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TALITA HELENA ARAÚJO DE OLIVEIRA

AVALIAÇÃO DA PRESENÇA E ATIVIDADE DO HPV NO CÂNCER DE PULMÃO

Recife

2020

TALITA HELENA ARAÚJO DE OLIVEIRA

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Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética

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"A ciência sem religião é manca, a religião sem a ciência é cega”

(Albert Einstein)

RESUMO

Devido a presença do HPV em alguns tumores de pulmão, existe possibilidade do vírus estar não apenas presente, mas também exercendo sua atividade carcinogênica e contribuindo para o desenvolvimento do câncer. Com o objetivo de analisar a presença e atividade do HPV em pacientes com câncer de pulmão, 63 amostras, incluindo parafinadas e frescas, foram coletadas e o HPV detectado e genotipado através de PCR e sequenciamento, respectivamente. Em seguida, foi feita análise de expressão gênica das oncoproteínas virais através de qPCR e imuno-histoquímica, e foi avaliada a expressão de genes-alvo do HPV como p16, p21 e PCNA. O DNA viral foi detectado em 38 amostras das 63, onde 31/38 foram positivas para o HPV 16 e 7/38 foram positivas para o HPV 18. Os tipos de câncer pulmonar HPV-positivo foram: Carcinoma de células escamosas (39,39%), adenocarcinoma (33,33%), Carcinoma de células pequenas (18,18%) e carcinoma de células não-pequenas (9,1%). A presença de E6 e E7 foi detectada em 28/33 e 25/33 amostras, respectivamente, tanto no núcleo como no citoplasma das células tumorais de amostras parafinadas e E5, E6 e E7 foi quantificado em 10/19, 2/19 e 4/19 amostras frescas, respectivamente. PCNA, p21 e p16 mostrou expressão diferenciada entre amostras HPV+ e HPV-, além de correlação com a expressão das oncoproteínas. Com esses resultados, podemos concluir que o HPV além de estar presente em tumores pulmonares também está também ativo.

Palavras-chave: Carcinoma. Papilloma vírus. Carcinogênese. Oncoproteínas.

ABSTRACT

HPV are widely known as a necessary condition of cervical cancer although currently there is evidence that they are associated with several other types of cancer and may be also involved in lung cancer development. So, we evaluated the presence and activity of HPV in lung cancer. Methods: The detection of HPV was performed by PCR, followed by genotype and immunohistochemical stain of HPV oncoproteins, in tumor tissue samples, fresh and paraffin-embedded from 63 patients. Then, the expression of p16, p21 and PCNA key HPV targets was evaluated. Results: HPV was found to be present in 38 of the 63 samples, and types 16 and 18 were detected in 31/33 and 7/33, respectively. About the presence of the virus in different histological types of tumