase, resultado da expressão aumentada da subunidade catalítica hTERT.

É interessante ressaltar que diferentes proteínas celulares interagem com E6 e E7 de HPVs de alto e baixo risco oncogênico (Gupta *et al.*, 2003; revisado por Pim e Banks, 2010). Essas proteínas estão localizadas em diferentes compartimentos celulares e devem contribuir para a atividade transformante de HPV de formas diversas. A relevância fisiológica de todas essas interações ainda não está totalmente elucidada.

Diversos estudos sugerem que a ligação da oncoproteína E6 aos integrantes da família PDZ e a ativação de hTERT são necessárias para o processo de imortalização (Kyono *et al.*, 1997; revisado por Pim e Banks, 2010; Klingelhutz *et al.*, 1996; Schütze *et al.*, 2014). Além disso, a degradação de p53, mediada por E6 de HPVs de alto risco oncogênico, é o fator mais importante para

o processo de transformação celular induzida por estes vírus (Scheffner *et al.*, 1993; Werness *et al.*, 1990; revisado por Longworth e Laimins, 2004a).

Um dos principais alvos da oncoproteína E7 é a forma hipofosforilada de pRb, resultando na degradação desta proteína pela via de proteólise dependente de ubiquitinação (Wang *et al.*, 2001). Assim, a célula perde um dos principais reguladores negativos do ciclo celular, uma vez que ocorre a liberação dos fatores de transcrição da família E2F, antes associados em complexos com pRb (Dyson *et al.*, 1989). Quando o fator de transcrição E2F está livre, liga-se ao DNA resultando e ativa o processo de replicação. Em consequência, as células suprabasais infectadas são capazes de reativar a proliferação no tecido (Cheng *et al.*, 1995). Além disso, também foi demonstrado que a oncoproteína E7 é capaz de associa-se a outras proteínas envolvidas no ciclo celular como: parceiros de pRb (p.e., p105, p107, p130), inibidores de quinase dependente de ciclina (p.e., p27, p21), histonas deacetilases (HDAC), ciclinas E e A, entre outros (revisado por Longworth e Laimins, 2004b; Zerfass *et al.*, 1995) (figura 2).

Ainda assim, a atividade biológica das proteínas virais E6 e E7 depende da célula hospedeira infectada pelo vírus ou transfectada com genes de HPV. Células escamosas epiteliais são o hospedeiro natural de HPV, e culturas de queratinócitos genitais humanos têm sido utilizadas para estudar as consequências da expressão de E6 e E7. Estes estudos mostraram que ambas as proteínas cooperam na imortalização de PHKs e na inibição da diferenciação terminal destas células induzida por soro e cálcio (Barbosa e Schlegel, 1989; Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989; Schlegel *et al.*, 1988). Entretanto, embora possuam crescimento e diferenciação alterados, estas células imortalizadas por HPV não são capazes de crescer independentemente de

ancoragem e de induzir o crescimento de tumores em camundongos atímicos (Schlegel *et al.*, 1988), sendo que mudanças genéticas adicionais parecem ser necessárias para a progressão maligna. Entretanto, quando culturas de queratinócitos imortalizados por HPV-16 e HPV-18 são utilizadas em culturas organotípicas, observa-se que os tecidos formados exibem anormalidades histológicas semelhantes às observadas em neoplasias intra-epiteliais do colo do útero (Blanton *et al.*, 1991; Garner-Hamrick *et al.*, 2004).

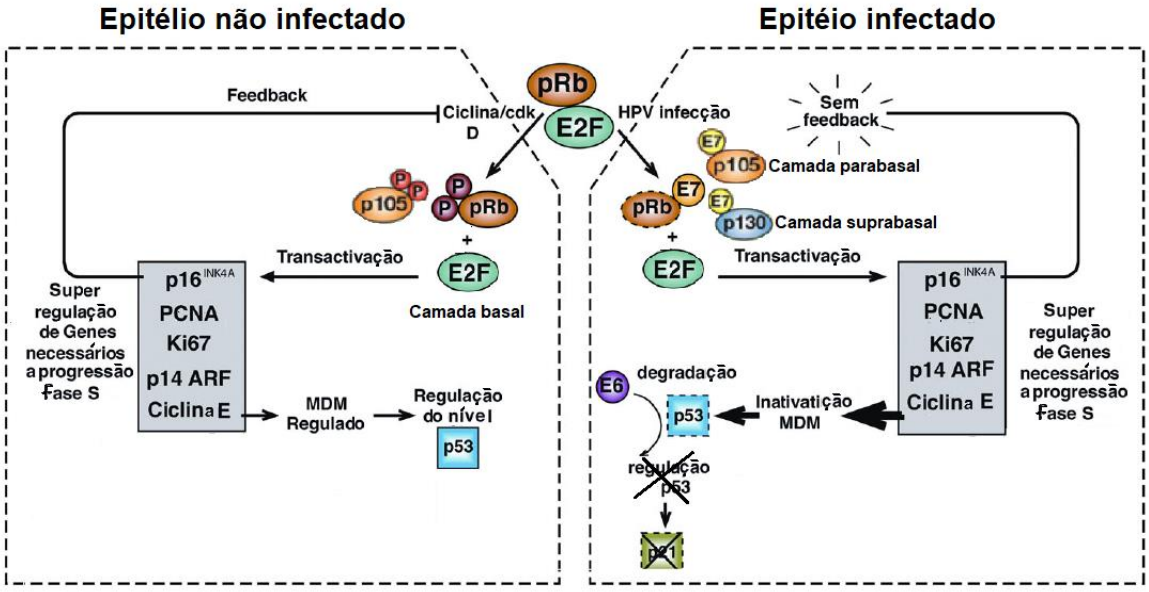

Figura 2 – Infecção pelo papilomavírus humano de alto risco (HPV) nas vias moleculares que regulam a diferenciação epitelial e a proliferação celular. A progressão do ciclo celular é regulada nas diferentes camadas epiteliais pelos membros da família de proteínas pRb (retinoblastoma). As proteínas E7 dos tipos de HPV de alto risco são capazes de degradar os membros desta família e liberar fatores de transcrição E2F que permite que células basais e parabasais entrem na fase S. (Fonte: adaptado de Doorbar *et al.*, 2012,2015).

2. Variabilidade genética intra-típica de HPV-18

A classificação filogenética do HPV é baseada na identidade da sequência do gene *L1*, uma das regiões mais conservada do genoma viral, permitindo o agrupamento dos vírus em diferentes níveis taxonômicos. Novos

tipos virais são definidos por variações maiores que 10%, subtipos por variações entre 2-10% e variantes por substituições menores que 2% (revisado por Bernard, 2005). No que concerne à análise do genoma viral completo, foi demonstrado que 4% e 9,9% das sequências nucleotídicas e de aminoácido são variáveis, respectivamente, entre as variantes moleculares de HPV-16 (Chen *et al.*, 2005). Maior conservação é observada entre as variantes de HPV-18, sendo descrito que 3,8% e 4,7% das sequências nucleotídicas e de aminoácido são variáveis, respectivamente (Chen *et al.*, 2013). É importante ressaltar que a variabilidade genética nos diferentes genes é filogeneticamente compatível, ou seja, mudanças nucleotídicas em um gene encontram correspondência com alterações nucleotídicas em outros genes (Chen *et al.*, 2005; Smith *et al.*, 2011; Yamada *et al.*, 1995).

A análise da variabilidade nucleotídica intra-típica de HPV-18 tem sido estudada servindo como ferramenta importante para traçar a filogenia e evolução destes vírus, além de ser utilizada em estudos sobre a história natural das infecções por HPV. No início dos anos 90, um fragmento da LCR foi sequenciado de uma compilação de amostras HPV-18 positivas oriundas de quatro continentes (Ong *et al.*, 1993). As sequências geradas foram comparadas àquela denominada de protótipo (primeiro isolado de HPV-18 a ser completamente sequenciado), a qual foi isolada de um tumor de colo uterino obtido de uma paciente do Nordeste do Brasil (Boshart *et al.*, 1984). A partir das sequências geradas, foi construída uma árvore filogenética que se observou ser composta por três ramos distintos, cuja nomenclatura foi baseada na origem da maior parte dos isolados que os compõe: ramo Africano (Af), Ameríndio (As+AI) e Europeu (E). A variante protótipo de HPV-18 é um membro do ramo As+AI. Mais recentemente, a análise

do genoma completo de HPV-18 de espécimes oriundos de todo o mundo revelou que a maior parte das alterações nucleotídicas detectadas separa as variantes E e As+AI daquelas do ramo Af (Chen *et al.*, 2013). Nesta nova análise, as variantes do ramo As+AI passaram a ramificar-se na linhagem A, dentre as sublinhagens 1 e 2 (A1 e A2); as variantes do ramo E foram reclassificadas entre as sublinhagens A3 a A5; e as variantes Af são agora identificadas nas sublinhagens B1 a B3, além da linhagem C (figura 3) (Chen *et al.*, 2013). Enquanto que algumas variantes são encontradas por todo o mundo, outras parecem estar associadas a determinados grupos étnicos (Chen *et al.*, 2015). A dispersão e a baixa taxa de evolução de HPV-18 detectada sugerem que estes co-evoluíram com os seus hospedeiros naturais em um período de milhões de anos. Desta maneira, a colonização das Américas por europeus e africanos é refletida na composição das variantes de HPV destes continentes (Arias-Pulido *et al.*, 2005; Calleja-Macias *et al.*, 2004; Chen *et al.*, 2015; Sichero *et al.*, 2007; Ong *et al.*, 1993).

3. Variabilidade intra-típica de HPV-18 e risco de neoplasia do colo do útero

No que concerne à história natural das infecções por HPV, o estudo de variabilidade genética é fundamental para distinguir a infecção persistente da reaquisição da infecção por diferentes variantes moleculares do mesmo tipo viral (Mayrand *et al.*, 2000; Villa *et al.*, 2000). Adicionalmente, alguns estudos tem visado analisar a associação de variantes moleculares de HPV-18 específicas com o risco diferencial de persistência da infecção viral e de desenvolvimento de lesão do colo do útero maligna (revisado por Sichero e Villa, 2006).

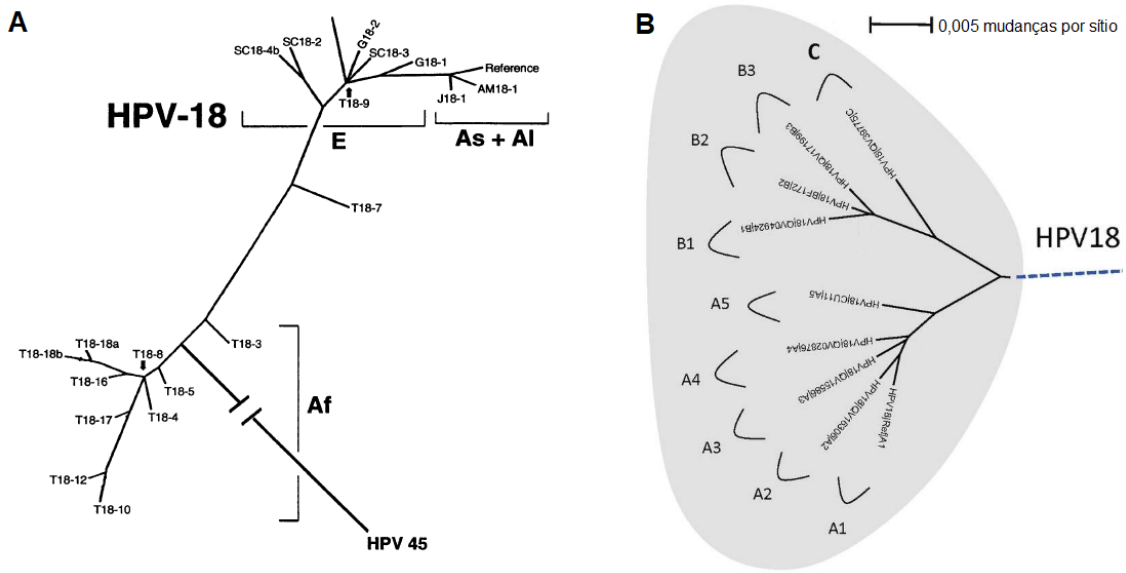

Figura 3 – Árvores filogenéticas de HPV-18. (A) Árvore filogenética desenvolvida por Ong e col (1993) baseou-se em fragmento de LCR de 321 pb identificados em amostras de pacientes de coortes representativas na África (Tanzânia, amostras T), Europa (Alemanha, amostras G; Escócia, amostras SC) e Ásia Oriental (Japão, amostras J). Os colchetes Af, E e As+Al referem-se a ramos com variantes que ocorrem com maior frequência em pacientes africanos, europeus, do leste asiático e indianos americanos. A amostra HPV-18 T18-8 é o clone referência (protótipo). (B) Árvore filogenética desenvolvida por Chen e col. (2013) a partir do alinhamento de sequências completas do genoma circular linearizadas. A linha sólida preta representa a distância entre os clados, com o número de alterações descrito em legenda no canto superior direito da figura (Fonte: adaptado de Ong *et al.*, 1993; Chen *et al.*, 2013)

A maior parte dos estudos epidemiológicos define a infecção persistente e transiente por HPV ao nível de tipo viral. Entretanto, a análise de variantes moleculares poderia revelar taxas de persistência menores que aquelas medidas em estudos baseados apenas na tipagem (Franco *et al.*, 1994). No nosso meio, foi conduzido um estudo epidemiológico prospectivo sobre a história natural das infecções por HPV (estudo Ludwig/McGill) abrangendo aproximadamente 2.500 mulheres residentes na cidade de São Paulo (Franco *et al.*, 1999). A análise da sequência nucleotídica das amostras HPV-18 positivas oriundas deste estudo revelaram a presença de variantes moleculares dos três ramos filogenéticos nesta população, sendo que variantes do ramo E foram as mais prevalentes (80% das

amostras). Foi também observado que mulheres em que foi detectado DNA de HPV-18 em mais de uma visita, eram sempre positivas para mesma variante molecular. Ainda mais, foi observado que variantes E de HPV-18 estão significativamente mais associadas à persistência da infecção viral (Sichero *et al.*, 2007). Devido ao pequeno número de mulheres neste estudo em que foi detectado DNA de HPV-18 e que desenvolveram lesão intra-epitelial cervical, infelizmente não foi possível analisar o risco de desenvolvimento de lesão atribuível às diferentes variantes moleculares.

Embora hoje não exista mais dúvida de que variantes As+AI de HPV-16 estão mais associadas ao risco de desenvolvimento de neoplasia cervical (Berumen *et al.*, 2001; Hildesheim *et al.*, 2001; Sichero *et al.*, 2007, Xi *et al.*, 2007), os dados acerca da relevância clínica da variabilidade genética de HPV-18 são ainda limitados. Inicialmente, a análise da sequência nucleotídica de um fragmento do gene *E2* sugeriu que variantes Af de HPV-18 têm menor potencial oncogênico em amostras oriundas dos Estados Unidos, embora os achados não atingissem significância estatística (Hecht *et al.*, 1995). Em outro estudo realizado em mulheres de Portugal foi observado uma tendência, embora também não estatisticamente significativa, de variantes As+AI de HPV-18 estarem associadas com risco aumentado de desenvolvimento de câncer de colo do útero quando comparadas às variantes do ramo E (Pista *et al.*, 2007). Adicionalmente, outro estudo conduzido nos Estados Unidos revelou que, em relação às variantes do ramo Af, o risco de desenvolvimento de lesão intra-epitelial neoplásica de grau III (CIN3) foi 3,8 e 4,8 vezes maior em mulheres em que foram detectadas variantes dos ramos E e As+AI, respectivamente (Xi *et al.*, 2007). Em conjunto, estes dados indicam que as variantes do ramo As+AI de HPV-18 poderiam representar maior

potencial oncogênico se comparadas às variantes do ramo Af. Ao contrário, foi observado em um estudo conduzido na Espanha que variantes Af estavam associadas a um risco aumentado, porém não significativo, de desenvolvimento de CIN3 em comparação com variantes E e As+AI (Pérez *et al.*, 2014). Cabe ressaltar que nenhuma diferença significativa no risco de desenvolvimento de CIN3 atribuível às diferentes variantes moleculares de HPV-18 foi observada em outros estudos conduzidos na Costa Rica (Schiffman *et al.*, 2010), nos Estados Unidos (Arias-Pulido *et al.*, 2005) e em outras regiões geográficas (Chen *et al.*, 2015). Pelo exposto, torna-se difícil concluir se há evidências contundentes de que diferentes variantes de HPV-18 estão associadas a diferentes riscos de desenvolvimento de lesão e câncer de colo uterino.

A associação entre as diversas variantes moleculares de HPV-18 e a histopatologia dos cânceres do colo do útero foi também descrita em diferentes estudos. Foi observado que variantes Af de HPV-18 são exclusivamente detectadas entre as amostras de carcinoma escamoso invasivo do colo do útero, ao passo que variantes E e As+AI são mais prevalentes em amostras de adenocarcinoma e carcinoma adenoescamoso, sugerindo que variantes Af podem estar associadas com tumores com comportamento menos agressivo, uma vez que os carcinomas escamosos tem um prognóstico relativamente melhor quando comparado aos adenocarcinoma e carcinoma adenoescamoso (Burk *et al.*, 2003; de Boer *et al.*, 2005; Lizano e García-Carrancá , 1997). Entretanto, outros estudos não apoiam essa associação (Arias-Pulido *et al.*, 2005; Chen *et al.*, 2015).

4. Variabilidade genética de HPV-18 e potencial oncogênico

A baixa taxa geral de substituições não-sinônimas observadas em HPVs sugere que estes vírus estão sob forte pressão seletiva. Além disso, a reduzida variação de aminoácido também pode ser atribuída ao fato de que estes vírus utilizam a maquinaria de replicação de DNA celular que é caracterizada por alta fidelidade, capacidade de revisão e mecanismos de reparo pós-replicação.

A observação de que apenas as proteínas E6 e E7 oriundas de HPVs de alto risco oncogênico são capazes de imortalizar PHKs em cultura e de alterar as funções normais de proteínas supressoras de tumor, já sugeria a importância da variabilidade genética no potencial oncogênico dos HPVs (Elbel *et al.*, 1997; revisado por zur Hausen, 2002). Embora HPV-16 e HPV-18 sejam capazes de inibir a diferenciação terminal de queratinócitos induzida por soro e cálcio, foi observado que o HPV-18 é aproximadamente 10 vezes mais potente na inibição que o HPV-16. Essa diferença foi mapeada na LCR-E6-E7 e indica que a variabilidade genética nessa região do genoma viral deve ser de grande significância funcional (Villa e Schlegel, 1991). Corroborando este dado, um estudo em que foi realizada inserção do genoma completo de HPVs mucosos (HPV-6, -11, -16, -18) em queratinócitos primários (colo do útero, tonsilar, prepúcio) demonstrou que o HPV-18 induz crescimento estendido com maior eficiência em comparação aos outros tipos virais, independente da variante molecular testada (Lace *et al.*, 2009).

De fato, durante a última década, a comunidade científica tem-se interessado em inferir funcionalmente a importância da variabilidade genética sob o potencial oncogênico das diferentes variantes moleculares, sendo a maior parte dos estudos realizados com HPV-16. Foram observadas diferenças na atividade

transcricional entre as variantes E e As+AI de HPV-16 (Kämmer *et al.*, 2000; Veress *et al.*, 1999). Adicionalmente, foi descrito que variantes naturais de E6 de HPV-16 apresentam diferenças quanto a ligação às proteínas celulares E6BP e hDlg, a degradação de p53 e BAX, a inibição da transativação por p53, a alteração da diferenciação de queratinócitos em cooperação com a proteína E7 protótipo, além de diferenças no potencial transformante (Asadurian *et al.*, 2007; Lichtig *et al.*, 2006; Richard *et al.*, 2010; Sherman *et al.*, 1997; Sichero *et al.*, 2012; Stöppler *et al.*, 1996; Zehbe *et al.*, 2011). Dados do nosso laboratório indicam que PHKs transduzidos com E6/E7 da variante As+AI de HPV-16 superaram aqueles infectados com a variante E na proliferação celular, na habilidade de crescer independente de ancoragem (Sichero *et al.*, 2012), e na migração e invasão através de uma matriz de colágeno (Hochmann *et al.*, 2016). Ainda mais, estes queratinócitos imortalizados pela variante As+AI de HPV-16 apresentam maior ativação das vias de sinalização induzidas por MAPK e AKT/PI3K.

Em relação às variantes moleculares de HPV-18, a variante Af demostrou uma maior capacidade de ativar a origem de replicação (ori) viral quando a região da LCR-*E1* das diferentes variantes foi inserida em células C33 (carcinoma de colo uterino HPV negativo, p53 mutado), HaCaT (queratinócitos humanos imortalizados espontaneamente) e HEK-293 (células embrionárias de rim humano) (Amador-Molina *et al.*, 2013). Entretanto, foi reportado que a LCR de variantes do ramo As+AI possuem maior atividade transcricional quando comparadas às variantes do ramo E em células C33 (Sichero *et al.*, 2005). Corroborando este achado, foi observado que a LCR de HPV-18 do ramo Af apresentou menor atividade transcricional quando comparada às demais

variantes não somente em células C33, mas também em células de adenocarcinoma cervical HPV-18 positivo (HeLa), e naquelas oriundas de carcinoma de células escamosas HPV-16 (SiHa) e HPV-18 (CaLo) positivas (López-Saavedra *et al.*, 2009). Ademais, este mesmo grupo identificou variações na sequência proteica de E2 que não alteraram a atividade transcricional viral, entretanto a capacidade de induzir apoptose era maior nas variantes Af (López-Saavedra *et al.*, 2009; Fuentes-González *et al.*, 2019).

No que se refere à avaliação da atividade da proteína E6 de HPV-18 em modelos funcionais, foi observado que células MCF-7 (adenocarcinoma de mama), NIH3T3 (fibroblastos imortalizados de camundongo) e HaCat expressando E6 da variante As+AI transcreveram altos níveis de E6 completo resultando em baixos níveis da proteína p53 e altos níveis de p14ARF; ao contrário de células contendo variantes Af em que o transcrito mais abundantemente detectado foi E6*I, associado a altos níveis de p53 e altos níveis de p14arf (de la Cruz-Hernández *et al.*, 2005; Vazquez-Vega *et al.*, 2013). Ademais, esse estudo revelou que células NIH3T3 expressando E6/E7 da variante As+AI de HPV-18 apresentaram maior capacidade de formação de tumor em camundongos atímicos. Foi também observado que células MCF-7 expressando a proteína E6 da variante As+AI de HPV-18 não possuem PTEN fosforilado, ao contrário de células com a variante Af, em que baixos níveis de PTEN fosforilado foram detectados comparado a células MCF-7 não transfectadas que possuem altos níveis de PTEN ativo (Contreras-Paredes *et al.*, 2009). Os níveis de PTEN fosforilado detectados nas diferentes linhagens foram observados estar inversamente correlacionados aos níveis de PI3K e ERK2 fosforilados, além da capacidade proliferativa destas células. Mais recentemente,

a expressão gênica global foi analisada em células HaCat expressando E6 de variantes moleculares de HPV-18 dos três ramos filogenéticos. Foi observado em células HaCat contendo E6 da variante Af um aumento de transcritos celulares associados ao ciclo celular e migração, cujo efeito biológico foi também demonstrado funcionalmente (Fragoso-Ontiveros *et al.*, 2012). Entretanto, cabe ressaltar que estes achados contrastam com os resultados observados pelo mesmo grupo utilizando células MCF-7 infectadas por E6 (Contreras-Paredes *et al.*, 2009). Assim, estudos adicionais são necessários para avaliar o significado da variabilidade intra-típica de HPV-18 para a possível correlação destes achados aos dados epidemiológicos.

5. Justificativa e objetivo

Mais de 25 anos de estudos acerca do ciclo de vida e das propriedades transformantes de tipos de HPV que infectam a mucosa epitelial estabeleceram a relação causal direta entre a infecção por HPV de alto risco oncogênico e o carcinoma do colo uterino (revisado por IARC, 2012). Mundialmente, o HPV-16 é o tipo mais prevalente tanto em neoplasias pré-invasivas quanto em carcinomas invasivos do colo do útero, seguido pelo HPV-18 (van den Marel *et al.*, 2012), entretanto o HPV-18 é muito prevalente em adenocarcinomas (56%) e tumores adenoescamosos (39%) (Bosch *et al.*, 1995).

Variantes moleculares de tipos de HPV evoluíram principalmente através de variações de apenas um nucleotídeo com um padrão de fixação de linhagem destas variações. Ainda se debate se variantes moleculares de HPV-18 evoluíram de maneira a obter diferenças claras em relação ao risco de persistência do DNA viral, de desenvolvimento de lesão do colo uterino de alto grau e de associação

aos diferentes tipos histopatológicos tumorais com diferenças na agressividade (revisado por Sichero e Villa, 2006). No que concerne à análise das diferenças biológicas e bioquímicas atribuíveis à heterogeneidade genética intra-típica de HPV-18, é fundamental ressaltar que os poucos estudos conduzidos até o momento exploraram exclusivamente a variabilidade da oncoproteína viral E6, e, mais importante, nenhum dos estudos foi realizado em modelos de células hospedeiras naturais de HPV-18, isto é, PHKs.

Pelo exposto, esta tese teve por objetivo caracterizar de maneira abrangente a atividade funcional das proteínas E6/E7 das variantes As+AI, E e Af de HPV-18 em PHKs transduzidos com estas proteínas.

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2 MANUSCRITO 1

Propriedades funcionais das variantes naturais do Papilomavirus Humano 18.

Emily M. Nunes, Valéria Talpe Nunes, João S P Sobrinho, Silvaneide Ferreira, Vanesca Lino, Lara Termini, Gabriela Á. F. Silva, Enrique Boccardo, Luisa L. Villa, Laura Sichero.

Para submissão a mBio (*Research Article*)

Propriedades funcionais das variantes naturais do Papilomavírus Humano 18

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Título corrido: Propriedades das variantes de HPV-18.

Resumo:

As variantes africanas (Af) de HPV-18 são exclusivamente detectadas em carcinoma cervical escamoso, enquanto que variantes europeias (E) e ameríndias (As+AI) prevalecem em adenocarcinoma e carcinoma adenoescamoso. No que concerne às diferenças biológicas entre estas variantes, há poucos estudos conduzidos até o momento e estes exploraram somente a variabilidade de E6, sendo que nenhum destes trabalhos foi realizado em um modelo de células hospedeiras naturais. Neste estudo, os PHKs foram transduzidos com E6/E7 das variantes As+AI e Af de HPV-18 (PHK18AA e PHK18Af) e subcultivados até p30, quando foram consideradas imortalizadas. Os PHK18AA atingiram a imortalização significativamente mais rápido do que os PHK18Af, ainda que uma “crise” não fosse observada nos PHKs transduzidos. No entanto, não houve diferença nas taxas de proliferação entre os PHK18AA e PHK18Af em p30. Os PHK18AA imortalizados foram mais eficientes em formar colônias e esferoides em monocamada e cultura tridimensional, respectivamente, em diferentes experimentos. Ademais, os PHK18AA imortalizados apresentaram maior capacidade de invadir através de uma matriz de colágeno, ainda que diferenças na migração não fossem observadas entre as variantes. Por outro lado, os PHK18Af imortalizados apresentaram maior resistência à diferenciação em meio enriquecido por soro e cálcio. Finalmente, culturas organotípicas derivadas dos PHKs imortalizados por ambas as variantes de HPV-18 eram semelhantes na morfologia e apresentaram níveis equivalente de proteínas associadas a diferenciação e proliferação. Em conjunto, nossos dados indicam que a variante As+AI de HPV-18 tem um maior potencial oncogênico em comparação à variante Af.

Importância:

O papilomavírus humano (HPV) está virtualmente associado a todos os casos de câncer de colo uterino. Sugeriu-se que variantes ameríndias (As+AI) e europeias

(E) de HPV-18 apresentem maior potencial oncogênico do que as variantes africanas (Af). Neste estudo foram avaliadas as propriedades funcionais associadas à carcinogênese de queratinócitos humanos primários (PHK) imortalizados por E6/E7 das variantes As+AI e Af de HPV-18. Nós observamos que os PHK18AA atingiram a imortalização mais rápido do que os PHK18Af, além de apresentarem maior eficiência em formar colônias, esferoides e em invadir matriz de colágeno. Ainda que os PHK18Af imortalizados apresentassem maior capacidade em induzir resistência à diferenciação em meio enriquecido com soro e cálcio, culturas organotípicas derivadas dos PHKs imortalizados não exibiram diferenças histopatológicas. Assim, sugere-se que a variante As+AI de HPV-18 tenha maior potencial carcinogênico quando comparada a variante Af. Este estudo é único em analisar a função de E6/E7 de HPV-18 em células hospedeiras naturais do HPV.

Palavras- chaves: HPV, potencial oncogênico, variante, transformação celular, imortalização.

1. Introdução

O câncer de colo de útero (CCU) é o quarto câncer com maior incidência entre mulheres no mundo, sendo que o papilomavírus humano (HPV) está virtualmente associado a todos os casos (IARC, 2020). Enquanto os CCU de origem escamosa estão primordialmente associados à infecção por HPV-16 (80%), os adenocarcinomas estão similarmente associados às infecções por HPV-16 e -18 (Bruni *et al.*, 2010; de Sanjosé *et al.*, 2007; revisado por Bouvard *et al.*, 2009; Walboomers *et al.*, 1999).

Baseado em um fragmento da LCR, as variantes moleculares de HPV-18 (definidas por variação genômica menor que 2%) foram incialmente agrupadas em três ramos filogenéticos nomeados de acordo com a origem da maior parte das amostras que os compõe: africano (Af), ameríndio (As+AI) e europeu (E) (Ong *et al.*, 1993). O protótipo (primeiro tipo isolado e sequenciado) de HPV-18 pertence ao ramo As+AI. Posteriormente, um estudo baseado no genoma completo destes isolados revelou que a maior parte das alterações nucleotídicas separa as variantes E e As+AI de HPV-18 daquelas do ramo Af (Chen *et al.*, 2013). Mais recentemente, a nomenclatura das variantes de HPV-18 foi reorganizada em linhagens (nominadas por letras) e sublinhagens (nominadas por números) que divergem em 1-2% e 0,5-1% no genoma completo, respectivamente (Chen *et al.*, 2013; Burk *et al.*, 2013). Assim, as variantes As+AI equivalem às sublinhagens A1 e A2; o ramo E engloba as sublinhagens A3 a A5; e as Af classificam-se nas linhagens B/C.

Diversos estudos tem sugerido que variantes As+AI de HPV-16 e HPV-18 são mais oncogênicas que variantes E e Af, (Berumen *et al.*, 2001; de Boer *et al.*,2005; Lizano e García-Carrancá, 1997; Tornesello *et al.*, 2004; Villa *et al.*,

2000; Xi *et al.*,1997, 2007; Sichero *et al.*, 2007). Estudos realizados em Portugal e Estados Unidos demostraram uma tendência, ainda que não significativa, de variantes As+AI de HPV-18 estarem associadas ao risco aumentado de desenvolvimento de lesão intra-epitelial neoplásica de grau III (NIC3) e CCU (Pista *et al.*, 2007; Xi *et al.*, 2007). Por outro lado, na Espanha, foi observado que variantes Af estão associadas a um risco aumentado, ainda que não significativo, de desenvolvimento de NIC3 em comparação com as variantes E e As+AI (Pérez *et al.*, 2014). Cabe ressaltar que nenhuma diferença significativa no risco de desenvolvimento de NIC3 atribuível às diferentes variantes moleculares de HPV-18 foi observada em outros estudos conduzidos na Costa Rica (Schiffman *et al.*, 2010), nos Estados Unidos (Arias-Pulido *et al.*, 2005) ou mesmo em um projeto multinacional que reuniu mais de 700 amostras HPV-18 positivas oriundas de 39 países (Chen *et al.*, 2015). Portanto, estudos adicionais devem ser conduzidos a fim de melhor avaliar as discrepâncias reportadas.

A associação entre as diversas variantes moleculares de HPV-18 e a histopatologia dos CCU foi também descrita. Alguns estudos reportaram que, enquanto as variantes Af de HPV-18 foram exclusivamente detectadas entre amostras de carcinoma cervical escamoso, as variantes E e As+AI foram mais prevalentes em amostras de adenocarcinoma e carcinoma adenoescamoso. Estes dados indicam que variantes Af podem estar associadas a tumores com comportamento menos agressivo, uma vez que os carcinomas escamosos tem um prognóstico relativamente melhor quando comparado aos adenocarcinoma e carcinoma adenoescamoso (Burk *et al.*, 2003; de Boer *et al.*, 2005; Lizano e García-Carrancá, 1997). Cabe ressaltar que estes achados ainda são motivo de debate (Arias-Pulido *et al.*, 2005; Chen *et al.*, 2015).

As proteínas E6/E7 de HPVs de alto risco cooperam na imortalização de queratinócitos humanos primários (PHKs) e na inibição da diferenciação destas células induzidas por soro e cálcio (Münger *et al.*, 1989; Hawley-Nelson *et al.*, 1989; revisado por Stanley, 2010). Isso se deve à continua expressão destes oncogenes que interagem com diversas proteínas celulares, incluindo p53 e pRB, resultando na desregulação da apoptose, proliferação e senescência (Gupta *et al.*, 2003; revisado por Pim e Banks, 2010; Dyson *et al.*, 1989; Scheffner *et al.*, 1993). Estas células imortalizadas por E6/E7 de HPV-16 e HPV-18, quando utilizadas em culturas organotípicas, apresentaram anormalidades histológicas semelhantes às observadas em neoplasias cervicais intra-epiteliais em humanos (Blanton *et al.*, 1991). Ainda que ambos os HPV-16 e HPV-18 sejam capazes de inibir a diferenciação terminal de PHKs induzida por soro e cálcio, o HPV-18 é aproximadamente 10 vezes mais potente na inibição que o HPV-16 (Villa e Schlegel, 1991), indicando que a variabilidade genética destes vírus impacta substancialmente sobre o potencial transformante. Baseado nisso, nas últimas duas décadas a comunidade científica tem-se interessado em avaliar a importância da variabilidade genética sob o potencial oncogênico das diferentes variantes moleculares.

No que concerne aos estudos com HPV-18, foi demonstrado que variantes Af apresentam menor atividade transcricional (Sichero *et al.*, 2005; López-Saavedra *et al.*, 2009) em comparação às variantes As+AI e E. Foi também reportado que células MCF-7 (adenocarcinoma de mama), NIH3T3 (fibroblastos imortalizados de camundongo) e HaCat expressando E6 da variante As+AI transcrevem altos níveis de E6 completo resultando em baixos níveis da proteína p53 e altos níveis de p14ARF; ao contrário de células expressando E6 da variante

Af nas quais o transcrito mais abundantemente detectado foi E6*I associado aos altos níveis de p53 e baixos níveis de p14ARF (de la Cruz-Hernández *et al.*, 2005; Vazquez-Vega *et al.*, 2013). Ademais, células NIH3T3 expressando *E6/E7* da variante As+AI apresentaram maior capacidade de formação de tumor em camundongos atímicos (de la Cruz-Hernández *et al.*, 2005). Finalmente, foi descrito que células MCF-7 expressando E6 da variante As+AI de HPV-18 não possuíam PTEN fosforilado, ao contrário de células expressando E6 da variante Af que apresentavam baixos níveis de PTEN fosforilado e das células MCF-7 não transfectadas que exibiram elevados níveis de PTEN ativo (Contreras-Paredes *et al.*, 2009). É importante ressaltar que altos níveis de PTEN fosforilado são inversamente correlacionados aos níveis de PI3K e ERK2 fosforilados e, consequentemente, à capacidade de proliferação celular.

É crucial observar que, no que concerne à análise das diferenças biológicas e bioquímicas atribuíveis à heterogeneidade genética intra-típica de HPV-18, os poucos estudos conduzidos até o momento exploraram exclusivamente a variabilidade da oncoproteína viral E6 e, mais importante, nenhum dos estudos foi realizado em modelos de células hospedeiras naturais de HPV-18, isto é, queratinócitos humanos primários (PHK). Assim, este estudo objetivou avaliar as diferenças nas propriedades de variantes naturais de E6/E7 de HPV-18 a fim de melhor compreender a associação destas com possíveis diferenças no risco de desenvolvimento de neoplasias e associação com o grau histológico observados. Mais especificamente, avaliamos diferenças nas propriedades funcionais associadas à carcinogênese das variantes As+AI e Af nos hospedeiros naturais destes vírus, os PHKs.

2. Resultados

As variantes de E6/E7 de HPV-18 diferem no potencial de imortalização de PHKs.

Sabe-se que o HPV-18 apresenta alta capacidade de imortalização de PHKs quando comparado aos outros tipos oncogênicos, entretanto ainda não é conhecido se há diferença no potencial oncogênico entre as distintas variantes deste tipo viral (Sichero *et al.*, 2005; Lace *et al.*, 2009). Uma vez que a expressão de *E6/E7* é necessária para a célula infectada ultrapassar as barreiras de senescência e crise, e alcançar a imortalização, nossa hipótese é que as alterações nucleotídicas não-sinônimas observadas em ambos os genes das variantes de HPV-18 tenham impacto sobre estes processos (Kaur e McDougall, 1989).

De forma generalizada, as variantes de HPV-18 divergem em três ramos filogenéticos: As+AI, E e Af. Uma vez que alterações não-sinônimas não foram observadas entre os isolados As+AI e E nas amostras de esfregaços genitais previamente caracterizadas (Franco *et al.*, 1999; Sichero *et al.*, 2007), as análises neste estudo foram restritas às variantes As+AI e Af. Estas apresentam uma alteração não-sinônima no gene *E6* (C491A), resultando na substituição N129K na proteína da variante Af; e uma alteração no gene *E7* (C593T), resultando na substituição H2Y na proteína da variante Af.

A fim de investigar possíveis diferenças entre as variantes de HPV-18 no que se refere ao potencial de imortalização e à capacidade de ultrapassar um período de crise (início da morte celular após a parada do ciclo celular), dois lotes distintos de *pools* de PHKs obtidos comercialmente foram transduzidos com títulos equivalentes (MOI=10) de partículas virais contendo plasmídeos

recombinantes pLNSX E6/E7 das variantes As+AI e Af (nomeados PHK18AA e PHK18Af, respectivamente, daqui em diante). Após cinco passagens em cultura, o DNA dos PHKs transduzidos foi isolado e realizou-se sequenciamento para confirmação das sequências de *E6* e *E7* de HPV-18 transduzidas (dados não apresentados).

Em seguida, em garrafas de cultivo celular de 25 cm^2 foram plaqueados $1,25 \times 10^5$ PHKs transduzidos. As células foram subcultivadas ao atingirem 80% confluência, sendo as mesmas consideradas imortalizadas ao alcançarem a passagem 30 (Halbert *et al.*, 1991). Foi observado que os PHKs não-transduzidos morreram na passagem nove, enquanto que os *pools* de PHK18AA e PHK18Af atingiram a passagem 30 em média após 134 e 186 dias em cultura, respectivamente (figura 1A). Para ambos os lotes de *pools* de células utilizados, foi observado que os PHK18AA atingiram p30 significativamente mais rápido que os PHK18Af (lote 1, PHK18AA *versus* PHK18Af: p= 0,00; lote 2 PHK18AA *versus* PHK18Af p= 0,00). Cabe ressaltar que não foram observadas diferenças significativas no que concerne ao crescimento dos diferentes *pools* de PHKs transduzindo a mesma variante de HPV-18 (PHK18AA lote 1 *versus* lote 2: p= 0,53; PHK18Af lote 1 *versus* lote 2: p= 0,29).

O tempo de duplicação dos PHKs transduzidos ao longo dos dias em cultura foi calculado a fim de avaliar se a diferença observada entre as variantes de HPV-18 em relação ao tempo necessário para atingir p30 estava atrelada à distinta capacidade de superar um período de crise. Entretanto, observou-se que os PHK18AA e PHK18Af não apresentaram um período de crise evidente durante a imortalização, assim como também não foi identificada nenhuma diferença significativa entre os lotes de PHKs transduzidos com a mesma variante viral em

relação aos tempos de dobramento medidos (figura 1B). Assim, apesar da variante As+AI ter apresentado maior eficiência em imortalizar PHKs quando comparada à variante Af, esta habilidade parece não estar relacionada com a capacidade de ultrapassar um período de crise. Ademais, não foram observadas diferenças morfológicas entre os PHK18AA e PHK18Af, quer seja na área ou perímetro celular, quando comparados aos PHKs não-transduzidos, independentemente do lote, passagem em cultura ou confluência na qual as células se encontravam (área média de 1 272 ± 126 μm²; perímetro médio de 152 ± 7 μm) (figura 1C).

Os PHK18AA e PHK18Af não apresentam diferenças na capacidade proliferativa.

Uma vez que não foram observadas diferenças entre os dois lotes de PHKs transduzidos com a mesma variante de HPV-18 no que concerne ao tempo requerido para atingir p30, o tempo de duplicação e a morfologia celular, optou-se por dar continuidade aos demais experimentos utilizando apenas um dos lotes de PHKs (lote 1). A capacidade proliferativa dos PHKs transduzidos foi avaliada de três formas distintas e complementares: (1) medida dos níveis proteicos de PCNA (antígeno nuclear de proliferação celular) por *Western blotting*, (2) contagem de células ao longo de vários dias em cultura, seguida pela coleta das células e (3) análise dos níveis de Ki-67 (proteína nuclear expressa em níveis elevados a partir da fase G1 do ciclo celular) por citometria de fluxo.

Inicialmente os PHK18AA e PHK18Af nas passagens 5, 15 e 30, além dos PHKs não-transduzidos em passagem 5, foram cultivados em placas de 100 mm e, ao alcançarem 80% de confluência, a proteína total foi extraída, fracionada em

SDS-PAGE e analisada por *Western blotting*. Observou-se um aumento crescente dos níveis de PCNA no decorrer das passagens em cultura, sem diferença significativa entre as variantes de HPV-18 analisadas independentemente da passagem avaliada (PHK18AA *versus* PHK18Af p5: p= 0,23; p15: p= 0,89; p30: p= 0,66) (figura 1D).

Em seguida, 5 × 10^3 PHK18AA e PHK18Af imortalizados (p30), além dos PHKs não-transduzidos (p5), foram semeados em placas de 12 poços e, após dois, quatro e seis dias em cultura, triplicatas das células foram contadas. Apesar de ambos os PHKs transduzidos proliferarem significativamente mais rápido do que os PHKs não-transduzidos (PHK18AA *versus* PHKs: p= 0,00; PHK18Af *versus* PHKs: p= 0,01), as taxas de proliferação não foram significativamente diferentes entre os PHKs imortalizados com *E6/E7* das diferentes variantes de HPV-18 (PHK18AA *versus* PHK18A: p= 0,38) (figura 1E).

Por fim, foram analisados os níveis da proteína Ki-67 nas células imortalizadas contadas nos dias dois, quatro e seis. Para tanto, após a coleta e fixação das células, estas foram incubadas com anti-Ki-67 e analisadas por citometria de fluxo. Observou-se que os níveis de Ki-67 aumentaram com o decorrer dos dias em cultura celular, sendo que, a partir do quarto dia, os níveis de Ki-67 aumentaram substancialmente nos PHKs imortalizados por ambas as variantes de HPV-18. Entretanto, após seis dias em cultura, não foi observada diferença significativa nos níveis de Ki-67 medidos nos PHK18AA e PHK18Af (p= 0,05). Ainda assim, os níveis de Ki-67 após seis dias em cultura foram significativamente maiores nas células imortalizadas em comparação aos PHKs não-transduzidos (PHK18AA *versus* PHKs: p= 0,02; PHK18Af *versus* PHKs: p= 0,00) (figura 1F).

Os PHK18AA e PHK18Af apresentam níveis semelhantes de E6, E6*I, p53, p16, pRb e p21.

A fim de melhor compreender os mecanismos envolvidos na diferença no potencial de imortalização observados entre os PHK18AA e PHK18Af, nós avaliamos os níveis dos transcritos de E6, E6*I e E7, além da expressão proteica de E6 e de diferentes alvos moleculares destas proteínas virais. Para isso, os PHK18AA e PHK18Af (p5, 15 e 30), além dos PHKs não-transduzidos (p5), foram cultivados em placas de 100 mm até atingirem 80% confluência, quando RNA e proteína total destas células foram extraídos. O RNA foi utilizado em reações de qRT-PCR para determinação dos níveis dos mRNA de E6 e E7. Além disso, parte do RNA (1 µg) foi convertido a cDNA e submetido à RT-PCR com a finalidade de detectar e comparar os níveis dos transcritos E6 e E6*I simultaneamente. Já as proteínas extraídas foram submetidas ao fracionamento em SDS-PAGE e analisadas por *Western blotting* para avaliação dos níveis de E6, assim como das proteínas supressoras de tumor p53 e pRb e dos inibidores de quinase dependente de ciclina p16 e p21.

Nós observamos níveis semelhantes do transcrito completo de E6 e do produto de processamento alternativo E6*I nas células transduzidas com *E6/E7* de HPV-18, independente do lote de PHKs (dados não apresentados), variante ou passagens em cultura (figura 2A). De fato, foi descrito que o HPV-18 é o tipo de alto risco em que se observam os maiores níveis do transcrito E6*I (Tang *et al.*, 2006).

O motivo pelo qual o transcrito E6*I é produzido nas células hospedeiras infectadas não está completamente elucidado (Evans *et al.*, 2016; Schneider-Gädicke e Schwarz, 1986; Smotkin e Wettstein, 1986). Entretanto, sugere-se que

o processamento alternativo que resulta no aumento de E6*I favoreça a expressão de E7 (Sedman *et al.*, 1991; Cheng *et al.*, 1995; Brant *et al.*, 2018). Pelo exposto, reações de qRT-PCR foram realizadas a fim de avaliar os níveis de expressão de E6 e E7. Células HeLa foram usadas como controle positivo e a expressão gênica da proteína ribossômica mitocondrial S18 foi usada como normalizador. Como esperado, não foram detectados E6 e E7 nos PHKs não-transduzidos (figura 2B). Por outro lado, níveis elevados e similares de E6 e E7 foram observados nos PHKs transduzidos e nas células da linhagem HeLa independentemente da variante de HPV-18 e da passagem em cultura analisada.

Entretanto, é conhecido que nem sempre existe uma correlação direta entre os níveis de transcrito e da proteína correspondente em uma célula. Ainda mais, embora não esteja completamente elucidada a razão pela qual células infectadas por HPVs de alto risco expressem E6*I, já foi observado que esta proteína truncada é capaz de ligar-se à proteína E6 e inibir suas funções, incluindo a ubiquitinação da proteína supressora tumoral p53 (Scheffner *et al.*, 1993; Sedman *et al.*, 1991; Shally *et al.*, 1996; Pim e Banks., 1997; de la Cruz-Hernández *et al.*, 2005). Pelo exposto, foram realizados ensaios de *Western blotting* para a avaliação dos níveis de E6 e p53. Nós observamos que ambos os PHK18AA e PHK18Af expressaram níveis similares da proteína E6 nas células em passagens cinco e 30, embora menores que nas células em passagem 15 (figura 2C). Complementarmente, nós observamos um aumento dos níveis de p53 nas células transduzidas com HPV-18 em passagem 30, apresentando uma linearidade entre os níveis de E6 e os de p53 (figura 2D).

Os níveis da proteína viral E7 foram indiretamente avaliados pelos níveis de seus alvos celulares pRb, p16 e p21 por *Western blotting*. Observou-se que as

células imortalizadas (p30) por HPV-18 apresentaram níveis reduzidos de pRB em comparação aos PHKs não-transduzidos, ainda que similares entre as variantes estudadas (figura 2E). Foi também observado que o nível de p16 estava mais elevado nos PHK18AA e PHK18Af em relação aos PHKs não-transduzidos, sem diferença entre as passagens em cultura ou entre as variantes estudadas (figura 2F). Finalmente, níveis semelhantes de p21 foram observados entre os PHK18AA e PHK18Af, embora menores que do que nas células não-transduzidas (figura 2G).

Os PHK18AA e PHK18Af diferem no potencial de formação de colônias em experimentos em monocamada.

A infecção por HPVs de alto risco impacta sobre diferentes proteínas envolvidas na regulação do ciclo celular e apoptose levando à imortalização celular. Contudo, essas células não são capazes de formar tumores em camundongos atímicos (DeFilippis *et al.*, 2003), indicando a necessidade de alterações genéticas adicionais para indução da tumorigênese (White *et al.*, 1994; Duensing *et al.*, 2000; Demeret *et al.*, 2003). Uma vez que as variantes As+AI e Af apresentaram diferenças no que se refere à imortalização dos PHKs, nós objetivamos avaliar a capacidade destas células imortalizadas em formar colônias em modelos de cultivo de células em monocamada através de ensaio clonogênico e de resistência à diferenciação terminal induzida por soro e cálcio.

Inicialmente, 500 PHK18AA e PHK18Af imortalizados, além dos PHKs não-transduzidos (p5), foram semeados em placas de seis poços e a formação e o crescimento de colônias foram acompanhados por 10 dias, quando as células foram coradas com solução de cristal violeta. Como esperado, os PHKs não-

transduzidos geraram um número baixo de colônias quando plaqueados em baixa densidade (média de 4,2 colônias). Por outro lado, foi observado que os PHK18AA foram capazes de induzir o crescimento de um maior número de colônias (média de 52 colônias) quando comparados aos PHK18Af (média de 36,7 colônias), embora essa diferença não fosse estatisticamente significativa (p= 0,13) (figura 3A). Isso se deve em parte à observação de que as colônias formadas pelos PHK18Af eram muito menos coesas quando comparadas àquelas advindas dos PHK18AA, o que dificultou substancialmente a identificação de colônias verdadeiras. De fato, a área média das colônias geradas pelos PHK18AA foi significativamente maior que àquelas advindas dos PHK18Af (PHK18AA: média de 0,25 mm^2, PHK18Af: média 0,16 mm^2, p= 0,00).

Em seguida, a fim de avaliar se as variantes de HPV-18 diferem na capacidade de resistir à diferenciação celular, 1 × 10^3 PHK18AA e PHK18Af imortalizados, além dos PHKs não-transduzidos (p5), foram cultivados em placas de seis poços e, após sete dias, foram cultivados ou não em meio DMEM acrescido de 10% de soro fetal bovino e 1 µg/mL de hidrocortisona por mais sete dias. As células foram então incubadas com MTT, fotografadas e posteriormente diluídas em DMSO, seguido por leitura de absorbância a 570 nm. Pode-se observar na figura 3B que ambos os PHK18AA e PHK18Af eram significativamente mais viáveis que os PHKs não-transduzidos, independentemente do meio de cultura utilizado. Adicionalmente, observou-se que os PHK18Af apresentaram maior capacidade de resistir à diferenciação na presença de meio enriquecido com soro e cálcio, em comparação com os PHK18AA (p=0,00), embora diferenças na viabilidade destas não foram observadas quando as células foram cultivadas em meio não-enriquecido.

Os PHK18AA e PHK18Af diferem na formação de esferoides em cultura tridimensional (3D).

Apesar das culturas em monocamadas serem amplamente utilizadas para avaliar alterações morfológicas e bioquímicas representativas, estas carecem de interações complexas célula-célula ou células-matriz extracelular (MEC) que tem papel importante na tumorigênese e migração celular. Estas deficiências podem ser superadas pelo emprego de culturas tridimensionais (3D) que permitem que as células se organizem e formem uma estrutura que melhor reproduz o microambiente *in vivo*. Para isso, os PHKs não-transduzidos (p5), além dos PHK18AA e PHK18Af imortalizados, foram cultivados em três distintas condições: placas de baixa aderência, meio semi-sólido (*soft agar*), além de placas de baixa aderência com uso concomitante de força magnética para estímulo da formação de esferoides.

Inicialmente, 2×10^4 PHKs não-transduzidos, PHK18AA e PHK18Af foram semeados em placa de seis poços de baixa aderência. Após 14 dias, os esferoides formados foram analisados macro e microscopicamente. Os PHK18AA foram capazes de formar um maior número de esferoides (média de 12,6 esferoides) em comparação aos PHK18Af (média de 11,9 esferoides), embora esta diferença observada não fosse estatisticamente significativa (p= 0,70) (figura 3C). Entretanto, assim como no ensaio clonogênico (figura 3A), observou-se microscopicamente que os esferoides derivados dos PHK18Af eram muito menos coesos quando comparados àqueles advindos dos PHK18AA, o que dificultou substancialmente a contagem dos esferoides. De fato, a área dos esferoides derivados dos PHK18AA (média de 0,28 mm²) foi significativamente maior que a área daqueles derivados dos PHK18Af (média de 0,09 mm²) (p= 0,01). Assim, os

PHK18AA apresentaram uma maior eficiência de formação de esferoide (η) em comparação aos PHK18Af quando cultivados em placas de baixa aderência (η PHK18AA *versus* η PHK18Af: 0,20% *versus* 0,09%). Os PHKs não-transduzidos apresentaram 0,002% de eficiência de formação de esferoides nessa condição.

Em seguida, 5 × 10^3 PHKs não-transduzidos (p5), além dos PHK18AA e PHK18Af imortalizados, foram cultivados em placa de 24 poços em meio semi-sólido constituído de *soft* agar. A formação e o crescimento de esferoides foram acompanhados por 15 dias, quando estes foram corados com MTT e as imagens registradas macro e microscopicamente. Neste ensaio, nós observamos que os PHK18AA formaram um número maior de esferoides em relação aos PHK18Af, entretanto a diferença não foi estatisticamente significativa (PHK18AA média de 13,4 esferoides *versus* PHK18Af média de 8,7; p= 0,10) (figura 3D). Ademais, observou-se que a área média dos esferoides derivados dos PHK18AA e PHK18Af foi similar (média de 0,41 mm^2; p= 0,67).

Finalmente, utilizamos a tecnologia de bioimpressão magnética de células (n3D *Biosciences*) para avaliação da capacidade dos PHK18AA e PHK18Af imortalizados em formar esferoides. Para tanto, 1 × 10^4 PHKs não-transduzidos (p5), além dos PHK18AA e PHK18Af imortalizados, e das células da linhagem HeLa (controle positivo), foram incubados com solução contendo nanopartículas magnéticas e em seguida cultivados em placa de baixa aderência com 96 poços. Posteriormente, uma placa magnética foi inserida abaixo das células plaqueadas por 24 horas e o desenvolvimento dos esferoides foi acompanhado por mais 15 dias. Assim como observado nos experimentos anteriores, os esferoides formados pelos PHK18Af apresentaram um aspecto “poroso” que impossibilitava a caracterização como um esferoide único, semelhante àqueles observados

quando na utilização de PHK18AA e HeLa (figura 3E). Ainda que estes sejam resultados advindos de apenas um experimento em que se realizou uma média de 20 replicatas para cada linhagem celular estudada, esses dados corroboraram e expandiram os dados obtidos em cultura em monocamada e 3D (figura 3A-D).

Os PHK18AA e PHK18Af diferem no potencial de invasão, mas não na capacidade de migração.

Foi descrito que células tumorais apresentam as mesmas estratégias básicas para migração que às observadas em PHKs que recebem estímulos pró-migratórios, porém não observadas em queratinócitos já diferenciados. Entretanto, o desequilíbrio de sinais migratórios que ocorrem nos PHKs imortalizados por HPVs permite a continua migração e invasão favorecendo assim a expansão tumoral (revisado por Friedl e Wolf, 2003). Com o intuito de avaliar a capacidade migratória dos PHKs imortalizados com as diferentes variantes de HPV-18, 5×10^5 PHKs não-transduzidos (p5), além dos PHK18AA e PHK18Af imortalizados, foram semeados em placas de 12 poços e, após 24 horas, as células foram tratadas com mitomicina C 10 µM para a indução da parada do ciclo celular. Após seis horas de tratamento, foi realizado um risco no centro dos poços utilizando-se uma ponteira de micropipeta e a migração celular, ainda em presença de mitomicina C, foi acompanhada por 12 horas. Observou-se que os PHKs imortalizados com as diferentes variantes de HPV-18 apresentaram uma capacidade de migração similar: enquanto os PHK18AA cobriram 49,7% da área da ferida em 12 horas, os PHK18Af cobriram 48,5% da área neste período ($p= 0,28$) (figura 4A). Além disso, não foram observadas diferenças significativas nas taxas de parada do ciclo celular no tratamento com mitomicina C, nem entre os

PHK18AA e 18Af (p= 0,65), nem entre os PHKs não-transduzidos e PHK18AA (p= 0,84) ou PHKs não-transduzidos e PHK18Af (p= 0,50) (figura S1A). Entretanto, cabe ressaltar que diferenças foram observadas na maneira como estas células migraram (figura S1B-D). Nós observamos que os PHK18AA migraram de maneira coletiva, ou seja, foi observada uma movimentação de um agrupado de células em direção à área da ferida, comumente observado em tumores de origem epitelial (revisado por Friedl e Wolf, 2003). Por outro lado, os PHK18Af moveram-se individualmente para à área aberta de maneira normalmente observada em tumores pouco diferenciados (revisado por Friedl e Wolf, 2003).

A invasão celular é definida pela penetração das barreiras teciduais, como a membrana basal e o estroma intersticial, e requer não apenas uma alta capacidade migratória, mas também adesão e proteólise de componentes da matriz extracelular. Apesar da invasão ocorrer durante a morfogênese celular e a cicatrização de feridas, é também uma capacidade adquirida initerruptamente por células malignas (revisado por Friedl e Wolf, 2003).

Para avaliar o potencial invasivo das diferentes variantes de HPV-18, foi utilizado o *QCM High sensitivity non-crosslinked collagen invasion assay* que emprega um tipo de colágeno não reticulado, fornecendo um ambiente que permite a ocorrência de altas taxas de invasão celular. Foram semeados $2,5 \times 10^5$ PHK18AA e PHK18Af imortalizados nos insertos contendo o colágeno previamente hidratado com meio de cultura KSFM. A fim de estimular a invasão, adicionou-se meio de cultura acrescido de 15% de soro fetal bovino do lado de fora dos insertos. Após 72 horas, os insertos foram corados e as células na parte interna do inserto foram removidas. Os insertos foram fotografados e

posteriormente imersos em meio de extração para análise indireta da capacidade de migração por medida de absorbância a 540 nm.

A coloração dos insertos revelou diferenças no modo de invasão entre as células imortalizadas: enquanto foi observada invasão radial e central nos PHK18AA, os PHK18Af exibiram apenas invasão radial (figura 4B; insertos). Ainda mais, observou-se a invasão de grandes agrupados dos PHK18AA nas imagens microscópicas, enquanto os PHK18Af pareceram invadir em pequenos conjuntos de células (figura 4B; magnificação 4× e 10×). Finalmente, as medidas de absorbância indicaram que os PHK18AA possuíam um potencial de invasão significativamente maior que os dos PHK18Af (PHK18AA *versus* PHK18Af = média de absorbância 0,99 e 0,81, respectivamente; p= 0,00).

Os PHK18AA e PHK18Af induzem padrões de diferenciação celular semelhantes às lesões cervicais de alto grau.

O sistema convencional de cultura de queratinócitos em monocamada seleciona apenas as células capazes de proliferar e limita o processo de diferenciação celular, o qual é de extrema importância para que o ciclo de vida do HPV complete-se nas células hospedeiras. Visando avaliar o padrão de diferenciação induzido pelas diferentes variantes de HPV-18, nós utilizamos o modelo de cultura organotípica ou *raft* que permite a estratificação em camadas e a diferenciação celular semelhante à observada nos tecidos epiteliais (revisado por Termini e Boccardo, 2018). Para isso, os PHKs não-transduzidos (p0), além dos PHK18AA e PHK18Af imortalizados, foram cultivados sobre um equivalente dérmico composto de colágeno tipo I de cauda de rato e fibroblastos irradiados (Boccardo *et al.*, 2004). A cultura foi mantida na interface meio-ar e nutrida com

meio de cultura acrescido de fatores de crescimento, soro e alta concentração de cálcio. Após 12 dias, os epitélios formados foram fixados em formaldeído 2% e embebidos em parafina. A partir destes tecidos parafinizados, técnicas histológicas foram realizadas a fim de avaliar não apenas a estrutura tecidual após coloração com hematoxilina-eosina (HE), mas também os níveis das proteínas PCNA, p16 e citoqueratina 10 (CK10).

A coloração com HE dos tecidos derivados dos PHKs não-transduzidos revelou que estes epitélios apresentaram uma estratificação ordenada e condizente com a pele saudável, sendo possível observar uma camada basofílica composta por células basais indiferenciadas, além de várias camadas de células suprabasais eosinofílicas facilmente distinguíveis (figura 4C).

Por outro lado, os epitélios formados a partir dos PHK18AA e PHK18Af eram hiperplásicos, apresentaram estratificação irregular, desordenada e não foi possível distinguir a camada basal das suprabasais. As células imortalizadas por ambas as variantes de HPV-18 originaram tecidos com alterações epiteliais comparáveis a uma lesão intraepitelial escamosa de alto grau e diferenças na espessura ou morfologia dos epitélios formados não foram observadas.

Em seguida, nós realizamos ensaios de imuno-histoquímica utilizando anticorpo contra o biomarcador de diferenciação epitelial CK10. Pode-se observar nos PHKs não-transduzidos níveis de CK10 similares em todas as camadas suprabasais. O mesmo padrão foi observado nos epitélios derivados dos PHKs imortalizados, independentemente da variante de HPV-18. Cabe ressaltar, que a CK10 constitui um marcador não apenas de diferenciação, mas também de hiperplasia das células de reserva, metaplasia escamosa e da zona de

transformação cervical, a qual constitui a região em que a maior parte dos carcinomas cervicais se origina (Maddox *et al.*, 1999).

A proteína p16 é um importante biomarcador da infecção por HPVs, sendo superexpresso em amostras de CCU (Khleif *et al.*, 1996; Klaes *et al.*, 2001). Assim como detectado por *Western blotting* utilizando as proteínas extraídas de cultivo celular em monocamada, observou-se alta expressão de p16 através de todas as camadas dos epitélios derivados de PHK18AA e PHK18Af imortalizados; ao contrário dos epitélios advindos dos PHKs não-transduzidos, que apresentaram baixos níveis de p16 confinados apenas nas células não-proliferativas das camadas intermediárias (figura 2F e 4C, respectivamente). Ainda assim, diferenças relevantes entre os epitélios derivados das células imortalizadas pelas duas variantes não foram observadas.

Os níveis de proliferação das células nos epitélios gerados foram mensurados indiretamente pela avaliação da localização e intensidade dos sinais obtidos em ensaios de imuno-histoquímica utilizando um anticorpo anti-PCNA. Analogamente aos dados obtidos por *Western blotting* apresentados na figura 1D, observou-se elevada expressão de PCNA nas culturas organotípicas derivadas dos PHK18AA e PHK18Af em todas as camadas epiteliais. Por outro lado, baixos níveis de PCNA foram observados nos epitélios advindos dos PHKs não-transduzidos, sendo estes restritos à camada basal proliferativa (figura 4C).

É importante salientar que nos epitélios derivados das células imortalizadas, foram observados concomitantemente altos níveis de CK10 e PCNA na maior parte das células das camadas suprabasais indicando que as proteínas E6/E7 das duas variantes de HPV-18 analisadas são capazes de induzir a proliferação e diferenciação em uma mesma célula, como já observado em

estudos acerca da avaliação do potencial oncogênico das variantes de HPV-16 (Richard *et al.*, 2010). Ainda assim, não foram observadas diferenças suficientes na morfologia e níveis de diferentes proteínas entre os epitélios derivados de PHK18AA e PHK18Af imortalizados que pudesse indicar lesões com graus de agressividade distintos.

3. Discussão

Este estudo visou a avaliação abrangente do potencial oncogênico de duas variantes naturais de HPV-18 no *background* genético de PHKs, que consistem das células hospedeiras naturais da infecção por este vírus. Em comparação à variante As+AI, a variante Af de HPV-18 apresenta uma alteração não-conservativa em cada uma das proteínas virais: E6 (N129K) e E7 (H2Y). Inicialmente, foram construídos vetores retrovirais recombinantes contendo os oncogenes virais de ambas as variantes de HPV-18 que, em seguida, foram utilizados para transdução de PHKs comercialmente disponíveis. A transdução adequada foi confirmada através da análise dos níveis de transcritos virais por RT-PCR, além de proteínas virais e celulares por *Western blotting*.

Os PHKs transduzidos com as diferentes variantes de HPV-18 foram continuamente cultivados até a passagem 30, quando foram considerados imortalizados. O estudo dessa cinética revelou que os PHK18AA atingiram p30 em um período significativamente menor que PHK18Af. Ademais, nós não observamos diferenças na morfologia entre as células imortalizadas por ambas as variantes de HPV-18 quando comparadas aos PHKs não-transduzidos. No que concerne ao tempo de dobramento, nós não observamos diferenças significativas entre as células transduzidas pelas variantes de HPV-18. Nossos resultados

corroboram aos achados de Lace e col. (2009) que também não observaram um período de crise evidente quando PHKs de diferentes origens anatômicas (prepúcio, cérvice uterina e tonsilas) foram transduzidos com o genoma de HPV-16 ou HPV-18; embora contrastem com Richard e col. (2010) que transduziu PHKs oriundos de prepúcio transduzidos com *E6/E7* das diferentes variantes de HPV-16 e observou um aumento no tempo de duplicação celular entre as passagens 10 e 11, evidenciado um período de crise anterior à imortalização.

Nós detectamos a proteína viral E6 nas células transduzidas com ambas as variantes de HPV-18 por *Western blotting*, embora se observe oscilação dos níveis desta proteína através das passagens em cultura: baixos nas células recém-infectadas (p5), aumentados em p15 e novamente diminuídos em p30, quando as células foram consideradas imortalizadas. Como esperado, os níveis de p53 apresentaram correlação linear inversa aos de E6, dados estes semelhantes aos previamente descrito por Pim e col. (1997) e de la Cruz-Hernández e col. (2005). Ainda que os níveis proteicos de E6 estejam diminuídos em p30, em comparação aos observados em p15, os níveis dos transcritos E6 e E6*I medidos foram similares através das passagens em cultura, assim como os níveis do transcrito E7, independentemente da variante avaliada. Estes resultados contrastaram com estudos prévios conduzidos empregando-se células MCF-7 e C33 (carcinoma de colo uterino HPV negativo, p53 mutado) que reportaram maiores níveis de E6*I nas células transfectadas com apenas *E6* da variante Af em comparação à variante As+AI (Vazquez-Vega *et al.*, 2013; de la Cruz-Hernández *et al.*, 2005). Entretanto, é importante ressaltar que o *background* genético das células, assim como os modelos utilizados para superexpressão de E6, diferem entre os estudos e dificultam a comparação. Já os níveis proteicos de

E7 foram avaliados indiretamente pela mensuração dos níveis de seus principais alvos celulares. Nós observamos que os níveis de p16 e pRb nas células transduzidas com HPV-18 estavam aumentados e diminuídos, respectivamente, em relação aos detectados nos PHKs não-transduzidos, independentemente da variante avaliada. Adicionalmente, os níveis de p21 estavam diminuídos em todas as passagens das células transduzidas, independentemente da variante avaliada. Nós também não observamos diferenças significativas entre os PHK18AA e PHK18Af imortalizados no que concerne às taxas de proliferação celular, quer seja quando as células foram contadas ou quando a proliferação foi avaliada indiretamente pelos níveis das proteínas PCNA e Ki-67.

Além do importante papel de E6 na imortalização celular, está consolidado que esta oncoproteína é crucial para a resistência à diferenciação terminal de queratinócitos induzida por soro e cálcio *in vitro* (Stöppler *et al.*, 1996; Sherman e Schlegel, 1996; Asadurian *et al.*, 2007). Neste trabalho, nós observamos que, quando os PHKs imortalizados pelas variantes de HPV-18 foram mantidos em meio de cultura enriquecido com soro e cálcio, os PHK18Af apresentaram maior resistência à diferenciação quando comparados aos PHK18AA. Embora, diversos estudos indiquem que a capacidade de E6 em degradar e/ou transativar p53 poderia estar relacionada ao potencial de resistência à diferenciação, não é possível descartar a importância de outras vias celulares neste processo (Stöppler *et al.*, 1996; Sherman *et al*., 1997; Asadurian *et al.*, 2007). Uma vez que nós não observamos diferenças nos níveis de p53 entre os PHK18AA e PHK18Af, estudos adicionais são necessários para melhor compreender as vias pelas quais as proteínas E6/E7 da variante Af conferem maior resistência à diferenciação em relação à variante As+AI. A resistência à

diferenciação induzida por soro e cálcio pode ser avaliada através da coloração das células com o corante metileno blue, seguida pela contagem das colônias formadas; ou, como realizado neste estudo, através da medição colorimétrica após incubação das células com MTT, o qual consiste de um experimento clássico utilizado para aferir a viabilidade celular. Ambos os corantes precisam ser processados por mitocôndrias para que seja possível a discriminação das colônias de células resistentes à diferenciação celular. Assim, existe ainda a possibilidade de que as oncoproteínas virais advindas das diferentes variantes impactem diferentemente sobre as vias de respiração celular, podendo influenciar desde a resposta ao estresse oxidativo até a morte celular. Embora diversos estudos conduzidos até o momento tenham avaliado o papel de E6, assim como de E6*I, sobre a atividade mitocondrial associada ao estresse oxidativo e à morte celular, não existem relatos acerca da influência das funções mitocondriais na diferenciação de queratinócitos infectados por HPVs (de la Cruz-Hernández *et al.*, 2005; Vogt *et al.*, 2006; Evans *et al.*, 2016; Cabeça *et al.*, 2019).

Em seguida, objetivou-se avaliar o potencial oncogênico das diferentes variantes de HPV-18 através da análise de diversas propriedades intrínsecas às células carcinogênicas (Hanahan e Weinberg, 2011). Em conjunto, os ensaios realizados neste trabalho indicaram que os PHK18AA imortalizados apresentaram maior eficiência de formação de colônias quando cultivados em baixa densidade ou independente de adesão ao substrato (avaliados por cultivo celular em placas de baixa aderência celular ou em meio semi-sólido) em comparação aos PHK18Af. Cabe ainda ressaltar, a dificuldade enfrentada na identificação de colônias e esferoides verdadeiros advindos dos PHK18Af, fato este observado em todos os experimentos realizados, uma vez que houve tendência destas células

formarem estruturas de aglomerados celulares não-coesos como claramente evidenciado nas imagens microscópicas, com realce ao uso da tecnologia de bioimpressão magnética de células. Mesmo que em diferente *background* genético, foi também observado por de la Cruz-Hernández e col. (2005) um maior número de esferoides advindos de células NIH3T3 expressando *E6* da variante As+AI em meio semi-sólido (*soft* agar) em comparação às células expressando *E6* de outras variantes de HPV-18.

Em seguida, duas outras importantes características intrínsecas às células tumorigênicas foram avaliadas nos PHKs imortalizados por *E6/E7* das diferentes variantes de HPV-18: migração e invasão celular. Nós não observamos diferenças na capacidade de migração entre os PHK18AA e PHK18Af empregando o ensaio de cura de ferida, ao contrário de Fragoso-Ontiveros e col. (2012) que observaram um potencial de migração significativamente maior nas células expressando *E6* da variante Af de HPV-18, em comparação àquelas expressando *E6* da variante As+AI. É importante ressaltar as diferenças entre os modelos experimentais utilizados: nos ensaios realizados por Frangoso-Ontiveros e col. (2012), foi avaliada apenas a atividade da proteína E6 e em um *background* genético diferente dos PHKs utilizados por nós; ademais, não foi utilizada mitomicina C para indução da parada do ciclo celular durante a avaliação da migração, o que dificulta a discriminação das células que migraram daquelas que proliferaram.

A invasão de células tumorais depende não apenas da atividade migratória destas, mas também de outros fatores cruciais que permitem a penetração de barreiras teciduais para que estas células possam estabelecer metástases a distância (revisado por Friedl e Wolf, 2003). Ainda que não foi

possível observar diferenças no potencial migratório dos PHKs imortalizados pelas diferentes variantes, nossos resultados demonstraram que os PHK18AA apresentaram uma maior capacidade de invasão em matriz de colágeno quando comparados aos PHK18Af. Assim, poderíamos supor que possivelmente as proteínas E6/E7 das distintas variantes de HPV-18 apresentam diferentes capacidades em interagir com proteínas alvo que compõe a membrana basal, estroma intersticial e/ou matriz extracelular. Até o momento, alguns estudos foram realizados a fim de compreender o papel de E6/E7 na capacidade de invasão de células infectadas por HPVs de alto risco. Yoshida e col. (2008) provaram que PHKs transduzidos com *RAS* e *E7* de HPV-18 apresentaram maior potencial de invasão em matriz de colágeno e *rafts* em comparação a PHKs transduzidos apenas com *E7* de HPV-18. Já Wang e col. (2019) transfectaram *E6* de HPV-16 ou *E6* de HPV-16 com o motivo de ligação a PDZ (PBM) deletado nas linhagens de células CaSki (derivadas de carcinoma de colo do útero HPV-16 positivo), HeLa, C33 e HaCaT (derivadas de queratinócitos humanos imortalizados) e observaram que a região PBM do E6 de HPV-16 contribuiu significativamente para na promoção da migração e invasão das células de CCU. Entretanto, ainda não há trabalhos publicados que tenham comparado a atividade das oncoproteínas advindas de diferentes variantes moleculares no potencial de invasão ou na capacidade de interagir com as mesmas proteínas celulares importantes neste processo.

Embora os PHK18Af apresentaram maior capacidade de resistir à diferenciação em meio enriquecido com soro e cálcio, nós não observamos diferenças relevantes entre as células imortalizadas pelas duas variantes de HPV-18 em formarem epitélios nos sistemas de culturas organotípicas. Todos os

epitélios derivados das células imortalizadas pelas diferentes variantes de HPV-18 apresentaram hiperplasia, além de altos níveis de CK10, PCNA e p16 por todo o tecido gerado, sendo compatíveis com o esperado em neoplasias intra-epiteliais cervicais. Ainda assim, os diferentes parâmetros avaliados nestes epitélios não foram suficientes em indicar lesões com graus de agressividade distintos.

Em conjunto, nossos dados indicam que a expressão de ambas as oncoproteínas E6 e E7 das variantes As+AI e Af de HPV-18 interferem em diferentes aspectos associados à carcinogênese. Ainda que a expressão de *E6/E7* de ambas as variantes de HPV-18 foi capaz de induzir a imortalização de PHKs, a variante As+AI imortalizou estas células em um período menor. Ademais, nós também observamos que os PHK18AA geraram maior número de colônias e esferoides em diversos experimentos, além de apresentar um maior potencial de invasão em comparação aos PHK18Af. Entretanto, não foram observadas diferenças significativas no que se refere ao potencial de proliferação e migração dos PHKs imortalizados pelas duas variantes. Finalmente, os PHK18Af apresentaram maior capacidade de inibir a diferenciação celular em meio enriquecido com soro e cálcio, ainda que não fossem observadas diferenças nos epitélios derivados dos PHK18AA e PHK18Af em cultura organotípica. Embora, como todos nossos dados apontem para um maior potencial oncogênico atribuível à variante As+AI em relação à variante Af de HPV-18, estudos adicionais são necessários para melhor compreender os mecanismos bioquímicos por trás das diferenças observadas.

4. Materiais e métodos

Cultura de células

Células das linhagens celulares HeLa (ATCC CCL-2), derivada de adenocarcinoma de colo de útero HPV-18 positivo; NIH3T3 (ATCC CRL 1658), advinda de fibroblasto murino; Bosc-23 (ATCC CRL 11270) e Am-12, ambas advindas de rim humano e gentilmente cedidas pela Profa. Dra. Louise Chow (Universidade de Alabama, Birmingham, EUA), foram cultivadas em meio *Dulbecco's Modified Eagle Medium* (DMEM, Invitrogen, Califórnia, EUA), contendo 10% de soro fetal bovino (SFB), e incubadas a 37 °C em 5% CO_2.

Dois lotes distintos de *pools* de queratinócitos humanos primários (PHK) oriundos de prepúcio de recém-nascidos de doadores distintos e adquiridos da Clonetics (Nova Jersey, EUA, cat # 00192906, lotes # 355184 e 357479), além dos fibroblastos de camundongo (p.e., Swiss 3T3 J2 ou Balb/C 3T3 JCA 31), foram cultivados em meio de manutenção de queratinócitos livre de soro (*keratinocyte serum free medium*, KSFM, Invitrogen, Califórnia, EUA), suplementado com 5 ng/mL de fator de crescimento epidermal (EGF, Invitrogen, Califórnia, EUA) e 50 mg/mL de extrato de pituitária bovina (Invitrogen, Califórnia, EUA), e incubados a 37 °C em 5% CO_2.

Plasmídeos

A sequência completa dos genes *E6/E7* (812 pb) das duas variantes de HPV-18 foi amplificada por PCR a partir de amostras de esfregaços genitais previamente caracterizadas (Franco *et al.*, 1999; Sichero *et al.*, 2007). Os fragmentos gerados foram clonados no vetor retroviral pLNSX (GenBank: M28248.1) entre os sítios das endonucleases de restrição *EcoRI* e *BamHI*. A ausência de inserção de mutações durante a construção e clonagem dos vetores retrovirais recombinantes foi confirmada por sequenciamento empregando-se o

BigDye® Terminator v3.1 Cycle Sequencing kit (AB Applied Biosystems, Califórnia, EUA) em um sequenciador automático *ABI 3130XL Genetic Analyser* (AB Applied Biosystems, Califórnia, EUA).

Foram construídos vetores retrovirais recombinantes contendo a sequência de *E6/E7* das variantes As+AI e Af (Ong *et al.*, 1993), hoje reclassificadas como sublinhagens A1 e B1, respectivamente (Chen *et al.*, 2013). Em comparação à sequência da variante As+AI, apenas uma alteração não-conservativa foi observada em cada um dos genes *E6* e *E7* (E6: N129K e E7: H2Y) da variante Af de HPV-18. Grandes quantidades de DNA destes vetores foram obtidas por maxi-preparação utilizando-se *o EndoFree Plasmid Maxi kit* (Qiagen, Hilden, Alemanha), de acordo com as instruções do fabricante.

Transdução dos PHKs

Os vetores retrovirais recombinantes pLNSX E6/E7 das duas variantes moleculares de HPV-18, além do vetor pLNSX vazio, foram transfectados em células da linhagem celular empacotadora ecotrópica Bosc-23 utilizando-se o *Fugene 6 Transfection reagent* (Promega, Madison, EUA). Aproximadamente 48 horas após a transfecção, o sobrenadante das células Bosc-23 foi utilizado para infectar células da linhagem celular empacotadora anfotrópica Am-12 na presença de 10 µg/mL de polibreno (Sigma, Missouri, EUA). Após 48 horas, as células Am-12 infectadas foram selecionadas utilizando-se 500 µg/mL de geneticina (Invitrogen, Califórnia, EUA) por uma semana. O título viral destas células foi avaliado empregando-se o protocolo de contagem de colônias de células NIH3T3 infectadas (Ausubel *et al.*, 2001) no qual quantidades equivalentes de partículas retrovirais (contendo os vetores recombinantes pLNSX *E6/E7* das variantes As+AI

ou Af de HPV-18, ou apenas o vetor pLNSX vazio) foram utilizadas para transduzir as células dos dois lotes distintos de PHKs (PHK#1 e PHK#2).

Após seleção dos PHKs infectados utilizando-se 300 µg/mL de geneticina por duas semanas, as células transduzidas foram repicadas semanalmente (1:6) até a quinta passagem a fim de se obter grande quantidade de uma população pura de células infectadas. Os PHKs transduzidos foram denominados: PHK *pool* 1 (lot # 355184) e PHK *pool* 2 (lot # 357479) (vetor vazio); PHK18AA *pool* 1 e PHK18AA *pool* 2 (PHKs transduzindo *E6/E7* da variante As+AI), PHK18Af *pool* 1 e PHK18Af *pool* 2 (PHKs transduzindo *E6/E7* da variante Af). Uma vez que os PHKs transduzidos com o vetor pLNSX vazio cessaram a proliferação na passagem cinco, os PHKs *pool* 1 e PHKs *pool* 2 não-transduzidos foram utilizados como controles nos diferentes experimentos.

Cinética de crescimento e tempo de duplicação dos PHKs transduzidos

A cinética de crescimento dos PHKs transduzidos com as duas variantes de HPV-18 foi avaliada por plaqueamento sucessivo de 1,25 × 10^5 células em garrafa de 25 cm², sendo estas células contadas e subcultivadas quando atingiam 80% de confluência. As células foram cultivadas até passagem 30 quando foram consideradas imortalizadas (Halbert *et al.*, 1991). O tempo de duplicação foi calculado utilizando-se o programa disponível no sítio http://www.doubling-time.com/compute.php.

Ensaio de proliferação e expressão de Ki-67

Os PHK18AA e PHK18Af (ambos em p30), além dos PHKs não-transduzidos (p5), foram cultivados (5 × 10^3 células) em placas de 12 poços e,

após dois, quatro e seis dias, as células foram tripsinizadas e contadas utilizando-se o *Countess™ automated cell counter* (Invitrogen, Califórnia, EUA). Após a contagem, as células foram coletadas, fixadas com etanol 70% e mantidas a -20°C por no mínimo uma hora. Em seguida, as células foram lavadas com PBS 1×, para retirada do etanol, e incubadas com o anticorpo monoclonal de coelho recombinante anti-Ki67 (1:50, ab92742, Abcam, Massachusetts, EUA) a temperatura ambiente por uma hora. Após lavagem com PBS 1×, as células foram incubadas com o anticorpo secundário de cabra anti-IgG de coelho marcado com Alexa Fluor 633 (1:100, A-21070, Thermo Scientific, Wilmington, EUA) a temperatura ambiente por uma hora. As amostras foram avaliadas no citômetro de fluxo *Attune Acoustic Focusing Flow Cytometer* (Thermo Scientific, Wilmington, EUA), e os dados gerados foram analisados no programa *FlowJo X 10.0.7r2* (Tree Star, Oregon, EUA).

Extração de RNA, RT-PCR e qRT-PCR

O RNA total dos PHK18AA e PHK18Af (ambos p5, 15 e 30), além dos PHKs não-transduzidos (p5), foi extraído utilizando-se o reagente Trizol (Invitrogen, Califórnia, EUA) e clorofórmio (Merck, Darmstadt, Alemanha), seguido por precipitação com etanol 75% diluído em água tratada com dietilpirocarbonato (DEPC) por duas horas a -80 °C e ressuspensão em água deionizada e destilada. A quantidade de RNA extraído foi avaliada em espectrofotômetro NanoDrop® (Thermo Scientific, Wilmington, EUA) a 260 nm. Um micrograma de RNA foi transcrito reversamente à cDNA empregando-se o *High-Capacity RNA-to-cDNA kit* (Applied Biosystems, Foster City, EUA) como recomendado pelo fabricante. Na reação de RT-PCR (reação em cadeia da polimerase via transcriptase reversa)

foram utilizados iniciadores capazes de amplificar o transcrito completo de E6, além do produto de processamento alternativo E6*I (nts 105-581). Os produtos amplificados foram visualizados no fotodocumentador *UVP GelDoc-It2 310 Imaging System* (Thermo Scientific, Wilmington, EUA) após eletroforese em gel de acrilamida (acrilamida:bisacrilamida 29:1) 8% por 1 hora a 100 V e posterior coloração com nitrato de prata.

Os RNAs extraídos foram ainda utilizados em uma reação de qRT-PCR (reação em cadeia da polimerase via transcriptase reversa quantitativa) empregando-se o *kit GoTaq® 1-Step RT-qPCR System* (Promega, Madison, EUA) em equipamento *Applied Biosystems 7500 Real-Time PCR System* (Thermo Scientific, Wilmington, EUA) para avaliação da expressão de *E6* (nts 111-180) e *E7* (nts 750-852). Os níveis de mRNA da proteína ribossômica mitocondrial S18 (nts 192-339) foram utilizados como controle endógeno da reação.

Extração de proteínas e *Western blotting*

Proteína total dos PHKs não-transduzidos (p5), além dos PHK18AA e PHK18Af (ambos p5, 15 e 30), foi extraída utilizando-se tampão RIPA (Tris-HCl 20 mM, pH 7,5, NaCl 150 mM, deoxicolato de sódio 0,5%, SDS 0,1%, NP-40 1%) suplementado com um coquetel de inibidores de protease e fosfatase (Roche, Basel, Suíça), após cultivo até 80% de confluência em placas de 100 mm. Em seguida, a quantificação da proteína extraída foi realizada utilizando-se o reagente Bradford (Bio-Rad, Califórnia, EUA) em um leitor de placa *Bio-Rad Benchmark Microplate Reader* (Bio-Rad, Califórnia, EUA).

As proteínas extraídas (60 - 120 µg) foram fracionadas em SDS-PAGE (eletroforese em gel de poliacrilamida na presença de dodecil sulfato de sódio) e

posteriormente transferidas para membranas de PVDF (fluoreto de polivinilideno, *GE Healthcare Life Sciences*, Buckinghamshire, Inglaterra). Após incubação com os anticorpos primários (*overnight* a 4 °C) e secundários (uma hora a temperatura ambiente), os sinais foram revelados utilizando-se o *Amersham ECL™ Western Blotting Detection Reagent* (*GE Healthcare Life Sciences*, Buckinghamshire, Inglaterra) no equipamento *ImageQuant LAS 4.000* (*GE Healthcare Life Sciences*, Buckinghamshire, Inglaterra). As imagens foram analisadas no programa *Image J 1.52p* (*National Institute of Health*, EUA).

Os seguintes anticorpos primários foram utilizados: anticorpo monoclonal de rato anti-E6 de HPV-18 (1,8 µg/mL, AVC#1006, *Arbor Vita Corporation*, Califórnia, EUA), anticorpo monoclonal de rato anti-p53 (1:500, sc-126, Santa Cruz, Texas, EUA), anticorpo monoclonal de rato anti-pRb (1:250, ab24, Abcam, Massachusetts, EUA), anticorpo monoclonal de rato anti-CDKN2A/p16INK4a (1:1 000, ab16123, Abcam, Massachusetts, EUA), anticorpo policlonal de coelho anti-p21 (1:250, ab7960, Abcam, Massachusetts, EUA) e anticorpo monoclonal de rato anti-PCNA (1:1 000, ab29, Abcam, Massachusetts, EUA). Para a normalização dos níveis proteicos totais, as membranas foram adicionalmente incubadas com o anticorpo monoclonal de rato anti-α-tubulina (1:5 000, T9026, Sigma-Aldrich, Missouri, EUA).

Ensaio clonogênico e análise da morfologia celular

Foram semeados 500 PHK18AA e PHK18Af (ambos p5 e 30), além dos PHKs não-transduzidos (p5), em placa de seis poços. A formação e o crescimento das colônias de células foram acompanhados por 10 dias através de registro fotográfico de ao menos três colônias por poço usando-se os equipamentos Primo

Vert (*Zeiss*, Jena, Alemanha) e *EVOS FL Auto Imaging System* (*Life Technologies*, Califórnia, EUA). Posteriormente, as células foram coradas com cristal violeta (Sigma-Aldrich, Missouri, EUA) e a imagem da placa foi registrada no fotodocumentador *UVP GelDoc-It2 310 Imaging System* (*Thermo Scientific*, Wilmington, EUA). O perímetro e área das células, assim como o número de colônias formadas e a área das mesmas, foram avaliados no programa *Image J 1.52p* (National Institute of Health, EUA).

Ensaio de diferenciação por soro e cálcio

Foram semeados 1 × 10^3 PHK18AA e PHK18Af imortalizados (p30), além dos PHKs não-transduzidos (p5), em placa de seis poços. Após sete dias, as células foram cultivadas ou não em meio de diferenciação (DMEM, SFB 10%, 1 µg/mL de hidrocortisona). Sete dias após a troca ou não do meio, as células foram incubadas com 0,5 mg/mL de MTT (brometo de 3-(4,5-dimetil-2-tiazolil)-2, 5-difenil-2H-tetrazólio, Sigma-Aldrich, Missouri, EUA) por três horas. Posteriormente, as células foram fotografadas no fotodocumentador *UVP GelDoc-It2 310 Imaging System* (Thermo Scientific, Wilmington, EUA). Finalmente, as células foram diluídas em DMSO (dimetilsulfóxido) e medidas de absorbância em comprimento de onda à 570 nm foram realizadas no leitor de placa *Bio-Rad Benchmark Microplate Reader* (Bio-Rad, Califórnia, EUA).

Ensaio de baixa aderência

Foram semeados 2 × 10^4 PHK18AA e PHK18Af imortalizados, além dos PHKs não-transduzidos (p5), em placa de seis poços de baixa aderência (Corning Inc., Nova Iorque, EUA). Após 14 dias, os esferoides formados foram analisados

macro e microscopicamente. Registros fotográficos em magnitude 4× e 10× foram realizados no microscópio *EVOS FL Auto Imaging System* (Life Technologies, Califórnia, EUA). A eficiência de formação de esferoides foi calculada dividindo-se o número de esferoides formados pelo número de células plaqueadas (Vishinoi *et al.*, 2016; Dontu *et al.*, 2003). O número e área dos esferoides formados foram avaliados no programa *Image J 1.52p* (*National Institute of Health*, EUA).

Crescimento independente de ancoragem (ensaio de *soft* agar)

Foram cultivados 5 × 10^3 PHK18AA e PHK18Af imortalizados, além dos PHKs não-transduzidos (p5), em meio semi-sólido (*soft* agar de baixo ponto de fusão a 0,5%) em placa de 24 poços previamente cobertos por meio contendo *soft* agar a 1%. A formação e o crescimento dos esferoides foram acompanhados por 15 dias, quando estes foram corados com solução contendo 1 mg/mL de MTT (Sigma-Aldrich, Missouri, EUA) e as imagens foram registradas macroscopicamente, usando o fotodocumentador *UVP GelDoc-It2 310 Imaging System* (Thermo Scientific, Wilmington, EUA), e microscopicamente, utilizando-se o Primo Vert (Zeiss, Jena, Alemanha). O número e área dos esferoides formados foram aferidos no programa *Image J 1.52p* (*National Institute of Health*, EUA).

Bioimpressão magnética de células

Os PHK18AA e PHK18Af imortalizados, além dos PHKs não-transduzidos (p5), foram cultivados em placa de 100 mm até atingirem 80% de confluência. Seguiu-se a incubação com uma solução de nanopartículas magnéticas biocompatíveis *Nanoshuttle- PL* (Greiner Bio-One GmbH, Frickenhausen, Alemanha) por 16 horas. Após este período, 1 × 10^4 células foram semeadas em

placa de 96 poços de baixa aderência (Greiner Bio-One GmbH, Frickenhausen, Alemanha). Em seguida, a placa magnética *96-well spheroid drive* (Greiner Bio-One GmbH, Frickenhausen, Alemanha) foi inserida abaixo da placa contendo as células e mantida por 24 horas. O desenvolvimento dos esferoides foi acompanhado por 15 dias através de registro fotográfico utilizando-se o microscópio Primo Vert (Zeiss, Jena, Alemanha).

Ensaio de migração celular

Foram semeados 5 × 10^5 PHK18AA e PHK18Af imortalizados, além dos PHKs não-transduzidos (p5), em placa de 12 poços. Após 24 horas, a fim de inibir o ciclo celular, as células foram incubadas por seis horas com mitomicina C 10 µM (Sigma-Aldrich, Missouri, EUA) e em seguida um risco com a ponta de uma ponteira de 200 µL foi realizado no centro através de todo o poço. A partir deste momento, acompanhou-se a migração celular por um período de 12 horas. As imagens e vídeos foram registrados utilizando-se o microscópio *EVOS FL Auto Imaging System* (*Life Technologies*, Califórnia, EUA). A área das feridas foi aferida no programa *Image J 1.52p* (*National Institute of Health*, EUA).

Em seguida, para demostrar a parada do ciclo celular, as células foram coletadas, fixadas com etanol 70%, mantidas a -20 °C por no mínimo duas horas, sendo em seguida lavadas com PBS 1× para retirada do etanol e incubadas com solução de iodeto de propídio (0,1% v/v de Triton X-100, RNase A 20 mg/mL, iodeto propídeo 5 mg/mL, em PBS 1×) por 20 minutos a 37 °C. As amostras foram avaliadas no citômetro de fluxo *Attune Acoustic Focusing Flow Cytometer* (*Thermo Scientific*, Wilmington, EUA) e os dados gerados foram analisados no programa *FlowJo X 10.0.7r2* (*Tree Star*, Oregon, EUA).

Ensaio de invasão celular

O potencial de invasão foi avaliado usando o *kit QCM High sensitivity non-crosslinked collagen invasion assay* (Millipore, Darmstadt, Alemanha) seguindo as indicações do fabricante. Primeiramente, o inserto contendo a matriz de colágeno foi hidratado com meio de cultura carenciado de fatores de crescimento e extrato de pituitária bovina. Em seguida, foram adicionados 2,5 × 10^5 PHK18AA e PHK18Af imortalizadas, além dos PHKs não-transduzidos (p5), em meio carenciado. A fim de estimular a invasão, foi adicionado meio de cultura acrescido de 15% de soro fetal bovino do lado de fora dos insertos. Após 72 horas, os insertos foram corados (corante fornecido pelo fabricante) e as células na parte interna do inserto foram removidas cuidadosamente com cotonete. Os insertos foram registrados fotograficamente utilizando-se o microscópio Primo Vert (Zeiss, Jena, Alemanha) e, em seguida, imersos em meio de extração (fornecido pelo fabricante) para análise indireta da capacidade de invasão por medida de absorbância a 540 nm no leitor de placa *Bio-Rad Benchmark Microplate Reader* (Bio-Rad, Califórnia, EUA).

Cultura organotípica e imuno-histoquímica

Os PHK18AA e PHK18Af imortalizados, além dos PHKs não-transduzidos em passagem zero, foram semeados sobre um equivalente dérmico composto por colágeno tipo I, oriundo de cauda de rato (BD Biosciences, Califórnia, EUA), contendo fibroblastos de camundongo irradiados incapazes de proliferar ou migrar para a superfície dérmica (Boccardo *et al.*, 2004). A cultura foi mantida na interface meio-ar e nutrida por capilaridade com meio acrescido de fatores de crescimento, soro e alta concentração de cálcio por 12 dias. Os epitélios formados

foram fixados em formaldeído 2%, embebidos em parafina e passados por sucessivos cortes para confecção de lâminas que foram submetidas à coloração com hematoxilina-eosina (a fim de analisar a estrutura tecidual) ou à imuno-histoquímica para avaliação dos níveis de PCNA, p16 e citoqueratina 10 (CK10). Para detecção destas proteínas, as lâminas foram desparafinizadas, re-hidratadas e hibridizadas empregando-se o *kit Novolink-Polymer-Detection-Systems* (Novocastra, Leica, Wetzlar, Alemanha) e os anticorpos primários específicos, como sugerido pelo fabricante. Os seguintes anticorpos primários foram utilizados: anticorpo monoclonal de coelho anti-citoqueratina 10 (1:2 500, ab76318, Abcam, Massachusetts, EUA), anticorpo monoclonal de rato anti-p16 (1:500, sc-56330, Santa Cruz, Texas, EUA) e anticorpo monoclonal de rato anti-PCNA (1:250, ab29, Abcam, Massachusetts, EUA).

Análise estatística

A análise estatística dos dados gerados foi conduzida utilizando-se o programa Statistix 8 para *Windows* (*Analytical Software*, FL, US) e empregando-se o teste T para comparar os resultados obtidos no grupo controle (PHKs não-transduzidos) aos obtidos utilizando-se os PHK18AA e PHK18Af. Todos os gráficos foram construídos no Microsoft Excel 2016 (Microsoft, Washington, EUA) com valores médios e erro padrão entre as replicatas realizadas para cada experimento. O valor de p foi considerado significativo quando menor do que 0,05.

5. Agradecimento

Nós gostaríamos de agradecer ao Instituto do Câncer do Estado de São Paulo (ICESP) e Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) por fornecer as condições necessárias ao desenvolvimento deste

trabalho. Este projeto foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo (Protocolo de Pesquisa nº 255/16) e financeiramente apoiado pelos Processos FAPESP nº 2007/58590-7 a LS e 2015/26346-6 a EMN. Nenhum conflito de interesse foi declarado pelos autores.

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Lista de figuras

das proteínas celulares p53 (D), pRb (E), p16 (F) e p21 (G) foram aferidos a partir do fracionamento de 60 µg de proteína total de PHKs não-transduzidos (p5), além dos PHK18AA e PHK18Af (ambos em p5, 15 e 30) em SDS-PAGE seguido de análise por *Western blotting*. Os níveis de tubulina foram usados como controle da quantidade de proteína aplicada em cada poço do gel. Está apresentado um experimento representativo de três experimentos realizados.

Figura 3 – Formação de colônias em culturas em monocamada e esferoides em culturas 3D gerados a partir dos PHKs transduzindo *E6/E7* das variantes de HPV-18. Para todos os experimentos, foram utilizados PHKs não-transduzidos (p5), além dos PHK18AA (p30) e PHK18Af (p30). O Teste T foi utilizado na análise estatística. (A) Eficiência de formação de colônias após plaqueamento em baixa densidade. Foram semeadas 500 células em placa de seis poços, em triplicata. Após sete dias, as células foram fixadas e coradas com cristal violeta. As imagens ilustram uma placa representativa de três experimentos independentes realizados, além de exemplos das colônias geradas em magnificação 4× no microscópio *EVOS FL Auto Imaging System* (Life Technologies). Os gráficos mostram a média do número de colônias formadas assim como a área média das colônias formadas. (B) Eficiência de formação de colônias resistentes à diferenciação induzida por soro e cálcio. Foram cultivadas 1 × 10^3 células em placa de seis poços, em triplicatas. Após sete dias, o meio de cultura ou foi mantido (controle) ou alterado para meio indutor de diferenciação. Após sete dias, as colônias foram coradas com MTT e as placas foram fotografadas. Posteriormente, as células foram diluídas em DMSO e a absorbância foi medida em comprimento de onda à 570 nm. As imagens ilustram uma placa representativa de três experimentos independentes realizados. Os gráficos mostram as médias de absorbância. (C) Eficiência de formação de esferoides em placas de baixa aderência. Foram semeadas 2 × 10^4 células em placa de seis poços de baixa aderência, em triplicatas. Após 14 dias, os poços foram fotografados. As imagens ilustram poços representativos de três experimentos independentes, em triplicata, além de imagens em magnificação 10× realizadas utilizando-se o microscópio Primo Vert (Zeiss, Jena, Alemanha). Os gráficos mostram a média do número de esferoides formados, assim como a área média dos esferoides formados. (D) Eficiência de formação de esferoides em meio semi-sólido (*soft* agar). Foram cultivadas 5 × 10^3 células em meio semi- sólido em placa de 24 poços, em triplicatas. A formação e o crescimento dos esferoides foram acompanhados por 15 dias. Após coloração com MTT, os esferoides foram avaliados micro e macroscopicamente. As imagens ilustram poços representativos de três experimentos independentes, realizados em triplicata, além de imagens dos esferoides em magnificação 4× e 10× no microscópio Primo Vert (Zeiss, Jena, Alemanha). Os gráficos mostram a média do número de esferoides formados, assim como a área média dos esferoides formados. (E) Eficiência de formação de esferoides através de bioimpressão magnética de células. Os PHKs não-transduzidos, além dos PHK18AA, PHK18Af e células da linhagem HeLa, foram incubados com solução *Nanoshuttle- PL* (Greiner Bio-One GmbH) e 1 × 10^4 células foram semeadas em placa de 96 poços não aderentes sobre placa magnética *96-well spheroid drive* (Greiner Bio-One GmbH) por 24 horas. Posteriormente, a placa magnética foi retirada e o crescimento dos esferoides foi acompanhado por 15 dias. Um experimento foi realizado com uma média de 20 replicatas para cada linhagem celular avaliada.

Figura 4 – Potencial de migração, invasão e desenvolvimento de culturas organotípicas a partir dos PHKs transduzindo *E6/E7* das variantes de HPV-18. Todos os experimentos foram conduzidos empregando-se PHKs não-transduzidos (p5 em A e p0 em C), PHK18AA (p30) e PHK18Af (p30). O Teste T foi utilizado na análise estatística. (A) Ensaio de cura de ferida usado para avaliar a capacidade de migração. Foram semeadas 5 × 10^5 células em placas de 12 poços e um risco foi realizado no meio do poço. As imagens ilustram poços representativos de três experimentos independentes realizados em triplicata. Em magnificação 10×, pode-se observar a cobertura da ferida seis e 12 horas após a realização do risco. O gráfico mostra a porcentagem média da área coberta 12 horas após a realização do risco. (B) Potencial de invasão de PHK18AA e PHK18Af foi estimado usando o *QCM High sensitivity non-crosslinked collagen invasion assay* (Millipore). Foram plaqueadas 2,5 × 10^5 células em insertos cobertos por colágeno previamente hidratados com meio carenciado e a invasão foi estimulada com a adição de meio suplementado com 15% de soro fetal bovino na parte externa dos insertos. A invasão celular foi acompanhada por 72 horas. As imagens dos insertos a olho nu, a junção das imagens realizadas em magnificação 4× e microfotografias realizadas em magnificação 10× são representativas de três experimentos independentes realizados em triplicata. O gráfico mostra a média de absorbância a 540 nm. (C) Culturas organotípicas geradas a partir dos PHKs não-transduzidos, PHK18AA e PHK18Af. Micrografias dos tecidos corados com hematoxilina-eosina (HE), e posterior realização de imuno-histoquímica para avaliação dos níveis de citoqueratina 10 (CK10), p16 e PCNA. Experimento representativo de dois ensaios independentes realizados.

Figura suplementar 1 – Potencial de migração dos PHKs não-transduzidos, PHK18AA e PHK18Af. (A) Porcentual de células nas fases G0/G1, S e G2/M do ciclo celular medido por citometria de fluxo após incubação com iodeto de propídio (5 mg/mL). Os PHKs (p5), PHK18AA (p30) e PHK18Af (p30) foram tratados com mitomicina C (10 µM por 18 horas) durante o ensaio de migração. O gráfico apresenta a média de três experimentos independentes realizados em triplicata. O Teste T foi utilizado na análise estatística. (B-D) Vídeos gerados a partir de microfotografias obtidas através de 12 horas após a realização dos riscos. As microfotografias foram realizadas com magnificação 4× no microscópio *EVOS FL Auto Imaging System* (*Life Technologies*). Os vídeos advindos das linhagens PHK (B), PHK18AA (C) e PHK18Af (D) são representativos de três experimentos independentes realizados em triplicata.

Figura 1.

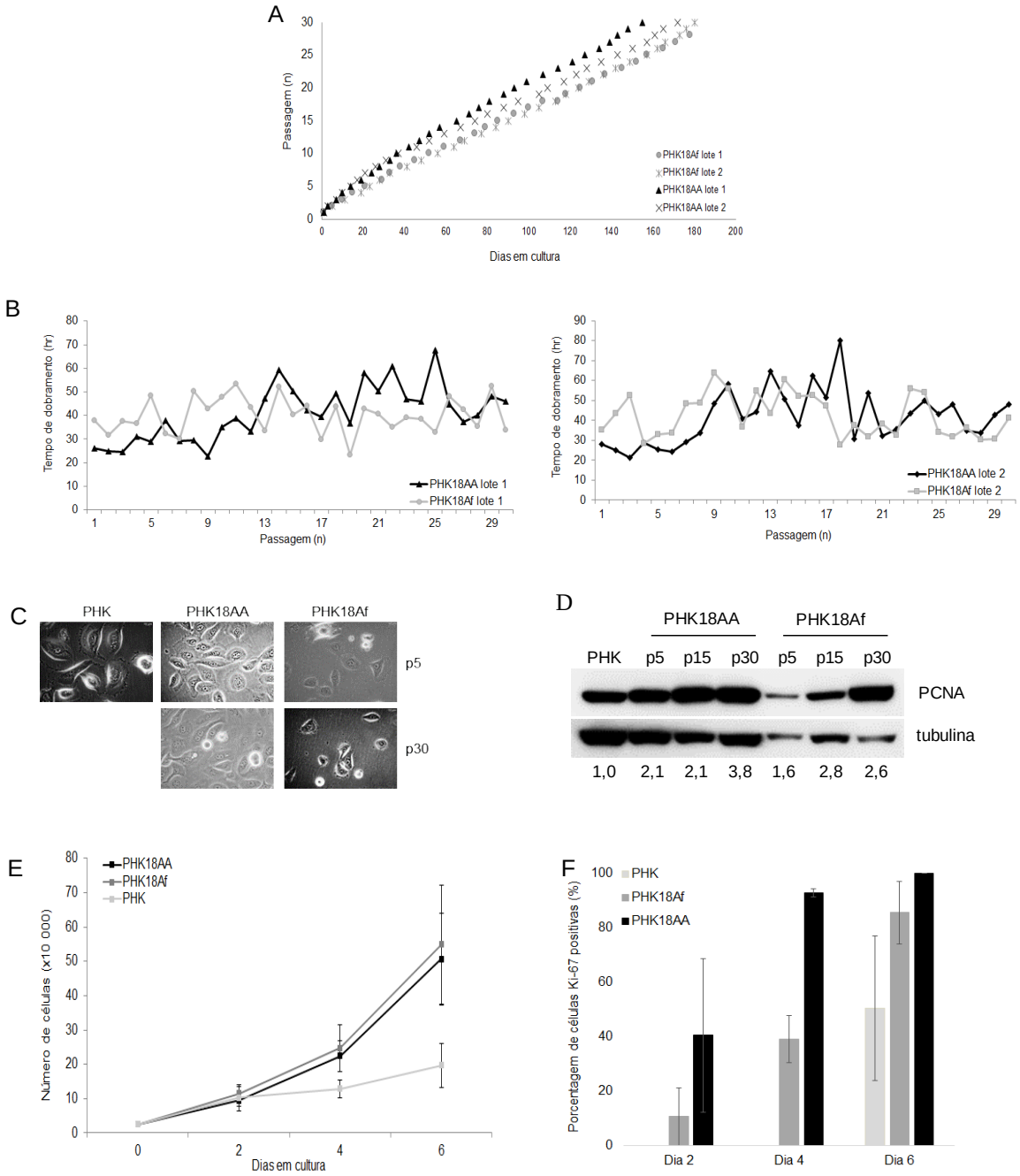

A
Passagem (n)
Dias em cultura
PHK18Af lote 1
PHK18Af lote 2
PHK18AA lote 1
PHK18AA lote 2
B
Tempo de dobramento (hr)
Passagem (n)
PHK18AA lote 1
PHK18Af lote 1
PHK18AA lote 2
PHK18Af lote 2
C
PHK
PHK18AA
PHK18Af
p5
p30
D
PHK18AA
PHK18Af
PHK
p5
p15
p30
p5
p15
p30
PCNA
tubulina
1,0
2,1
2,1
3,8
1,6
2,8
2,6
E
PHK18AA
PHK18Af
PHK
Número de células (x10 000)
Dias em cultura
F
PHK
PHK18Af
PHK18AA
Porcentagem de células Ki-67 positivas (%)
Dia 2
Dia 4
Dia 6

Figura 2.

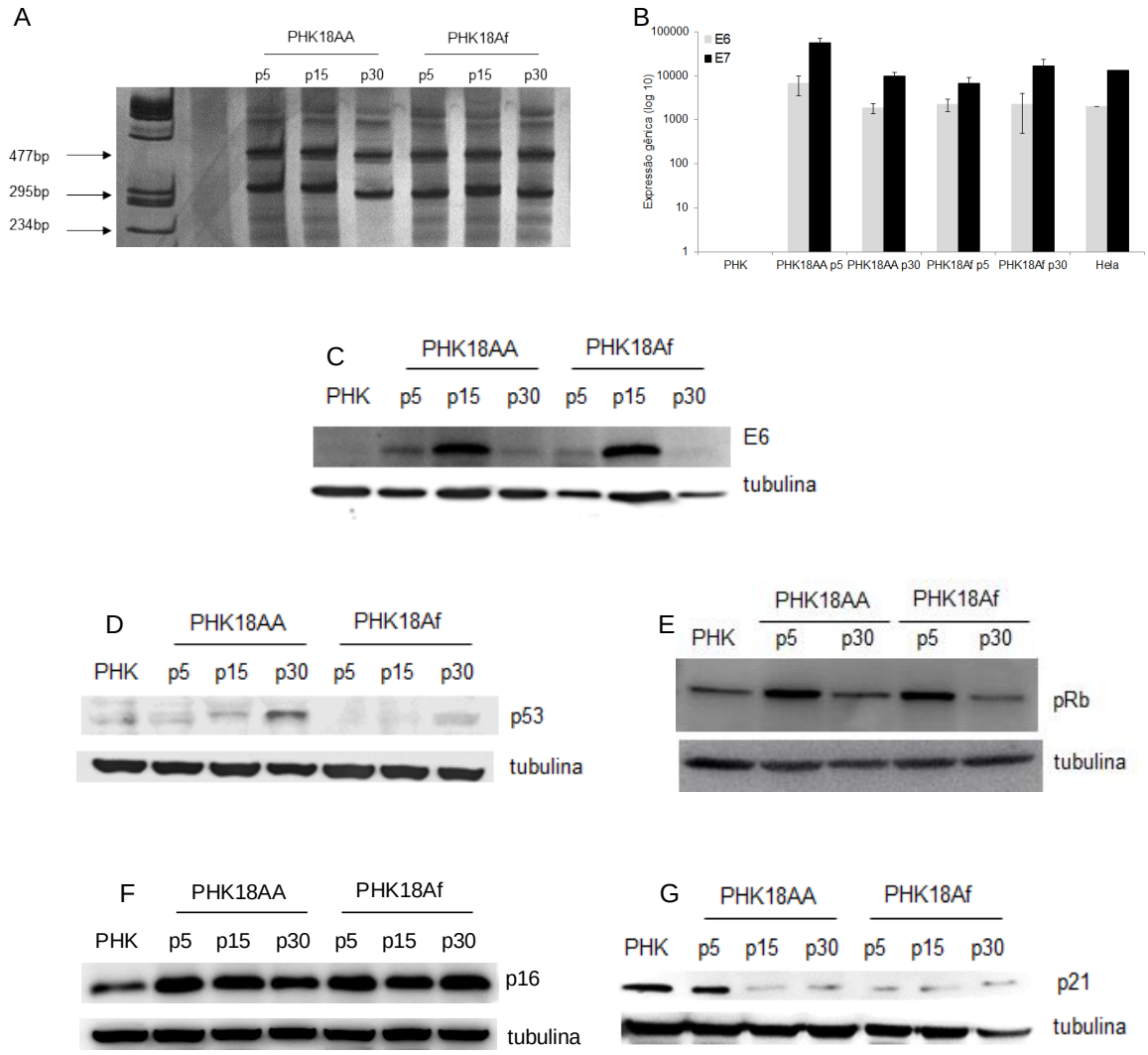

A
PHK18AA
PHK18Af
p5 p15 p30 p5 p15 p30
477bp
295bp
234bp
B
E6
E7
Expressão gênica (log 10)
100000
10000
1000
100
10
1
PHK
PHK18AA p5
PHK18AA p30
PHK18Af p5
PHK18Af p30
Hela
C
PHK18AA
PHK18Af
PHK p5 p15 p30 p5 p15 p30
E6
tubulina
D
PHK18AA
PHK18Af
PHK p5 p15 p30 p5 p15 p30
p53
tubulina
E
PHK18AA
PHK18Af
PHK p5 p30 p5 p30
pRb
tubulina
F
PHK18AA
PHK18Af
PHK p5 p15 p30 p5 p15 p30
p16
tubulina
G
PHK18AA
PHK18Af
PHK p5 p15 p30 p5 p15 p30
p21
tubulina

Figura 3.

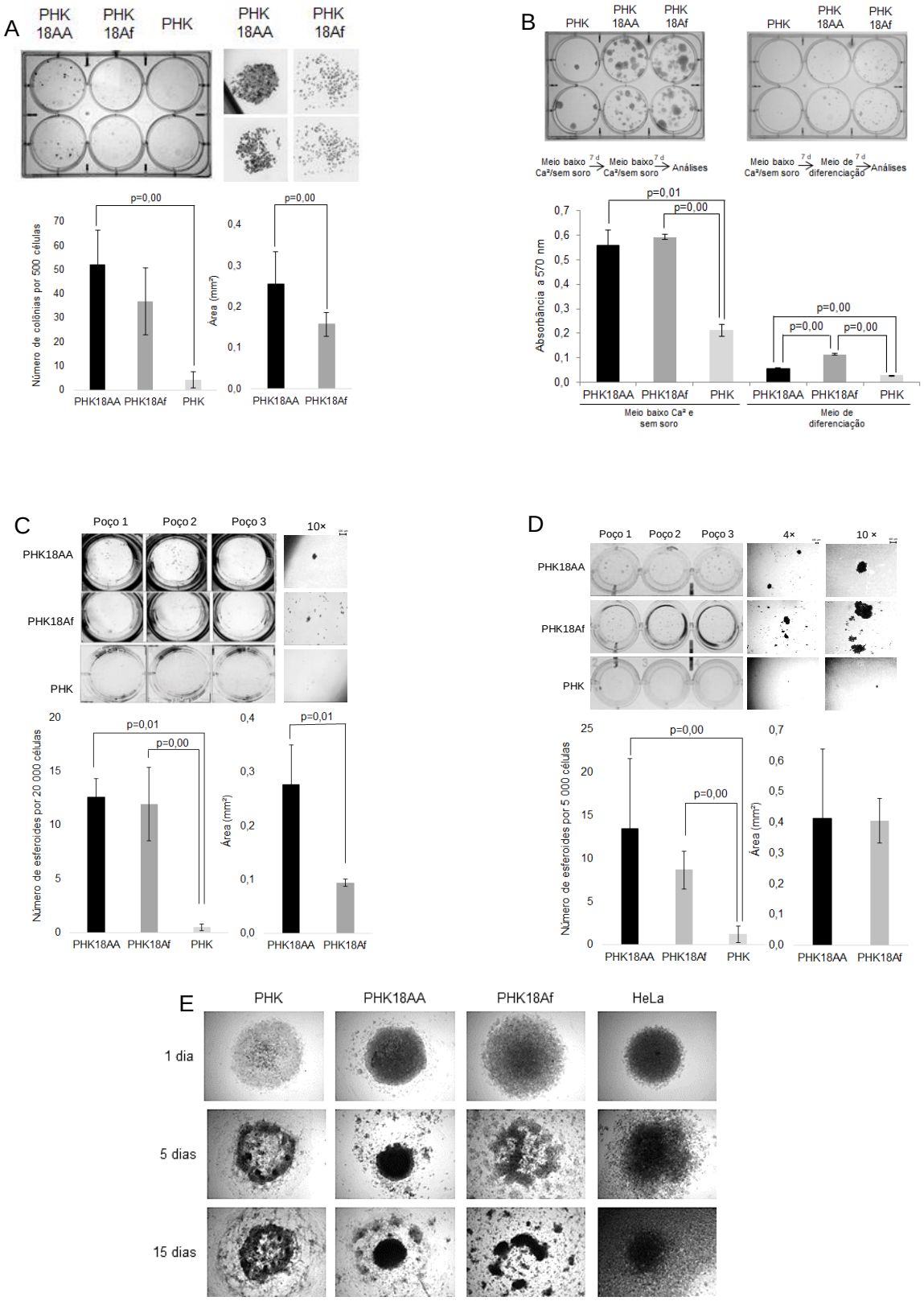

A
PHK 18AA
PHK 18Af
PHK
PHK 18AA
PHK 18Af
Número de colônias por 500 células
p=0,00
p=0,00
Área (mm²)
PHK18AA PHK18Af PHK
PHK18AA PHK18Af
B
PHK
PHK 18AA
PHK 18Af
Meio baixo Ca²/sem soro
7 d
Meio baixo Ca²/sem soro
Análises
Meio baixo Ca²/sem soro
7 d
Meio de diferenciação
7 d
Análises
Absorbância a 570 nm
p=0,01
p=0,00
p=0,00
p=0,00
p=0,00
PHK18AA PHK18Af PHK
PHK18AA PHK18Af PHK
Meio baixo Ca² e sem soro
Meio de diferenciação
C
Poço 1
Poço 2
Poço 3
10×
PHK18AA
PHK18Af
PHK
Número de esferoides por 20 000 células
p=0,01
p=0,00
Área (mm²)
p=0,01
PHK18AA PHK18Af PHK
PHK18AA PHK18Af
D
Poço 1
Poço 2
Poço 3
4×
10 ×
PHK18AA
PHK18Af
PHK
Número de esferoides por 5 000 células
p=0,00
p=0,00
Área (mm²)
PHK18AA PHK18Af PHK
PHK18AA PHK18Af
E
PHK
PHK18AA
PHK18Af
HeLa
1 dia
5 dias
15 dias

Figura 4.

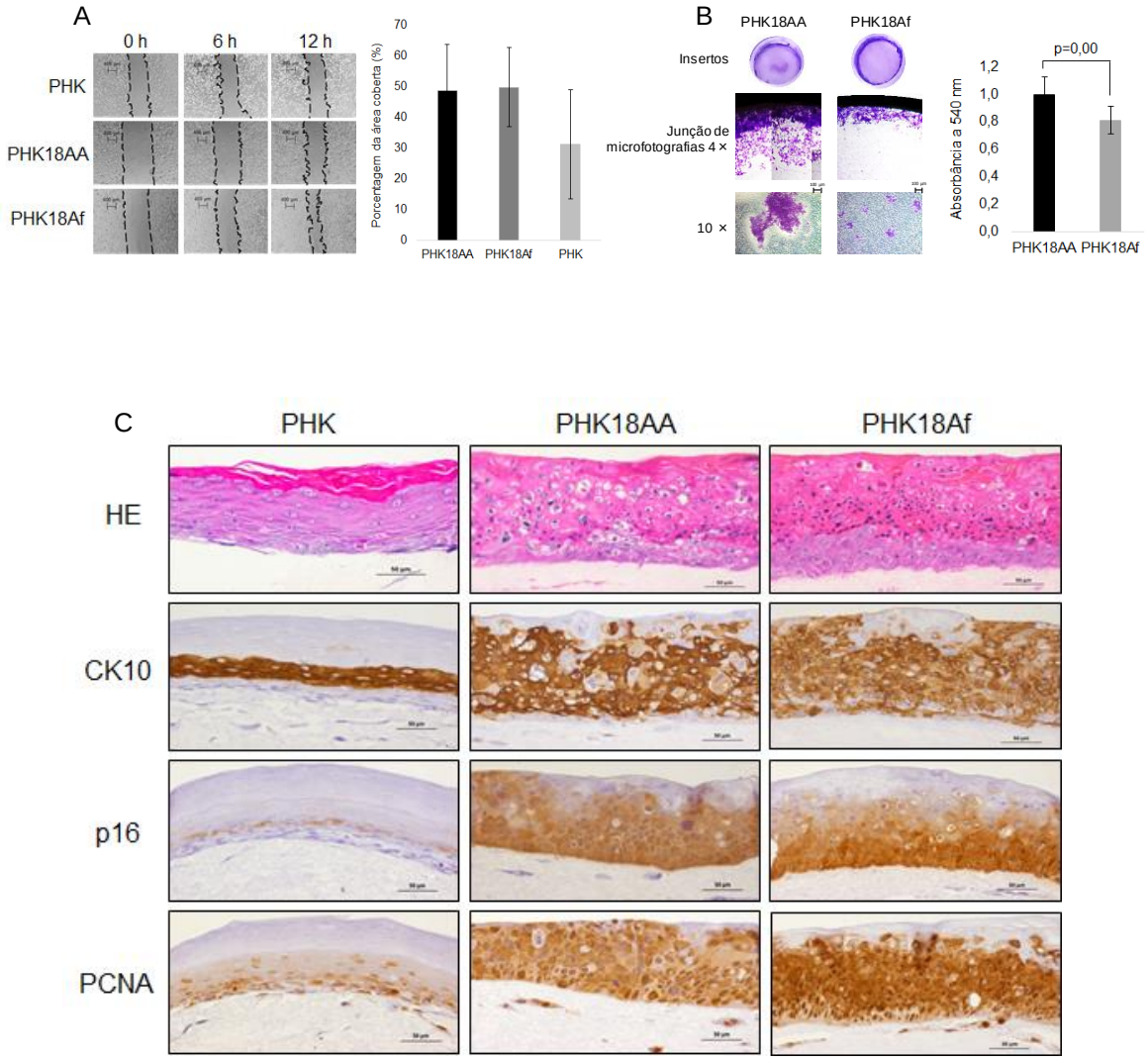
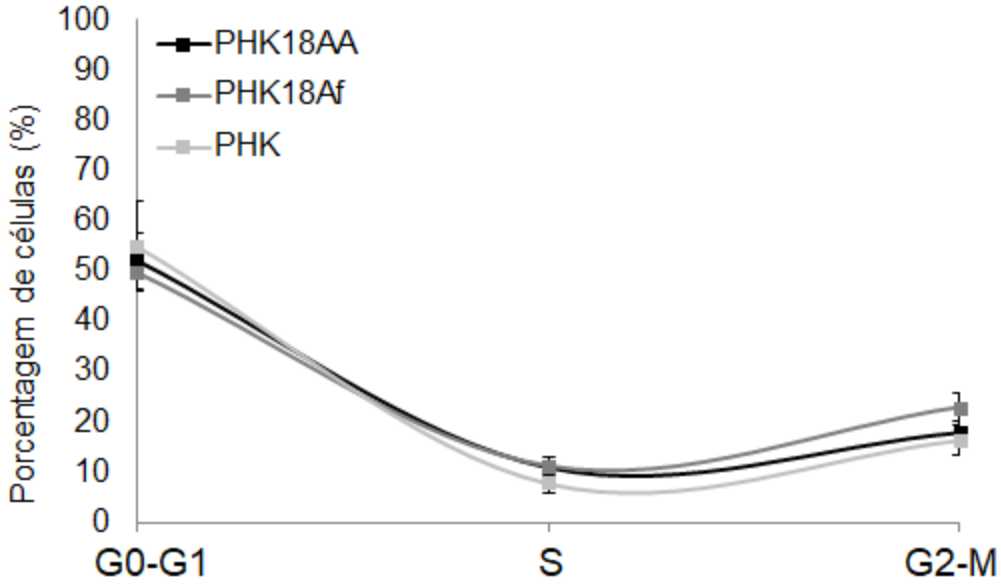

A
0 h
6 h
12 h
PHK
PHK18AA
PHK18Af
Porcentagem da área coberta (%)
70
60
50
40
30
20
10
0
PHK18AA
PHK18Af
PHK
B
PHK18AA
PHK18Af
Insertos
Junção de microfotografias 4 ×
10 ×
Absorbância a 540 nm
1,2
1,0
0,8
0,6
0,4
0,2
0,0
p=0,00
PHK18AA
PHK18Af
C
PHK
PHK18AA
PHK18Af
HE
CK10
p16
PCNA

Figura S1.

A



B

PHKs (video 1) disponível em https://www.4shared.com/s/fIDNPwEvwiq

PHKs (video 2) disponível em https://www.4shared.com/s/fS99W0t3Tea

C

PHK18 (video 1) disponível em https://www.4shared.com/s/fKBcg0K89ea

PHK18AA (video 2) disponível em https://www.4shared.com/s/fMHUyYprTiq

D

PHK18Af (video 1) disponível em https://www.4shared.com/s/ffYiE3LIoea

PHK18Af (video 2) disponível em https://www.4shared.com/s/fmSru_lGWea

3 MANUSCRITO 2

Variante molecular Africana de Papilomavírus Humano 18 não é capaz de degradar a proteína supressora tumoral PTPN14.

Emily Montosa Nunes, Oscar Trejo, Om Basukala, Laura Sichero, Lawrence Banks.

Para submissão a Oncogene (*Article*)

Variante molecular Africana de Papilomavírus Humano 18 não é capaz de degradar a proteína supressora tumoral PTPN14.

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Resumo:

PTPN14 é uma proteína supressora tumoral que é alvo celular da oncoproteína E7 de HPVs de alto risco oncogênico. A interação entre a ubiquitina ligase UBR4 e E7 é necessária para a degradação de PTPN14. Nós avaliamos a habilidade de E7 de duas variantes naturais de HPV-18 em degradar PTPN14 e gerar colônias a partir de células primárias de roedores (BRK) transformadas. Os genes *PTPN14* e *E7* das variantes ameríndia (As+AI) ou africana (Af) de HPV-18 foram co-expressos em células HEK293 e os níveis de PTPN14 foram avaliados por *Western blot*. Ademais, experimentos de imunofluorescência foram conduzidos para verificar a localização celular das proteínas PTPN14, UBR4 e E7. Apenas E7 As+AI foi capaz de induzir a degradação de PTPN14. Os níveis de co-localização de E7 As+AI e UBR4 ou PTPN14 foram inferiores àqueles observados para E7 Af em tratamento controle, enquanto que os níveis entre as variantes e estas proteínas celulares foram similares quando a atividade proteossomal foi inibida. Finalmente, células primárias de roedores (BRK) transfectadas com E7 Af geraram um menor número de colônias em comparação àquelas transfectadas com E7 As+AI. Em conjunto, nossos dados indicam que apenas a substituição H2Y em E7 é capaz de impactar sobre as propriedades bioquímicas e biológicas das variantes naturais de HPV-18.

Título corrido: HPV-18 Af não degrada PTPN14.

Palavras- chave: HPV, PTPN14, UBR4.

1. Introdução

O câncer de colo de útero é o quarto câncer mais comum entre as mulheres e virtualmente todos os casos estão associados à infecção por papilomavírus humano (HPV) (INCA, 2020). Mundialmente, o HPV-16 é o tipo mais prevalente em carcinomas invasivos de células escamosas do colo uterino, seguido por HPV-18, cuja prevalência se assemelha a de HPV-16 em amostras de adenocarcinomas (Walboomers *et al.*, 1999; Arends *et al.*, 1993; revisado por IARC, 2012).

O estudo das variantes nucleotídicas de HPV-18 vem resultando em importantes achados no que concerne à evolução e filogenia viral, assim como a história natural da infecção por HPV. As variantes de HPV são caracterizadas por uma variação menor que 2% na sequência do genoma completo (revisado por Bernard, 2005). As variantes de HPV-18 estão agrupadas em ramos filogenéticos nominados a partir da origem da maior parte das amostras que os compõe: Ameríndio (As+AI), Africano (Af), e Europeu (E) (Ong *et al.*, 1993; Chen *et al.*, 2013). Ainda assim, alta conservação da sequência de nucleotídeos e aminoácidos é observada entre as variantes de HPV-18: as sequências de aminoácidos de E6 e E7 são idênticas entre as variantes As+AI e E, enquanto que a variante Af apresenta apenas uma alteração não-sinônima na sequência do gene de *E6* (N129K) e uma em *E7* (H2Y).

A oncoproteína E7 de HPVs é capaz de interagir com diversos alvos celulares e mais recentemente foi identificada a associação desta com a proteína citoplasmática tirosina quinase PTPN14, que foi reconhecida como um potencial supressor tumoral (Rozenblatt-Rosen *et al.*, 2012; revisado por White e Howley, 2013). Szalmás e col. (2017) não apenas descreveram a forte associação física

entre PTPN14 e E7 de HPV-16, mas também a degradação de PTPN14 induzida por esta oncoproteína viral, sendo os resíduos de aminoácidos 80, 81 e 84 da região conservada 3 (CR3) de E7 cruciais para esta interação. Adicionalmente, White e col*.* (2016) reportaram que, para que E7 de HPV-16 e HPV-18 reduza os níveis de PTPN14, deve haver interação entre as proteínas PTPN14 e UBR4 E3 ligase (originalmente nomeada p600). Foi ainda demonstrado que a proteína UBR4 é capaz de interagir com a região CR1 de E7 de HPV-16, assim como com esta região dos tipos de baixo risco HPV-6 e HPV-11 (Huh *et al.*, 2005; White *et al.*, 2016). Pelo exposto, o presente estudo objetivou avaliar o impacto da variabilidade intratípica da proteína E7 de HPV-18 sobre a degradação de PTPN14 mediada por UBR4.

2. Resultados

A atividade proteossomal está relacionada a incapacidade de E7 de HPV-18 Af em degradar PTPN14.

Inicialmente, a fim de avaliar a habilidade de E7 das variantes naturais As+AI e Af de HPV-18 em degradar a proteína supressora tumoral PTPN14, células epiteliais embrionárias de rim humano (HEK293) foram co-transfectadas com *V5-PTPN14* e *FLAG-E7* das diferentes variantes independentemente. Após 24 horas, a proteína total das células foi extraída e analisada por *Western blotting* usando anticorpos específicos. Nós observamos que a degradação de PTPTN14 diferiu entre as variantes: enquanto E7 As+AI foi capaz de degradar esta proteína supressora tumoral ($p< 0,02$), E7 Af não apresentou a mesma habilidade ($p= 0,07$) (figura 1).

Em seguida, objetivamos avaliar os níveis de co-localização das proteínas PTPN14 e E7 das variantes de HPV-18, além da influência da atividade do proteossomo sobre os níveis de co-localização destas. Para tanto, células advindas de osteosarcoma

humano (U2OS) foram co-transfectadas com *V5-PTPN14* e *FLAG-E7* das diferentes variantes independentemente. Após 24 horas, as células foram tratadas com o inibidor de proteossomo Z-Leu-Leu-Leu-al (CBZ) ou DMSO (controle) por três horas. A determinação dos níveis de co-localização foi realizada utilizando a ferramenta *Colocalization Threshold* (*Image J*, NIH) que fornece o coeficiente de co-localização *Mander's Overlap* (R= 0, 0% sobreposição; R= 1, 100% sobreposição). Foi observado que a localização de PTPN14 e E7 de HPV-18 de ambas as variantes é principalmente citoplasmática, independentemente do tratamento (DMSO ou CBZ). Na ausência do inibidor de proteossomo, foram observados altos níveis de PTPN14 co-localizando com E7 Af (R médio= 0,55), porém não com E7 As+AI (R médio= 0,29) (p= 0,01) (figura 2A-C). Entretanto, quando as células foram tratadas com o inibidor de proteossomo CBZ, observou-se aumento dos níveis de co-localização das proteínas PTPN14 e E7 As+AI (R médio= 0,66) (figura 2D e 2E) comparado ao tratamento controle. Por outro lado, não foi observada diferença nos níveis de co-localização de PTPN14 e E7 Af nas células transfectadas e tratadas (R médio= 0,63) (figura 2D e 2F) ou não com CBZ (R médio= 0,62). Diferente do observado quando as células foram tratadas somente com DMSO, nós não observamos diferença significativa no valor de R quando as células co-transfectadas com PTPN14 e E7 das variantes As+AI ou Af foram tratadas com CBZ (p= 0,99). Em conjunto, esses dados mostram que há um aumento nos níveis de co-localização de E7 As+AI e PTPN14 por imunofluorescência quando a degradação de PTPN14 está inibida, isto é, quando o complexo proteossomal está inibido. Por sua vez, os níveis de co-localização de E7 Af e PTPN14 parecem não ser afetados pela atividade proteossomal.

A proteína E7 de variantes naturais de HPV-18 apresentam diferenças nos níveis de co-localização com UBR4.

Com o objetivo de analisar a co-localização de UBR4 e E7 das variantes de HPV-18, células U2OS foram co-transfectadas com *V5-UBR4* e *FLAG-E7* das diferentes

variantes independentemente. Após 24 horas, as células foram tratadas com o inibidor de proteossomo Z-Leu-Leu-Leu-al (CBZ) ou DMSO (controle) por três horas. Similarmente ao observado com PTPN14, a localização de UBR4 é principalmente citoplasmática em células expressando ou não E7 das variantes de HPV-18, independentemente do tratamento. Ainda mais, observou-se que nas células tratadas com DMSO houve menor co-localização de UBR4 e E7 As+AI (R médio= 0,07) em comparação à UBR4 e E7 Af (R médio= 0,53) (p= 0,03) (figura 3A-C). Já nas células tratadas com CBZ, observou-se aumento da co-localização de UBR4 e E7 As+AI (R médio= 0,52) (figura 3D e 3E) quando comparado às células tratadas com DMSO. Por outro lado, não foi observada diferença nos níveis de co-localização de UBR4 e E7 Af nas células tratadas (R médio = 0,50) (figura 3D e 3F) ou não com CBZ. Diferente do observado quando as células foram tratadas com DMSO, nós não observamos diferença significativa no valor de R quando as células co-transfectadas com UBR4 e E7 das variantes As+AI ou Af foram tratadas com CBZ (p= 0,88). Em conjunto, esses achados mostraram aumentos nos níveis de co-localização de E7 As+AI e UBR4 por imunofluorescência quando o complexo proteossomal está inibido. Por sua vez, a co-localização entre E7 Af e UBR4 pareceu não ser afetada pela atividade proteossomal.

A proteína E7 Af apresenta menor potencial de formação de colônias quando comparada a proteína E7 As+AI.

Por fim, nós objetivamos comparar o potencial da proteína E7 oriunda das variantes As+AI e Af de HPV-18 em induzir a formação de colônias em células primárias de roedores (Massimi e Banks, 2005). Para tanto, células primárias de rim de ratos neonatos (BRK) foram extraídas e cultivadas em meio DMEM suplementado com 10% de soro fetal bovino. Após 24 horas, as células foram co-transfectadas com plasmídeos de expressão do oncogene ativador de transformação celular *EJ-RAS* e *E7* As+AI ou Af. Posteriormente, as células foram mantidas em cultura por três semanas na presença de

200 µg/mL de G418 quando as colônias oriundas das células selecionadas foram visualizadas após coloração com Giemsa 10%.

Nós observamos que as células primárias BRK transfectadas com *E7* As+AI produziram um número significativamente maior de colônias quando comparada as células transfectadas com E7 Af ($p > 0,05$). Estes dados indicam que o aminoácido presente no resíduo dois da proteína E7 tem papel importante na indução de formação de colônias em células primárias BRK.

3. Discussão

Neste trabalho, nós demostramos que a proteína citoplasmática tirosina fosfatase PTPN14, que foi observada ser parceira de E7 de HPVs, não é degradada pela proteína E7 da variante Af de HPV-18. Adicionalmente, nós observamos que a co-localização de E7 com UBR4 ou PTPN14 é influenciada pela atividade do proteossomo apenas nas células expressando E7 da variante As+AI. Foi reportado que a degradação de PTPN14 induzida por E7 depende da ligação de PTPN14 a UBR4 (White *et al*., 2016; Huh *et al.*, 2005). Pelo exposto, nós sugerimos que a variação natural que ocorre na proteína E7 da variante Af (H2Y) poderia, não apenas afetar a habilidade desta oncoproteína em degradar PTPN14, devido à incapacidade de formar o complexo UBR4/E7, mas também alterar a capacidade de formar colônias a partir de células primárias de roedores.

A proteína E7 de HPVs é uma pequena fosfoproteína nuclear ácida de 21 kDa. Foi demonstrado que a proteína E7 de HPVs de alto-risco oncogênico é necessária e suficiente para a transformação de fibroblastos de roedores (Watanabe e Yoshiike, 1988), para a imortalização de queratinócitos humanos primários (PHKs) (Halbert *et al.*, 1991), além de cooperar com o oncogene *RAS* ativado para a transformação de células primárias de roedores (Matlashewski *et*

al., 1987). A região amino terminal da proteína E7 apresenta elevada similaridade com duas regiões não contiguas da proteína E1A de Adenovírus denominadas de CR1 e CR2 (regiões conservadas 1 e 2) (Phelps *et al.*, 1992). A sequência de aminoácidos no domínio CR1, a qual engloba os resíduos 1 a 15 de E7 de HPV-16, foi demonstrada ser necessária para a transformação celular (Edmonds e Vousden, 1989). Ainda que o papel de CR1 não seja completamente conhecido, alguns estudos mostraram a interação de aminoácidos desta região com proteínas envolvidas na formação de complexos de ubiquitinação, como a cullin-2 (Huh *et al.*, 2007; revisado por Songock *et al.*, 2016; Todorovic *et al.*, 2012), assim como com diversos outros ligantes celulares (Banks *et al.*, 1990a; revisado por Songock *et al.*, 2016; Heck *et al.*,1992; Lee *et al.*, 2016; Calçada *et al.*, 2013). Por conseguinte, a variação de aminoácido no resíduo dois pode impactar em diferenças na interação com estes ligantes celulares.

Uma histidina (H) no resíduo dois de E7 é encontrada não apenas nos HPV-6, HPV-11, HPV-16 e HPV-18 (variante As+AI), mas também em outros 58 dos 145 genomas de HPVs já completamente sequenciados e depositados no sítio *The PapillomaVirus Episteme (PaVE)* (revisado por Songock *et al.*, 2016; PaVE, 2020). Por outro lado, um resíduo de isoleucina (I) é encontrado na posição dois da proteína E7 nos tipos virais pertencentes ao gênero beta-papilomavírus (associados às lesões cutâneas). Nos demais tipos de HPV, independentemente do gênero ou potencial oncogênico associado, os aminoácidos arginina (30 tipos), metionina, lisina, glutamina, tirosina ou valina são também detectados nesta posição (revisado por Songock *et al.*, 2016; PaVE, 2020). Ainda mais, assim como observado na variante Af de HPV-18, uma tirosina

(Y) nessa posição é encontrada nos HPV-91 (espécie Alfa-8) e HPV-41 (espécie Nu-1) (PaVE, 2020).

O aminoácido histidina apresenta uma cadeia lateral de imidazol que, além de ser relevante para a atividade catalítica de enzimas, serve como ligante de metaloproteínases, proteínas estas que possuem grande importância na degradação da matriz extracelular necessária nos processos de migração e metástase em tumores (revisado de Friedl e Wolf, 2003). Já o aminoácido tirosina é encontrado principalmente em proteínas envolvidas em processos de transdução de sinal, atuando como receptor de grupos fosfato que são transferidos devido à atividade de proteínas quinases. Assim, a divergência de aminoácido observada entre as proteínas E7 das variantes As+AI e Af de HPV-18 poderiam resultar na interação com proteínas celulares diferentes, além de impactar no desenvolvimento de lesões com potencial oncogênico distinto.

Alguns estudos descreveram alterações nas funções biológicas e bioquímicas de E7 resultantes da alteração no segundo aminoácido desta proteína em HPV-16. Por exemplo, Watanabe e col. (1990) introduziram por mutagênese sítio-dirigida uma substituição para ácido aspártico (D) nesta posição e observaram redução no potencial de E7 no que concerne à transformação de células primárias BRK. Adicionalmente, foi observado que a substituição deste aminoácido por prolina (H2P) não impactou sobre a interação de E7 com pRb ou p107 (Banks et al., 1990b; Demers *et al.*, 1996), embora resultou na eliminação da capacidade desta proteína em inativar a função de pRb ou reduzir os níveis proteicos de p107 ou p130 (Helt e Galloway, 2001; Bodily *et al.*, 2011; Jones e Münger, 1997). Adicionalmente, foi observado que a proteína E7 H2P apresentou: (1) reduzida capacidade de imortalização e transformação de células BRK (Banks

et al., 1990a; (2) baixa eficiência na formação de colônias quando expressa em PHKs (Bodily *et al.*, 2011); (3) falha na interrupção da quiescência de PHKs na camada suprabasal dos epitélios gerados em cultura organotípica (Demers *et al.*, 1996); (4) resistência à apoptose induzida por dano ao DNA causado pela incubação com actinomicina D em células epiteliais de mama humana (Demers *et al.*, 1996).

No que se refere à interação de E7 de HPV-16 com proteínas ubiquitinas, Huh e col. (2005) expressaram *E7* de HPV-16 em fibroblastos embrionários de camundongo (NIH3T3) e observaram que a depleção de UBR4 por RNA de interferência resultou na redução significativa do número de colônias formadas quando estas células foram cultivadas em meio semi-sólido (*soft* agar). Adicionalmente, ensaios de co-imunoprecipitação realizados empregando-se o mutante H2P de E7 de HPV-16 revelaram que o resíduo dois desta proteína é crucial não só para a interação de E7 com cullin-2, mas também para a indução da degradação de pRb (Huh *et al.*, 2007). Por sua vez, White e col. (2016) utilizou o mutante E7 H2P de HPV-16 para comprovar a importância de UBR4 na degradação de PTPN14.

Em conjunto, os dados gerados por nós ressaltam a importância do segundo aminoácido da proteína E7 dos HPVs na interação com alvos. Ademais, a mutação natural H2Y na proteína E7 da variante Af de HPV-18 poderia refletir em um potencial oncogênico reduzido comparado à E7 da variante As+AI de HPV-18. De fato, embora ainda seja motivo de debate, os diversos estudos funcionais e epidemiológicos conduzindo até o momento sugerem que a variante Af de HPV-18 representa um genótipo com menor potencial carcinogênico (Sichero *et al.*, 2005; López-Saavedra *et al.*, 2009; Fuentes-González *et al.*, 2019;

de la Cruz-Hernández *et al.*, 2005; Vazquez-Vega *et al.*, 2013; Pista *et al.*, 2007; Xi *et al.*, 2007, Fragoso-Ontiveros *et al.*, 2012; Pérez *et al.*, 2014; Chen *et al.*, 2015). É importante ressaltar que este estudo é pioneiro em descrever diferenças nas propriedades bioquímicas da proteína E7 advinda de diferentes variantes de HPV-18. Assim, a diferença na degradação da proteína supressora tumoral PTPN14 pela proteína E7 advinda das diferentes variantes de HPV-18 abre espaço para novos estudos referentes à importância da caracterização de variantes moleculares na compreensão da gênese dos tumores.

4. Materiais e Métodos

Células e plasmídeos

Células epiteliais embrionárias de rim humano (HEK293) e células advindas de osteosarcoma humano (U2OS) foram cultivadas em meio *Dulbecco's modified Eagles's medium* (DMEM) (GIBCO, *Thermo Fisher Scientific*, Massachusetts, EUA) suplementado com 10% de soro fetal bovino (GIBCO, *Thermo Fisher Scientific*, Massachusetts, EUA), penicilina-streptomicina (100 U/mL) e glutamina (300 µg/mL) (GIBCO, *Thermo Fisher Scientific*, Massachusetts, EUA). As células primárias de rim de ratos neonatos (BRK) foram obtidas de extração tecidual de rins de ratos Wistar Hannover (Harlan Sprague Dawley Inc., Milão, Itália) (Massimi e Banks, 2005) e cultivadas em meio DMEM suplementado com 10% de soro fetal bovino.

O plasmídeo contendo a sequência completa de *E7* da variante Af foi preparado a partir da sequência de *E7* da variante As+AI usando o kit *GeneArt*® *Site-Directed Mutagenesis PLUS kit* (Invitrogen, Califórnia, EUA) e os iniciadores 18E7F C593T (5'-ATGTATGGACCTAAGGCAACATTGCAAGAC-3') e 18E7R

C593T (5’-GTCTTGCAATGTTGCCTTAGGTCCATACAT-3’). Ambos os plasmídeos pCMV FLAG-HPV-18 E7 As+AI e pCMV FLAG-HPV-18 E7 Af foram utilizados para expressão de E7 nas células HEK293 e U2OS.

O plasmídeo pcDNA3 V5-PTPN14 foi gentilmente cedido pelo Dr. Jianmin Zhang (Roswell Park Cancer Institute, New York, EUA) para expressão de PTPN14 nas células HEK293 e U2OS. O plasmídeo pcDNA V5-UBR4 foi gentilmente cedido pelo Dr. Takafumi Tasaki (Kanazawa Medical University, Ishikawa, Japão) para expressão de UBR4 nas células HEK293 e U2OS. O plasmídeo pCDNA His-LacZ foi obtido comercialmente (Invitrogen, Califórnia, EUA) e usado para avaliar a eficiência da transfecção celular.

O plasmídeo pJ4Ω 18E7 As+AI foi construído a partir da técnica de clonagem sem restrição (van den Ent e Löwe, 2006). Primeiramente, realizou-se à amplificação da sequência de *E7* As+AI de HPV-18 a partir do plasmídeo pGEX-18E7, gentilmente cedido pelo Dr. Karl Munger (Tufts University, Massachusetts, EUA), utilizando os iniciadores 5’-ACTCTAGAGAATCCGATGCATGGACCTAAG-3’ e 5’-ATCAGCCATGGTAGATTACTGCTGGGATGC-3’. Em seguida, o produto desta amplificação foi fracionamento em gel de agarose 1,5% e a banda de interesse com aproximadamente 300 pb foi purificada. Finalmente, o fragmento purificado (E7 As+AI) foi inserido no plasmídeo pJ4Ω 16E7 através da técnica de clonagem sem restrição utilizando-se o kit *Stratagene’s QuikChange™ Site-Directed Mutagenesis* (Biocompare, Califórnia, EUA) e os iniciadores supracitados.

O plasmídeo pJ4Ω 18E7 Af foi construído utilizando-se o kit *Stratagene’s QuikChange™ Site-Directed Mutagenesis* (Biocompare, Califórnia, EUA) e os iniciadores 5’-CTAGAGAATCCGATGTATGGACCTAAGGCAACA-3’ e 5’-

TGTTGCCTTAGGTCCATACATCGGATTCTCTAG-3'. Os plasmídeos pJ4Ω 18E7 As+AI e pJ4Ω 18E7 Af foram utilizados para expressão de *E7* nas células primárias BRK.

Western blotting

Foram semeadas 7,5 × 10^5 células da linhagem HEK293 em placas de 100 mm. Após 24 horas, as células foram co-transfectadas empregando protocolo de precipitação com fosfato de cálcio (Wigler *et al.*, 1979) com 1 µg de pcDNA3 V5-PTPN14 e 1 µg pCMV FLAG-HPV-18 E7 As+AI ou pCMV FLAG-HPV-18 E7 Af.

A extração da proteína celular total foi realizada utilizando-se o tampão de amostra 2× SDS-PAGE (Tris 167 mM pH= 6,8; SDS 1,7%; 2-mercaptoetanol 1,17%; glicerol 50%). Os ensaios de *Western blotting* foram realizados como descrito anteriormente (Tomaić e Banks, 2009) usando o anticorpo monoclonal de rato anti-V5 (1:5 000, R96025, Sigma-Aldrich, Missouri, EUA) e o anticorpo monoclonal de rato anti-β-galactosidase (1:2 500, Z3783, Promega Corp., Wisconsin, EUA). O anticorpo secundário anti-IgG de rato conjugado ao HRP (1:1 000, Dako, P0260, Agilent, Califórnia, EUA) foi usado em seguida. As membranas foram reveladas usando o reagente *ECL detection system* (Amersham, GE Healthcare Life Sciences, Illinois, EUA), e as quantificações foram conduzidas usando o *software* Image J 1.52p (National Institutes of Health, EUA).

Immunofluorescência

Foram semeadas 1,5 × 10^5 células da linhagem U2OS em lamínulas de vidro dispostas sobre placas de seis poços. Após 24 horas, as células foram co-

transfectadas usando protocolo de precipitação com fosfato de cálcio com 4 µg de pcDNA3 V5-PTPN14 ou pcDNA V5-UBR4 e 4 µg de pCMV FLAG-HPV-18 E7 As+AI ou pCMV FLAG-HPV-18 E7 Af. Após cinco horas, uma solução de glicerol 15% (em PBS 1×) foi adicionada por um minuto a fim de induzir o choque osmótico e aumentar a eficiência da transfecção. Após 24 horas, as células foram tratadas por três horas com DMSO (controle) (Sigma-Aldrich, Missouri, EUA) ou com o inibidor de proteoassomo Z-Leu-Leu-Leu-al ([CBZ] (MG-132; Sigma-Aldrich, Missouri, EUA) dissolvido em DMSO e usado a 10 µM. Em seguida, as células foram fixadas com paraformaldeído 4% (em PBS 1×) por 15 minutos, permeabilizadas com Triton X-100 0,1% (em PBS 1×) por cinco minutos, e lavadas rapidamente em solução de glicina 100 mM (em PBS 1×).

As células foram posteriormente incubadas com o anticorpo monoclonal de rato anti-V5 (1:100, R96025, Sigma-Aldrich, Missouri, EUA) e/ou o anticorpo policlonal de coelho anti-FLAG (1:600, F7425-.2MG, Sigma-Aldrich, Missouri, EUA) em PBS 1× a 37 °C por uma hora. As células foram lavadas extensivamente com PBS 1× e incubadas com os anticorpos secundários de cabra anti-IgG de rato conjugado à *Rhodamine Red* (R6393, *Life Technologies*, Califórnia, EUA) ou de burro anti-IgG de coelho conjugado ao Alexa Fluor 488 (A-21206, *Life Technologies*, Califórnia, EUA) por 30 minutos a 37 °C. Finalmente, as células foram lavadas e as lâminas preparadas com reagente de montagem Vectashield (Vector Laboratories, Califórnia, EUA), sendo posteriormente seladas. Registros fotográficos das células foram realizados no microscópio confocal Zeiss LSM 510 (Zeiss, Jena, Alemanha) usando óleo de imersão e objetiva 60×. As imagens foram analisadas usando o *software Image J 1.52p* (*National Institutes of Health*, EUA).

Ensaio de formação de colônias em células primárias de roedores

Células primárias BRK foram extraídas e semeadas em placas de 100 mm. Após 24 horas, as células foram co-transfectadas usando protocolo de precipitação com fosfato de cálcio com 3 µg de *EJ-RAS* (Massimi e Banks, 2005) e 4 µg de pJ4Ω 18E7 As+AI ou pJ4Ω 18E7 Af. Cinco horas depois, uma solução de glicerol 15% (em PBS 1×) foi adicionada por um minuto a fim de induzir o choque osmótico e aumentar a eficiência da transfecção. Posteriormente, as células foram cultivadas em DMSO suplementado com 10% de soro fetal bovino e 200 µg/mL de G418 por três semanas para seleção das células transfectadas. Finalmente, as colônias formadas foram visualizadas após coloração com Giemsa 10%.

Análise estatística

A análise estatística foi conduzida usando o *software* Statistix 8 para *Windows* (*Analytical Software*, Flórida, EUA). O teste T foi utilizado para: (1) comparar os níveis de PTPN14 nas células HEK293 expressando ou não E7 das variantes de HPV-18; (2) comparar os níveis de co-localização das proteínas PTPN14 ou UBR4 com E7 das variantes de HPV-18 nos ensaios de imunofluorescência; (3) comparar o número de colônias oriundas das células primárias BRK selecionadas após a transfecção de *E7* das variantes de HPV-18 individualmente. O valor de p foi considerado significativo quando menor que 0,05.

5. Agradecimento

Nós gostaríamos de agradecer ao Centro Internacional de Engenharia Genética e Biotecnologia (ICGEB) e FAPESP por fornecer as condições necessárias ao desenvolvimento deste trabalho. Este projeto foi financeiramente apoiado pelo Processo FAPESP nº 2018/14697-7.

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Lista de figuras:

Figura 4 – Formação de colônias em células primárias de roedores após a transfecção por E7 de variantes de HPV-18. Células primárias de rim de ratos neonatos (BRK) foram estabelecidas e transfectadas com E7 (4 µg) das diferentes variantes de HPV-18 independentemente empregando um protocolo de precipitação com fosfato de cálcio. Todas as placas também foram transfectadas com o oncogene cooperante *EJ-RAS* (3 µg). As células transfectadas cresceram na presença de 200 µg/mL de G418 e as colônias de células selecionadas foram coradas com Giemsa 10% após três semanas do início da transfecção. *$p < 0,05$.

Figura 1.

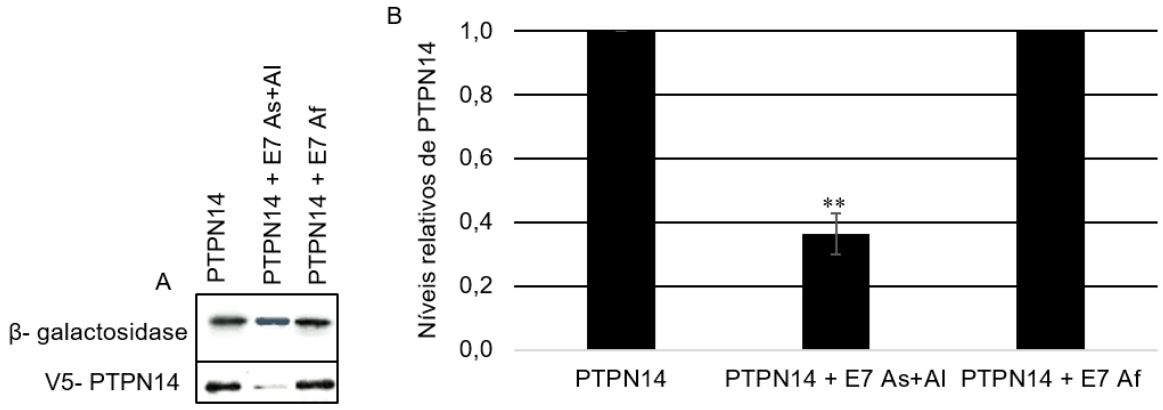

A
PTPN14
PTPN14 + E7 As+Al
PTPN14 + E7 Af
β- galactosidase
V5- PTPN14
B
Níveis relativos de PTPN14
1,0
0,8
0,6
0,4
0,2
0,0
**
PTPN14
PTPN14 + E7 As+Al
PTPN14 + E7 Af

Figura 2.

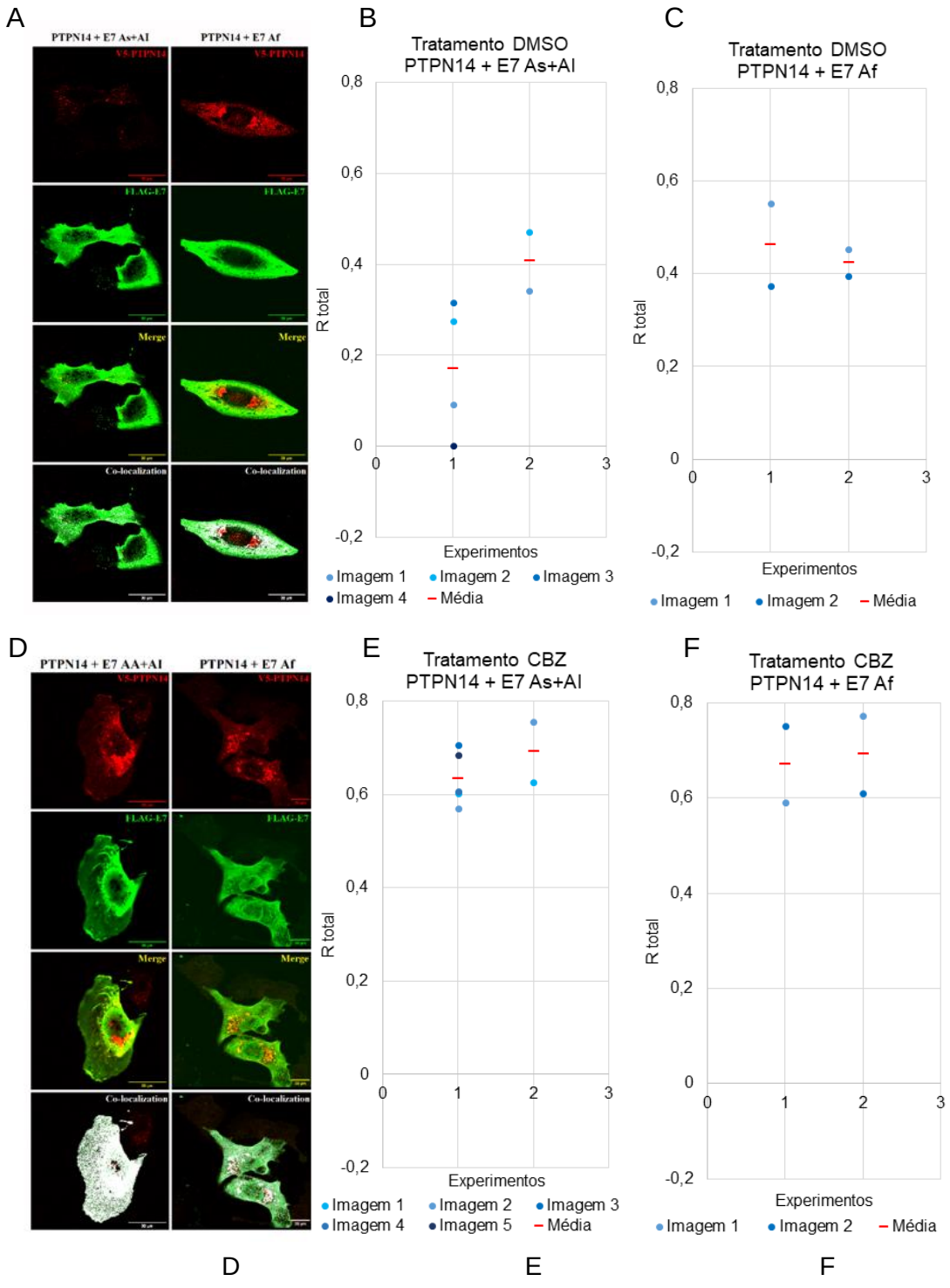

A
PTPN14 + E7 As+AI
PTPN14 + E7 Af
V5-PTPN14
FLAG-E7
Merge
Co-localization
B
Tratamento DMSO
PTPN14 + E7 As+AI
R total
Experimentos
Imagem 1
Imagem 2
Imagem 3
Imagem 4
Média
C
Tratamento DMSO
PTPN14 + E7 Af
R total
Experimentos
Imagem 1
Imagem 2
Média
D
PTPN14 + E7 AA+AI
PTPN14 + E7 Af
V5-PTPN14
FLAG-E7
Merge
Co-localization
E
Tratamento CBZ
PTPN14 + E7 As+AI
R total
Experimentos
Imagem 1
Imagem 2
Imagem 3
Imagem 4
Imagem 5
Média
F
Tratamento CBZ
PTPN14 + E7 Af
R total
Experimentos
Imagem 1
Imagem 2
Média
D
E
F

Figura 3.

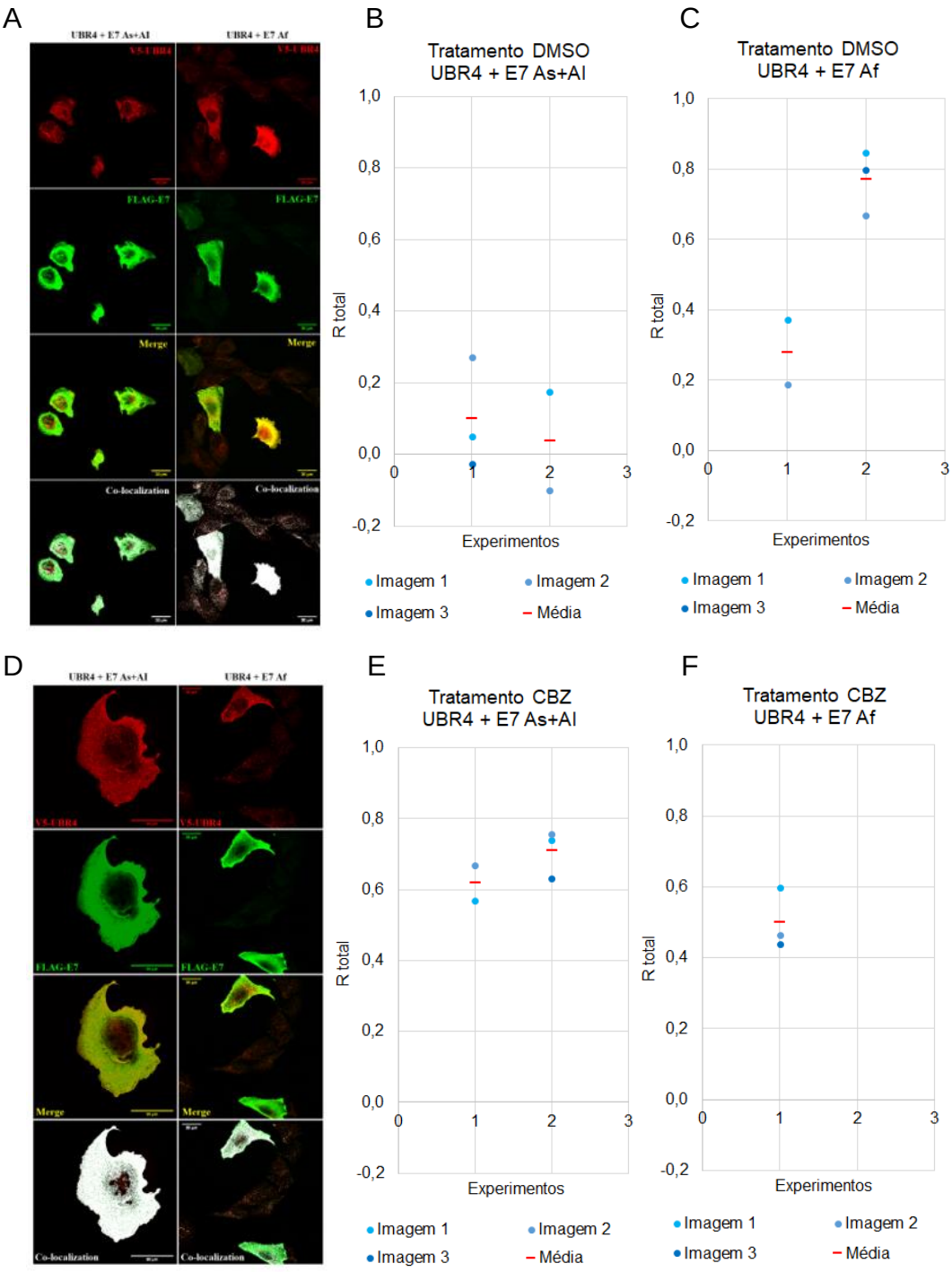
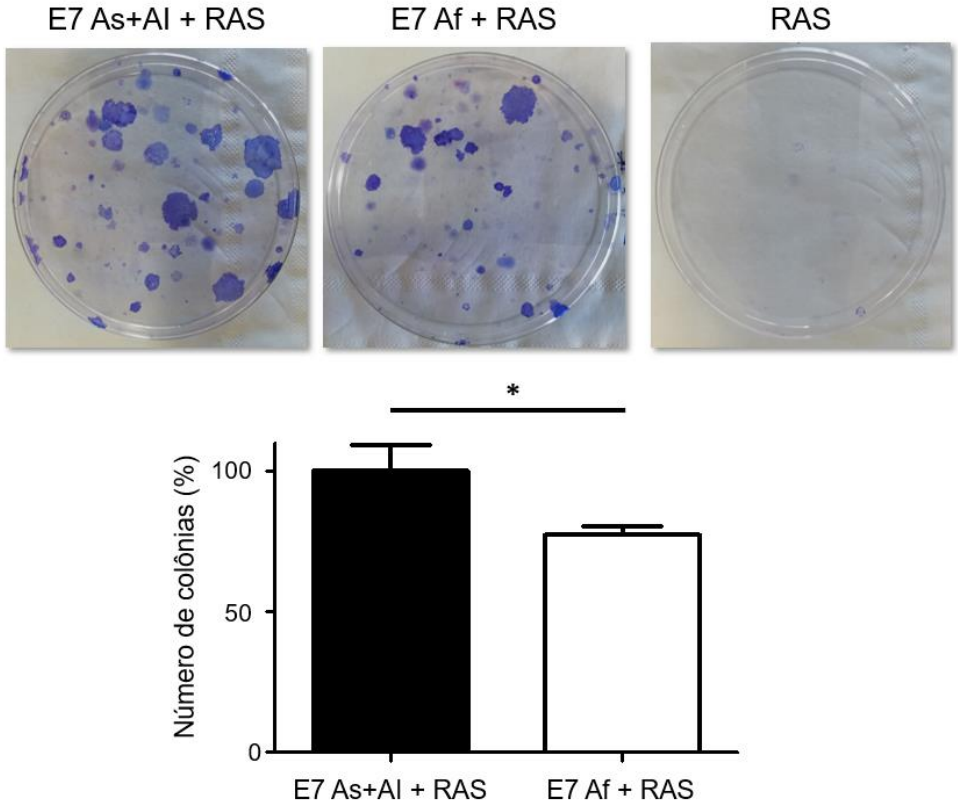

A
UBR4 + E7 As+AI
UBR4 + E7 Af
V5-UBR4
FLAG-E7
Merge
Co-localization
B
Tratamento DMSO
UBR4 + E7 As+AI
R total
Experimentos
Imagem 1
Imagem 2
Imagem 3
Média
C
Tratamento DMSO
UBR4 + E7 Af
D
E
Tratamento CBZ
UBR4 + E7 As+AI
F
Tratamento CBZ
UBR4 + E7 Af

Figura 4.


E7 As+AI + RAS
E7 Af + RAS
RAS
*
Número de colônias (%)
100
50
0
E7 As+AI + RAS
E7 Af + RAS

4 DISCUSSÃO E CONCLUSÃO

.

1. Discussão e conclusão

O HPV-18 é o segundo tipo de HPV mais prevalente em carcinomas escamosos invasivos do colo de útero e apresenta prevalência semelhante à de HPV-16 em adenocarcinomas. Ainda que a análise da variabilidade nucleotídica intra-típica de HPV-18 tenha resultado em importantes achados no que se refere à epidemiologia e evolução viral, estudos acerca da relevância clínica ainda são limitados. Até o momento, os estudos mostraram que as variantes As+AI e E estão associadas a um maior potencial oncogênico, além de serem mais prevalentes em adenocarcinoma e carcinoma adenoescamoso, quando comparadas a variante Af que tem sido detectada principalmente em amostras de carcinoma escamoso invasivo do colo uterino. Pelo exposto, este estudo objetivou avaliar de forma abrangente o potencial oncogênico atribuível às oncoproteínas E6/E7 das variantes naturais As+AI e Af de HPV-18 no background genético de PHKs, os quais consistem nas células hospedeiras naturais destes vírus.

Em comparação à variante As+AI, a variante Af de HPV-18 apresenta uma alteração não-conservativa em cada uma das oncoproteínas virais: E6 (N129K) e E7 (H2Y). Nós construímos vetores retrovirais recombinantes com o intuito de transduzir E6 e E7 destas variantes em PHKs comercialmente disponíveis, os quais foram subcultivados até p30, quando foram considerados imortalizados. Os PHK18AA atingiram a imortalização significativamente mais rápido do que os PHK18Af. Ainda assim, nós não observamos diferenças na morfologia entre as células imortalizadas por ambas as variantes de HPV-18 quando comparadas aos PHKs não-transduzidos, diferentemente de Richard e col. (2010) que observaram diferenças fenotípicas entre os PHKs transduzindo estas oncoproteínas das diferentes variantes de HPV-16.

Nós não observamos um período de crise evidente em nenhum dos PHKs transduzidos por HPV-18, corroborando com os resultados obtidos por Lace e col. (2009) ao transfectar PHKs de diferentes origens anatômicas com o genoma do HPV-18. Ainda que nós não tenhamos observado diferenças no potencial proliferativo entre os PHK18AA e PHK18Af imortalizados, os PHK18AA foram mais eficientes na formação de colônias e esferoides, como também observado por de la Cruz-Hernandéz e col. (2005) em ensaios empregando outros tipos celulares e avaliando somente a atividade da oncoproteína E6.

Neste estudo, nós também demostramos que a proteina E7 das diferentes variantes de HPV-18 diverge no que se refere à capacidade de interagir com proteínas celulares (p.e. PTPN14, UBR4) e à habilidade de induzir a formação de colônias em células primárias de roedores co-transfectadas com EJ-RAS. Após co-expressarmos PTPN14 e E7 das variantes As+AI ou Af de HPV-18 em células HEK293 ou U2OS, nós observamos que células transfectadas com E7 Af não eram capazes de induzir a degradação de PTPN14, ao passo que as células expressando E7 As+AI apresentaram níveis reduzidos da proteína PTPN14. Uma vez que já é conhecido que a interação de E7 e PTPN14 depende de resíduos contidos na extremidade carboxiterminal da proteína E7 e que para degradação desta proteína supressora tumoral é necessária a interação entre a proteína UBR4 e a extremidade aminoterminal da proteína E7, nós avaliamos os níveis de co-localização entre E7 das diferentes variantes e as proteínas PTPN14 e UBR4 (White et al., 2016; Huh et al., 2005). Nós observamos que, enquanto PTPN14 ou UBR4 apresentaram altos níveis de co-localização com E7 As+AI somente quando as células foram tratadas com um inibidor do complexo proteossomal, altos níveis de co-localização da oncoproteína E7 Af com PTPN14 ou UBR4

foram observados independente das células serem tratadas ou não com um inibidor de proteossomo. Ademais, nós demonstramos que células primárias BRK transfectadas com E7 Af geraram um menor número de colônias comparado às células expressando E7 As+AI. Estes dados corroboram àqueles oriundos dos PHKs imortalizados por E6/E7 das duas variantes de HPV-18 que revelaram que os PHK18AA foram capazes de gerar um maior número de colônias e esferoide em ensaios realizados em culturas de células em monocamada e 3D, respectivamente. Assim, é razoável supor que a alteração de apenas um aminoácido em E7 de HPV-18 interfere na capacidade deste vírus em induzir o crescimento de colônias.

Nós também avaliamos a capacidade dos PHKs imortalizados pelas diferentes variantes de HPV-18 em migrarem e invadirem. Apesar de não observamos diferenças entre os PHK18AA e PHK18Af imortalizados no que se refere à migração celular, os PHK18AA apresentaram um maior potencial invasivo quando comparados aos PHK18Af.

Finalmente, nós avaliamos a capacidade dos PHK18AA e PHK18Af imortalizados em induzirem a diferenciação celular, tanto em células cultivadas em monocamada em meio enriquecido por soro e cálcio, quanto em epitélios gerados em cultura organotípica. Nós observamos que os PHK18Af imortalizados apresentaram um maior potencial de resistência à diferenciação terminal em meio com soro e cálcio, ao passo que os PHKs imortalizados não exibiram diferenças na morfologia dos epitélios originados ou nos níveis de proteínas associadas à diferenciação (CK10) e a proliferação celular (PCNA e p16).

Em vista dos dados apresentados, nós acreditamos que a expressão conjunta de E6/E7 das variantes de HPV-18 podem interferir em diferentes

aspectos associados a carcinogênese. Assim como sugerido por diversos estudos conduzidos até o momento, nossos resultados também demostraram que a variante As+AI apresentam um maior potencial oncogênico em relação à variante Af de HPV-18. Por conseguinte, nosso estudo estimula novas investigações referentes à importância da caracterização de variantes moleculares de HPVs na compreensão da gênese dos tumores associados.

2. Referências

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5 ANEXOS

Anexo A -

Documento fornecido pelo Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo declarando ciência perante a execução do Protocolo de Pesquisa nº 255/16.

MEDICINA
USP
COMITÊ DE ÉTICA EM PESQUISA

APROVAÇÃO

O Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo, em sessão de 20/07/2016, APROVOU o Protocolo de Pesquisa nº 255/16 intitulado: "PROPRIEDADES BIOLÓGICAS E BIOQUÍMICAS DE VARIANTES NATURAIS DE HPV-18" apresentado pelo Departamento de RADIOLOGIA E ONCOLOGIA

Cabe ao pesquisador elaborar e apresentar ao CEP-FMUSP, os relatórios parciais e final sobre a pesquisa (Resolução do Conselho Nacional de Saúde nº 466/12, inciso IX.2, letra "c").

Pesquisador (a) Responsável: Laura Cristina Sichero Vettorazzo
Pesquisador (a) Executante: Emily Montosa Nunes

CEP-FMUSP, 20 de Julho de 2016.

Profa. Dra. Maria Aparecida Azevedo Koike Folgueira
Coordenador
Comitê de Ética em Pesquisa

Anexo B

Documento fornecido pela Faculdade de Medicina da Universidade de São Paulo atestando disciplinas cursadas pela aluna Emily Montosa Nunes na qualidade de aluna regular do programa de pós-graduação em Oncologia.

Universidade de São Paulo
Brasil

Janus
Sistema administrativo da Pós-graduação

Faculdade de Medicina

ATESTADO

Atestamos, para os devidos fins que, até o dia 10/02/2020, o(a) senhor(a) Emily Montosa Nunes, de número USP 9035801, cursou a(s) disciplina(s) abaixo na qualidade de aluno(a) regular do programa de pós-graduação em Oncologia.

Disciplina: Mecanismos de Carcinogênese
Sigla: MCM5923-1/1 **Carga Horária:** 60 **Conceito:** A **Frequência:** 88 **Créditos:** 4
Início: 21/09/2016 **Término:** 04/10/2016

Disciplina: Seminários sobre Vírus Persistentes de Importância em Saúde Pública
Sigla: IMT5124-1/3 **Carga Horária:** 60 **Conceito:** A **Frequência:** 100 **Créditos:** 4
Início: 16/03/2017 **Término:** 26/04/2017

Conceito a partir de 02/01/1997:
A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.
Um(1) crédito equivale a 15 horas de atividade programada.

Este documento eletrônico dispensa carimbo e assinatura. Sua autenticidade pode ser comprovada fornecendo-se o código de controle na seguinte página da Universidade de São Paulo: http://uspdigital.usp.br/webdoc

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Código de controle: 5GFD - JY43 - MFMX - 87PA
Código de controle válido até: 10/02/2021

Anexo C

Súmula Curricular

Emily Montosa Nunes (Nunes EM)

1. Formação

2010	Bacharelado em Biotecnologia	Centro de Desenv. Tecnol., UFPel, Pelotas
2016	Mestrado em Ciências	PPG Oncologia, ICESP, FMUSP, São Paulo
2016(em curso)	Doutorado em Ciências	PPG Oncologia, ICESP, FMUSP, São Paulo

2. Histórico Profissional

2016 - atual
Aluna de Doutorado do Laboratório de Biologia Molecular do Centro de Investigação Translacional em Oncologia do Instituto do Câncer do Estado de São Paulo, ICESP.

2014 - 2016
Aluna de Mestrado do Laboratório de Biologia Molecular do Centro de Investigação Translacional em Oncologia do Instituto do Câncer do Estado de São Paulo, ICESP.

2013 - 2013
Aluna de Iniciação Científica do Laboratório de Biologia Molecular do Centro de Investigação Translacional em Oncologia do Instituto do Câncer do Estado de São Paulo, ICESP.

2010 - 2013
Aluna de Iniciação Tecnológica do Laboratório de Genômica Funcional do Grupo de Pesquisa em Oncologia no Centro de Desenvolvimento Tecnológico, UFPel.

3. Publicações

3.1. Revistas Especializadas

1. Lorenzi AT, Fregnani JH, Villa LL, Sichero L, **Nunes EM**, Longatto-Filho A. Diversity of human papillomavirus typing among women population living in rural and remote areas of Brazilian territory. Papillomavirus Research. 2019; 8: 1-4.
2. Silva EM, Mariano VS, Pastrez PRA, Pinto MC, **Nunes EM**, Sichero L, Villa LL, Scapulatempo-Neto C, Syrjanen KJ, Longatto-Filho A. Human papillomavirus is not associated to non-small cell lung cancer: data from a prospective cross-sectional study. Infect. Agent Cancer. 2019; 14(18): 1-7.
3. Pimentel DRN, **Nunes EM**, Termini L, Nunes RAL, Lopez RVM, Ferreira S, Boccardo E, Prado JCPM, Enokihara MMSS, Sichero L, Tomimori J. Detection of human papillomaviruses and human polyomaviruses in

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4. **Nunes EM**, Talpe-Nunes V, Sichero L. Epidemiology and biology of cutaneous Human Papillomavirus. Clinics. 2018; 73:1-9.
5. da Costa AM, Fregnani JHTG, Pastrez PRA, Mariano VS, Neto CS, Guimarães DP, de Oliveira KMG, Neto SAZ, **Nunes EM**, Ferreira S, Sichero L, Villa LL, Syrjanen KJ, Longatto-Filho A. Prevalence of high risk HPV DNA in esophagus is high in Brazil but not related to esophageal squamous cell carcinoma. Histol Histopathol. 2018; 33(4): 357-363.
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7. Pastrez PRA, Mariano VS, da Costa AM, Silva EM, Scapulatempo-Neto C, Guimarães DP, Fava G, Neto SAZ, **Nunes EM**, Sichero L, Villa LL, Syrjanen KJ, Longatto-Filho A. The Relation of HPV Infection and Expression of p53 and p16 Proteins in Esophageal Squamous Cells Carcinoma. J Cancer. 2017; 8 (6): 1062-1070.
8. Sichero L, El-Zein M, **Nunes EM**, Ferreira S, Franco EL, Villa LL. Cervical Infection with Cutaneous Beta and Mucosal Alpha Papillomaviruses. Cancer Epidemiol Biomarkers Prev. 2017; 1: cebp.0081.2017.
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11. Sichero L, Nyitray AG, **Nunes EM**, Nepal B, Ferreira S, Sobrinho JS, Baggio ML, Galan L, Silva RC, Lazcano-Ponce E, Giuliano AR, Villa LL. Diversity of human papillomavirus in the anal canal of men: The HIM study. Clin Microbiol Infect. 2015 May; 21(5): 502–509.
12. Hartwig FP, Entiauspe LG, **Nunes EM**, Rodrigues FM, Collares T, Seixas FK, Da Silveira MF. Evidence for an Epistatic Effect between TP53 R72P and MDM2 T309G SNPs in HIV Infection: A Cross-Sectional Study in Women from South Brazil. *Plos One*. 2014; 9: e89489-11.
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14. Entiauspe LG, Silveira MF, **Nunes EM**, Basgalupp SP, Stauffet D, Dellagostin AO, Collares T, Seixas FK. High incidence of oncogenic HPV genotypes found in women from Southern Brazil. Braz. J. Microbiol. 2014; 45 (2): 689-694.

15. Entiauspe LG, **Nunes EM**, Collares T, Silveira MF, Seixas FK. Comparison between two methods for molecular characterization of human papillomavirus. DST - J Bras Doenças Sex. Transm. 2013; 25 (1): 13-15.

3.2. Capítulo de livros

1. **Nunes EM**, Kremer FS, Debom GN, Plaça JR, Souza JD, Goedert L. Banco de Dados Forense. In: Sibele Borsuk (Org.). Biotecnologia Forense. Pelotas: Editora UFPel, 2014, p. 9-33.
2. Valls JPH, **Nunes EM**, Sichero L. Carcinogênese Biológica. In: Renata de Freitas Saito, Marlous Vinícius Gomes Lana, Ruan F. V. Medrano, Roger Chammas (Org.). Fundamentos em Oncologia Molecular para Graduação. São Paulo: Ed. Atheneu, 2015, p. 53-75.

4. Auxílios Recebidos

1. FAPESP – Processo nº 2015/26346-6, Doutorado, projeto “Propriedades biológicas e bioquímicas de variantes naturais de HPV-18”, no valor de R$ 150.967,44, durante o período de 1 junho de 2016 a 29 de fevereiro de 2020.
2. FAPESP – Processo nº 2018/14697-7, Bolsa de Estágio no Exterior – Doutorado, projeto “Propriedades biológicas e bioquímicas de variantes naturais de HPV-18”, no valor de R$ 95.344,48, durante o período de 16 outubro de 2018 a 15 de julho de 2019.
3. FAPESP – Processo nº 2015/12557-5, Bolsa de Estágio no Exterior – Mestrado, projeto “Prevalência e diversidade de Beta-papilomavírus em amostras anogenitais e orais de homens do estudo HIM”, no valor de R$ 2.965,00, durante o período de 10 outubro de 2015 a 9 de novembro de 2015.
4. FAPESP - Processo nº 2013/20470-1, Mestrado, projeto “Prevalência e diversidade de Beta-papilomavírus em amostras anogenitais e orais de homens do estudo HIM”, no valor de R$ 44.542,08 durante o período de 1 de março de 2014 a 31 de março de 2016.
5. CNPq - Proc. 121393/2013-5, Iniciação Científica, projeto “INCT das Doenças do Papilomavirus”, no valor de R$ 2.400,00 durante o período de 01 de setembro de 2013 a 28 de fevereiro de 2014.
6. CNPq - Proc. 800226/2012-7, Iniciação Tecnológica, com o projeto “Nanooncologia: identificação de novos compostos na terapêutica do câncer”, no valor de R$ 4.520,00 durante o período de 01 de setembro de 2012 a 31 de julho de 2013.
7. CNPq - Proc. 800483/2011-1, Iniciação Tecnológica, com o projeto “Nanooncologia: identificação de novos compostos na terapêutica do câncer”, no valor de R$ 3.960,00 durante o período de 01 de setembro de 2011 a 31 de agosto de 2012.
8. CNPq - Proc. 144877/2010-4, Iniciação Tecnológica, com o projeto “Vacina Recombinante contra leptospirose para uso veterinário: avaliação do

antígeno ligani conjugado a microesferas de PLGA e Lipossomos”, no valor de R$ 1.440,00 durante o período de 01 de agosto de 2010 a 31 de julho de 2011.

5. Prêmios

1. Isenção de pagamento de inscrição no ICGEB DNA Tumour Virus Meeting com os trabalhos " "Biological properties of natural variants by Human Papillomavirus 18" e "The Human Papillomavirus type 18 African genetic variant is not able to degrade the PTPN14 tumor suppressor" pelo International Center for Genetic Engineering and Biotechnology (ICGEB), Trieste, Itália, no período de 9 a 14 de julho de 2019.
2. Menção honrosa em Prêmio Destaque 2018 com a publicação "Concordance of Beta-papillomavirus across anogenital and oral anatomic sites of men: The HIM Study" pelo Instituto do Câncer do Estado de São Paulo (ICESP), São Paulo.
3. Menção honrosa no “VI Prêmio Maria Mitzi Brentani” com o trabalho "Biological and biochemical properties of natural variants of HPV-18" pelo Instituto do Câncer do Estado de São Paulo (ICESP), São Paulo, em 2017.
4. Isenção de pagamento de inscrição no “3rd ICGEB Workshop on HPV and associated malignancies” com o trabalho "Concordance of Beta-papillomavirus across anogenital and oral sites is low in males from the HIM Study" pelo International Center for Genetic Engineering and Biotechnology (ICGEB), São Paulo, no período de 2 a 5 de setembro de 2017.
5. Prêmio Destaque 2016 com o capítulo "Carcinogênese Biológica " no livro “Fundamentos de Oncologia Molecular (Editora Atheneu)” pelo Instituto do Câncer do Estado de São Paulo (ICESP), São Paulo.
6. Prêmio Destaque 2016 com a publicação "Diversity of Beta-papillomavirus at anogenital and oral anatomic sites of men: The HIM Study" pelo Instituto do Câncer do Estado de São Paulo (ICESP), São Paulo.
7. Ajuda de custo para viagem e participação no 31st International Papillomavirus Conference "Concordance of Beta-papillomavirus in anogenital and oral samples of males from the HIM Study" pela International Papillomavirus Society (IPVS), Cidade do Cabo, África do Sul, no período de 28 de fevereiro a 4 de março de 2017.
8. Prêmio “Hélio Gelli Pereira” dado pela Sociedade Brasileira de Virologia no XXVII Congresso Brasileiro de Virologia, pelo trabalho “Diversity of Beta-papillomavirus at anogenital and oral anatomic sites of men: The HIM Study”, em Pirenópolis, Goiás, no período de 18 a 21 de setembro de 2016.
9. Primeiro lugar na categoria ciência da saúde no “XXI Congresso de Iniciação Científica da Universidade Federal de Pelotas (UFPel)” pela apresentação oral do trabalho “Papilomavírus Humano oncogênico relacionado à citologia oncológica em mulheres HIV positivo na cidade de Pelotas”, em Pelotas, Rio Grande do Sul, no período de 20 a 23 de novembro de 2012.
10. Primeiro lugar na modalidade livre do “Desafio Mural G- Biotec - A Biotecnologia e Você” da Universidade Federal de Pelotas (UFPel) pelo

trabalho “Visita Virtual Biotecnologia/CDTec”, em Pelotas, RS, no período de 01 de setembro a 01 de outubro de 2012.

6. Indicadores Quantitativos

6.1. Publicações em periódicos	15
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Contents lists available at ScienceDirect

Papillomavirus Research

journal homepage: www.elsevier.com/locate/pvr

Diversity of human papillomavirus typing among women population living in rural and remote areas of Brazilian territory

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ARTICLE INFO

Keywords:
Cervical cancer
Cervix neoplasms prevention
Genotype
Human *papillomavirus* DNA tests
Self-examination
Screening

ABSTRACT

Objectives: Genotyping HPV from samples tested positive to careHPV™ assay in rural and remote areas of Brazilian territory.
Methods: A total of 5079 women were enrolled in an opportunistic screening from the Barretos Cancer Hospital, through mobile units or ambulatory unit. All careHPV™ hr-HPV positive samples were tested by a Luminex-based protocol in order to evaluate the HPV infecting types.
Results: Positive hr-HPV results were obtained in 10.6% (536/5068) of women. Among these cases, HPV-56 and HPV-51 were the most common types detected in 32.3% and 31.4%, respectively. HPV-53 (20.5%), HPV-18 (18.5%), HPV-58 (17.6%), HPV-52 (16.0%) and HPV-16.6%) were the other most frequent types detected. These frequencies represent prevalences of 2.35%, 2.12%, 2.02%, 1.84% and 1.80% respectively, within the population studied. Regarding low-risk HPVs, HPV-6 was detected in 12.9% of the samples. The less frequent types (< 3%) were: HPV-70, HPV-11 and HPV-26.
Conclusions: The most frequent types detected were: HPV-56, HPV-51, HPV-53, HPV-18, HPV-58, HPV-52 and HPV-16 according to decreasing rates.

1. Introduction

Cervical cancer incidence and mortality still represents a critical issue for Public Health authorities with almost 570 thousand new cases and 311 thousand deaths, annually. This is particularly disturbing if considers that the majority of these cases are found in developing and poor countries of Africa and South America [1]. The limitations of screening effectiveness in poor countries are usually related to the scarcity of qualified human resources, lack of governmental planning for organized screening programs, lower quality control in cytology performance and absence of HPV testing, in addition to other reasons generally related to the lower economic incoming to support prevention cancer programs. This set of variables represents a huge barrier in the eradication of a potentially preventable malignancy that affects mostly women living in rural and remote areas and accentuates ethnical and racial inequities regarding the opportunities to reduce cancer mortality [2,3].

HPV vaccination is a well-tailored option for primary prevention tool of HPV-related cancer. Currently, a number of evidences strongly suggest that HPV vaccination is an important implement to reduce the prevalence of malignancies induced by persistent HPV oncogenic types infection in women underprivileged of medical assistance. Recently, an elegant report conducted by Hall and colleagues indicates the possibility of cervical cancer eradication within the next 20 years in Australia by the high-coverage vaccination and screening. The aim is to decrease the rates to four new cases per 100,000 women per year, to consider

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https://doi.org/10.1016/j.pvr.2019.100186
Received 13 November 2018; Received in revised form 4 July 2019; Accepted 16 September 2019
Available online 17 September 2019

cervical cancer as an extinguished problem of public health in Australia [4]. Arbyn and co-workers at Cochrane publication demonstrated the efficacy of HPV vaccination, which the data available about HPV vaccine have shown a high-certainty evidence of the protective effect of HPV vaccines against cervical intraepithelial neoplasia in female adolescents and young women aged 15 to 26 [5].

However, despite to the scientific encouragement towards vaccine implementation, there are many restrictive elements for HPV vaccines application with evident reluctance in many settings of the general population, which represent a serious obstacle for HPV-induced lesions reduction, which must be overcomes to increase the vaccine coverage that comprise psychological, religious and/or cultural reactions [6].

The report of Muñoz and colleagues defined with robust data from different countries the most prevalent HPV types worldwide [7]. This is relevant because HPV vaccines were tailored based on the most important HPV types related to cervical cancer development, and the results achieved clearly demonstrated the significant contribution of these vaccines to reduce HPV-related lesions in women cervix [8–10]. Conversely, some HPV types distribution are unequal worldwide, and these variations can induce alterations on the efficacy of HPV vaccine [11]. Moreover, some specific HPV types present to biological behavioral differences that can correspond to better or worse outcome for the women affected with cervical cancer [12].

Considering all these facts, we sought to investigate specific HPV types prevalence in rural and remote areas of Brazilian territory in order to draw a map of HPV types distribution in a country with continental dimension.

2. Methods

This was a retrospective evaluation of individual HPV types prevalence performed within a casuistic planned for a prospective study carried out from March to December 2010 of women attended at the Ambulatory of Prevention Department and the mobile units of Barretos Cancer Hospital (BCH), which assisted the Brazilian rural and remote areas, and these samples were previously underwent to Surepath Liquid-based Papanicolaou, and the HPV detection was performed using the careHPV™ molecular test [3].

The project was approved by the Research Ethics Committee of the Barretos Cancer Hospital - Pio XII Foundation, protocol number 404/2010.

2.1. The mobile units platform

A total of 3079 volunteer women aged over than 18 were invited to participate in a opportunistic screening available at the mobile units of the Barretos Cancer Hospital, which assisted the states of Goiás, Minas Gerais, Mato Grosso and São Paulo. At this stage, there was no randomization of the studied group and the samples were collected by health professionals (careHPV™ test and Liquid-based Papanicolaou test).

2.1.1. Collections carried out in mobile units

The samples collected to careHPV ™ medium were initially analyzed in the mobile unit in ten out 63 cities visited by the unit: Mineiros (GO), Portelândia (GO), Alto Araguaia (MT), Alto Garças (MT), Alto Taquari (MT), Rondonópolis (MT), Pedra Preta (MT), Guiratinga (MT), São José do Povo (MT), Itiquira (MT); totalizing over than 400 samples analyzed *in loco*; the remaining samples were tested at the Research Molecular Center of BCH.

2.2. Laboratory conditions for samples preservation

Cervico-vaginal samples tested for careHPV™ (Digene, Qiagen, Gaithersburg, MD, USA) were maintained under at 15 °C to 30 °C for two weeks, and/or 2 °C to 8 °C for four weeks or −20 °C for two months. Those samples that were tested positive in the careHPV test were maintained 2 °C to 8 °C.

2.3. DNA extraction

DNA from the positive samples maintained on the careHPV™ medium were extracted using the QIAmp MinElute Virus Vaccum kit (Qiagen Co) according to the manufacturer's protocol. Extracted DNA were kept in at −20 °C.

2.4. HPV genotyping

HPV positive samples using the careHPV™ test were further genotyped using the Luminex methodology (Luminex Corporation, TX, USA). This methodology uses oligonucleotide probes coupled polystyrene beads containing two different fluorophores inside that allows the simultaneous detection of at most 100 different specific HPV types. In this study, probes used allowed the identifications of 21 viral types: i) low risk: 6, 11 and 70, ii) high risk: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. PCR reactions were performed using the Qiagen Multiplex PCR kit (Qiagen, Dusseldorf, Germany) and a mixture of specific biotinylated primers capable of amplifying a fragment of the E7 gene, followed by bead-based Luminex technology (Gheit et al., 2007; Nunes et al., 2016; Schmitt et al., 2006). Primers for the amplification of β-globin were also added to evaluate the quality of template DNA. For each probe, MFI (mean fluorescence intensity) values obtained when no PCR product was added to the hybridization mixture was considered the background values. Cutoffs were computed by adding 20 MFI to 1.1X the median background value. Whenever a positive signal for HPV and β-globin was not obtained, the sample was considered inadequate. Those cases in which the positive signal for HPV was not observed, but β-globin was detected, samples were considered negative as well.

2.5. Statistical analysis

The prevalences of HPV specific types were computed. Statistical analysis was performed using IBM® SPSS® Statistics (Statistical Package for Social Sciences) version 20.1 for Windows (IBM Corporation, Route 100, Somers, NY 10589) and MedCalc® version 11.1 for Windows (Broekstraat 52, B −9030 Mariakerke, Belgium).

3. Results

Women tested positive for HPV (10.0% or n = 307) using the careHPV test showed mean age of 41.0 years. The socio-demographic findings achieved previously did not show any statistically significant association between HPV positivity and tobacco use, number of parities, number of sexual partners and contraceptive [3].

3.1. HPV types

From 443 samples available for analysis, high-risk HPV was detected in 369 samples, 72 were positive for high and low risk HPV simultaneously and 2 cases tested positive for low risk HPV (both HPV6).

Single HPVs infections were observed in 25.3% (112/443) of the samples, two of them by low-risk HPV (HPV 6). Conversely, 74.7% (331/443) of the samples had multiple infections solely with high-risk types or with high- and low-risk HPVs.

HPV-56 and HPV-51 were the most common types, and were detected in 32.3% and 31.4% of the samples, respectively. This represents 3.7% of HPV prevalence, considering the percentage of HPV positive women found in this study (11.5%). Following HPV-53 (20.5%), HPV-18 (18.5%), HPV-58 (17.6%), HPV-52 (16.0%) and HPV-16 (6%) were the most frequent, which represents a prevalence of 2.35%, 2.12%, 2.02%, 1.84% and 1.80%, respectively, according to the percentage of HPV positive women studied. Regarding low-risk HPVs, HPV-6 type

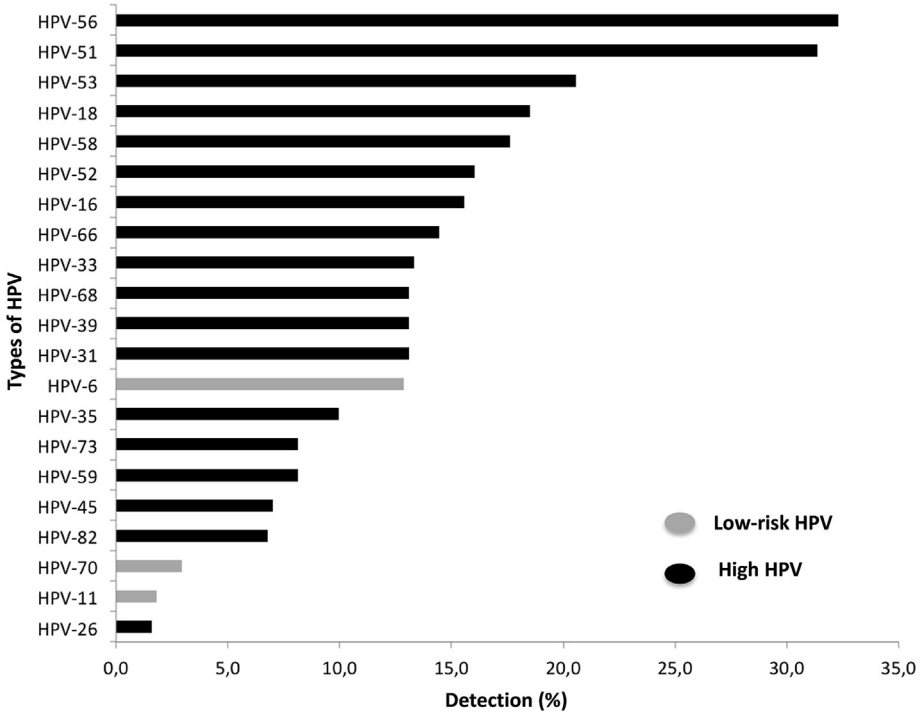

Fig. 1. General distribution of HPV genotypes detected in the cervicovaginal samples. The different genotypes are marked on the "Y" axis, while the frequency of the cases (shown in percentage) are on the "X" axis.

was detected in about 12.9% of the samples. The less frequent detected types (< 3%) were: HPV-70, HPV-11 and HPV-26. Fig. 1 depicts HPV types distribution.

4. Discussion

The general positivity for high-risk HPV reported in this current study was 10.6%: 11.5% observed in the casuistic of cases collected at the Prevention Department of HCB and 10% detected in women examined at mobile units, respectively. Data documented by other Brazilian studies showed positive rates ranging from 9.7% to 10.5% [13–17]. Recently, Torres and colleagues reported HPV-DNA frequency of 19.1% in women of remote areas of the Amazon, a region reputed as the site with the highest indexes of HPV prevalence in the Brazilian territory [18]. The HPV prevalence found in the current study was, however, slightly lower than those found in previous studies, with a prevalence superior to 15% [19] and quite similar to the prevalence of HPV infection of 9.6% found in Malasy population [20].

HPV genotyping performed within the positive samples showed in descending order, higher frequencies of the HPV-53, HPV-18, HPV-58, HPV-52, and HPV-5 and HPV- HPV-16, with frequencies of detection of 32.3%, 31.4%, 20.5%, 18.5%, 17.6%, 16.0% and 15.6%, respectively. Moreover, the overall HPV typing demonstrated that HPV types frequencies is particularly different if compared to the frequencies reported in literature, once HPV56, HPV51 and HPV53 were significantly more prevalent than HPV 16 and HPV18, presumed as the more prevalent HPV types worldwide [7]. Interesting, however, is that fact that previous studies, including a Canadian study, have reported a HPV DNA detection rate of 1 to 7% of HPV-56 in CIN2 and CIN3 patients [21,22]. In a retrospective study in Brazilian Bahia State, about 14% of women with high-grade lesions and 18% with low-grade lesions were identified with HPV-16 and HPV-56 infection [23]. These data differ substantially from another study carried out in the Northern region of Brazil that place HPV-16 and HPV-18 as the most prevalent types in women infected by HPV [24]. We speculate that these variations in prevalence of HPV types are certainly particular to each different region within the Brazilian territory; once this is a country of continental dimensions and formed by a complex network of different cultures and races, which may present more variations regarding HPV type regionally distribution. However, it is important to emphasize that the distribution of different HPV types is not homogeneous worldwide. In this field, HPV16 and HPV18 still show an important and significant protagonism even in Brazil [25]. Some studies have demonstrated that the five most common HPV types in the order of decreasing prevalence are HPV 16, 18, 31, 45 and 52 [26].

In addition, it is necessary to note that differences in the methodologies used to detect and type HPVs can certainly influence data of the different studies regarding HPV types prevalence.

Finally, the importance of HPV typing seems to be closed related to the contribution that these observations can provide to the strategies of vaccination in different regions of the world. This issue is not negligible if one takes in account that HPV vaccination should be planned for certain regions of the world, considering that the variation of HPV typing could infer a bias of HPV vaccine efficacy if the most prevalent types are not included among types selected for bivalent, quadrivalent or nonavalent vaccine currently available in the medical armamentarium. Cross-protection is presently and potentially expectable for all of the 3 licensed HPV vaccines provide protection against HPV types not included in the vaccines. It is well-stablished that bivalent and quadrivalent HPV vaccines provide some level of cross-protection against high-risk HPV types: 31, 33 and 45 HPV types. However, the degree of cross-protection against HPV types of nonavalent vaccine is not yet documented [27].

In conclusion, our study demonstrated that HPV types have a significative variation prevalence in Brazilian territory; data can be improved with robust increasing casuistry.

Disclosure

The careHPV ™ test kits, as well as equipment, preservation media, consumables and brushes for molecular testing, were provided by Qiagen® Brazil.

Funding statement

The study was supported financially (grant-aid) of the National Institute of Research and Technology in HPV (INCT - HPV) of São Paulo, through the CNPq process nº 573799/2008-3, FAPESP process nº 2008/57889-1, through and CNPq process nº 203348/2014-1.

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Silva et al. Infectious Agents and Cancer (2019) 14:18
https://doi.org/10.1186/s13027-019-0235-8

Infectious Agents and Cancer

RESEARCH ARTICLE Open Access

Human papillomavirus is not associated to non-small cell lung cancer: data from a prospective cross-sectional study

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Abstract

Background: The pathogenesis of lung cancer is triggered by a combination of genetic and environmental factors, being the tobacco smoke the most important risk factor. Nevertheless, the incidence of lung cancer in non-smokers is gradually increasing, which demands the search for different other etiological factors such as occupational exposure, previous lung disease, diet among others. In the early 80's a theory linked specific types of human papillomavirus (HPV) to lung cancer due to morphological similarities of a subset of bronchial squamous cell carcinomas with other HPV-induced cancers. Since then, several studies revealed variable rates of HPV DNA detection. The current study aimed to provide accurate information on the prevalence of HPV DNA in lung cancer.

Methods: Biopsies were collected from 77 newly diagnosed non-small cell lung cancer (NSCLC) patients treated at the Thoracic Oncology Department at Barretos Cancer Hospital. The samples were formalin fixed and paraffin embedded (FFPE), histologic analysis was performed by an experienced pathologist. DNA was extracted from FFPE material using a commercial extraction kit and HPV DNA detection was evaluated by multiplex PCR and HPV16 specific real-time PCR.

Results: HPV was not identified in any of the samples analysed (69).

Conclusions: Our data demonstrated a lack of HPV DNA in a series of NSCL cancers.

Keywords: Non-small cell lung cancer, Lung neoplasms, Papillomaviridae, Papillomavirus DNA

Background

Lung cancer is the major cause of cancer-related death worldwide. The International Agency for Research on Cancer (IARC) estimated 1.8 million new cases for 2016 [1]. Although the pathogenesis of lung cancer is complex and arises due to a combination of genetic and environmental factors [2], tobacco smoke is consensually assumed as the strongest factor associated to this disease development, worldwide [3]. However, epidemiologic data obtained from different geographical regions have revealed that approximately 25% of all cases of lung cancer are not attributable to tobacco use [4]. Other risk factors have attracted the attention of physicians because the incidence of this disease among non-smokers has increased in the past years [5–7]. Consequently, other etiological factors have been investigated including occupational exposure, previous lung disease, diet, among others [4, 8]. Additional, human papillomavirus (HPV) infection has emerged as a potential etiological agent for certain types of bronchogenic carcinomas. Syrjänen and col. (1979) [9] suggested in the early 80's that oncogenic HPVs could be responsible for lung cancer pathogenesis [10] due to the morphological resemblances within a subset of bronchial squamous cell carcinomas compared with the clinical manifestations of HPVs in the female genital tract [9, 11, 12]. Since then, numerous studies

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have pointed out an inquisitive heterogeneity on HPV DNA detection rates in lung cancers, with the highest rates detected among Asians [13–18]. European studies reported low or no HPV prevalence (0–10%) compared with the 22% (4/18) of Asian study. In the latter, HPV16 and HPV18 prevalence were 11.6 and 8.8%, respectively. However, the authors suggested more attention to study design and laboratory detection methods for analyzing this theme [16].

Syrjanen (2012) [19] performed a systematic review and formal meta-analysis of the literature reporting on HPV detection in lung cancer using MEDLINE and Current Contents platforms and found that geographical origin, histopathological types of cancer and HPV detection methods were not significant co-variates accounting for the heterogeneity of the HPV prevalence in lung cancer. Related to HPV infection as risk for lung cancer development, in 3,083 cases and 4,328 controls in two retrospective case-control studies and, one prospective nested case-control study, no association was observed between HPV infections and lung cancer development; also, viral oncogenes, HPV antibodies and DNA HPV were not related to lung cancer survival [20].

HPV infection in lung tissue was evaluated by another meta-analysis using the search terms “lung cancer”, “human papillomavirus”, “HPV” and their combinations and, antagonistically, the results suggested that HPV infection in lung tissue have a strong association with lung cancer development [21]. Another meta-analysis based on PubMed, Ovid and Web of Science to identify case-control studies and cohort studies that detected HPV in lung carcinomas included 30 or more cases published before Feb 28, 2017 and corroborated that HPV16 and HPV18 infection significantly increase the risk of lung cancer [22].

Additionally, a Chinese a study developed to identify the association between HPV positive rate and smoking in lung cancer (LC) patients observed that HPV infections are associated with smoking in LC patients and the association may relate to different regions [23]. Lung cancer study of Brazilian patients, the presence of HPV was detected by PCR followed by genotyping, found HPV in 33 of the 63 samples, and HPV types 16 and 18 were detected with frequencies of 81% (27/33) and 19% (6/33), respectively. Also, the expression of the E6 and E7 oncoproteins HPV type specific, evaluated by immunohistochemical, was detected in 28/33 samples and 25/33 samples, respectively [24].

Due to discrepancies among the results across the world and previous work from our country, we aimed to provide detailed additional data on the HPV burden in lung cancer by evaluating the prevalence of HPV DNA in samples of non-small cell lung cancer (NSCLC) using a specific method for this. Furthermore, p16 expression was accessed by immunohistochemistry (IHC) to further address if it’s immunoexpression could be considered as a surrogate marker of HPV activity in NSCLC specimens.

Materials and methods

Inclusion criteria

Samples were achieved from a prospective analysis of patients submitted to the bronchoscopy admitted at Barretos Cancer Hospital between 2013 and 2015. All biopsies were performed for diagnosis and all the patients (77) were naïve of treatment. Patients with NSCLC diagnosis that agreed to participate were included.

Ethics approval and consent to participate

Written informed consent was provided by all patients and the study was approved by Barretos Cancer Hospital Ethic Committee (number 920.225).

Patient characteristics

All 77 patients were subjected to a detailed trained interviewer assisted interview for assessment of demographic, NSCLC risk factors and previous history of HPV infection variables.

Tumor samples

Tumor samples were obtained by endobronchial and transbronchial biopsies. Specimens were routinely processed for histological diagnosis, HPV testing and for immunohistochemical analysis of p16. Pathologic stages were determined according to the American Joint Committee on Cancer (AJCC) [25], and histological classification according to criteria of the World Health Organization (WHO) [26].

DNA extraction

Formalin-fixed, paraffin-embedded (FFPE) tumor tissue sections, previously evaluated and delimited by an expert pathologist (CSN), were deparaffinized, and using QIAamp DNA Micro kit (QIAGEN, Hilden, Germany) DNA was extracted according to the manufacturer’s recommendations. Briefly, 4–6 10 μm FFPE sections were submitted to deparaffinization with 100% xylene, followed by washes of ethanol (100, 90, and 50%) and incubation with DNA extraction buffers overnight.

β-Globin polymerase chain reaction (PCR)

All 77 DNA samples were quantified by NanoDrop 2000 (ThermoScientific) and subjected to β-globin gene amplification using PCO3 (ACACAACTGTGTTCACTAGC 5′-3 ‘) and PCO4 (CAACTTCATCCACGTTCACC 5’-3′) primers. The amplification of a short fragment of 110 pair base of human β-globin gene indicates the integrity of

DNA for subsequent analysis. Only β-globin positive samples (62) were used for HPV analysis.

HPV analysis

HPV DNA in lung tumors was independently assessed using two different techniques based on real-time PCR. The first one consisted of TaqMan singleplex based type-specific real-time PCR targeting the E7 region of HPV 16 type using specific TaqMan probe (5′- 6FAMCAAGCAGAACCGGACAG-MGBNFQ-3′) and primers (forward 5′-GATGAAATAGATGGTCCA GC-3′ and reverse 5′- GCTTTGTACGCACAACCG AAGC-3′) [27]. Among 77 lung samples, 62 were analyzed by this assay and the reaction mixture was prepared as follows: TaqMan Universal PCR Master Mix 1 x (Applied Biosystem, Inc., EUA), 400 nM of each primer, 200 nM TaqMan probe and DNAse/RNAse free water. PCR amplifications were carried out using 5 μL of template DNA in a final volume of 25 μL in an ABI Prism 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, USA). The amplification conditions were as follows: initial denaturation for 10 min at 95 °C, followed by 40 amplification cycles of 15 s each at 95 °C and 1 min at 60 °C (annealing-extension step). Each PCR reaction included a negative (water) and a positive control (DNA extracted from CaSki cell line). All samples and controls were tested in duplicate and were considered positive when both replicates amplified in a cycle < 38.

Additionally, 29 samples were randomly chosen for testing using a type-specific PCR bead-based multiplex genotyping assay that combine multiplex PCR and bead-based Luminex technology (Luminex Corp., Austin, TX, USA) that is able to identify 21 HPV types [28, 29], as previously described [28] and, based on available biological data [30]. Additionally, the assay has a positive control for the quality of the template DNA represented by β-globin gene primers. PCRs were performed with 10 μL of template DNA in a 96-well format in 25-μl/well final reaction volume. HPV multiplex PCR were performed using QIAGEN Multiplex PCR Kit (Qiagen, Dusseldorf, Germany), according to manufacturer's instructions. Each reaction consisted of 45 cycles: 94 °C for 30 s, 63 °C for 3 min, and 72 °C for 90 s. The first cycle was preceded by incubation at 95 °C for 15 min and the last cycle was extended for 10 min at 72 °C. PCR negative control consisted of a reaction mix without DNA. Hybridizations were performed according to Schmitt et al. (2006) [31]. For each HPV type-specific probe, the mean fluorescence intensity (MFI) values obtained when no PCR product was added to the hybridization mixture was considered as background. The cutoffs were calculated by adding 5 MFI to 1.1 times the value of the median background. MFI values > 20 were considered positive.

Immunohistochemistry

Sections of 4 μm containing representative tumour areas were used for IHC, which was performed using Ventana Benchmark ULTRA automated system (Ventana Medical Systems, Inc., Tucson, AZ). Antigen retrieval was performed using cell conditioning 1 buffer (CC1) at 95 °C for 64 min. For detection of immune reaction we used the ultraView Universal DAB Detection Kit polymer amplification system (Ventana Medical Systems, Mannheim, Germany) according to manufacturer's instructions. Mouse monoclonal anti-human antibody against p16INK4A protein, Clone E6H4™, ready for use (Ventana, USA) was used as primary antibody. Samples with strong and diffuse nuclear and cytoplasmic staining in more than 70% of the cells were considered positive [25, 26]. All slides were analyzed by two observers (CSN and ALF) who revised discordant cases for achieving consensus. A cervical adenocarcinoma was used as a positive control for p16 staining and negative controls were obtained by omitting the primary antibodies [32, 33].

Statistical analyses

Frequencies were obtained using the IBM® SPSS® Statistics 21.0 software for Windows (IBM Corporation, Somers, NY, USA).

Results

Among 77 samples included in this study, 15 were excluded due to the lack of β-Globin amplification and 62 were suitable for further analysis: 41 (66.1%) squamous cell carcinomas (SCC) and 21 (33.9%) adenocarcinomas. The SCC staging was as follow: 2 (3.4%) stage I, 4 (6.8%) stage II, 26 (44.1%) stage III and 27 (45.8%) stage IV. Additionally, 27 tissue samples (51.9%) were classified as well or moderately differentiated, and 25 (48.1%) as poorly differentiated. Most patients were male (n = 47, 75.8%), married (n = 40, 64.5%), and 54 (87.1%) have lived in rural area. As a consequence, 21 (33.9%) patients were farmer. Further characteristics of the patients included in this study are summarized in Table 1. The immunohistochemical staining of tumor for p16 and Ki-67 are illustrated in Figure 1.

None of the 62 NSCLC samples tested was positive for HPV using any of the techniques. Furthermore, p16 immunoreaction of all NSCLC was analyzed and it was observed positive expression in 10 (14,3%) cases. The analysis of positive cases for the expression of p16 (10/70) in relation to socio-demographic information, lifestyle and clinical-pathological findings showed no statistically significant correlation (Table 2).

Table 1 General Characteristics of NSCLC patients

Characteristic	Frequency (*n*)	Valid Percent (%)
Alcohol intake		
No	7	11.3
Yes	55	88.7
Smoking habits		
No	9	14.5
Yes	53	85.5
Exposure to pesticide and insecticides		
No	32	51.6
Yes	30	48.4
Exposure to asbestos		
No	45	80.4
Yes	11	19.6
Quantity of sexual partners[a]		
1–10	34	56.6
11–100	22	36.6
> 100	4	6.7
Oral sex[a]		
No	42	71.2
Yes	17	28.8
History of Sexually transmitted disease[a]		
No	51	83.6
Yes	10	16.4
History of Genital warts[a]		
No	57	93.4
Yes	4	6.6
Total	62	100

[a]Missing data

Discussion

The main goal of this study was to evaluate the presence of HPV DNA in NSCLC. Many contradictory results have been found among the reports published about this issue; the majority of discordances may likely be attributable to the differences in methodology used to identify HPV and the fact that many studies have no control regarding the quality of the samples preservation retrospectively selected for analysis. After all, it is not a surprise that an assertive attention about the real meaning of HPV in lung cancer is currently questionable. We did not find any NSCLC positive sample for HPV DNA among the samples we evaluated, despite the use of a very sensitive methodology we used to identify HPV, and the stringent conditions to preserve the samples prospectively collected, including rigorous control of quality in each step of the study. This final result is in agreement with some results obtained worldwide from different studies groups [34, 35]. Ywakawa et al. [36], e.g., analysed HPV-16, 18 and 33 DNA in 275 lung adenocarcinoma samples using 2 different methodologies (PCR multiplex and nested PCR) and also found no positive samples. Additionally, in another series [37] comprising 196 samples (100 adenocarcinoma and 96 squamous cell carcinoma), using in situ hybridization (ISH) capable of detecting high-risk HPV DNA (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68 and 70) no HPV DNA was observed. These data argue against the possible participation of HPV in NSCLC carcinogenesis [36, 37].

On the other hand, some studies have reported correlation between HPV and lung cancer [21]. Syrjänen et al. [38] detected HPV DNA in 4/77 (5.2%) NSCLC samples, three of which were positive for HPV-16 and one sample for HPV -6 / -16 coinfection, using multiplex PCR (Luminex®). Sarchianaki et al. [2] analysed 100 samples of NSCLC using real-time PCR methodology (GP5 + / GP6 +) and, 19 (19%) samples tested positive for HPV DNA. In order to genotype the positive samples, LINEAR ARRAY HPV Genotyping (Roche) capable of detecting 37 high and low risk HPV genotypes was also used, and 42.1% (8/19) of the positive samples were HPV-16 [2]. Moreover, Yu et al. [39] analysed 261 samples (107 squamous cell carcinoma, 63 adenocarcinoma and 91 non-tumour samples as control) by real-time PCR and INNO LIPA; 59.8% (64/107) in SCC, 17.5% (11/63) of the adenocarcinoma samples and 23.1% in the control samples were positive for HPV DNA. HPV-16 and / or 18 were found in 79.7% in SCC, 72.7% in adenocarcinoma and 14.3% in the control samples [39].

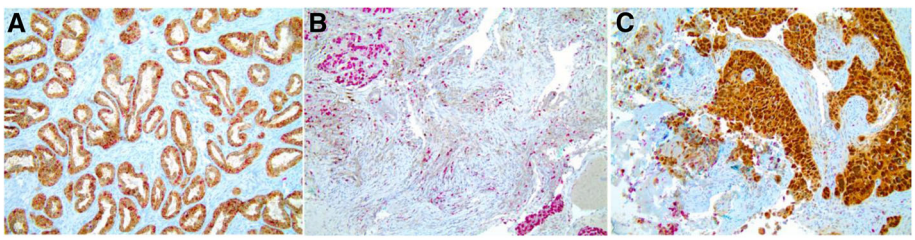

Fig. 1 Immunohistochemical staining of tumor for p16 expression. **a** Adenocarcinoma cervical used as positive control. **b** NSCLC sample scored as negative p16 expression. **c** NSCLC sample considered as positive p16 expression. As cell proliferation nuclear marker was used Ki-67. Magnification: × 20

Table 2 Characterization of the case group in relation to the expression of p16 and socio-demographic variables, lifestyle and clinical data. Cancer Hospital of Barretos, January/2013 to October/2015

Variable	Category	p16 expression				p
		Positive		Negative		
		n (*)	(%)	n (*)	(%)	
Gender	Female	4	(40,0)	13	(21,7)	0,242
	Male	6	(60,0)	47	(78,3)	
Race	Não Branco	3	(30,0)	12	(20,3)	0,679
	Branco	7	(70,0)	47	(79,7)	
Alcohol intake	Yes	8	(80,0)	53	(88,3)	0,607
	No	2	(20,0)	7	(11,7)	
Smoking habits	Yes	5	(50,0)	36	(60,0)	0,731
	No	5	(50,0)	24	(40,0)	
Histological types	Squamous cell carcinoma	6	(60,0)	37	(61,7)	0,999
	Adenocarcinoma	4	(40,0)	23	(38,3)	
Differentiation Degree	Well or Moderate	3	(30,0)	25	(52,1)	0,301
	Poor	7	(70,0)	23	(47,9)	
T	T1 – T2	2	(20,0)	12	(20,7)	0,999
	T3 – T4	8	(80,0)	46	(79,3)	
N	N0	1	(10,0)	10	(17,2)	0,999
	N positive	9	(90,0)	48	(82,8)	
M	M0	4	(40,0)	28	(48,3)	0,739
	M1	6	(60,0)	30	(51,7)	
Stage	I – II	0	(0,0)	6	(10,5)	0,580
	III – IV	10	(100,0)	51	(89,5)	
Total		10	(100)	60	(100)	

(*) Cases with missing values were excluded from the analysis
n number; TNM staging: a system based on the size and/ or the extension of the primary tumor (T), the number of compromised lymph nodes (N) and the presence of metastasis (M). Fisher's exact test was used. It was considered statistically significant $p < 0.05$

Together, these data indicate a wide rate of variation in the frequency of HPV among lung cancers with the highest frequency of virus being reported in East Asian countries, with a prevalence variation of 11.8–55.0% [16]. Finally, in a recent meta-analysis [40], 46 studies that demonstrated a higher prevalence of HPV in Asian countries (28.1%) when compared with European countries (8.4%) and countries from North and South America (21.3%), with regional differences between countries being observed. When the analysis was limited to HPV-16 and 18, which are the HPV types of higher oncogenic risk, a significant higher prevalence was observed in Asia (23.1%) in relation to Europe (4.4%) or the Americas (15.6%). This is interesting because some studies suggest that the heterogeneity in the prevalence of HPV in lung cancer is mainly due to the geographic differences, the different histological types analysed and the different detection methods used [19], besides the sample size, demographic composition of each study and host-specific factors [14, 16]. There is a consensus among most of the studies that it is necessary to investigate more cases to understand the real role (if any) of HPV in pulmonary carcinogenesis [14, 16, 20, 37, 40].

Still in an attempt to explore the relationship of HPV with NSCLC, IHC was performed in NSCLC paraffin samples to evaluate the expression of p16, a protein considered an indirect marker for HPV infection, since in SCC and adenocarcinoma of uterine cervix and, in a fraction of oropharyngeal squamous cell carcinomas, the overexpression of p16 is strongly related to HPV infection [37]. However, the relationship between p16 expression and NSCLC is not well established [41] and our data were not able to demonstrate the presumed relationship. Chang et al. [37], in addition to the evaluating the presence of HPV DNA, also evaluated the expression of p16 on NSCLC by IHC, and as a result, no correlation between the HPV and p16 expression was determined. Other authors, such as van Boerdonk et al. [42], Bishop et al. [43] and Doxtader et al. [41], presented similar results, with 20 to 30% of NSCLC, both adenocarcinoma and squamous cell carcinoma, positive for p16 and negative for HPV DNA. In addition, these authors concluded

that IHQ for p16 cannot be considered a surrogate method for assessing the presence of HPV in lung cancer.

Conclusion

Our data shows lack of HPV infection in a series of NSCL cancers. The few p16-positive cases are therefore unrelated to HPV. Several reports evaluating the prevalence of HPV DNA are increasing and new evidences with the HPV infection and NSCL coexistence are emerging [40–43]. However, there is still a lack of robust evidences of the possible participation of HPV in NSCLC carcinogenesis [44].

Acknowledgements

The authors thank the Endoscopy Department of Barretos Cancer Hospital where bronchoscopy were performed.

Authors' contributions

VSM and ALF wrote the paper and discussed results; EMS, PRAP, MCP, VSM collected samples; CSN and ALF did the p16 reading and interpretation. EMS, PRAP and VSM isolated DNA; EMS, EMN, VSM and LS carried out HPV genotype detection; KJS and ALF designed the study; ALF supervised all experiments performed as principal investigator. LS and LLV discussed an interpreted HPV results and the final text of the manuscript. All participants contributed commentary on and corrected the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by Conselho Nacional de Desenvolvimento Científico e Tencnológico (CNPq) [Grants number 401775/2012–7 to ALF; 573799/2008–3 to LLV]; Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [Grants numbers 2014/23414–8 to EMS; 08/57889–1 to LLV].

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 16 January 2019 Accepted: 24 July 2019
Published online: 02 August 2019

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Publisher's Note

LETTER TO THE EDITOR

Detection of human papillomaviruses and human polyomaviruses in immunosuppressed and immunocompetent individuals with actinic cheilitis: a case series

Editor

Actinic cheilitis (AC) is considered a precancerous lesion of the lip.[1] Renal transplant recipients (RTR) have a higher susceptibility to human papillomavirus (HPV) infections and lip squamous cell carcinoma (SCC).[2] Over 200 HPV types were described and most cluster within the α-, β- and γ-HPV genera.[3] Merkel cell polyomavirus (MCPyV) and several HPVs are associated with skin affections including cancer.[2] We evaluated the association of MCPyV, HPVs and related biomarkers with AC.

We analysed samples from 30 patients clinically diagnosed with AC attended at Unifesp and Hospital São Paulo between 2013 and 2016. The study was approved by the Research Ethics Committee of Unifesp. Most lesions affected individuals from Fitzpatrick phototypes I and II (66.7%) and developed within the lower lip (93.4%) corroborating previous studies.[1,4] Additionally, most subjects were male (66.7%) and reported chronic sun exposition (70.0%). The only variable significantly different between RTRs and ICs (immunocompetents) was age: ICs (mean age 67 years) were older than RTRs (mean age 55 years) ($P = 0.016$). Although IC and RTRs exhibited a difference in previous history of non-melanoma skin cancer (NMSC) (54.4% vs. 84.2%, respectively), it did not reach statistical significance ($P = 0.104$) which may reflect our small sample size. Two RTRs also reported history of lip SCC.

α-HPVs are commonly detected in the oral cavity, and HPV-16 responds for a fraction of oral cavity SCCs.[5] However, data does not support a role for α-HPVs in lip SCC.[6] β- and γ-HPVs are ubiquitously distributed throughout the body, including the oral cavity.[2,5] We observed that the majority of AC samples (90%) were positive for DNA of at least one HPV type, and over 70% of both RTRs and ICs harboured > one type (Table 1). Although individual HPVs were more prevalent among RTRs, no significant difference between RTRs and ICs regarding specific HPVs was found. Furthermore, no correlation between HPV detection, immune status and previous history of NMSC was observed. It is plausible that the high prevalence of HPVs detected in AC samples results from its proximity to the oral cavity. Alternatively, HPVs could have been shed from other regions of the face, predominantly eyebrows hairs which have been reported to have a high prevalence of cutaneous HPVs.[2] MCPyV DNA was detected in 3 RTRs; one also harboured γ-HPV-197 and -14 different β-HPVs DNA, and another harboured HPV-16 and HPV-18 in addition to nine different β-HPVs. The latter patient recently developed lip SCC.

$p16^{INK4}$ is a surrogate biomarker of α-HPVs infection in cervical cancer.[3] However, increased expression of $p16^{INK4a}$ during the progression from actinic keratosis to skin SCC was reported and was not associated with the detection of HPV DNA.[7,8] High levels of p53 were also detected in studies focused on AC and lip SCC.[9] However, in contrast to cervical cancer, no association was found between p53 expression and HPV status in skin SCC.[3,8,10] We observed no correlation between p53 or $p16^{INK4a}$ staining, HPV detection and immunosuppression in AC (Table 2), even when analysis was restricted to HPV-16-positive specimens. Nevertheless, a significant higher p53 positivity within individuals previously diagnosed with NMSC was observed ($P = 0.049$), suggesting that p53 may be a putative marker of lip malignancies, reflecting inactivating p53 UVR-induced mutations that increase protein stability.

This is the first study to report high prevalence of HPVs and simultaneous detection of MCPyV in AC. Although no significant correlation between specific viral types, immune status and history of NMSC was found, the potential role of these agents in this pathology cannot be discarded. Further investigation including a larger, multicentric casuistic is warrantee.

Funding source: This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [Grant number 2008/57889-1 to INCT-HPV]; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [Grant number 573799/2008-3 to INCT-HPV]; and Coordenação de Aperfeiçoamento de Pessoal do Nível Superior (CAPES) [to JT].

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Table 1 The prevalence of α-HPV, β-HPV, γ-HPV and polyomavirus in renal transplant recipients (RTRs) and immunocompetent (IC) individuals diagnosed with actinic cheilitis

Virus status	RTR (*N* = 19) *n* (%)	IC (*N* = 11) *n* (%)	*P*-value*
Any-HPV†			
Negative	1 (5.3)	2 (18.2)	0.783
1 type	5 (26.3)	2 (18.2)	
2 types	6 (31.6)	4 (36.4)	
3 types	7 (36.8)	3 (27.3)	
α-HPV			
Any α-HPV	13 (68.4)	6 (54.5)	0.696
HPV-16	9 (47.4)	4 (36.4)	0.708
HPV-18	6 (31.6)	1 (9.1)	0.215
HPV-31	0 (0.0)	1 (9.1)	0.367
HPV-39	0 (0.0)	1 (9.1)	0.367
HPV-51	1 (5.3)	0 (0.0)	1.000
HPV-52	0 (0.0)	1 (9.1)	0.367
HPV-58	1 (5.3)	4 (36.4)	0.047
HPV-66	0 (0.0)	1 (9.1)	0.367
β-HPV			
Any β-HPV	12 (63.2)	6 (54.5)	0.712
HPV-5	5 (26.3)	2 (18.2)	1.000
HPV-8	1 (5.3)	1 (9.1)	1.000
HPV-9	2 (10.5)	0 (0.0)	0.520
HPV-12	1 (5.3)	1 (9.1)	1.000
HPV-17	4 (21.1)	0 (0.0)	0.268
HPV-19	1 (5.3)	0 (0.0)	1.000
HPV-23	4 (21.1)	0 (0.0)	0.268
HPV-24	1 (5.3)	1 (9.1)	1.000
HPV-36	1 (5.3)	0 (0.0)	1.000
HPV-38	5 (26.3)	4 (36.4)	0.687
HPV-49	1 (5.3)	3 (27.3)	0.126
HPV-76	2 (10.5)	0 (0.0)	0.520
HPV-80	1 (5.3)	0 (0.0)	1.000
HPV-98	1 (5.3)	0 (0.0)	1.000
HPV-100	2 (10.5)	0 (0.0)	0.520
HPV-105	1 (5.3)	0 (0.0)	1.000
HPV-107	1 (5.3)	0 (0.0)	1.000
HPV-110	1 (5.3)	0 (0.0)	1.000
HPV-111	1 (5.3)	0 (0.0)	1.000
HPV-113	1 (5.3)	0 (0.0)	1.000
HPV-124	0 (0.0)	1 (9.1)	0.367
HPV-145	1 (5.3)	0 (0.0)	1.000
HPV-151	1 (5.3)	0 (0.0)	1.000
HPV-159	2 (10.5)	0 (0.0)	0.520
HPV-174	1 (5.3)	1 (9.1)	1.000
γ-HPV			
Any γ-HPV	4 (21.1)	1 (9.1)	0.626
HPV-50	1 (5.3)	0 (0.0)	1.000
HPV-108	1 (5.3)	0 (0.0)	1.000
HPV-116	1 (5.3)	1 (9.1)	1.000
HPV-131	1 (5.3)	0 (0.0)	NA
HPV-132	2 (10.5)	0 (0.0)	0.520
HPV-162	1 (5.3)	0 (0.0)	1.000
HPV-167	1 (5.3)	0 (0.0)	1.000
HPV-169	0 (0.0)	1 (9.1)	0.367
HPV-179	1 (5.3)	0 (0.0)	1.000
HPV-197	1 (5.3)	0 (0.0)	1.000
HPV-200	1 (5.3)	0 (0.0)	1.000
HPV-201	1 (5.3)	0 (0.0)	1.000
HPV-202	1 (5.3)	0 (0.0)	1.000
Polyomavirus‡			
MCPyV	3 (15.8)	0 (0.0)	0.279
HPyV6	0 (0.0)	0 (0.0)	
HPyV7	0 (0.0)	0 (0.0)	

NA, Not available.
*Fisher's exact test using the SPSS v.25 for Windows.
†DNAs were obtained by organic extraction, and α-, β- and γ-HPVs DNA detection and typing was accessed using an *E7* type-specific PCR bead-based multiplex genotyping assay.
‡The presence of MCPyV, HPyV6 and HPyV7 infection was accessed by PCR.

Table 2 Immunohistochemical analysis of $p16^{INK4}$ and p53 levels in renal transplant recipients (RTRs) and immunocompetent (IC) individuals diagnosed with actinic cheilitis

	RTR (*N* = 19) *n* (%)	IC (*N* = 11) *n* (%)	*P*-value*
P53†			
Negative	6 (31.6)	7 (63.6)	0.132
Positive	13 (68.4)	4 (36.4)	
P16‡			
Negative	8 (42.1)	6 (54.5)	0.707
Positive	11 (57.9)	5 (45.5)	
HPV-16 positive	**(*N* = 9)**	**(*N* = 4)**	
P53			
Negative	7 (77.7)	3 (75.0)	1.000
Positive	2 (22.3)	1 (25.0)	
P16			
Negative	6 (66.7)	1 (25.0)	0.266
Positive	3 (33.4)	3 (75.0)	

*Fisher's exact test using the SPSS v.25 for Windows.
†Immunohistochemistry for p53 was achieved using an anti-p53 antibody (Clone DO, Dako, CA, USA) and the EnVision System (CAT# GV804; Dako Omnis). p53 expression was subjectively scored as positive when nuclear staining was moderate to strong in cells of the basal and suprabasal layers within several areas of the fragment analysed and negative when staining was low or absent, or solely within the basal layer.
‡Immunohistochemistry for $p16^{INK4a}$ was performed using the CINtec® $P16^{INK4a}$ Histology Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) in a Ventana Benchmark GX equipment (Ventana Medical Systems, Tucson, AZ, USA). Expression of $p16^{INK4a}$ was subjectively scored as positive when nuclear or nuclear/cytoplasmic staining was moderate to strong in several areas of the fragment analysed and negative when staining was low or absent.

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DOI: 10.1111/jdv.15779

CLINICS

REVIEW ARTICLE

Epidemiology and biology of cutaneous human papillomavirus

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Nunes EM, Talpe-Nunes V, Sichero L. Epidemiology and biology of cutaneous human papillomavirus. Clinics. 2018;73(suppl 1):e489s

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Cutaneous human papillomaviruses (HPVs) include β- and γ-HPVs, in addition to a small fraction of α-HPVs. β-HPVs were first isolated from patients with the rare genetic disorder *Epidermodysplasia verruciformis*, and they are associated with the development of nonmelanoma skin cancer at sun-exposed skin sites in these individuals. Organ transplant recipients also have greater susceptibility to β-HPV infection of the skin and an increased risk of developing nonmelanoma skin cancer. In both immunosuppressed and immunocompromised individuals, cutaneous HPVs are ubiquitously disseminated throughout healthy skin and may be an intrinsic part of the commensal flora. Functional analysis of E6 and E7 proteins of specific cutaneous HPVs has provided a mechanistic comprehension of how these viruses may induce carcinogenesis. Nevertheless, additional research is crucial to better understand the pathological implications of the broad distribution of these HPVs.

KEYWORDS: Human Papillomavirus; Cutaneous; Prevalence; Nonmelanoma Skin Cancer.

INTRODUCTION

Human papillomavirus (HPV) represents a diverse group of viruses infecting mainly epithelial and mucosal tissues (1). Based on the identity of the *L1* major capsid gene sequence, the majority of the over 200 viral types characterized to date cluster within the alpha (α)-, beta (β)-, or gamma (γ)-HPV genus (2, 3). While the great majority of α-HPVs are mucosal types isolated from the anogenital epithelia, some viral types in this genus (e.g., HPVs 2, 3 and 10) and β- and γ-HPVs were originally designated cutaneous types (Figure 1). To date, 54 β-HPVs (subdivided into 5 species, β1-5) and 98 γ-HPVs (subdivided into 27 species, γ1-27) have been fully sequenced and characterized (http://www.nordicehealth.se/hpv center/reference_clones/), and it is expected that these numbers will further increase once partial sequences of putative novel types are described (4). Some cutaneous HPVs are clearly associated with the development of various skin lesions, from warts to carcinomas, in restricted populations (1,5). Nevertheless, it has proven difficult to determine the role of particular β-HPVs in cutaneous malignancies because of the high viral diversity and ubiquity of multiple types throughout healthy skin, the oral cavity, the nasal mucosa and the anogenital region (6-11).

No potential conflict of interest was reported.

Received for publication on December 1, 2017. **Accepted for publication on** February 6, 2018

Commemorative Edition: 10 years of ICESP

DOI: 10.6061/clinics/2018/e489s

Prevalence and distribution of cutaneous HPVs among immunosuppressed individuals

The first record concerning the association of HPV with papillomatous skin lesions that harbored carcinogenic potential dates to the early 1920s, when Lewandowsky and Lutz (12) first described a hereditary condition named *Epidermodysplasia verruciformis* (EV) that is characterized by extensive warts throughout the body (Table 1). Later, Jablonska et al. (13) observed that EV individuals infected with β-HPV 5 and 8 had a higher risk of developing nonmelanoma skin cancer (NMSC), particularly in ultraviolet (UV)-exposed sites. Together, both viral types are detected in approximately 90% of skin squamous cell carcinomas (SCCs) in EV patients. In these cases, β-HPVs are actively transcribed and generally persistent at high copy number (14). Currently, β-HPV 5 and 8 are accepted as possible etiological agents (carcinogen group 2B) of cutaneous SCC (cSCC) in immunosuppressed EV individuals by the International Agency for Research on Cancer (IARC) (15).

In the following years, several studies focused on analyzing the oncogenic potential of cutaneous HPVs in other immunosuppressed individuals, among which organ transplant recipients (OTRs) were the most extensively investigated. OTRs slightly resemble EV patients in that they are often covered with wart-like skin lesions and actinic keratosis (AK). Furthermore, OTRs have up to a 100-fold increased risk of developing NMSC compared to the general population (16-17). Importantly, the increased risk of NMSC is mostly associated with a higher incidence of cSCC (18-20). Clinical and histological features of these lesions suggest that cSCCs occasionally develop from viral warts or other precursor lesions (21,22). NMSC in OTRs often presents as multiple lesions and is usually confined to UV-exposed anatomical sites, most likely associated with local immunosuppression (23,24). These tumors are also more aggressive

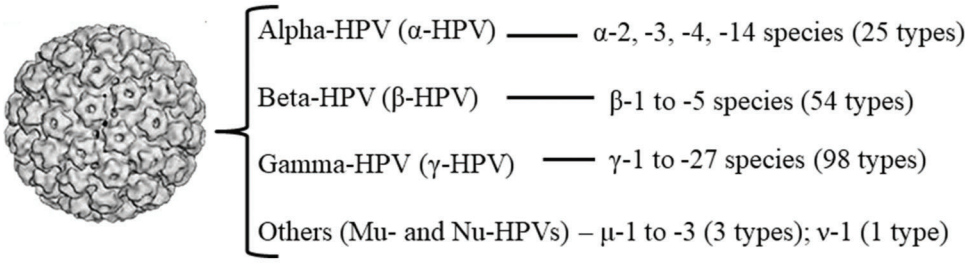

Figure 1 - Distribution of cutaneous viral types within different HPV genera. The number of cutaneous viral types within each genus is indicated.

Table 1 - Select studies on the prevalence and distribution of cutaneous HPVs among immunosuppressed individuals.

Year	Author (s) (reference number)	Data
1922	Lewandowsky and Lutz (12)	First description of epidermodysplasia verruciformis (EV).
1972	Jablonska et al. (13)	β-HPVs 5 and 8 infected EV individuals had a higher risk of developing NMSC (after UV exposition).
1974	Koranda et al. (26)	Cutaneous warts were detected in 43% of ORT individuals after 3 months to 9 years following transplant.
1976 1977 1980	Mullen et al. (20) Hoxtell et al. (19) Hardie et al. (18)	Increased risk of NMSC is mostly associated to higher incidence of cSCC.
1978 1989	Sbano et al. (22) Barr et al. (21)	cSCC occasionally develop from viral warts or other precursor lesions.
1980	Hardie et al. (18)	The incidence of skin cancer increases 5% per year after the first year of transplant, with a cumulative risk of 44% after 9 years.
1980	Hardie et al. (18)	Tumors are more aggressive in OTRs than in the general population.
1984	Boyle et al. (27)	18% of renal transplant patients who were highly exposed to UV developed carcinogenic lesion in the skin.
1995 2000	Birkeland et al. (16) Lindelöf et al. (17)	OTRs have until 100 fold increased risk of developing NMSC as compared to the general population.
1997 2000 2003	Boxman et al. (35) Harwood et al. (36) Meyer et al. (37)	β-HPV is more prevalent in skin warts biopsies than in both the normal skin and plucked hairs among OTRs.
2000	Antonsson et al. (6)	Among OTRs, dialysis patients, and healthy controls, solely the first group reported ever having skin cancer (11.5%).
2000	Lindelöf et al. (17)	Within 15 years of transplantation, up to 90% of OTRs develop warts and/or cSCC.
2000 2007	Berkhout et al. (29) Hazard et al. (30)	Infections of cutaneous HPVs frequently persist in OTRs.
2003	Feltkamp et al. (40)	There is a positive epidemiological association between β-HPV seroreactivity and cSCC development.
2004 2004 2007	Termorshuizen et al. (24) Harwood et al. (34) Forslund et al. (23)	NMSCs among OTRs are often multiple and usually confined to UV-exposed anatomical sites.
2005 2008 2011 2016	Weissenborn et al. (46) Rollison et al. (45) Arron et al. (43) Chahoud et al. (44)	Data regarding the association between cutaneous HPV infection and cSCC is still inconclusive.
2007	Nindl et al. (28)	OTRs have higher cutaneous HPV prevalence rate up to 90% in cSCC compared to the normal skin (11-32%).
2007 2014	Hazard et al. (30) Hampras et al. (31)	Older ages and history of sunburn are associated to an elevated risk of β-HPV persistent infection.
2008 2010	Michael et al. (39) Antonsson et al. (38)	Seroconversion to β-HPV increases with age.
2009	Bouvard et al. (15)	β-HPVs 5 and 8 are accepted as possible etiological agents (carcinogens group 2B) of cSCC in immunosuppressed EV individuals.
2011	Proby et al. (33)	Individuals with concordant β-HPV DNA in plucked eyebrow hairs and serologic tests had a significantly increased risk of developing SCC.
2013	Neale et al. (32)	There is a significant association between the number of β-HPVs detected at eyebrow hair follicles and the increased risk of cSCC among OTRs.

in OTRs than in the general population and form metastases more readily (18,25).

Among OTRs, cutaneous warts were detected in 43% of individuals at 3 months to 9 years following transplant (25,26). Additionally, within 15 years of transplantation, up to 90% of OTRs develop warts and/or cSCC (17). Upon analyzing skin smears from OTRs, dialysis patients and healthy controls, Antonsson et al. (6) observed that 11.5% of OTRs reported ever having skin cancer, whereas no cases of NMSC were observed in the other groups studied. NMSC incidence in OTRs varies depending on the duration of immunosuppression; Hardie et al. (18) demonstrated that the incidence of skin cancer increased 5% per year after the first year of transplant, with a cumulative risk of 44% after 9 years.

The incidence of NMSC is also related to long sun exposure. Boyle et al. (27) observed that 18% of renal transplant patients with high exposure levels to sunshine (>3 months in a tropical or subtropical climate or >5 years in an outdoor occupation) developed carcinogenic alterations in their skin: two patients were diagnosed with cSCC, and seven were diagnosed with AK, whereas neither lesion type was noted in the other patients or in the control group.

Although cumulative sun exposure is the major risk factor for NMSC, recent studies have revealed a role for HPV as a cofactor in association with UV radiation in cSCC in OTRs. OTRs have a higher cutaneous HPV prevalence rate in cSCC (up to 90%) than in normal skin (11-32%) (28). These infections frequently persist (29,30), and it has been observed that older age and a history of sunburn are associated with an elevated risk of persistent β-HPV infection (30,31). Furthermore, a significant association between the number of β-HPVs detected in eyebrow hair follicles and an increased risk of cSCC was reported among OTRs from Europe (the Netherlands, the United Kingdom, France and Italy) (32). Moreover, individuals with concordant β-HPV DNA in plucked eyebrow hairs and serologic tests had a significantly increased risk of developing SCC (33).

Among immunosuppressed OTRs, the occurrence of multiple cutaneous HPV infections is common (34), but high viral loads were shown to be associated with an increased risk of SCC development, with total load seemingly more important than the individual load of any specific type (32). It should be noted, however, that β-HPV is more highly prevalent in skin wart biopsies from OTRs than in either normal skin or plucked hairs from these patients (29,35-37). Serological studies have also demonstrated that seroconversion to β-HPV increases with age (38,39) and have revealed a positive epidemiological association between β-HPV seroreactivity and cSCC development (40), even though not every infection is accompanied by a detectable or relevant seroresponse.

Nevertheless, given the high incidence of cSCCs in OTRs, identifying a clear link between β-HPV infection and cSCC would have important implications for therapy and prevention (41,42). Therefore, more recent case-control studies are ongoing, focusing on the association of cutaneous HPV in the early stages of NMSC carcinogenesis in immunosuppressed individuals. However, the data are inconclusive (42-46).

Prevalence and distribution of cutaneous HPVs among immunocompetent individuals

Cutaneous β- and γ-HPV DNA can be detected beginning in early infancy and may be detected in 70% of children by 4 years of age (47). Additionally, β-HPV types detected on parents are more commonly found on their babies (47,48). Viral transmission seems to occur inevitably through direct skin contact (10,49-51), and these viruses have been suggested to be commensal to humans (38,52,53) (Table 2).

Table 2 - Select studies on the prevalence and distribution of cutaneous HPVs among immunocompetent individuals.

Year	Author (s) (reference number)	Data
1997	Boxman et al. (35)	Cutaneous HPVs detection in eyebrow hairs seems to reflect infections in other parts of the body (useful in epidemiological studies).
2000	Antonsson et al. (6)	Sun exposure and history of skin cancer are risk factors associated to β-HPVs detection in IC individuals.
2000	Harwood et al. (36)	Cutaneous HPVs prevalence was higher among individuals who reported ever having skin lesions.
2000 2003 2007 2009 2009	Antonsson et al. (6) Struijk et al. (56) Hazard et al. (30) Weissenborn et al. (48) de Koning et al. (55)	β-HPV DNA detection and seroprevalence increases with age.
2003	Antonsson et al. (47)	The presence of cutaneous β- and γ-HPVs DNA is observed since early infancy.
2003 2009	Antonsson et al. (47) Weissenborn et al. (48)	β-HPVs types detected on parents are also more commonly found in their babies.
2003 2010 2014	Antonsson et al. (52) Antonsson et al. (38) Bzhalava et al. (53)	β- and γ-HPVs may be commensal to humans.
2004	Termorshuizen et al. (24)	Severe sunburns are associated with the presence of β-HPV DNA.
2004	Stockfleth et al. (60)	HPVs 5 and 8 were the most frequently found in premalignant lesions, SCC and BCC.
2004 2006 2011 2017 2017 2017	Smith et al. (75) Fakhry et al. (73) Termine et al. (77) Hampras et al. (74) Steinau et al. (76) Nunes et al. (10)	Simultaneous oral-genital type-specific β-HPV infections are relatively rare.

Table 2 - Continued.

Year	Author (s) (reference number)	Data
2005 2011	Weissenborn et al. (46) Arron et al. (43)	Higher viral loads are detected within pre-malignant skin lesions as compared to SCC.
2007	Forslund et al. (23)	Most viruses detected on the external skin surface may reflect HPV deposition.
2007 2009	Köhler et al. (54) de Koning et al. (55)	The prevalence of β-HPVs DNA in plucked hairs from different body sites of IC individuals is approximatelly 90%.
2008	Patel et al. (59)	β-HPVs may play a role in the pathogeny of NMSC also in healthy individuals.
2008 2017 2017	Feltkamp et al. (49) Moscicki et al. (51) Nunes et al. (10)	Viral transmission seems to occur through direct skin contact.
2009	Bouvard et al. (15)	Epidemiological evidence concerning the association between specific β- and γ-HPVs and the development of skin cancer in IC is inconclusive.
2009	Weissenborn et al. (48)	UV radiation may be a putative viral detection-related risk factor.
2010 2010	Antonsson et al. (38) Iannacone et al. (62)	High overall seropositivity (>90%) to at least one viral β-HPV is observed within healthy individuals.
2010 2011	Michael et al. (63) Proby et al. (33)	Low overall seropositivity to at least one viral β-HPV is observed within healthy individuals.
2011	Proby et al. (33)	β-HPV types most commonly detected in the skin also have the highest seroprevalence.
2011 2013 2013 2013 2013 2014 2014 2015 2015 2016 2017	Bottalico et al. (7) Forslund et al. (8) Pierce Campbell et al. (68) Sichero et al. (65) Paolini et al. (70) Hampras et al. (31) Sichero et al. (66) Sichero et al. (69) Donà et al. (67) Nunes et al. (9) Moscicki et al. (51)	Analysis of β- and γ-HPVs DNA and antibodies prevalence among series of samples (anogenital, oral, skin, nasal cavity from women and men).
2013	Neale et al. (32)	Cutaneous HPVs target the hair follicle bulge, which is probably the reservoir of these viruses.
2013	Sichero et al. (65)	The majority of male genital samples could not be classified using technologies widely used for typing of α-HPVs.
2013 2016	Pierce Campbell et al. (68) Rahman et al. (71)	Male external genital lesions (EGL) are not associated to β-HPVs infections.
2013 2014	Sichero et al. (65) Sichero et al. (66)	Most samples from the male genitals were positive for multiples cutaneous HPV DNA.
2013 2014 2015	Sichero et al. (65) Sichero et al. (66) Sichero et al. (69)	The detection of β-HPVs in one anatomic site may also represent deposition of virions shed from other anatomic sites.
2013 2014 2015 2015 2016	Sichero et al. (65) Sichero et al. (66) Donà et al. (67) Torres et al. (72) Nunes et al. (9)	The detection of cutaneous HPVs DNA is not associated to sexual risk factors. Other routes of transmission such as autoinoculation and non-penetrative sexual activities could be associated.
2016	Chahoud et al. (44)	Analyses of association between the detection of antibodies to β-HPVs with SCC risk development.
2017	Hampras et al. (74)	The occurrence of concordant β-HPV infections seem to be higher across keratinized tissues than across mucosal sites.
2017	Moscicki et al. (51)	The transmission rate of β-HPVs between anogenital sites from men-to-women and women-to-men was similar, suggesting these are sexually transmitted.

β-HPVs are widespread in immunocompetent (IC) individuals within the general population: when plucked hairs from different body sites are tested, the prevalence is approximately 90% (54,55). It is believed that cutaneous HPVs target the hair follicle bulge, which is probably the reservoir of these viruses (32). Therefore, eyebrow hairs have served as an easily obtained material for marker analysis in several epidemiological studies and seem to reflect infection in other parts of the body (35).

Advanced age has been shown to be the most important factor influencing the presence of β-HPV DNA in IC individuals (56,57). Furthermore, sun exposure and a history of skin cancer are risk factors associated with β-HPV detection in these individuals (6). For OTRs, some studies have investigated the prevalence of cutaneous HPVs among IC individuals of different ethnicities and residing under different climate conditions (52). It was reported that the prevalence of HPV DNA was lower in samples from Zambia than in those

collected in Sweden ($p<0.01$) and Bangladesh ($p<0.05$) (52). β-HPV prevalence and distribution studies have shown that viral positivity was, on average, higher on the forehead (36%) and back of the hand (38%) than on the buttocks (26%), indicating that UV radiation may be a putative risk factor for viral infection, even though sun exposure data were not collected in this specific study (48). In fact, severe sunburns have been associated with the presence of β-HPV DNA (24). Second-degree burns and repetitive sunburns, with skin regeneration of the underlying capillary bulb, may result in the amplification of β-HPV DNA by activating the HPV life cycle (24). In fact, the risk for SCC development among Australian or Netherlander IC individuals is higher for those in which β-HPV DNA was detected at high loads in plucked eyebrow hairs (32,56,58).

β-HPV persistence was more commonly observed in adults (92%) than in children (66%), and although multiple β-HPVs can infect persistently, no specific type seems to predominate in such infections (48). Nevertheless, it must be highlighted that the prevalence of β-HPVs significantly decreases after tape stripping, indicating that only a small number of epithelial cells are in fact infected, and most of the detected viruses may reflect deposition throughout the external skin surface (23).

The IARC recognizes the need for further research on cutaneous HPVs to better understand the widespread distribution of these viruses. β-HPVs may also play a role in the pathogeny of NMSC in healthy individuals (59); however, to date, epidemiological evidence is inconclusive concerning the association between specific β- and γ-HPVs and the development of skin cancer in IC individuals (14,15).

Regarding the HPV status in NMSCs from immunosuppressed and IC individuals, viral prevalence was higher in the former group for all lesion types analyzed: premalignant lesions (88% among immunosuppressed *vs* 54% among IC), SCC (84% *vs* 27%) and basal cell carcinoma (BCC, 75% *vs* 36%) (36). Nevertheless, the prevalence and spectrum of HPV types detected within the two populations were equivalent among premalignant lesions, SCC and BCC, and HPVs 5 and 8 were the most frequently identified types (60). Interestingly, β-HPV prevalence was reported to be higher in premalignant AK than in cSCC, and real-time PCR analysis indicated higher viral loads in premalignant lesions (46) than in SCC, in which viral load rarely reaches the level of one viral copy per cell (43,46). This scenario is compatible with a carcinogenic role for HPV at the early stages of skin carcinogenesis. Because cSCC most commonly develops in sun-exposed anatomical sites, it is reasonable to suppose that UV radiation may impede HPV antigen presentation by suppressing local cell immunity (61). In addition, several studies have suggested the importance of β-HPVs as cofactors to UV radiation in the development of SCC by facilitating the accumulation of UV-induced mutations, which can ultimately lead to cell transformation.

In addition to HPV DNA detection, the detection of antibodies to β-HPVs and their association with SCC risk development have been evaluated in several studies (44). Nevertheless, overall, serological studies show considerable heterogeneity in the results: whereas high overall seropositivity (>90%) to at least one viral β-HPV has been described (38,62), a lower prevalence is observed in other studies (33,63). The divergence in the data obtained in these studies could be attributed not only to differences in serological methods but also to the range of cutaneous HPVs tested. With β-HPV DNA detection, the seroprevalence has been shown to increase with age (6,30,55). Notably, β-HPV types most commonly detected in the skin have the highest seroprevalence worldwide (33).

Due to the wide distribution of cutaneous HPVs in the skin, several groups, including ours, have recently focused on investigating the distribution of cutaneous HPVs in other anatomical sites, including the anogenital area. Within the HIM (HPV Infection in Men) cohort study (64), we initially observed that most of the ~15% of male genital samples that could not be classified with widely used α-HPV typing technologies harbored β- and γ-HPVs, as evidenced by using a PCR sequencing protocol (65). We further observed that most samples were positive for HPV DNA of multiple cutaneous types using a sensitive Luminex-based methodology, suggesting that the former protocol could underestimate the true prevalence of cutaneous β- and γ-HPVs in the male genital region (65,66). In order to better understand the prevalence and distribution of cutaneous HPVs, we and others further analyzed β- and γ-HPV DNA and antibodies by Luminex methodology in a series of samples obtained from the anogenital region of both men and women (9,51,65-69), the oral cavity (7,9,70), the skin (31), and the nasal cavity (8). Taken together, these studies corroborate that cutaneous HPVs are ubiquitously disseminated throughout healthy skin and may be an intrinsic part of the commensal flora.

We further observed that male external genital lesions (EGLs) are not associated with β-HPV infections (68,71) and that the detection of DNA from these viruses is not associated with sexual risk factors, indicating other routes of transmission, such as autoinoculation and nonpenetrative sexual activities (9,65-67,72). Alternatively, the detection of β-HPVs at one anatomic site may indicate the deposition of virions shed from other anatomic sites (50,65,66,69). Nevertheless, it was recently reported that among heterosexual couples, the transmission rate of β-HPVs between anogenital sites was 15.9 per 100 person-months from men-to-women, with a similar risk for women-to-men transmission, suggesting that β-HPVs can be sexually transmitted (51). Lastly, the few reports in which the oral and anogenital regions were analyzed concurrently indicated that simultaneous oral-genital type-specific β-HPV infections are relatively rare (10,73-77) but seem to be higher across keratinized tissues than across mucosal sites (74). Nevertheless, given the large number of samples and β-HPV types analyzed, it is unlikely that the same HPV type will be found at a distant skin site by chance.

Functional analysis of cutaneous HPVs

HPVs are small, nonenveloped viruses with circular double-stranded DNA of approximately 8000 bp. The viral genome is divided into three regions: the long control region (LCR) contains cis-responsive elements for viral and cellular proteins that regulate viral gene expression and replication; the early region (E), which encodes proteins crucial to viral transcription and replication; and the late region (L), which encodes the viral capsid structural proteins (78-80). Although the viral genome structure and organization are highly conserved among HPVs, the LCR of β-HPVs is shorter than that of α-HPVs, and the E5 gene is absent from the β-HPV genome (81-83).

The HPV life cycle is tightly associated with the differentiation of the stratified squamous epithelium. HPV infection begins with entrance of the virus into the basal layer of the epithelia due to microtrauma (79,84,85). At least for high-risk α-HPV-infected tissues, the differentiation process is altered

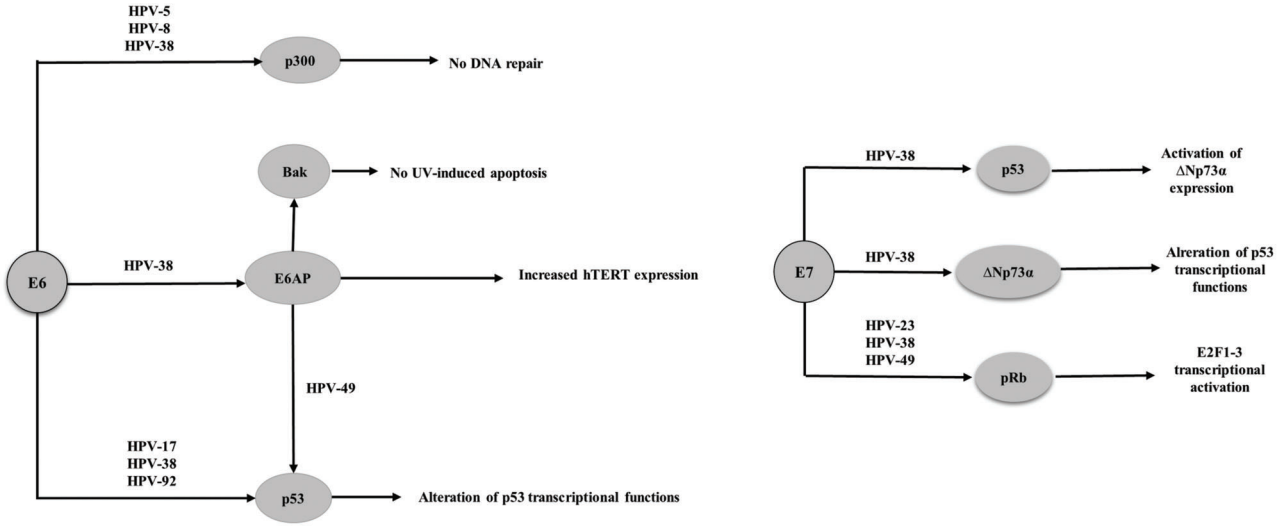

Figure 2 - Cellular targets of the E6 and E7 proteins from specific β-HPVs.

Table 3 - Highlights regarding the epidemiology and biology of cutaneous human papillomavirus in immunosuppressed and immunocompetent individuals.

- β- and γ-HPVs, in addition to few α-HPVs are originally designated "cutaneous types".
- The IARC classified β-HPVs 5 and 8 as possible etiological agents of skin SCC in EV individuals.
- OTRs not only have a higher susceptibility to β-HPVs but further attain an ~100 fold increased risk of developing NMSC as compared to the general population.
- Among OTRs and IC individuals cutaneous HPVs are ubiquitously spread throughout the body and may be an intrinsic part of the commensal flora.
- Although cumulative sun exposure is the major risk factor for NMSC, studies points towards β-HPV infections as co-factors in skin SCC in association with UV radiation.
- β-HPVs most probably play a role in the initiation of skin SCC rather than in the maintenance of the transformed phenotype.
- Function analysis of E6 and E7 proteins of specific cutaneous HPVs indicate that the biology involved in β-HPV mediated skin carcinogenesis differ from that induced by high-risk α-HPV types.

by expression of the E6 and E7 viral oncoproteins, which interact principally with the TP53 and pRb suppressor proteins, respectively, but also interact with a broad spectrum of other cellular proteins, altering the biological properties of the host cell (79,83,86-89).

As previously discussed in this review, β-HPVs most likely play a role in the initiation of cSCC rather than in the maintenance of the transformed phenotype (14). It is hypothesized that β-HPV infections destabilize the host genome, allowing tumors to further develop in the absence of the viral genome (90-92). Some studies have provided a mechanistic comprehension of how these viruses induce carcinogenesis and have indicated that the biology involved in β-HPV-mediated skin carcinogenesis differs from that induced by high-risk α-HPVs (80,91,93,94).

Studies have shown that β-HPVs 38 and 49 are able to immortalize primary human keratinocytes, whereas HPVs 10, 14, 22, 23, 24 and 36 do not have this ability (95-97). It has also been reported that transgenic mice expressing the HPV 38 E6 and E7 proteins under control of a keratinocyte-specific promoter exhibit epidermal hyperplasia and are susceptible to the development of cutaneous tumors promoted by chemical carcinogens and UV radiation (98-101). Although the E6 protein from HPVs 8, 24 and 38 binds *in vitro* to E6-AP (E6-associated protein), p53 degradation was observed in the presence of only HPV 49 (95-97,102) (Figure 2). The E6 protein from β-HPVs 5, 8, and 38 attenuates p53 phosphorylation and ubiquitination in response to UV exposure, resulting in less efficient repair of damaged cellular DNA (90,96). Additionally, HPV 38 induces telomerase by a mechanism dependent on E6-AP (95). HPV 38 E6 also alters the capacity of p53 to activate proteins involved in apoptosis and suppress proliferation by inducing the accumulation of ΔNp73, a p53 isoform that antagonizes p53 (96). The E6 protein from HPVs 5, 8 and 38 was shown to bind p300, preventing p53 acetylation and p53 -induced repair and transcriptional transactivation, thus contributing to the accumulation of mutations and chromosomal abnormalities (90). Furthermore, as with α-HPV, the E6 protein of some β-HPVs induces BAK degradation, thus preventing the release of pro-apoptotic mitochondrial factors (103). The interaction of E6 with E6-AP is required not only for BAK degradation but also for hTERT (human telomerase reverse transcriptase) induction (104,105).

The E7 proteins from the cutaneous HPVs 154, 22, 23, 24, 36, 38 and 49 bind *in vitro* to pRb but are unable to induce pRb degradation when expressed in human keratinocytes (97,106-108) (Figure 2). Nevertheless, in human keratinocytes transduced with HPV 38 and 39 E6 and E7 proteins, E2F-induced transcription is likely activated because these viral proteins induce pRb hyperphosphorylation (80,95,97).

Although most research on oncogenic potential and disease association has focused on α-HPVs, there is interest in identifying a role of non-α HPV types in the pathogenesis of benign and malignant lesions (Table 3). Challenges in finding relevant associations between cutaneous HPV infection and NMSC

Table 4 - Challenges/critical open questions regarding the epidemiology and biology of cutaneous human papillomavirus in immunosuppressed and immunocompetent individuals.

• Are cutaneous HPVs associated to the development of non-melanoma skin cancer among immunosuppressed and immunocompetent individuals?
• Why are cutaneous HPVs more diverse than their mucosal counterparts?
• Do cutaneous HPVs contribute to carcinogenesis associated to other carcinogens at skin and non-skin sites?
• Do cutaneous HPVs contribute to high-risk mucosal carcinogenesis in cases of co-infections?
• Why are cutaneous HPVs more prevalent in precursor lesions compared to malignant lesions?
• Why do specific β-HPVs deregulate fundamental cellular events intimately linked to transformation *in vitro*, but do not cause cancer in humans?

development include the multiplicity and ubiquity of these viruses throughout the human body, the high probability of viral transmission (including autoinoculation), and differences observed in the carcinogenic potential of individual β-HPVs (Table 4). The last IARC monograph (100B) was unable to identify consistent epidemiological evidence for an etiological role attributable to any specific cutaneous HPV type or species in NMSC development. In addition, biological mechanisms explaining the oncogenicity of these viruses have not been fully elucidated.

AUTHORS' CONTRIBUTIONS

Nunes EM, Talpe-Nunes V and Sichero L critically discussed and wrote the manuscript.

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Histol Histopathol (2018) 33: 357-363

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Prevalence of high risk HPV DNA in esophagus is high in Brazil but not related to esophageal squamous cell carcinoma

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Summary. Background. The first publication that associated Human Papillomavirus (HPV) infection and esophageal cancer was published in 1982. However, data are still contradictory and require further investigation. The aim of this study was to identify high risk HPV DNA in esophageal tissue of patients with and without esophageal squamous cell carcinoma (ESCC) and correlate HPV presence with classical risk factors. Methods. Invited patients signed the informed consent form, and interviews were conducted in order to obtain information about sociodemographic and lifestyle behavior. During endoscopy, esophageal biopsies were collected from case and controls. Multiplex polymerase chain reaction genotyping was conducted on endoscopic biopsies to identify HPV types and HPV-16 was further evaluated by specific PCR real time. Results. Among 87 cases, 12 (13.8%) had tumors harboring high risk HPV DNA and among 87 controls, 12 (13.8%) had high risk HPV DNA (OR:1.025 [CI:0.405:2.592]). Variables regarding consumption of alcohol and use of tobacco continued to characterize risk factors even after adjustments by presence or absence of high risk HPV. Conclusion. HPV was demonstrated to be frequently and similarly associated to normal and malignant esophageal tissues, but not as an independent risk factor to esophageal cancer. Impact. To contribute to the Brazilian population data on this subject, which is still contradictory.

Key words: Human papillomavirus, Esophageal neoplasms, Risk factors, Cancer Epidemiology, Esophageal cancer

Introduction

Currently, esophageal cancer (EC) is an important public health problem worldwide. According to estimates by GLOBOCAN 2012, EC is the eighth most common cancer worldwide and the sixth most common cause of cancer death (Ferlay et al., 2013).

Risk factors related to, and those suspected of being involved with EC, have been widely studied; however,

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DOI: 10.14670/HH-11-929

Abbreviations. AME, Medical Specialties Ambulatory; BCH, Barretos Cancer Hospital; CI, Confidence Interval; EC, Esophageal cancer; ESCC, Esophageal squamous cell carcinoma; HPV, Human papillomavirus; OR, Odds Ratio; PCR, Polymerase chain reaction

little has been achieved since the classic studies of Wynder and Bross (1961) and Silber (1985), suggesting an association of some chemicals (nitrosamines, mycotoxins, cigarette smoke, excessive intake of alcohol and opium abuse), physical (solid and hot food) and nutritional deficiencies (particularly vitamins A, B, C and certain trace elements such as molybdenum and zinc) with the development of this malignant neoplasm (Wynder and Bross, 1961; Silber, 1985).

Recently, evidence suggests that human papillomavirus (HPV) may play an etiological role in esophageal carcinogenesis, in at least one subtype of esophageal carcinoma, the squamous cell carcinoma; the number of studies in this area have increased steadily, as evidenced in several reviews (Syrjänen, 1987, 2000a,b, 2002, 2003; Syrjänen et al., 1996). The first descriptions of oral lesions associated with HPV were preceded by reports that suggested the involvement of viruses in the development of benign (Syrjänen et al., 1982) and malignant (Syrjänen, 1982) lesions of the squamous epithelium of the esophagus. These initial observations were based on the discovery of morphological similarities between HPV lesions in the genital tract (warts) and esophageal lesions (papillomas) (Syrjänen, 1982; Syrjänen et al., 1982).

Although the first report on the presence of HPV in esophageal squamous cell carcinoma (ESCC) occurred more than 30 years ago (Syrjänen et al., 1982), its real prevalence is still poorly known and its role in carcinogenesis is questionable. Although the number of studies and interest in the subject have increased in recent years, the literature is scarce and controversial (Kamangar et al., 2009). Data accumulated reflects a trend linking HPV and EC in high risk areas, whereas in low risk areas such association was not evident (Antunes et al., 2013).

Knowing that esophageal carcinogenesis is complex, dependent on different risk factors and considering the controversial results described in reports that correlate high risk HPV infection with the development of ESCC, we aimed, in this study, to characterize individuals without cancer and patients with ESCC in relation to the presence of high risk HPV DNA and well known risk factors for both EC as well as for HPV infection.

Materials and methods

This is an observational case-control study. Cases were defined as patients of both genders, aged above 18 years, who were admitted to the Barretos Cancer Hospital (BCH), with histopathological confirmation of ESCC, clinical indication for endoscopy and no previous treatment for cancer. Controls were defined as individuals without cancer of the esophagus, having had clinical indication for endoscopy for benign disease of the digestive system, examined in the Endoscopy Department from Medical Specialties Ambulatory (AME, acronym in Portuguese) in Barretos, São Paulo, Brazil. The sample size of that prospective and controlled study was based on earlier published work (Guo et al., 2012). Patients with limited understanding of research objectives during the consent, submitted to adjuvant and/or neoadjuvant therapy previous to sample collection, mental disability, unfavorable clinical conditions to undergo medical procedures, or insufficient amount of sample and/or inadequate quality were excluded.

Endoscopic examination and sample collection

The procedure for conducting the Digestive Endoscopy followed the routine of the Department of Endoscopy at BCH and Ambulatory Medical Specialties in Barretos, using sedation and flexible video endoscopes (Olympus 180, Japan; Fuginon 4400, Japan). The collection of biological samples was prospectively performed from esophageal biopsies. Samples were fixed in formalin and embedded in paraffin. The biological material was processed by the Department of Pathology, at BCH for histopathological diagnosis.

HPV detection and characterization

Specimens were digested with proteinase K-SDS 1% and HPV DNA was obtained by organic extraction (Green and Sambrook, 2012). HPV DNA was measured in all samples using type-specific PCR bead-based multiplex genotyping (TS-MPG) assays that combine multiplex polymerase chain reaction (PCR) and bead based Luminex technology (Luminex Corp., Austin, TX, USA), as described elsewhere (Nunes et al., 2016). This methodology is able to identify 21 HPV types including 13 of high-risk and 3 of low risk oncogenic, accordingly (Muñoz et al., 2006; Gheit et al., 2007).

As a positive control for the quality of the template DNA, primers targeting the β-globin gene were included. The assays were performed with 10μL DNA template in a 96-well format in 25-µl/well reaction volume. HPV multiplex PCR was performed with QIAGEN Multiplex PCR Kit (Qiagen, Dusseldorf, Germany), according to manufacturer's instructions. Each reaction consisted of 45 cycles: 94°C for 30 seconds, 63°C for 3 minutes, and 72°C for 90 seconds. The first cycle was preceded by incubation at 95°C for 15 minutes and the last one was extended for 10 minutes at 72°C. Negative controls consisted of reaction mixture without DNA.

The hybridization assay was performed according to Schmitt et al. (2006). For each HPV type-specific probe, mean fluorescence intensity (MFI) values obtained when no PCR product was added to the mixture of hybridization, was considered as background. The cutoff was calculated by adding 5 MFI for 1.1 X the value of median found. We considered positive values higher than 20 MFI.

Statistical analyses

Covariates associated with HPV detection and ESCC were analyzed and the results were expressed as odds ratio (OR) by simple logistic regression. Confirmatory

model were analyzed in multiple logistic regression between alcohol and tobacco interaction, where time and consumption were considered, using the HPV detection as variable of interest. The respective intervals with 95% confidence (95% CI) were also presented. The significance level was 0.05 (5%). For tabulation and statistical analysis we used IBM® SPSS® Statistics 20.0.1 software for Windows (IBM Corporation, Route 100, Somers NY 10589).

Results

During the period between February 2013 and August 2014, 174 individuals were enrolled, of which 87 (50.0%) were patients with ESCC (cases) and 87 (50.0%) was individuals without cancer (controls). Cases and controls were matched (1 case : 1 control) by gender and age (±3 years). The population of the study had the following characteristics: male (78.2%; n=136), self-

Table 1. Odds ratio associate with patients characteristics (n= 174).

Variable	Cases		Controls		OR [95% CI]
Category	n	%	n	%	Univariate
High Risk HPV					
Negative	75	86.2	75	86.2	Reference
Positive	12	13.8	12	13.8	1.00 [0.42 : 2.36]
Sociodemographic variables					
Sex					
Female	19	21.8	19	21.8	Reference
Male	68	78.2	68	78.2	1.00 [0.48 : 2.05]
Age					
≤60 years	46	52.9	42	48.3	Reference
>60 years	41	47.1	45	51.7	0.82 [0.45 : 1.51]
Race (self-reported) *					
White	59	71.1	51	64.6	Reference
Non-white	24	28.9	28	35.4	0.74 [0.37 : 1.44]
Marital status					
Not married	33	37.9	20	23.0	Reference
Married	54	62.1	67	77.0	0.48 [0.25 : 0.94]
Education					
Illiterate	15	17.2	14	16.1	Reference
Up to middle school	63	72.4	61	70.1	0.96 [0.42 : 2.16]
High school / Graduate	9	10.3	12	13.8	0.70 [0.22 : 2.16]
Life Habits					
Interaction time consumption of tobacco and alcohol					
Alcohol <15 years and Tobacco <15 years	3	3.4	21	24.1	Reference
Alcohol <15 years and Tobacco ≥15 years	11	12.6	8	9.2	9.6 [2.11 : 43.75]
Alcohol ≥15 years and Tobacco <15 years	15	17.2	19	21.8	5.52 [1.38 : 22.10]
Alcohol ≥15 years and Tobacco ≥15 years	58	66.7	39	44.8	10.40 [2.9 : 37.29]
Interaction consumption of tobacco and alcohol *					
Alcohol <30 g alcohol/day and Tobacco <40 packs/year	57	66.3	67	77.9	Reference
Alcohol <30 g alcohol/day and Tobacco ≥40 packs/year	12	14.0	6	7.0	2.35 [0.83 : 6.66]
Alcohol ≥30 g alcohol/day and Tobacco <40 packs/year	11	12.8	10	11.6	1.29 [0.51 : 3.26]
Alcohol ≥30 g alcohol/day and Tobacco ≥40 packs/year	6	7.0	3	3.5	2.35 [0.56 : 9.82]
Sexual habits					
Age at first intercourse *					
≤15 years old	29	34.9	25	29.1	Reference
>15 - ≤17 years old	21	25.3	22	25.6	0.79 [0.35 : 1.79]
>17 years old	33	39.8	39	45.3	0.71 [0.35 : 1.46]
Partner number *					
≤5	34	41.0	44	50.6	Reference
>5	49	59.0	43	49.4	1.47 [0.80 : 2.70]
Total	87	100	87	100	

* There are missing values.

reported white skin (67.9%; n=110); marital status (69.5%; n=121); low level of schooling (16.7% n=29) were illiterate and 71.3% (n=124) attended up to middle school. Age ranged from 41-83 years (mean=60.6 years, SD=10.1 years; median=60.0 years).

Table 1 shows the results of the comparison between cases and controls by high risk HPV DNA, correlated with social status, consumption of alcohol, tobacco and sexual habits variables.

Regarding the combined effect of tobacco use and alcohol consumption over the course of time, individuals who consumed alcohol for less than 15 years and tobacco for more than 15 years had 9.6 fold increased risk to develop ESCC relative to those who consumed alcohol and tobacco for less than 15 years; individuals who consumed alcohol more than 15 years and tobacco for less than 15 years had a 5.5 fold increased risk to develop ESCC relative to those who consumed alcohol and tobacco less than 15 years; individuals who consumed alcohol and tobacco for more than 15 years had a 10.4 fold increased risk to develop ESCC relative to those who consumed alcohol and tobacco for less than 15 years (Table 1).

There was no statistically significant difference between the variables age at first intercourse and number of partners (Table 1). Active oral sexual intercourse showed statistically significant differences (p=0.017). Individuals who reported engaging in oral sex once in their lives had a lower risk (OR=0.4) of having ESCC when compared to individuals who reported never having performed oral sex (Table 1).

In the multivariate analysis, using a confirmatory model including the Interaction of tobacco and alcohol, considering time and consumption, and included the HPV detection with interest variable, the HPV didn't show an increased risk to ESCC. The results of the multivariable regression analyses are shown in Table 2.

Discussion

The results herein reported revealed a high prevalence of high risk HPV DNA in esophageal mucosa; however, these occurrences did not seem to have the same causality observed in cervix carcinoma. This data is very interesting and intriguing because they stimulate provocative queries about the behavior of high risk HPV DNA in the esophageal tissue. Different risk factors were evaluated, especially those widely known to facilitate HPV infection. History of oral sexual intercourse, e.g., was not a discriminatory variable, indicating that for cases and controls, the presence of high risk HPV DNA can be a constant independent of the meaning of any injury. On the contrary, it was observed that alcohol and tobacco consumption still prevail as the classic risk factors for developing ESCC.

This was an observational case-control study; such a design is generally used for slow progressive disease and allows the evaluation of various risk factors for a particular outcome (Cousens and Balthazar, 1995). However, these studies are subject to criticism because they may contain limitations as related to the control group selection and also the interpretation of the results may be hampered by confounding factors (Pereira, 1995).

Selection of participants for the "case" group was performed at the Endoscopy Department of the BCH, an exclusively specialized oncology center. We included individuals for the "control" group in the Endoscopy Department of the AME, which is also located in the municipality of Barretos, São Paulo, Brazil. The differences between the general characteristics of the two groups generate results that cannot be sufficiently explained as what occurred with oral sex intercourse, evaluated as a protector factor. In order to balance the divergence between institutions and to assemble homogeneous groups, patients were matched by gender and age (±3 years).

Many studies about EC achieved data from retrospective cases and scarcely reported information about the population characteristics enrolled. The differential of the current work was the prospective collection of samples and detailed questionnaire about individual characteristics, which allows for the rigorous selection of the biopsy area and the range of risk factors evaluated. Consequently, several reports worldwide have

Table 2. Multivariable analyses.

Variable/ Category	p	OR [95% CI] Univariate
High Risk HPV		
Negative	0.959	Reference
Positive		1.025 [0.405 : 2.592]
Interaction time consumption of tobacco and alcohol		
Alcohol <15 years and Tobacco <15 years	0.007	Reference
Alcohol <15 years and Tobacco ≥15 years	0.005	9.423 [1.974 : 44.981]
Alcohol ≥15 years and Tobacco <15 years	0.016	5.572 [1.372 : 22.627]
Alcohol ≥15 years and Tobacco ≥15 years	0.001	9.581 [2.592 : 35.421]
Interaction consumption of tobacco and alcohol		
Alcohol <30 g Alcohol/day and Tobacco <40 packs/year	0.866	Reference
Alcohol <30 g Alcohol/day and Tobacco ≥40 packs/year	0.489	1.468 [0.495 : 4.355]
Alcohol ≥g Alcohol/day and Tobacco <40 packs/year	0.890	0.935 [0.358 : 2.441]
Alcohol ≥g Alcohol/day and Tobacco ≥40 packs/year	0.614	1.460 [0.335 : 6.359]

described heterogeneous data regarding HPV prevalence in the esophageal tissue that varied from the absence of virus (Ashworth et al., 1993; Rugge et al., 1997; Gao et al., 2006; Koh et al., 2008; Antunes et al., 2013), to prevalences up to 100% (Li et al., 2001) in ESCC. In control population, some studies also report the absence of the virus (Lenhart et al., 1991; Ashworth et al., 1993; Cooper et al., 1995; Fidalgo et al., 1995; Lambot et al., 2000; Souto Damin et al., 2006; Koh et al., 2008; Antonsson et al., 2010) and others reported prevalences as high as 59% (Li et al., 2001). It should be considered that this variation in prevalence can be explained by multiple factors such as the methodological differences used to detect HPV, the number of cases analyzed, as well as environmental, geographic and demographic differences among the populations evaluated (Takahashi et al., 1998; Kawaguchi et al. 2000; Matsha et al., 2002; Syrjänen, 2002, 2013; Far et al., 2007; Malik et al., 2011; Zhang et al., 2011).

In the Brazilian territory, the prevalence of HPV in ESCC was analyzed in four studies, ranging from 0% to 16% (Weston and Prolla 2003; Souto Damin et al., 2006; Herbster et al., 2012; Antunes et al., 2013). Several studies, including those performed in Brazil, have in common the fact that these were conducted retrospectively, performed with different HPV detection techniques and quite conflicting results. All the data supported that HPV, somehow, is not involved with the esophagus carcinogenesis, which is corroborated, in part, by our findings. Furthermore, our study showed the same positivity indexes for high risk HPV DNA, in controls and cases, also observed in other studies. The high sensitivity of the methodologies herein used, and the strict control of prospectively collected samples, are determinant variables to explain these differences among the results observed in Brazilian regions. It is important to emphasize, however, that in Brazil, HPV-related to esophageal cancer, does not have the same meaning as reported in other countries, such as China, for example (Guo et al., 2012).

HPV prevalence observed among cases is high when compared to global data accumulated so far, but reflect and corroborate the results described in many studies found in the literature. Studies conducted in South Africa, India, Greece and Japan found similar prevalences as we obtained, ranging from 15% to 25% (Williamson et al., 1991; Agarwal et al., 1998; Khurshid et al., 1998; Lyronis et al., 2005). In individuals without cancer, however, there is a higher prevalence rate when compared only to data published in Brazilian literature to date (Weston and Prolla, 2003; Souto Damin et al., 2006; Antunes et al., 2013).

It is well-known that three forms of HPV infection may occur: clinical, subclinical and latent. The latent virus can be diagnosed only by molecular techniques targeting HPV-DNA (Richart and Wright, 1991). Therefore, we hypothesize that this may be the form found in controls in this study. The mechanisms by which the HPV remains in this state of latency remain largely unknown, but it is believed that immunological factors are decisive for this condition. Often, HPV infection may regress spontaneously, but we have no further knowledge about how long the virus could remain in this state, nor about the rate of progression of this form of latency (Burd, 2003).

Alcohol consumption was considered a major risk factor for ESCC. The International Agency for Research on Cancer (IARC) classifies alcohol as a cause of esophageal cancer (IARC 2008) and ecological and case-control classical studies established that alcohol has a strong causal role in the development of ESCC in most regions of the world (Tuyns, 1970, 1983; Audigier et al., 1975; Tuyns et al., 1979). Tobacco use was also considered a risk factor for ESCC in this study, mainly when smoking hand-rolled cigarettes was considered separately. We can also observe the so called "dose response effect", in that the risk increased as the amount consumed also increased.

The difference between study groups engaged in active oral sex may be related to selection bias of the control population. As much as has been paired by sex and age to the homogenization of the groups, it is observed that the control group is markedly more urbanized, which, despite potential controversies, may directly reflect the data on the sexual habits obtained in this study.

HPV was demonstrated to be frequently associated to normal and malignant esophageal tissues despite the fact that esophageal HPV presence does not appear to be a risk factor for ESCC development, esophagus seems to be an important HPV-reservoir, in health or disease.

Acknowledgements: The authors would like to thank the BCH, Medical Specialties Ambulatory (AME) Clinic of Barretos, HPV Teams at BCH and Molecular Biology Laboratory, Center for Translational Research in Oncology, São Paulo Cancer Institute (ICESP), São Paulo, Brazil.

Funding statement: CNPq Universal for providing supplies to the largest study, of which this study is a part of, entitled "The role of human papillomavirus (HPV) as the etiologic agent of esophageal cancer. A cross-sectional study, case-control and longitudinal at Barretos Cancer Hospital"; (Grant number 482666/2012-9 to ALF); INCT HPV [Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [Grant number 08/57889-1 to LLV]; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant number 573799/2008-3 to LLV)].

Ethical Aspects and Consent to Participate: This study was approved by the Ethics Committee at BCH with register number 134471. Patients were referred to a private room and informed about the purpose of the study, procedures for biological sampling and the necessary information requested on the data collection instrument. Patients who agreed to participate signed the Informed Consent form. Participants, then, answered a questionnaire containing sociodemographic information and explaining the known risk factors for the HPV infection and EC. All interviews were conducted by previously trained members of the HPV Research Group at BCH - Pius XII Foundation. Interviews provided information to characterize the duration and type of exposure.

Conflict of interest: Nothing to declare.

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Accepted September 6, 2017

Virology 510 (2017) 55–59

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Concordance of Beta-papillomavirus across anogenital and oral anatomic sites of men: The HIM Study☆

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ARTICLE INFO

Keywords:
Cutaneous human papillomavirus
Males
HIM Study
Concordance
Anogenital
Oral

ABSTRACT

We evaluated the concordance between β-HPVs detected in external genital skin, anal canal, and oral cavity specimens collected simultaneously from 717 men that were participating in the multinational HIM Study. Viral genotyping was performed using the Luminex technology. Species- and type-specific concordance was measured using kappa statistics for agreement. Overall, concordance of β-HPVs across sites was low and mainly observed among paired genital/anal canal samples. When grouped by species, solely β-4 HPVs showed moderate concordance in genital/anal pairs (κ = 0.457), which could be attributed to the substantial concordance of HPV-92 in men from Brazil and Mexico (κ > 0.610). β-HPV type concordance was higher in Mexico, where HPV-19 was consistently concordant in all anatomic site combinations. Our analysis indicates that type-specific concordance across sites is limited to few viral types; however, these infections seem to occur more often than would be expected by chance, suggesting that although rare, there is agreement among sites.

1. Introduction

Most of over 200 human papillomavirus (HPV) types characterized to date cluster within the Alpha (α)-, Beta (β)-, or Gamma (γ)-HPV genus. Whereas α-HPVs are mainly mucosal types isolated from the anogenital epithelia, β- and γ-HPVs, originally classified as cutaneous types, are ubiquitously distributed throughout the body and may be an intrinsic part of the commensal flora (Antonsson et al., 2000, 2003; Bottalico et al., 2011; Hazard et al., 2007; Sichero et al., 2014, 2015). Additional research is crucial to better understand the pathological implications of the broad distribution of these viruses.

Several studies show that oral α-HPV infections are associated with immunosuppression and with sexual behavior resembling associations observed at the anogenital epithelia (Kreimer et al., 2004). In contrast, at the oral cavity and anogenital epithelia, β-HPVs seem to differ from that of α-HPVs, in the lack of association between β-HPV detection and sexual risk factors. This observation points toward other routes of transmission such as autoinoculation and non-penetrative sexual activities (Sichero et al., 2014, 2015; Nunes et al., 2016; Donà et al., 2015; Torres et al., 2015). Alternatively, detection of β-HPVs in one

Abbreviations: HPV, human papillomavirus; HIM Study, HPV Infection in Men Study; SS, skin swabs; EB, eyebrow hairs; NMSC, non-melanoma skin cancer

☆ Funding Statement: This work was supported by the Ludwig Institute for Cancer Research to [LLV]; Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [Grants numbers 2013/01440-4 to LS; 2013/20470-1 and 2015/12557-5 to EMN; 2008/57889-1 to LLV]; Conselho Nacional de Desenvolvimento Científico e Tencnológico (CNPq) [Grant number 573799/2008-3 to LLV]. The infrastructure of the HIM Study cohort was supported through a grant from the National Cancer Institute (NCI), National Institutes of Health (NIH) (R01 CA098803 to ARG).

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http://dx.doi.org/10.1016/j.virol.2017.07.006
Received 30 May 2017; Received in revised form 28 June 2017; Accepted 5 July 2017
Available online 11 July 2017

Table 1
Any β-HPV DNA detection at the genital region, anal canal, and oral cavity in paired samples from the same HIM participant.

	Any β-HPV - anal canal[a]		Any β-HPV - oral cavity[b]	
	Positive	**Negative**	**Positive**	**Negative**
Any β-HPV positive - genital (n=557)	318	239	181	376
Any β-HPV negative - genital (n=160)	71	89	29	131
total	389	328	210	507

Pearson chi-square test was used to compare the prevalence of any β-HPV at the anal canal or oral cavity among men positive or negative for any β-HPV at the genitals.
[a] $p = 0.004$.
[b] $p < 0.001$.

anatomic site may also represent deposition of virions shed from other anatomic sites (Sichero et al., 2014, 2015; Liu et al., 2015).

The few reports in which the oral and anogenital regions were analyzed concurrently indicate that simultaneous oral-cervical type-specific α-HPV infections are relatively rare (Fakhry et al., 2006; Smith et al., 2004; Steinau et al., 2017; Termine et al., 2011). Instead, specific β-HPV types are not only shared within family members, but are also commonly detected in different cutaneous anatomical sites within the same individual (Antonsson et al., 2000, 2003; Weissenborn et al., 2009). Nevertheless, the relation between oral and anogenital β-HPV infections remains poorly explored (Hampras et al., 2017; Moscicki et al., 2017). Therefore, the purpose of this study was to evaluate the concordance of 43 β-HPV types detected across samples simultaneously collected from the external genital skin, the anal canal, and the oral cavity among men enrolled in the multinational HPV Infection in Men (HIM) cohort study from three different countries.

2. Materials and methods

2.1. Clinical samples and β-HPV genotyping

Biological specimens originated from the HIM Study cohort (HPV Infection in Men) conducted among over 4000 men from São Paulo (Brazil), Cuernavaca (Mexico) and Tampa, Florida (US) (Nunes et al., 2016). Study methods and design are described in detail elsewhere (Giuliano et al., 2008, 2011; Kreimer et al., 2011; Nyitray et al., 2011). The study was approved by human research ethics committees at each study sites. Informed consent was obtained from all participants prior to enrollment.

The present analysis included 717 men (238 from the US, 241 from Brazil and 238 from Mexico) with adequate (β-globin positive and/or β-HPV positive) specimens collected from the oral cavity, anal canal and genital skin, collected at the same study visit. For HPV status assessment, PCRs were conducted using a mixture of specific biotinylated primers capable of amplifying a fragment of the *E7* gene of 43 β-HPV from five different species, followed by bead-based Luminex technology (Gheit et al., 2007; Nunes et al., 2016; Schmitt et al., 2006). Primers for the amplification of β-globin were also added to evaluate the quality of template DNA. For each probe, MFI (mean fluorescence intensity) values obtained when no PCR product was added to the hybridization mixture was considered the background values. Cutoffs were computed by adding 20 MFI to 1.1X the median background value.

2.2. Statistical analysis

Pearson chi-square test was used to compare the prevalence of any β-HPV at the anal canal or oral cavity among men positive or negative for any β-HPV at the genitals. Samples collected from the three anatomic sites were considered concordant if the same HPV species or genotype was detected at two or more anatomic sites simultaneously. HPV concordance between any two paired samples of the three different specimens (genital, anal canal, oral cavity) was calculated using kappa statistics (Cohen, 1960) which express the frequency of agreement beyond chance. Kappa-values less than 0.4 indicate poor agreement, a kappa estimate between 0.41 and 0.60 indicates moderate agreement, and a kappa estimate between 0.61 and 0.8 indicates substantial agreement (Landis and Koch, 1997). Precision, a measure of exactness, consists of the percentage of positive agreement between any two paired samples (either negative or positive for the same type specific β-HPV) among all cases analyzed. All analyses were conducted using SPSS 18.0 software (SPSS, Chicago, IL) to calculate kappa values and MedCalc (r) Version 16.8 (c) 1993–2016 MedCalc Software to calculate 95% confident interval [95%CI].

3. Results

We detected 3302 single β-HPV infections across all anatomic sites among the 717 men included in this analysis. At least one β-HPV was detected in 557 (77.7%), 389 (54.3%) and 210 (29.3%) specimens of the external genital skin, anal canal, and oral cavity, respectively. Detection of multiple β-HPVs was more common at the genitals than in the anal canal or oral cavity. A detailed distribution of β-HPVs in this population and population characteristics have been previously described (Nunes et al., 2016).

Having β-HPV DNA (any type) at all three anatomical sites was observed in 127 men (17.7%). Detection of any β-HPV at the anal canal was more common among men with any β-HPV at the genitals (57.1%) than among genital β-HPV DNA negative men (44%) ($p = 0.004$). Likewise, the prevalence of any β-HPV at the oral cavity was higher among men with any β-HPV at the genitals compared to men without genital β-HPV (32.5% versus 18%, $p < 0.001$) (Table 1).

Independent of the sample geographical origin (US, Brazil, Mexico), when analyzed any β-HPV or grouped by species, kappa values revealed only slight agreement for all three anatomic pair combinations analyzed (Table 2). The only exception was β-4 HPV species for which agreement in genital/anal canal samples was moderate ($\kappa = 0.457$). We next evaluated agreement strength for individual β-HPV types. Table 3 shows kappa values exclusively of viral types for which at least in one pair sample combination the agreement observed was moderate ($\kappa = 0.410–0.600$) or substantial ($\kappa = 0.610–0.800$). The highest kappa values were observed among Mexican men and among paired genital/anal canal samples. The majority of type-specific kappa values > 0.410 were restricted to men from one single geographic region. Exceptions were HPV-92 (β-4 species) that showed substantial concordance in paired genital/anal canal specimens from Brazil and Mexico ($\kappa > 0.610$), and HPV-93 (β-1 species) which presented moderate agreement in genital/anal canal and genital/oral pairs from Mexico ($\kappa = 0.434$, both), and substantial agreement in oral/anal canal regions in the US. Furthermore, in Mexico, HPV-19 (β-1) was consistently concordant in all anatomical sites combinations analyzed. Nevertheless, it is important to highlight that when considering individual β-HPV types, analyses were based in very few cases (Table 4) which precluded the evaluation of socio-demographic and behavior variables that could be associated to the risk of detecting the same viral type in more than one anatomic site.

Table 2
Kappa values and precision (by n of pairs) of Beta-HPV species agreement across anatomical regions among 717 men participating in the HPV Infection in Men Study.

Beta-HPV	Genital/Anal canal (95%CI)[a]	Precision (%)[b]	Genital/Oral (95%CI)	Precision (%)	Oral/Anal canal (95%CI)	Precision (%)
Any β-HPV	0.093 (0.028; 0.157)	407 (56.76)	0.086 (0.044; 0.127)	314 (43.79)	0.173 (0.110; 0.235)	410 (57.18)
Beta-1	0.112 (0.047; 0.177)	388 (54.11)	0.135 (0.084; 0.185)	388 (54.11)	0.200 (0.126; 0.273)	497 (69.32)
Beta-2	0.161 (0.100; 0.221)	394 (54.95)	0.086 (0.047; 0.125)	323 (45.05)	0.141 (0.076; 0.207)	462 (64.44)
Beta-3	0.285 (0.204; 0.365)	562 (78.38)	0.119 (0.056; 0.182)	551 (76.85)	0.110 (0.017; 0.203)	624 (87.03)
Beta-4	**0.457 (0.119; 0.795)**	710 (99.02)	NE	709 (98.88)	NE	712 (99.30)
Beta-5	−0.008 (−0.012; −0.003)	706 (98.47)	NE	711 (99.16)	NE	712 (99.30)

[a] Kappa coefficient of concordance; 95%CI- 95% confidence intervals; NE- could not be evaluated. Strength of agreement is poor when of value of kappa is < 0.000; slight when kappa among 0.000–0.200; fair when 0.210–0.400; moderate when 0.410–0.600; substantial when 0.610–0.800. Moderate or substantial agreements are in bold.
[b] Precision is percentage of agreement (number DNA negative or positive for the same type specific β-HPV in any two paired samples)/(total number of any two paired samples).

5. Discussion

We previously reported the presence of any β-HPV in 67.3% of normal skin swabs and 56.5% of eyebrow hairs within a subcohort of 209 US men enrolled to the HIM study (Hampras et al., 2014). Additionally, among 123 participants from the US, we observed that concordance of any β-HPV detection was greater (31.0%) across keratinized tissue sites (genital skin, eyebrow hairs, and forearm skin) than across mucosal sites (anal and oral mucosa, 6.9%) (Hampras et al., 2017). We now widen our analysis to access the agreement of β-HPV DNA detection across samples collected concurrently from the external genital skin, anal canal, and oral cavity in a HIM sub-cohort of 717 healthy men from three different countries (Brazil, US, Mexico). Despite the broad diversity of β-HPV types detected, and the fact that any β-HPV infection was higher at the anal canal and oral cavity of men in which any β-HPV was also detected in the genitals, species- and type-specific β-HPV agreement across two anatomic sites was a rare event.

Considering solely cutaneous anatomic regions (arms, forehead and thighs), high type-specific β-HPV agreement across sites has been described among healthy individuals (Antonsson et al., 2000, 2003; Weissenborn et al., 2009), and also among individuals affected with psoriasis (Cronin et al., 2008), epidermodisplasia verruciformis (Dell'Oste et al., 2009), actinic keratosis (Schneider et al., 2013), or squamous cell carcinoma (Plasmeijer et al., 2010). In contrast, in our study the strength of agreement across genital, anal canal, and oral cavity was poor for most individual viral types analyzed. Nevertheless, one should consider that given the large number of samples and β-HPV types analyzed, it is unlikely that the same HPV type will be found at a distant skin site by chance. Furthermore, since the prevalence of HPV in the oral cavity was less than a half of that observed at the genitals, the low positivity agreement measured was not surprising.

Table 3
Value of kappa and precision of Beta-HPV types in agreement of anatomical sites from 717 men participating in the HPV Infection in Men Study.

	US		Brazil		Mexico	
	Kappa[a] (95%CI)	**Precision[b] (%)**	**Kappa (95%CI)**	**Precision (%)**	**Kappa (95%CI)**	**Precision (%)**
Genital/anal canal						
Beta-1						
HPV-19	−0.011 (−0.023; −0.0002)	97.48	−0.007 (−0.021; 0.006)	96.27	**0.565 (0.123; 1)**	98.74
HPV-93	0.277 (−0.162; 0.716)	97.90	NE	99.59	**0.434 (0.024; 0.844)**	97.90
HPV-105	**0.415 (0.088; 0.741)**	96.64	0.146 (−0.061; 0.353)	92.12	0.396 (0.160; 0.632)	93.28
Beta-2						
HPV-22	0.163 (0.009; 0.318)	81.09	0.169 (0.010; 0.328)	85.48	**0.469 (0.327; 0.610)**	84.45
HPV-104	NE	97.90	−0.007 (−0.017; 0.004)	97.93	**0.663 (0.225; 1)**	99.16
Beta-3						
HPV-49	**0.454 (0.159; 0.750)**	96.22	0.229 (0.006; 0.451)	92.53	0.102 (0.087; 0.478)	88.66
HPV-76	0.244 (0.054; 0.434)	88.24	**0.525 (0.365; 0.685)**	90.04	0.236 (0.049; 0.422)	87.82
Beta-4						
HPV-92	−0.008 (−0.017; −0.0002)	98.32	**0.663 (0.225; 1)**	99.17	**0.665 (0.047; 1)**	99.58
Genital/oral cavity						
Beta-1						
HPV-19	−0.007 (−0.018; 0.004)	97.90	0.216 (−0.139; 0.572)	97.10	**0.798 (0.411; 1)**	99.58
HPV-36	NE	100	−0.008 (−0.017; −0.0002)	98.34	**0.497 (−0.103; 1)**	99.16
HPV-93	0.277 (−0.162; 0.716)	97.90	NE	99.17	**0.434 (0.024; 0.844)**	97.90
Beta-2						
HPV-15	0.176 (−0.123; 0.475)	96.22	0.231 (−0.049; 0.511)	95.02	**0.566 (0.127; 1)**	98.74
Oral cavity/anal canal						
Beta-1						
HPV-14	**0.665 (0.047; 1)**	99.58	0.189 (−0.141; 0.519)	96.68	NE	100
HPV-19	−0.006 (−0.014; 0.002)	98.74	−0.004 (−0.010; 0.002)	99.17	**0.663 (0.225; 1)**	99.16
HPV-47	**0.565 (0.123; 1)**	98.74	−0.006 (−0.016; 0.003)	98.34	0.111 (−0.124; 0.347)	94.54
HPV-93	−0.008 (−0.017; −0.0002)	98.32	**0.665 (0.047; 1)**	99.59	0.237 (−0.164; 0.638)	97.48
Beta-2						
HPV-75	**0.497 (−0.103; 1)**	99.16	−0.006 (−0.013; 0.002)	98.75	NE	98.74

[a] Kappa-kappa coefficient of concordance; 95%CI- 95% confidence intervals; NE-could not be evaluable. Strength of agreement is poor when of value of kappa is < 0.000; slight when kappa among 0.000–0.200; fair when 0.210–0.400; moderate when 0.410–0.600; substantial when 0.610–0.800. Moderate or substantial agreements are in bold.
[b] Precision is percentage of agreement (number DNA negative or positive for the same type specific β-HPV in any two paired samples)/(total number of any two paired samples).

Table 4
Detection of specific Beta-HPV types in different anatomical region among 717 men for which agreement of concordance was moderate or substantial[a].

	US			Brazil			Mexico			All		
	Genital	Anal	Oral	Genital	Anal	Oral	Genital	Anal	Oral	Genital	Anal	Oral
Beta-1												
HPV-14	6	2	1	23	8	2	7	0	0	36	10	3
HPV-19	4	2	1	2	1	1	3	4	2	9	7	4
HPV-36	0	3	0	2	0	2	1	0	3	3	3	5
HPV-47	6	3	4	19	1	3	24	11	4	49	15	11
HPV-93	5	2	2	0	1	2	5	4	4	10	7	8
HPV-105	10	4	0	18	5	1	18	10	1	46	19	2
Beta-2												
HPV-15	10	4	1	12	2	4	5	1	2	27	7	7
HPV-22	39	22	4	35	10	2	53	30	15	127	62	21
HPV-75	3	3	1	13	2	1	8	3	0	24	8	2
HPV-104	5	0	0	4	1	0	4	2	0	13	3	0
Beta-3												
HPV-49	12	5	0	20	4	3	25	16	5	57	25	8
HPV-76	27	13	6	39	17	4	28	13	9	94	43	19
Beta-4												
HPV-92	2	2	0	4	2	0	2	1	0	8	5	0

[a] Number of men in which the specific β-HPV type was detected at the anatomical site indicated. Each infection is counted as an individual event in multiple infections.

The high type-specific HPV discordance observed by us could derive either from differences in HPV natural history at each of three anatomic sites or from separate exposures events. The natural history of α-HPV infections at anogenital epithelia and the oral cavity are associated with sexual behavior as these infections are mostly sexually transmitted. Despite a common mode of transmission, oral-cervical type-specific α-HPV agreement is low among women (Fakhry et al., 2006; Steinau et al., 2017; Termine et al., 2009, 2011), and in men α-HPV agreement, although rare, is higher between the penile and anal sites (7%) compared to anal-oral concordance (2%) (King et al., 2015; Tsikis et al., 2017; van Rijn et al., 2014). Interestingly, among men with genital warts, agreement of α-HPV types between the anal canal and genital wart was 78.1%, while concordance between oral and genital wart types was 60.9% (Kofoed et al., 2014). Taken together, these data indicate that the low type-specific α-HPV agreement reported possibly derives independent infection events. For β-HPVs, autoinoculation and non-penetrative sexual activities may be plausible forms of viral transmission (Donà et al., 2015; Nunes et al., 2016; Sichero et al., 2014, 2015; Torres et al., 2015). However, among healthy women from Brazil, we observed no association of β-HPV infections at the cervix and certain hygienic/sexual behaviors including hygienic tampon or menstrual cloth use, masturbation frequency, or vagina douching (Sichero et al., 2017). Additionally, it was recently reported that among heterosexual couples, the transmission rate of β-HPVs between anogenital sites was 15.9 per 100 person months from men-to-women and that risk for women-to-men transmission was similar, suggesting that these can be sexually transmitted (Moscicki et al., 2017). Unfortunately, the low type-specific agreement between any two anatomic sites precluded an analysis to assess factors associated with concordance at any two anatomical regions. Finally, one may not exclude the possibility of differences in the susceptibility of the oral and anogenital mucosa to genotype specific β-HPV infections.

We observed that β-HPV type-specific agreement was more common in the Mexican population as compared to Brazil and the US populations. In fact, among men from the HIM study, we also recently reported significant geographic differences in the natural history of α-HPV types 6 and 16 genital infection (Sudenga et al., 2017). Interestingly, we observed that β-HPV types for which strength of agreement for any two anatomical sites was moderate or substantial were not the most prevalent in the populations analyzed (Nunes et al., 2016). For instance, in Mexico, the most prevalent β-HPV types were HPV-21 (β-1), −22 (β-2), 24 (β-1), and −38 (β-2) independent of the anatomic region, whereas HPV-19 (β-3) showed substantial agreement between genital-oral and anal-oral specimens of the same individual. Thus, although small in number, concurrent infections of the same viral types occurred more often than would be expected by chance. Further studies are warranted to elucidate the biological basis of agreements observed.

A limitation of our study is that we examined only 43 of the 51 β-HPV types characterized to date, and the current analysis was not extended to γ-HPV genomes. Assuming new HPV cutaneous types are continually identified, our study likely underestimated the prevalence of additional viral genotypes. Nevertheless, because the methodology used to access β-HPV DNA is a high-sensitive DNA-based assay one may be over detecting viral DNA that not necessarily represent true infection.

In conclusion, our data point towards a high prevalence of different β-HPV types at the external genital skin, as compared to the anal canal and oral cavity of men from different countries. Although type-specific concordance across sites was limited to few viral types, our analysis indicates that such infections are unlikely to be independent of one another. Further studies may allow a more detailed investigation of the biological relationship between any two anatomic sites, but also of risk factors of viral acquisition and modes of transmission in different populations.

Conflict of interest

ARG (IISP39582) and SLS (IISP53280) are current recipient of grant funding from Merck, and ARG and LLV are members of the Merck Advisory Board for HPV prophylactic vaccines. No conflicts of interest are declared for any of the remaining authors.

Ethical approval

Ethical approval was given by the University of South Florida (IRB# 102660), Ludwig Institute for Cancer Research, Centro de Referência e Treinamento em Doenças Sexualmente Transmissíveis e AIDS, and Instituto Nacional de Salud Pública de México.

Acknowledgements

The authors would like to thank the Infections and Cancer Biology Group (International Agency for Research on Cancer, Lyon, France), HIM Study Teams in the USA (Moffitt Cancer Center, Tampa, FL), Brazil (Centro de Referência e Treinamento em DST/AIDS, Fundação Faculdade de Medicina, Instituto do Câncer do Estado de São Paulo, Ludwig Institute for Cancer Research, São Paulo) and Mexico (Instituto Mexicano del Seguro Social, Instituto Nacional de Salud Pública, Cuernavaca). This work was supported by Ludwig Institute for Cancer Research to [LLV]; Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [Grants numbers 13/01440-4 to LS; 13/20470-1 and 15/12557-5 to EMN; 08/57889-1 to LLV]; Conselho Nacional de Desenvolvimento Científico e Tencnológico (CNPq) [Grant number 573799/2008-3 to LLV]. The infrastructure of the HIM Study cohort was supported through a grant from the National Cancer Institute (NCI), National Institutes of Health (NIH) (R01 CA098803 to ARG).

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IVYSPRING INTERNATIONAL PUBLISHER

Journal of Cancer

2017; 8(6): 1062 -1070. doi: 10.7150/jca.17080

Research Paper

The Relation of HPV Infection and Expression of p53 and p16 Proteins in Esophageal Squamous Cells Carcinoma

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Received: 2016.08.02; Accepted: 2016.12.23; Published: 2017.04.09

Abstract

GOAL: To investigate the HPV prevalence and characterize the expression of potential molecular surrogate markers of HPV infection in esophageal squamous cell carcinoma.

MATERIALS AND METHODS: The prevalence of HPV in individuals with and without esophageal cancer (EC) was determined by using multiplex PCR; p16 and p53 protein levels were assessed by immunohistochemistry (IHC).

RESULTS: High-risk HPV (hr-HPV) was found in the same frequency (13.8%) in esophageal squamous cell carcinoma (ESCC) and in healthy individuals. The p53 expression was positive in 67.5% of tumor tissue, 20.0% of adjacent non-tumoral tissue and 1.8% of normal esophageal tissue. p16 was positive in 11.6% of esophageal cancer cases and 4.7% of adjacent non-tumoral tissue. p16 was undetectable among control group samples. p53 and p16 levels were not significantly associated with the HPV status.

CONCLUSIONS: These results suggest that hr-HPV types are not associated with the development of ESCC and that p53 and p16 protein expression have no relationship with HPV infection in normal or cancerous esophagus.

Key words: Esophageal neoplasias, p16, p53, immunohistochemistry, HPV.

Introduction

Esophageal cancer (EC) represents the eighth highest incidence of cancer worldwide (456,000 cases/year), and is usually diagnosed at an advanced stage, showing rapid progression and extremely poor prognosis [1, 2]. For this reason, it is ranked as the sixth most common cause of cancer death (400,000/year) [1]. In Brazil, EC is among the ten most common incident cancers, ranking sixth among men and thirteenth among women, with the higher incidences occurring in southern and southeast Brazil [3], supposedly associated with the high consumption of mate tea [4, 5].

The etiology of esophageal squamous cell carcinoma (ESCC) is complex and the geographical, cultural and ethnic variations observed suggest an association of established risk factors for this disease,

such as alcohol consumption, smoking, malnutrition, environmental factors, and infectious pathogenic microorganisms [6, 7].

In the early 80s, Syrjänen *et al.* (1982) identified for the first time features of HPV cytopathic infection in benign and malignant esophageal tumors [8]. However, studies with populations of varied cultural characteristics, associated with the use of different viral detection methodologies resulted in significantly heterogeneous data concerning HPV prevalence and viral types identified in ESCC [9-11], something that encourages further research in this area and requires knowledge of both molecules activated by the virus, and the expression of surrogate infection markers [12].

During persistent high-risk HPV (hr-HPV) infection, the increased expression of viral oncoproteins E6 and E7 can interfere with cell cycle control and trigger chromosomal instability [13, 14] since they inactivate p53 and pRb tumor-suppressor protein (retinoblastoma protein), respectively, resulting in increased 16^{INK4A} and p53 expressions. Hence, immunohistochemistry (IHC) for p16 and p53 expression is considered as assay for detection of surrogate markers of HPV infection in genital and oropharyngeal carcinomas [15-18].

In HPV positive oropharyngeal, cervical and vaginal tumors [15, 17, 19], the p16 expression is altered, and abnormal p53 expression has also been reported in HPV-positive oropharyngeal carcinoma [19] and penile lesions [20]. Furthermore, clinical development of these HPV-induced tumors, related to the treatment response and overall survival, is favorable when compared to HPV negative tumors [21, 22]. For this reason, the investigation of these proteins in ESCC is appropriate and may be an efficient research tool to find potential tumor markers [23, 24].

Therefore, this study aimed at investigating the prevalence of HPV in ESCC and normal esophageal tissue and also evaluates p16 and p53 expression levels in HPV positive esophageal cancers.

Materials and Methods

From January 2013 to October 2014, patients newly admitted to the Upper Digestive Department at Barretos Cancer Hospital referred for upper digestive endoscopy were screened and a total of 101 patients diagnosed with primary ESCC were enrolled. For the control group, 101 individuals aged 18 years or over, undergoing upper gastrointestinal endoscopy for reasons other than esophageal malignancy and with no microscopic or gross esophageal pathology admitted to the Ambulatory of Medical Specialties (AME, acronym in Portuguese) in Barretos were screened and matched by gender and age (+/- 3 years) with the cases. Sample size of the prospective and controlled study was based on a previously published work [25]. All individuals participating in this study signed an informed consent prior to enrollment and completed a questionnaire regarding their socio-demographical and lifestyle information. Moreover, clinic-pathological information was obtained through medical record review. The protocol was approved by the Ethics Committee at Barretos Cancer Hospital.

Exclusion criteria

Volunteers that had been submitted to adjuvant and/or neoadjuvant therapy, displaying any mental disability or an unfavorable clinical condition that would render them unfit for procedures, or that could provide insufficient amount of sample and/or inadequate sample quality were excluded.

Samples collection

Samples used in this project were collected during endoscopy and processed by the Pathology Department for diagnosis. Of the 101 patients with EC initially recruited for this study, only 87 samples had the enough DNA quantity to be included in the present study. Therefore, 87 controls were matched and included in the analysis. During the endoscopy procedure, biopsies were preceded by visual examination, and sample collection was conducted in the following order: tumor tissue and adjacent non-tumoral tissue for cases; proximal and distal esophageal tissue for controls. Biopsies fixed in 10% buffered formalin and embedded in paraffin were examined by a senior pathologist (CSN).

HPV detection and characterization

Biopsy specimens were digested with proteinase K-SDS 1%; DNA was obtained by organic extraction [26]. HPV DNA was assessed in all samples using a type-specific PCR bead-based multiplex genotyping (TS-MPG) assay that combine multiplex polymerase chain reaction (PCR) and bead-based Luminex technology (Luminex Corp., Austin, TX, USA), as cited in another paper [27]. This methodology is able to identify 21 HPV types [28, 29]. Based on their frequency in cervical cancer and available biological data, 12 of these types, namely types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 have been defined as carcinogens (IARC Group 1A)—hereafter referred to as hr-HPV. For eight other types, in the high-risk clade, namely types 26, 53, 66, 67, 68, 70, 73 and 82, the combination of their low frequency, lack of data on their active transcription and their transforming potential in model systems, has led them to be classified as only probable/possible carcinogens

(IARC Groups 2A and 2B)—hereafter referred to as possible high-risk (pHR)-HPV types [30].

As a positive control for the quality of the template DNA, primers for β-globin gene were included in the reactions. PCRs were performed with 10μL of template DNA in a 96-well format in 25-μl/well final reaction volume. HPV multiplex PCR was performed with QIAGEN Multiplex PCR Kit (Qiagen, Dusseldorf, Germany), according to manufacturer's instructions. Each reaction consisted of 45 cycles: 94°C for 30 seconds, 63°C for 3 minutes, and 72°C for 90 seconds. The first cycle was preceded by incubation at 95°C for 15 minutes and the last cycle was extended for 10 minutes at 72°C. PCR negative control consisted of a reaction mix without DNA.

Hybridizations were performed according to Schmitt *et al* (2006) [31]. For each HPV type-specific probe, the mean fluorescence intensity (MFI) values obtained when no PCR product was added to the hybridization mixture was considered as background. The cutoffs were calculated by adding 5 MFI to 1.1 times the value of the median background. MFI values > 20 were considered positive.

Immunohistochemistry

Expressions of p16 and p53 proteins were assessed by IHC using anti-p16 (monoclonal mouse anti-human p16INK4A protein, Clone E6H4TM, ready for use, Ventana, Tucson, AZ, USA) and anti-p53 (monoclonal mouse anti-human p53 protein, Clone DO-7, dilution 1:1200, Cell Marque, Rocklin, CA, USA) in an automated system (Ventana Benchmark ULTRA, Tucson, AZ, USA). Briefly, formalin-fixed, paraffin-embedded tumor specimens (4 μm) were deparaffinized by heating (75°C for 4 minutes), and antigen retrieval was achieved by use of cell conditioning buffer 1 (CC1) at 95°C for 64 minutes. Specific primary antibodies were separately added, and ultraView Universal DAB Detection Kit polymer amplification system (Ventana Medical Systems, Tucson, AZ, USA) was used according to manufacturer's instructions.

A cervical adenocarcinoma was used as positive control for p16 staining (Figure 2 E) and negative controls were obtained by omitting the primary antibodies (Figure 2 D). Samples with strong and diffuse nuclear and cytoplasmic in more than 70% of the tissue were considered positive [32, 33]. All scorings were conducted blindly by two observers (CSN and ALF). A colon carcinoma sample with a diffuse nuclear p53 staining was used as a positive control for p53 labelling (Figure 2 B) whilst a breast carcinoma sample was used as a negative control (Figure 2 A). The slides were evaluated for nuclear expression, and samples with at least 10% of strong nuclear staining [34] were considered positive for the p53 expression. All scorings were conducted with no knowledge of clinical characteristics or outcome by two observers (CSN and ALF), independently. The scanning of histological images was performed using the Olympus BX43 microscope and the Olympus Soft Imaging Solution GmbH, version 5.2 software (Olympus Corporation, Shinjuku-ku, Tokyo, Japan).

The observers revised all immunohistochemical discordant cases and consensus was achieved.

Statistical Analyses

The description of the study population was made from frequency tables. Chi-square, Fisher's exact, McNemar and Q-Cochran tests were used for comparison between groups (cases x controls; negative HPV x positive HPV). The significance level was 0.05 (5%). All analyses were conducted using the IBM® SPSS® Statistics 21.0 software for Windows (IBM Corporation, Somers, NY, USA).

Results

Figure 1 shows the number of samples included in the HPV analysis and p53 and p16 IHCs. Then, the characteristics of population with 174 volunteers (87 cases and 87 controls) were described. Overall, the majority of the study population was composed by men (78.2%) with an average age of 60 years. However, when lifestyle was investigated, it was shown that the intake of alcohol (90.8%), tobacco smoking (81.6%) and alcohol intake associated with tobacco smoke (75.9%) was significantly higher among individuals in the case group compared to controls individuals (74.7%; 67.8%; 60.9%, respectively) (Table 1).

Table 1. Characterization of study population

Variable / Category	Cases		Controls		p*
	n	(%)	n	(%)	
Gender					
Male	68	78.2	68	78.2	1.000
Female	19	21.8	19	21.8	
Age					
≤ 60 years	46	52.9	42	48.3	0.544
>60 years	41	47.1	45	51.7	
Alcohol					
No	8	9.2	22	25.3	**0.005**
Yes	79	90.8	65	74.7	
Tobacco					
No	16	18.4	28	32.2	**0.036**
Yes	71	81.6	59	67.8	
Alcohol + Tobacco					
Never drank or smoked	3	3.4	16	18.4	**0.006**
Drinks and smokes	66	75.9	53	60.9	
Drinks or smokes	18	20.7	18	20.7	

*Used test: Qui-square

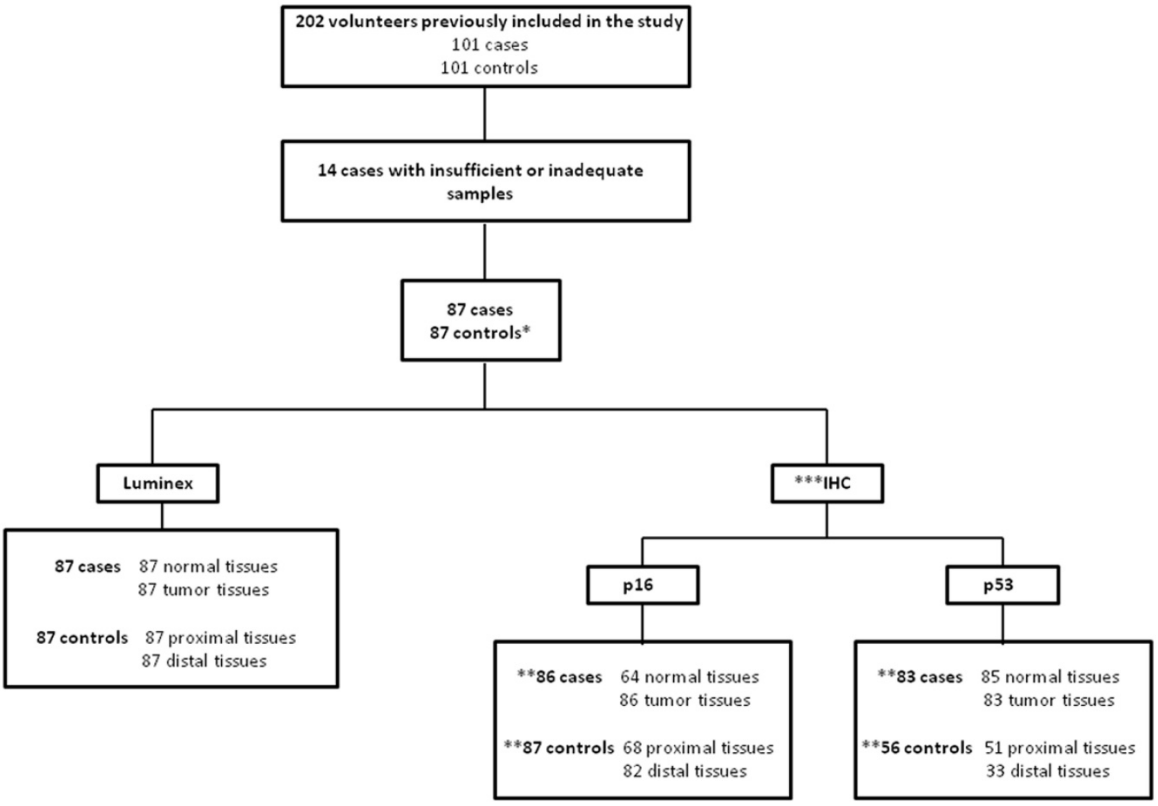

Figure 1. Clinical Samples included in the present study *As our study was case-control, only the 87 controls matched with cases were assessed in the work. ** For counting of subjects evaluated for p53 and p16 proteins expression by IHC was considered that individuals with at least one analysis to the tissues for the control group (proximal and/or distal esophageal tissues) and tumor tissue for case group. *** This analysis could not be performed for all individuals included in the study due to lack of material.

Among the 87 analyzed esophageal tumors, most of them (59%) were moderately differentiated, localized in the middle third of the esophagus (55.2%) and with TNM staging as advanced (73.5%). Moreover, a review of medical records held in May 2016 provided the information that 75.9% of patients in the study died due to the disease and only 14.9% were still alive without ESCC (Table 2).

Table 2. Characterization of tumor tissue

Variable	Category	n	%
Degree of differentiation	Well	11	13.3
	Moderate	**49**	**59.0**
	Little	23	27.7
Topography	Upper third	3	3.4
	Middle third	**48**	**55.2**
	Lower third	7	8.0
	More than one location	29	33.3
TNM Staging	I and II	22	26.5
	III and IV	**61**	**73.5**
T	T1/T2	10	13.0
	T3/T4	67	87.0
N	N0	22	38.6
	N positive	35	61.4
M	M0	65	79.3
	M1	17	20.7
Patient status	Death due to cancer	**66**	**75.9**
	Alive with disease	8	9.2
	Alive without disease	13	14.9

To evaluate HPV DNA prevalence in the esophagus, tissues were initially examined separately in tumor and adjacent non-tumoral tissue for cases and, proximal and distal esophageal tissues for controls. The results obtained were, respectively: 11.5% (10/87), 2.3% (2/87), 10.3% (9/87) and 4.6% (4/87). The overall frequency of HPV in each group was considered as positive when the individual had DNA HPV in at least one tissue. Thus, the frequency of DNA HPV was 13.8% for both of groups.

Table 3 shows the frequency of HPV types detected in each anatomical region analyzed. Regarding the adjacent non-tumoral tissue, two different hr-HPVs (HPV 31 and 66) were detected in equal proportion (1.15%). However, for tumor tissue, HPV 16 (2.3%), 18 (1.15%), 51 (1.15%), 31 (1.15%), 66 (1.15%) types, in addition to coinfections (4.6%) were detected. In controls tissues, HPV 56 (2.3%), 16 (1.15%), 31 (1.15%), 51 (1.15%), 26 (1.15%) types and coinfections (3.4%) were detected in proximal esophagus, whilst in the distal esophagus, only four coinfections (4.6%) were found. Importantly, no low-risk DNA HPV was detected in samples analyzed.

We observed no significant association between the detection of HPV DNA and any of the different

variables analyzed (gender, age and consumption of alcohol and tobacco, isolated or combined). In addition, HPV prevalence, clinicopathological characteristics (histological degree and TNM staging) and status of the patient were not significantly related (p=NA*, p=0.502, p=NA*, respectively) (*could not be calculated).

Table 3. Frequency of the HPV types detected by Luminex

Variable	Cases				Controls			
	Adjacent normal		Tumor		Proximal		Distal	
	n	(%)	n	(%)	n	(%)	n	(%)
HPV 51	0	0.0	1	1.15	1	1.15	0	0.0
HPV 31	1	1.15	1	1.15	1	1.15	0	0.0
HPV 66	1	1.15	1	1.15	0	0.0	0	0.0
HPV 56	0	0.0	0	0.0	2	2.3	0	0.0
HPV 18	0	0.0	1	1.15	0	0.0	0	0.0
HPV 16	0	0.0	2	2.3	1	1.15	0	0.0
HPV 26	0	0.0	0	0.0	1	1.15	0	0.0
Coinfecction	0	0.0	4*	4.6	3**	3.4	4***	4.6
Total	**2**	**2.3**	**10**	**11.5**	**9**	**10.3**	**4**	**4.6**

Frequencies refer to HPV types detected in each tissue analyzed by Luminex.
*56,66; 39,53,66; 53,66; 16,53.
**16,66; 18,31,33,53; 45,53.
***16,31; 31,59; 16,39,51; 31,45,53.

p53 and p16 immunohistochemistry

Regarding cellular proteins expression, we observed that p16 and p53 levels were significantly higher in tumor tissues (67.5% for p53 and 11.6% for p16) than in control group (1.8% for p53 and 0% for p16) ($p < 0.001$) (Figure 2). p53 expression was considered positive in 67.5% of tumor tissues (Figure 2C), 20.0% of adjacent non-tumoral tissue and 1.8% of normal esophageal tissue. Furthermore, p16 expression was observed in 11.6% of tumor tissues (Figure 2F) and 4.7% of adjacent non-tumoral tissue specimens. No p16 expression was observed in the control group. p53 and p16 expression levels were not significantly associated with HPV status.

Table 4 shows the number of samples that showed agreement and disagreement regarding the p16 expression and the presence of HPV. The majority of samples (80.9%) were double negative concordant (p16-/HPV-). Only one case (0.6%) showed concomitant concordance for positive p16 expression and the presence of HPV. Interestingly, this case did not show characteristics that distinguishes those negative for p16 since was a man aged 73, non-white, with ESCC moderately differentiated type, advanced stage (III), negative expression for p53, was alcohol intake associated with tobacco smoke and died due to cancer. Furthermore, 32 samples were discordant to p16 IHC and HPV results, wherein 9 p16 positive samples (5.2%) were HPV negative whilst 23 (13.3%) samples were negative for p16 expression, but positive for HPV.

Table 4. Agreement between p16 expression and HPV DNA in esophageal samples

Agreement	Samples	
	n	(%)
p16- HPV -	140	80.9
p16+ HPV +	1	0.6
p16+ HPV -	9	5.2
p16- HPV +	23	13.3

Frequency samples with concordant results between the HPV status and the expression of p16 protein for subjects in the study.

Discussion

The role of HPV in esophageal carcinogenesis is a controversial issue that has been described with significant discrepancies in results, which may be attributed to the different methodologies employed in the HPV DNA detection and, in the many ways, in which sample may be collected and preserved [35] and, maybe, the most important, the characteristics of individuals enrolled, including their sexual practices and social-demographic data [36]. Thus, to our knowledge, this is the first study having a prospective design and controlled in Brazil, which provides important reliability to the results. The goal of this project was to obtain Brazilian data that would help clarify the frequency of HPV in ESCC and the molecular pathways involved in this esophageal cancer development process, besides gathering evidences that could assist in the prognostic evaluation of patients affected by such a malignancy or the prevention of the disease through an HPV vaccine.

We demonstrated that HPV frequencies were identical in case and control groups (13.8%) by using Luminex® technology. The HPV frequency in esophageal tumors we detected is consistent with other studies conducted in Brazil in which the HPV16 and 18 prevalence found was 15.75% by using Nested PCR methodology [37] and 13.0% by using PCR followed by sequencing and in situ hybridization (HPV16 was the most frequent type) [38]. However, this prevalence is higher than others that described HPV prevalence in Brazilian cases of esophageal cancers around 2.5% of low-risk HPV using hybrid capture methodology [39] and 0.0% using nested PCR methodology [40]. Furthermore, in non-cancer volunteers, the HPV frequency of 13.8% was similar to the first Brazilian study carried out with samples from South of the country which found an HPV prevalence of 10% in normal esophageal samples by hybrid capture methodology [39]. However, all other Brazilian studies did not detect DNA HPV in healthy

esophageal samples analyzed [37, 38, 40], and this confirms unequivocally that the frequency of HPV in neoplastic and normal esophageal tissues are so variable, not only for any regional differences, but also very important, according to the methodological techniques used to address this goal [35, 36].

Differences in HPV prevalence largely depend on the test used and type of the samples and their conservation. The variation among the groups did not come as a surprise but the real meaning of the findings did. This point was elegantly reported in a recent review of the literature [35]; worldwide HPV-ESCC infection rates ranged from 11.7% to 39.9%, with the high-ESCC-incidence countries relating HPV tumor infections rates significantly higher in relation to low-ESCC-incidence countries. In China, in a high-ESCC-incidence region, for example, HPV infections in ESCC ranged from 32.8% to 63.6%, whereas in North America (low incidence region) rates ranged between 8.7% to 16.6% [35]. Li *et al.* (2014) also reported that the positive rate for HPV in ESCC was 14.0% for Europe and the Americas, according to our findings [11]. In relation to adjacent non-tumoral esophageal tissue, literature has shown rates as high as 58.9% in some studies, and as low as 0% in others. Such disparities may be justified by the small sample size, making it hard to answer questions of the HPV ESCC prevalence [35].

Global data shown by recent literature indicates that HPV has no significant etiological role in most cases of ESCC [35]. Another recent study showed that HPV infection may play a role in esophageal carcinogenesis only in high-incidence regions [41] and this conclusion corroborates, in part, the results demonstrated by our study, since we found identical HPV frequency in patients without EC group compared to the group with ESCC. To confirm, no correlation with p16 and p53 expression with tumor was observed and they are canonic pathways altered in HPV-cervical cancer.

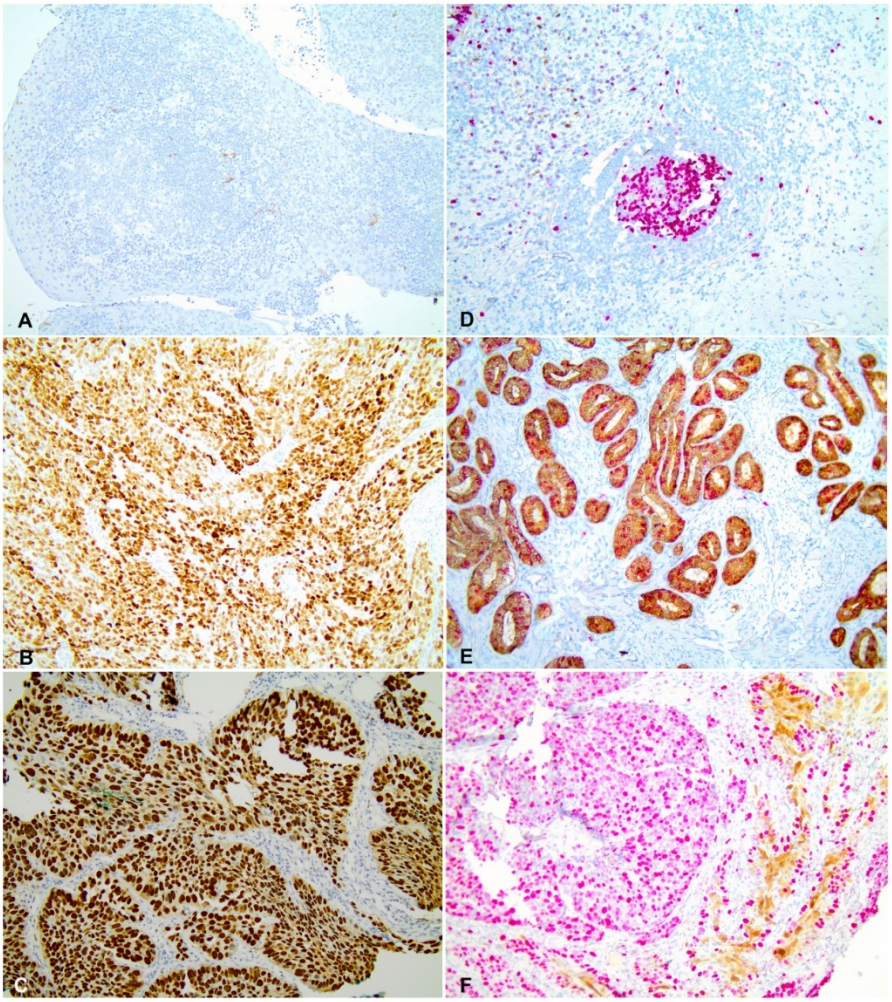

Figure 2. p53 and p16 immunohistochemistry in ESCC (A) Negative control for p53 staining. (B) Positive control for p53 staining. (C) Infiltrative moderately differentiated ESCC tissue with p53 strong nuclear expression. (D) Negative control for p16 staining. (E) Positive control for p16 staining. (F) Infiltrative poorly differentiated ESCC tissue with strong expression of p16. All images were obtained with 20X magnification.

Despite the HPV frequency be similar, the HPV types detected by us is different from those reported by other Brazilian studies, since the HPV 16 and 18 types were less frequent in our casuistry [39, 40]. However, although less frequent, the HPV types we detected had already been detected in ESCC in different geographic regions of the world [HPV 16 (11.4%), 18 (2.9%), 52 (1.1%), 33 (0.8%) and 31 (0.6%)] [11]. In South America, HPV types most often found in the cervix are HPV 16, 58, 18, 45 and 31, whereas, only HPV types 16, 18 and 31 are cited as frequently in cervical and esophageal cancer. From this information, it is strongly suggested that the main types of HPV that infect the esophageal mucosa in the Brazilian population are different from those that infect the lining of the cervix and esophagus in other regions the world [42].

In addition to detecting HPV presence in normal and tumoral esophageal tissues, we also evaluated the p53 and p16 proteins expression, since it is known that the altered expression (increased or reduced) of these proteins is directly related to tumor progression in several types of cancers, induced or not by HPV. Although there are a significant number of positive HPV samples, they were not associated with p16 and p53 expression in this study. However, the expression of these proteins increases progressively with the severity of esophageal injury, corroborating previous studies [43, 44].

According to p16 expression and HPV status in esophageal samples from patients with and without EC, we found only one sample (0.6%) with concordant results p16+/HPV+. This result corroborates recent systematic review, where it was shown that, unlike the results for cervical and oropharynx cancer, in EC there is slight or no correlation between p16 overexpression and the HPV positivity. By bringing together studies in EC, the authors concluded that the rate of double positivity (HPV+/p16+) is below 5% of cases [35]. Although some studies on head and neck show strong relationship between p16 expression and the HPV presence, it was shown that tests for HPV DNA and p16 immunohistochemistry were not satisfactorily consistent, and that the use of only one of these tests is inadequate to confirm the viral origin of oropharyngeal cancer [45].

We observed that 9 esophageal samples (5.2%) were p16+/HPV-, and similar result obtained by Fonmarty *et al.* (2015), who analyzed the p16 expression and HPV status of oropharyngeal tumors. The authors identified 11 discordant samples (p16+/HPV-) and according to authors, the p16 overexpression detected by IHC reflects an altered pRb function induced by E7 viral oncoprotein; and this altered function may be secondary to mutation of the Rb gene regardless of HPV persistent infection [45]. Another hypothesis for explain these results can be attributed to PCR's failure to detect the viral DNA, which can be supported by the non-recognition of L1 sequence of the viral genome with complementary primers used in the PCR [45]. Kawakami *et al.* (2003) showed that 11% of samples were p16+/HPV- and the authors suggested that these result reflect a deregulation in the Rb signaling pathway unrelated to HPV, as noted, for example, in small-cell lung cancer and lymphomas [46].

The inverse correlation between p16-/HPV+ is also questioned by some authors seeking to uncover if there is a chance of HPV playing its carcinogenic role in the esophagus without inducing the overexpression of p16. Among suggested hypotheses, one of them states that esophageal carcinogenesis may involve a high rate of p16 methylation promoters, thus inhibiting its expression in the oncogenic HPV infection [35]. It has been shown that there is loss of p16 expression secondary to promoter methylation in 72% of ESCC cases [47], as it has been shown for other tumor types [48]. The current study also found similar results, since 23 samples (13.3%) were p16-/HPV+. Kawakami *et al.* (2013) reported that 20% of esophageal tumors were p16-/HPV+, and that most of these tumors showed methylation of the p16 gene promoter [46]. Similar results in head and neck carcinomas also showed p16-/HPV+ cases, although in lower frequencies [49-51].

We concluded that in this particular series, the prevalence of HPV in ESCC and healthy esophagus in the Brazilian population was identical, which suggested that the HPV does not necessarily has a role in the development of esophageal cancer since there was no association between the status of HPV and the expression of p53 and p16.

Abbreviations

ESCC: Esophageal Squamous Cell Carcinoma
hr-HPV: High-risk HPV
PCR: Polymerase Chain Reaction
HPV: Human papillomavirus
EC: Esophageal Cancer
IHC: Imunohistochemistry
PRb: Retinoblastoma protein
AME: Ambulatory Medical Specialties
DNA: Deoxyribonucleic acid
TS-MPG: Type-specific PCR bead-based multiplex genotyping
MFI: Mean fluorescence intensity
CC1: Cell conditioning buffer
TNM: Classification of Malignant Tumours
ICESP: Center for Translational Research in Oncology, São Paulo Cancer Institute

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico
FAPESP: Fundação de Amparo à Pesquisa do Estado de São Paulo
CEP: Ethics committee for research

Acknowledgements

The authors would like to thank the Barretos Cancer Hospital, Ambulatory Medical Specialties (AME) Clinic of Barretos, HPV Teams in the Barretos Cancer Hospital and Molecular Biology Laboratory, Center for Translational Research in Oncology, São Paulo Cancer Institute (ICESP), São Paulo, Brazil. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tencnológico (CNPq) [Grants number 482666/2012-9 to ALF; 573799/2008-3 to LLV]; Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [Grants numbers 13/15968-0 to PRAP; 08/57889-1 to LLV].

Funding statement

CNPq Universal for promoting supplied to the largest study of which this study is part entitled "The role of human papillomavirus (HPV) as the etiologic agent of esophageal cancer. A cross-sectional study, case-control and longitudinal in Barretos Cancer Hospital" (Grant number 482666/2012-9 to ALF); Fundação de Amparo à Pequisa do Estado de São Paulo (FAPESP) (Grant number 13/15968-0 to PRAP); INCT HPV [Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [Grant number 08/57889-1 to LLV]; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant number 573799/2008-3 to LLV)].

Ethical approval

This project is part of a larger study approved by the CEP (134 471).

Competing Interests

The authors have declared that no competing interest exists.

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Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

Research Article

Cancer Epidemiology, Biomarkers & Prevention

Cervical Infection with Cutaneous Beta and Mucosal Alpha Papillomaviruses

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Abstract

Background: Alpha-human papillomavirus (α-HPV) plays a causal role in cervical cancer, but little is known about the epidemiology of genital Beta-human papillomavirus (β-HPV) infection.

Methods: We used Luminex and PCR hybridization to detect β- and α-HPVs prevalence at enrollment and 12-month follow-up in cervical samples from 505 women enrolled in the Ludwig-McGill cohort study. We compared epidemiologic correlates of both β- and α-HPVs and compared genotypes between these genera with respect to co-occurrence and association with cervical cytologic abnormalities.

Results: Infection with β-HPV types was more prevalent than that with α-HPV types at both visits (cumulative prevalences: 27.3% vs. 21.6%, respectively, $P = 0.034$). β-HPVs were mostly transient; however, only 1.98% women retained their original positivity at 12 months, whereas persistence was higher for α-HPVs (5.15%; $P = 0.007$). Age, parity, and sexual activity variables were predictors of α-HPV but not of β-HPV. α- and β-HPV types occurred independently. Increased risk of cervical abnormalities was restricted to women infected with α-9 or α-6 HPV types. We found no epidemiologic correlates for β-HPV infections.

Conclusions: Detection of β-HPV types in the cervix tends to occur as random and transient episodes not explained via the sexual-transmission correlates that characterize infections by α-HPVs.

Impact: Although it is plausible that β-HPVs may play a direct or indirect carcinogenic role, the lack of epidemiologic correlates for detection episodes of these viruses and lack of association with cervical lesions speak against their ancillary role as sexually transmitted agents in cervical carcinogenesis. *Cancer Epidemiol Biomarkers Prev; 26(8); 1312–20. ©2017 AACR.*

Introduction

More than 200 human papillomavirus (HPV) genotypes (types, for short) have been characterized, of which the great majority clusters phylogenetically within three genera of the *Papillomaviridae* family: Alpha (α)-, Beta (β)-, and Gamma (γ)-HPV (1). The α genus contains HPV types that infect mostly mucosal and genital regions, including 25 oncogenic types with an established, probable, or possible role in the etiology of cervical cancer (2). β- and γ genera include HPV types that commonly infect the dry skin; they are commonly referred to as cutaneous HPVs. To date, 52 β- and 82 γ- HPVs have been identified. Thus, in combination, cutaneous HPVs are a more diverse group than α-HPVs (65 types; http://www.hpvcenter.se/html/refclones.html). HPV5 and HPV8 (included in the β-1 species) are recognized as possible etiologic agents in cutaneous squamous cell carcinoma (SCC) in epidermodysplasia verruciformis primarily in sun-exposed areas (3). However, the role of specific β-HPV types in cutaneous SCC among immunocompromised non–epidermodysplasia verruciformis and immunocompetent individuals has proven difficult to demonstrate because of the high viral diversity and ubiquity of multiple types in healthy skin, oral cavity, male anogenital region and condylomas (3–8).

Although most research on oncogenic potential and disease association has focused on α-HPVs, there is interest in identifying a role of non-α-HPV types in the pathogenesis of benign and malignant lesions. It has been hypothesized that, instead of contributing directly to cancer development, β-HPVs may play a role solely at early stages of carcinogenesis, thus allowing the accumulation of mutations and destabilization of the host genome, ultimately driving tumorigenesis (9–13).

The objective of the current study was to describe the prevalence, distribution, and persistence of β-HPV types in DNA from cervical samples among asymptomatic women enrolled in the Ludwig-McGill Cohort Study, and compare these to cervical α-HPV types and species. We examined demographic and behavioral correlates of both β- and α-HPV types, and compared them with respect to their association with precancerous cytologic abnormalities.

Materials and Methods

Study design and participants

The study sample consisted of a subset of women enrolled in the Ludwig-McGill Cohort Study, a longitudinal investigation of the

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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doi: 10.1158/1055-9965.EPI-17-0081

Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

Table 1. Characteristics of the study sample at baseline in the Ludwig-McGill Cohort Study ($n = 505$)

Variables	Categories	*n* (%) [a]
Age (years)	18–22	62 (12.3)
	22–29	121 (24.0)
	30–39	186 (36.8)
	≥40	136 (26.9)
Ethnicity	White	318 (63.0)
	Nonwhite	187 (37.0)
Education	<Elementary	125 (24.8)
	Elementary	301 (59.6)
	High school	68 (13.5)
	College/University	9 (1.8)
Smoking	Never	245 (48.5)
	Current	172 (34.1)
	Former	88 (17.4)
Number of pregnancies	0–1	80 (15.8)
	2–3	225 (44.6)
	4–6	147 (29.1)
	≥7	50 (9.9)
Oral contraceptive use	Never	85 (16.8)
	<6 years	261 (51.7)
	≥6 years	159 (31.5)
Condom use	No	185 (36.6)
	Yes	320 (63.4)
Hygienic tampon use	No	454 (89.9)
	Yes	50 (9.9)
Menstrual cloth use	No	313 (62.0)
	Yes	191 (37.8)
Vaginal douching	Never/occasional	475 (94.1)
	Frequent	30 (5.9)
Age at first intercourse	20–50	142 (28.1)
	18–19	113 (22.4)
	16–17	113 (22.4)
	≤ 15	137 (27.1)
Lifetime number of sex partners	0–1	238 (47.1)
	2–3	176 (34.9)
	4+	91 (18.0)
Number of sex partners in the last 5 years	0–1	410 (81.2)
	≥2	95 (18.8)
Number of sex partners in the last year	0–1	483 (95.6)
	≥2	18 (3.6)
Anal sex practiced between visits	No	301 (59.6)
	Yes	204 (40.4)
Lifetime number of anal sex partners	0	319 (63.2)
	1	172 (34.1)
	2–3	14 (2.8)
Received oral sex	Never	239 (47.3)
	Ever	266 (52.7)
Annual frequency of masturbation acts in the last 5 years[b]	0	351 (69.5)
	<1	92 (18.2)
	1–9	27 (5.3)
	10–35	19 (3.8)
	≥36	16 (3.2)
History of sexually transmitted diseases	No	384 (76.0)
	HPV-related	20 (4.0)
	Other	100 (19.8)
Cytology grade[c]	NILM	471 (93.3)
	ASC-US	16 (3.2)
	LSIL	13 (2.6)
	HSIL	3 (0.6)

Abbreviations: HSIL, high squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy

[a]Frequencies may not add up to 505 women because of missing values for some variables.

[b]Question asked at the follow-up visit. Of those practicing masturbation, 147 reported using hands and 6 using objects (1 missing).

[c]Refers to the highest cytologic grade attained during the first year.

natural history of HPV infection and precursor lesions of cervical cancer. A detailed description of the study design and methods can be found elsewhere (14). Briefly, 2,462 women ages 18 to 60 years were recruited from family medicine, gynecology, and family planning clinics in Sao Paulo, Brazil, from 1993 to 1997. Participants were followed up every 4 months in the first year following enrollment, and then twice yearly, for up to 10 years. Questionnaires were administered and biological specimens were collected. The study was approved by ethical review boards of the participating institutions in Brazil and Canada, and informed consent was obtained from all participants. Supplementary Fig. S1 provides an overview of the sample selection strategy. Of the 2,462 women enrolled, only those with complete data (questionnaire and genotyping) at both visits, and whose samples were considered adequate (i.e., β-globin positive) were considered eligible for the current analysis. In addition, the enrollment and follow-up visits had to be within 10 days of one exact year apart (i.e., ≥355 days and ≤375 days) to permit the assessment of 12-month infection persistence. The analysis sample thus included 505 women randomly selected from 1,160 women who had completed the first and fourth visit (referred to hereafter as enrollment and follow up visit, respectively) within the first year. Samples from original cohort visits two and three were not tested.

HPV genotyping

DNA was extracted from exfoliated cervical cells by spin-column chromatography. Mucosal α-HPVs were tested by PCR amplification with MY09/11 and PGMY09/11 primers followed by genotyping via hybridization with HPV type–specific oligonucleotide probes and restriction fragment length polymorphism analysis. In combination, these two techniques allow the identification of potentially more than 40 genital α-HPV types, which were classified as per the following species: α-1: HPVs 32, 42; α-3: HPVs 61, 62, 72, 81, 83, 84, 89; α-4: HPV57; α-5: HPVs 26, 51, 69, 82; α-6: HPVs 53, 56, 66; α-7: HPVs 18, 39, 45, 59, 68, 70; α-8: HPV40; α-9: HPVs 16, 31, 33, 35, 52, 58, 67; α-10: HPVs 6, 11, 44; α-11: HPVs 34, 73; α-13: HPV54; and α-14: HPV71 (15). The presence of cutaneous β-HPVs was determined by a type-specific, multiplex genotyping PCR assay using a mixture of specific biotinylated primers that amplify a 180 to 280 bp fragment of the *E7* gene, followed by genotyping via a bead-based Luminex technology (16). This assay distinguishes 43 β-HPV types (species β-1: HPVs 5, 8, 12, 14, 19, 20, 21, 24, 25, 36, 47, 93, 98, 99, 105, 118 124, 143; species β-2: HPVs 9, 15, 17, 22, 23, 37, 38, 80, 100, 104, 107, 110, 111, 113, 120, 122, 145, 151; species β-3: HPVs 49, 75, 76, 115; species β-4: HPV92; and species β5: HPVs 96, 150). We included negative and positive controls to ascertain the quality of template DNA (17).

Statistical analysis

We calculated descriptive statistics to summarize the baseline characteristics of the study sample and prevalence rates at enrollment and follow-up for all individual HPV types and by grouping them as species within each genus. We constructed scatter plots to display the correlation between prevalence of individual HPV types between enrollment and one-year follow-up visits separately for each genus. We tested the statistical strength and significance of the correlations by calculating nonparametric Spearman's rank correlation coefficients and respective *P* values. We examined the tendency for infections of HPV types of each genus to persist by calculating ratios of observed to expected frequencies and

Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

Table 2. Prevalence of infection [*n* (%)] with β-HPV types and species in the Ludwig-McGill Cohort Study ($n = 505$)

Type-specific β-HPV	Positivity at enrollment	Positivity at 1-year follow-up	Positivity at enrollment AND follow-up	Positivity at enrollment OR follow-up
HPV5	5 (0.99)	4 (0.79)	0	9 (1.78)
HPV8	3 (0.59)	8 (1.58)	0	11 (2.18)
HPV9	1 (0.20)	1 (0.20)	0	2 (0.40)
HPV12	2 (0.40)	4 (0.79)	0	6 (1.19)
HPV14	0	1 (0.20)	0	1 (0.20)
HPV15	3 (0.59)	0	0	3 (0.59)
HPV17	1 (0.20)	1 (0.20)	1 (0.20)	1 (0.20)
HPV19	1 (0.20)	1 (0.20)	0	2 (0.40)
HPV20	0	0	0	0
HPV21	21 (4.16)	7 (1.39)	0	28 (5.54)
HPV22	8 (1.58)	13 (2.57)	1 (0.20)	20 (3.96)
HPV23	0	2 (0.40)	0	2 (0.40)
HPV24	3 (0.59)	7 (1.39)	0	10 (1.98)
HPV25	0	0	0	0
HPV36	0	10 (1.98)	0	10 (1.98)
HPV37	0	0	0	0
HPV38	14 (2.77)	8 (1.58)	0	22 (4.36)
HPV47	0	2 (0.40)	0	2 (0.40)
HPV49	1 (0.20)	0	0	1 (0.20)
HPV75	0	0	0	0
HPV76	3 (0.59)	6 (1.19)	0	9 (1.78)
HPV80	0	0	0	0
HPV92	0	1 (0.20)	0	1 (0.20)
HPV93	0	0	0	0
HPV96	1 (0.20)	2 (0.40)	0	3 (0.59)
HPV98	0	0	0	0
HPV99	0	0	0	0
HPV100	2 (0.40)	1 (0.20)	0	3 (0.59)
HPV104	0	0	0	0
HPV105	1 (0.20)	1 (0.20)	0	2 (0.40)
HPV107	0	2 (0.40)	0	2 (0.40)
HPV110	3 (0.59)	3 (0.59)	0	6 (1.19)
HPV111	10 (1.98)	2 (0.40)	0	12 (2.38)
HPV113	1 (0.20)	0	0	1 (0.20)
HPV115	0	0	0	0
HPV118	0	0	0	0
HPV120	2 (0.40)	1 (0.20)	0	3 (0.59)
HPV122	0	7 (1.39)	0	7 (1.39)
HPV124	1 (0.20)	0	0	1 (0.20)
HPV143	0	0	0	0
HPV145	0	0	0	0
HPV150	0	0	0	0
HPV151	1 (0.20)	0	0	1 (0.20)
β-HPV species[a]				
β-1	33 (6.53)	39 (7.72)	3 (0.59)	69 (13.66)
β-2	44 (8.71)	37 (7.33)	5 (0.99)	76 (15.05)
β-3	4 (0.79)	6 (1.19)	0	10 (1.98)
β-4	0	1 (0.20)	0	1 (0.20)
β-5	1 (0.20)	2 (0.40)	0	3 (0.59)
Any β-HPV	75 (14.85)	73 (14.46)	10 (1.98)	138 (27.33)

[a]β-HPV species: β-1 (HPVs 5, 8, 12, 14, 19, 21, 24, 36, 47, 105, 124); β-2 (HPVs 9, 15, 17, 22, 23, 38, 100, 107, 110, 111, 113, 120, 122, 151); β-3 (HPVs 49, 76); β-4 (HPV92); β-5 (HPV96).

respective 95% confidence intervals (CI). Expected frequencies were based on the assumption of independence of observations between the enrollment and one-year visits. We also assessed whether infections with β-HPV and α-HPV types tended to co-occur in the same women by comparing observed and expected frequencies based on the period prevalence data for both visits. This analysis was done for individual types and for types grouped within their respective species.

We used unconditional logistic regression to estimate OR and 95% CI for univariate associations between baseline characteristics, as independent variables, and the one-year period prevalence of HPV infection according to genus, as outcome. The referent category consisted of women without the respective genus-specific HPV infection. In separate logistic models, we examined the association between genus-specific HPV species (as independent variables) and cervical cytologic abnormalities (as outcome) using two definitions for an abnormal cytology: atypical cells of undetermined significance (ASC-US) or worse and low-grade squamous intraepithelial lesion (LSIL) or worse. For each, two types of analyses were performed; unrestricted and restricted. In the former, women with infections with types belonging to a given species were compared to all others as

Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

Table 3. Prevalence of infection [*n* (%)] with α-HPV types and species in the Ludwig-McGill Cohort Study ($n = 505$)

Type-specific α-HPV	Positivity at enrollment	Positivity at 1-year follow-up	Positivity at enrollment and follow-up	Positivity at enrollment or follow-up
HPV6/11	5 (0.99)	0	0	5 (0.99)
HPV16	19 (3.76)	12 (2.38)	6 (1.19)	25 (4.95)
HPV18	3 (0.59)	2 (0.40)	0	5 (0.99)
HPV26	0	0	0	0
HPV31	3 (0.59)	5 (0.99)	1 (0.20)	7 (1.39)
HPV32	0	1 (0.20)	0	1 (0.20)
HPV33	1 (0.20)	1 (0.20)	0	2 (0.40)
HPV34	0	0	0	0
HPV35	1 (0.20)	1 (0.20)	0	2 (0.40)
HPV39	0	2 (0.40)	0	2 (0.40)
HPV40	2 (0.40)	2 (0.40)	1 (0.20)	3 (0.59)
HPV42	0	0	0	0
HPV44	3 (0.59)	2 (0.40)	0	5 (0.99)
HPV45	0	1 (0.20)	0	1 (0.20)
HPV51	6 (1.19)	5 (0.99)	1 (0.20)	10 (1.98)
HPV52	5 (0.99)	4 (0.79)	1 (0.20)	8 (1.58)
HPV53	8 (1.58)	7 (1.39)	0	15 (2.97)
HPV54	2 (0.40)	4 (0.79)	1 (0.20)	5 (0.99)
HPV56	1 (0.20)	4 (0.79)	1 (0.20)	4 (0.79)
HPV57	0	0	0	0
HPV58	4 (0.79)	5 (0.99)	1 (0.20)	8 (1.58)
HPV59	2 (0.40)	1 (0.20)	0	3 (0.59)
HPV61	4 (0.79)	4 (0.79)	1 (0.20)	7 (1.39)
HPV62	0	0	0	0
HPV66	4 (0.79)	1 (0.20)	0	5 (0.99)
HPV67	0	0	0	0
HPV68	0	2 (0.40)	0	2 (0.40)
HPV69	0	0	0	0
HPV70	6 (1.19)	2 (0.40)	2 (0.40)	6 (1.19)
HPV71	1 (0.20)	4 (0.79)	0	5 (0.99)
HPV72	0	1 (0.20)	0	1 (0.20)
HPV73	0	0	0	0
HPV81	1 (0.20)	1 (0.20)	0	2 (0.40)
HPV82	1 (0.20)	0	0	1 (0.20)
HPV83	0	1 (0.20)	0	1 (0.20)
HPV84	2 (0.40)	2 (0.40)	0	4 (0.79)
HPV89	0	0	0	0
α-HPV species[a]				
α-1	0	1 (0.20)	0	1 (0.20)
α-3	7 (1.39)	9 (1.78)	1 (0.20)	15 (2.97)
α-4	0	0	0	0
α-5	7 (1.39)	5 (0.99)	1 (0.20)	11 (2.18)
α-6	13 (2.57)	12 (2.38)	1 (0.20)	24 (4.75)
α-7	11 (2.18)	9 (1.78)	2 (0.40)	18 (3.56)
α-8	2 (0.40)	2 (0.40)	1 (0.20)	3 (0.59)
α-9	32 (6.34)	26 (5.15)	9 (1.78)	49 (9.70)
α-10	8 (1.58)	2 (0.40)	0	10 (1.98)
α-11	0	0	0	0
α-13	2 (0.40)	4 (0.79)	1 (0.20)	5 (0.99)
α-14	1 (0.20)	4 (0.79)	0	5 (0.99)
Any α-HPV	69 (13.66)	66 (13.07)	26 (5.15)	109 (21.58)

[a]α-HPV species: α-1 (HPVs 32, 42); α-3 (HPVs 61, 62, 72, 81, 83, 84, 89); α-4 (HPV57); α-5 (HPVs 26, 51, 69, 82); α-6 (HPVs 53, 56, 66); α-7 (HPVs 18, 39, 45, 59, 68, 70); α-8 (HPV40); α-9 (HPVs 16, 31, 33, 35, 52, 58, 67); α-10 (HPVs 6, 11, 44); α-11 (HPVs 34, 73); α-13 (HPV54); α-14 (HPV71).

referent, that is, those who did not have infections with types of that species. In the restricted analysis, we used a fixed referent group of HPV negative women ($n = 396$ for α-species, $n = 367$ for β-species). Statistical analysis was performed using Stata version 13 (StataCorp).

Results

Table 1 presents characteristics of the study sample. The mean age of subjects was 33.5 years (range: 18–57). The majority was white and had at least elementary school education. Slightly more than half of the women reported ever smoking cigarettes. Only 15.8% of women were never or only once pregnant; 83.6% reported at least two pregnancies. Almost half of the women had none or one lifetime sexual partner and most reported no previous sexually transmitted diseases.

Tables 2 and 3 show the prevalence of infection with β- and α-HPVs, respectively, at each visit, as well as the positivity at both enrollment and follow-up (presumed as persistent infections) and positivity at either enrollment or follow-up (used for

Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

estimating period prevalence). Among the 43 β-HPVs detectable by the assay, 14 viral types were not found. The most prevalent types were HPV21 (β-1 species), HPV22 and HPV38 (both from β-2 species). The point prevalence of infection with any β-HPV type was just under 15% at each of the two visits, whereas the same estimates were just over 13% for any α-HPV. Overall, considering both visits as period prevalence, β-HPVs were more common than α-HPVs: 27.3% versus 21.6%, respectively ($P = 0.034$).

β-HPV infection was mostly a transient finding: 1.98% of women retained their original positivity at 12 months (Table 2). On the other hand, persistence was higher for α-HPVs (5.15%; $P = 0.007$; Table 3). When considering as denominators the women who had at least one of the visits positive for types of the two genera the differences became more pronounced: 7.25% and 23.85%, for β-HPV and α-HPV types, respectively ($P = 0.0005$).

By examining the strength of the correlation between prevalence estimates at enrollment and follow-up it is possible to further assess the relative transience or persistence of type-specific infection episodes by HPV genus. Figure 1 shows the between-visit scatter plots of type-specific prevalence for types that were detected in at least one of the visits (i.e., period prevalence > zero). β-HPV types (Fig. 1, top) had a scattered distribution, with no statistical evidence that the between-visit prevalence estimates were correlated (Spearman's correlation coefficient = 0.344; $P = 0.068$). On the other hand, the equivalent estimates for α-HPVs (Fig. 1, bottom) were significantly correlated (Spearman's correlation coefficient = 0.533; $P = 0.0035$).

On the basis of the assumption that detection of a given HPV type is independent between visits, we compared the observed and expected positivity for the most common HPV types, that is, those that were detected in at least 10 women in either the enrollment or follow-up visits (Supplementary Table S1). For none of the 7 β-HPV types included in the analysis was there evidence against the assumption of independence. On the other hand, among the 3 most common α-HPV types there was strong evidence for HPV16 that visit-specific prevalence estimates were not independent. Positivity for HPV16 in both visits was 13.3 times (95% CI, 4.87–29.02) more frequent than expected by chance. The ratio for HPV51 was 16.84 but largely imprecise (95% CI, 0.42–93.80).

We also tested whether positivity for β-HPV and α-HPV types were associated as coinfections both as individual types (Supplementary Table S2) and as grouped genus-specific species (Supplementary Table S3). None of the pairwise combinations of the most common β-HPV and α-HPV types seemed to be more common than expected (Supplementary Table S2). There was also no evidence that by grouping all type-specific episodes into species between-genus coinfections became observable because of the larger sample sizes (Supplementary Table S3).

Table 4 displays associations between candidate risk factors and the one-year combined positivity for α- and for β-HPVs, separately. Age and number of pregnancies were inversely associated with risk of α-HPVs. As expected, lifetime and recent (last one or five years) number of sex partners were strong predictors of an increased risk of detecting α-HPVs. Women who reported ≥ 2 sex partners in the last year were 5.03 times (95% CI, 1.94–13.11) more likely to be infected with any α-HPV type compared with women with at most one partner during the same period. In contrast, there were no clear

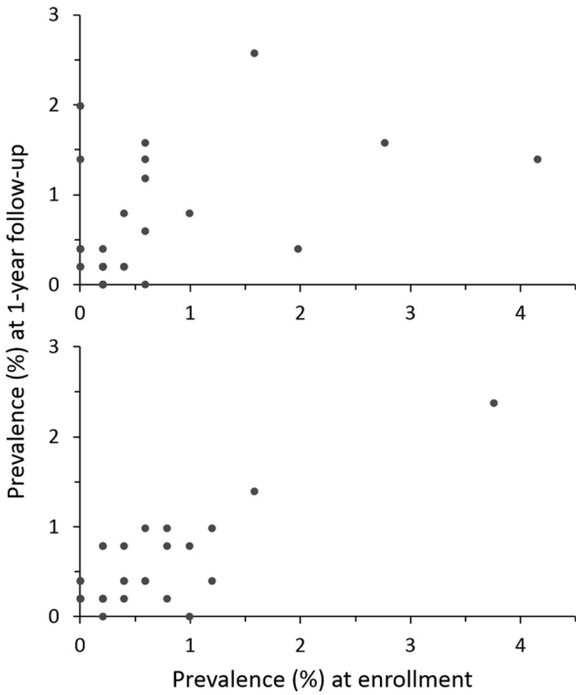

Figure 1.
Correlation between enrollment and one-year follow-up prevalence of type-specific HPV infections in the Ludwig-McGill Cohort Study ($n = 505$). Top, β-HPV types; Bottom, α-HPV types (only types detected in either of the two visits were included).

correlates of β-HPV infection among the same women, except for an implausible reduced risk among women reporting 4 or more lifetime sexual partners.

Table 5 presents the association between HPV infection status and lesion outcome based on the worst lesion grade observed by cervical cytology during the first year of follow up. Not surprisingly, women with HPV types of the α-9 species (includes HPVs 16 and 31) were at a higher risk of developing LSIL or worse compared with HPV negative women (restricted analysis, OR, 25.72; 95% CI, 7.70–85.95) or with those not harboring a viral type of α-9 species (unrestricted analysis, OR, 19.69; 95%CI, 6.79–57.12). Strong associations were also observed for women infected with HPV types of α-6 species (includes HPVs 53, 56, and 66). In contrast, no significant association was found between women infected with any β-HPV species and cervical lesion risk, regardless of grade and analysis type.

Discussion

We found that cervical infection with mostly cutaneous HPV types of the β-HPV genus were relatively common among low-income Brazilian women attending an opportunistic screening program and enrolled in our cohort study. In fact, β-HPV infections were more common than those by mucosotropic α-HPV types but appear to be mostly transient episodes. Interestingly, we found no epidemiologic correlates of cervical infections with β-HPV types. For construct validity, we conducted the same analyses with α-HPV types, which also served the purpose of assessing whether infection with the latter types, individually and

Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

Table 4. Univariate associations between subject characteristics and one-year period prevalence of HPV infection by genus in the Ludwig-McGill Cohort Study ($n = 505$)

		α-HPV positivity (n=109)		β-HPV positivity (n=138)	
Variable	**Categories**	***n* (%)**	**OR (95% CI)**	***n* (%)**	**OR (95% CI)**
Age, y	18–22	24 (22.02)	1	17 (12.32)	1
	22–29	24 (22.02)	0.39 (0.20–0.77)	37 (26.81)	1.17 (0.59–2.30)
	30–39	43 (39.45)	0.48 (0.26–0.88)	45 (32.61)	0.84 (0.44–1.62)
	≥40	18 (16.51)	0.24 (0.12–0.49)	39 (28.26)	1.06 (0.54–2.08)
Ethnicity	White	67 (61.47)	1	90 (65.22)	1
	Nonwhite	42 (38.53)	1.09 (0.70–1.68)	48 (34.78)	0.87 (0.58–1.32)
Education	<Elementary	21 (19.27)	1	32 (23.19)	1
	Elementary	65 (59.63)	1.36 (0.79–2.35)	81 (58.70)	1.07 (0.67–1.72)
	High School	21 (19.27)	2.21 (1.10–4.44)	23 (16.67)	1.49 (0.78–2.83)
	College/University	2 (1.83)	1.41 (0.27–7.29)	2 (1.45)	0.83 (0.16–4.20)
Smoking	Never	48 (44.04)	1	67 (48.55)	1
	Current	39 (35.78)	1.20 (0.75–1.94)	41 (29.71)	0.83 (0.53–1.30)
	Former	22 (20.18)	1.37 (0.77–2.43)	30 (21.74)	1.37 (0.81–2.32)
No. pregnancies	0–1	27 (24.77)	1	21 (15.22)	1
	2–3	49 (44.95)	0.55 (0.31–0.96)	65 (47.10)	1.14 (0.64–2.03)
	4–6	19 (17.43)	0.29 (0.15–0.57)	44 (31.88)	1.20 (0.65–2.21)
	≥7	13 (11.93)	0.69 (0.31–1.51)	7 (5.07)	0.46 (0.18–1.17)
Oral contraceptive use	Never	20 (18.35)	1	26 (18.84)	1
	<6 years	60 (55.05)	0.97 (0.54–1.73)	74 (53.62)	0.90 (0.53–1.53)
	≥6 years	29 (26.61)	0.73 (0.38–1.38)	38 (27.54)	0.71 (0.40–1.28)
Condom use	No	30 (27.52)	1	54 (39.13)	1
	Yes	79 (72.48)	1.69 (1.06–2.70)	84 (60.87)	0.86 (0.58–1.29)
Hygienic tampon use	No	89 (81.7)	1	128 (92.8)	1
	Yes	20 (18.3)	2.73 (1.48–5.04)	10 (7.2)	0.64 (0.31–1.31)
Menstrual cloth use	No	74 (67.9)	1	89 (64.5)	1
	Yes	35 (32.1)	0.72 (0.46–1.14)	49 (35.5)	0.87 (0.58–1.30)
Vaginal douching	Never/occasional	98 (89.9)	1	132 (95.7)	1
	Frequent	11 (10.1)	2.23 (1.03–4.83)	6 (4.3)	0.65 (0.26–1.62)
Age at first intercourse	20–50	27 (24.77)	1	38 (27.54)	1
	18–19	22 (20.18)	1.03 (0.55–1.93)	31 (22.46)	1.03 (0.59–1.80)
	16–17	27 (24.77)	1.34 (0.73–2.44)	39 (28.26)	1.44 (0.84–2.47)
	≤ 15	33 (30.28)	1.35 (0.76–2.40)	30 (21.74)	0.77 (0.44–1.33)
Lifetime number of sex partners	0–1	41 (37.61)	1	73 (52.90)	1
	2–3	39 (35.78)	1.37 (0.84–2.23)	53 (38.41)	0.97 (0.64–1.49)
	4+	29 (26.61)	2.25 (1.29–3.91)	12 (8.70)	0.34 (0.18–0.67)
Sex partners in the last 5 years	0–1	71 (65.14)	1	115 (83.33)	1
	≥2	38 (34.86)	3.18 (1.96–5.16)	23 (16.67)	0.82 (0.49–1.37)
Sex partners in the last year	0–1	96 (88.07)	1	134 (97.10)	1
	≥2	10 (9.17)	5.03 (1.94–13.11)	3 (2.17)	0.52 (0.15–1.83)
Anal sex practiced between visits	No	61 (56.0)	1	81 (58.7)	1
	Yes	48 (44.0)	1.21 (0.79–1.86)	57 (41.3)	1.05 (0.70–1.58)
Lifetime number of anal sex partners	Never	67 (61.5)	1	86 (62.3)	1
	1	37 (33.9)	1.03 (0.65–1.62)	48 (34.8)	1.05 (0.69–1.59)
	2–3	5 (4.6)	2.09 (0.68–6.44)	4 (2.9)	1.08 (0.33–3.55)
Received oral sex	Never	47 (43.1)	1	64 (46.4)	1
	Ever	62 (56.9)	1.24 (0.81–1.90)	74 (53.6)	1.05 (0.72–1.56)
Annual frequency of masturbation acts in the last 5 years	0	80 (73.4)	1	97 (70.3)	1
	<1	13 (11.9)	0.56 (0.29–1.05)	25 (18.1)	0.98 (0.58–1.64)
	1–9	6 (5.5)	0.97 (0.38–2.48)	8 (5.8)	1.10 (0.47–2.60)
	10–35	5 (4.6)	1.21 (0.42–3.46)	6 (4.3)	1.21 (0.45–3.27)
	≥36	5 (4.6)	1.54 (0.52–4.56)	2 (1.4)	0.37 (0.08–1.68)
History of sexually transmitted diseases	No	81 (74.31)	1	105 (76.09)	1
	HPV-STD	8 (7.34)	2.49 (0.99–6.31)	4 (2.90)	0.66 (0.22–2.03)
	Other STD	20 (18.35)	0.94 (0.54–1.62)	28 (20.29)	1.03 (0.63–1.69)

grouped as species, was predictive of β-HPV detection. Although we observed the expected reproductive health and sexual activity correlates for α-HPV infections, none of the sociodemographic, lifestyle, behavioral, and reproductive health variables that we collected via questionnaire was predictive of β-HPV infections. Likewise, infections with α-HPV species that include carcinogenic types were associated with cervical precancerous abnormalities, whereas none of the β-HPV species was statistically associated

Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

Table 5. Associations between positivity for HPV species by genus and cytologic abnormalities[a] in the Ludwig-McGill Cohort Study ($n = 503$)

		≥ASC-US ($n = 32$)			≥LSIL ($n = 16$)		
		Unrestricted analysis[b]		Restricted analysis[c]	Unrestricted analysis[b]		Restricted analysis[c]
Genus	Species	n	OR (95% CI)	OR (95% CI)	n	OR (95% CI)	OR (95% CI)
Alpha[d]	α-3	0	0.00 (0.00–4.19)	0.00 (0.00–7.90)	0	0.00 (0.00–9.12)	0.00 (0.00–42.26)
	α-5	2	3.42 (0.71–16.55)	5.63 (1.12–28.35)	1	3.18 (0.38–26.46)	9.78 (1.00–95.51)
	α-6	5	4.66 (1.61–13.51)	7.04 (2.30–21.51)	5	11.84 (3.72–37.67)	27.15 (6.72–109.79)
	α-7	1	0.86 (0.11–6.69)	1.49 (0.19–11.95)	0	0.00 (0.00–7.42)	0.00 (0.00–34.83)
	α-9	13	8.52 (3.89–18.69)	9.41 (4.15–21.35)	10	19.69 (6.79–57.12)	25.72 (7.70–85.95)
	α-10	1	1.66 (0.20–13.49)	2.81 (0.33–23.67)	0	0.00 (0.00–14.62)	0.00 (0.00–65.55)
Beta[e]	β-1	4	0.91 (0.31–2.68)	0.93 (0.31–2.79)	0	0.00 (0.00–1.64)	0.00 (0.00–1.61)
	β-2	6	1.34 (0.53–3.39)	1.30 (0.51–3.30)	2	0.81 (0.18–3.64)	0.69 (0.15–3.10)
	β-3	1	1.66 (0.20–13.49)	1.66 (0.20–13.65)	0	0.00 (0.00–14.62)	0.00 (0.00–12.52)

[a]Cytologic outcome defined at two cut-off points (ASC-US and LSIL). Two women had missing data on cytology.
[b]Unrestricted analysis compared women with a genus-specific species infection against a floating referent group of all women who did not have that particular species infection.
[c]Restricted analysis compared women with a genus-specific species infection against a fixed referent group of HPV-negative women (α referent group n=396, β negative group n=367).
[d]β-HPV species: β-1 (HPVs 5, 8, 12, 14, 19, 21, 24, 36, 47, 105, 124); β-2 (HPVs 9, 15, 17, 22, 23, 38, 100, 107, 110, 111, 113, 120, 122, 151); β-3 (HPVs 49, 76).
[e]α-HPV species: α-3 (HPVs 61, 62, 72, 81, 83, 84, 89); α-5 (HPVs 26, 51, 69, 82); α-6 (HPVs 53, 56, 66); α-7 (HPVs 18, 39, 45, 59, 68, 70); α-9 (HPVs 16, 31, 33, 35, 52, 58, 67); α-10 (HPVs 6, 11, 44).

with such findings. We also did not find any evidence that α-HPV and β-HPV types occur preferentially as coinfections.

The overall point prevalence of β-HPV infections in the cervix in the present investigation (15%) was considerably lower than those we observed using the same assay methodology in a study of healthy men for the anal canal (54.3%), genitals (77.7%), external genital lesions (61.1%), as well as the oral cavity (29.3%; refs. 5, 18, 19). Moreover, infection by two or more β-HPVs was not detected in the same cervical specimen despite the fact that multiple β-HPVs are commonly observed in the skin of healthy individuals and organ transplant recipients (20, 21), as well as in male genitals where we detected up to 19 viral types simultaneously (3, 19, 22).

A limitation of the current study is that we examined 43 out of the 52 β-HPV types described up until now and did not access γ-HPV genomes. Because new HPV cutaneous types are continually characterized, this analysis possibly underestimated the frequency of untested and unknown HPVs. In addition, short-term persistence of β-HPVs could not be evaluated as samples tested were collected 12 months apart.

Some studies have reported co-detection of α- and β-HPVs in the oral cavity and in penile cancer specimens (5, 23). However, a possible role of β-HPVs as carcinogenetic co-factors augmenting that of α-HPVs at these anatomic sites remains unclear. It is conceivable that cutaneous HPV infections could affect the acquisition and/or clearance of mucosal HPVs. With this hypothesis in mind, we examined the co-occurrence of α- and β-HPVs in our cohort in an attempt to identify a propensity for some types to occur more frequently as joint infections. Our findings do not support this hypothesis, as we observed that α- and β-HPV types and species occurred mostly independently of each other. This is in contrast with the recent report of a significant association between β-HPV detection and HPVs 16/18 infections at the anal canal among HIV-negative men who have sex with men (24). Similarly, in one study, detection of α-HPVs in the oral cavity significantly increased the odds of β-HPV detection (25).

Clinical manifestations of infections with certain β-HPV types include common, plantar, planar, and genital warts (26). Although recent functional in vitro studies reveal that some β-HPV types have intrinsic oncogenic potential (12, 27), they tend to cause neoplastic disease at anatomic sites typically exposed to ultraviolet radiation. However, β- and γ-HPV DNA have also been detected in condylomas and other genital lesions, and seem to play a carcinogenic role in head and neck cancers (23, 28, 29).

Although β-HPVs were more common than α-HPVs, the latter were more likely to persist and be associated with markers of sexual activity and with cervical lesions in the current study. In contrast, β-HPVs infections were not associated with cervical abnormalities or any of a long list of putative predictors. We had observed the same lack of epidemiologic or lesion correlates in a study of anogenital specimens of men (6, 7). It is reasonable to suppose that β-HPVs detection in mucosal surfaces, including cervicovaginal, may reflect deposition of virions shed from cutaneous body sites and introduced by hand as part of genital hygiene. Because hair follicles are potential reservoirs of persistent HPV infection, it cannot be ruled out that some instances of β-HPV detection in our subjects may have resulted from skin/anogenital hair contamination during specimen sampling or from recent sexual activity.

In summary, detection of cervical β-HPVs was more common than α-HPVs, but detection episodes were transient, seemed to occur at random, and were not associated with risk for cervical lesions. There was no correlation between β-HPV and α-HPV detection, either at the individual type level or as grouped species. In contrast with α-HPV, which were correlated with known sexual activity markers, we found no predictors of cervical β-HPV detection. Detection of cutaneous HPVs in the cervix may be unrelated to an active infectious process and may merely represent deposition of virions or contamination during cervical sampling.

Disclosure of Potential Conflicts of Interest

L.L. Villa reports receiving commercial research support and speakers bureau honoraria, and is a consultant/advisory board member for Merck, Sharp & Dohme. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The funders of the study had no involvement in study design; in the collection, analysis, and interpretation of data; neither in the writing of the report; and in the decision to submit the article. The corresponding author had

full access to all the data in the study and had final responsibility for the decision to submit for publication.

Authors' Contributions

Conception and design: L. Sichero, E.L. Franco, L.L. Villa
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Acknowledgments

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Grant Support

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (grant numbers 13/01440-4 to L. Sichero; 13/20470-1 to E.M. Nunes; 08/57889-1 to L.L. Villa); Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant number 573799/2008-3 to L.L. Villa). The Ludwig-McGill Cohort Study was funded by the Ludwig Institute for Cancer Research (intramural grant to L.L. Villa and E.L. Franco), the U.S. National Cancer Institute (grant CA70269 to E.L. Franco), and the Canadian Institutes of Health Research (grants MA-13647, MOP-49396, CRN-83320 to E.L. Franco).

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Received January 24, 2017; revised February 20, 2017; accepted March 24, 2017; published OnlineFirst April 4, 2017.

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Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

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Published OnlineFirst April 4, 2017; DOI: 10.1158/1055-9965.EPI-17-0081

Cancer Epidemiology, Biomarkers & Prevention

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Cancer Epidemiol Biomarkers Prev 2017;26:1312-1320. Published OnlineFirst April 4, 2017.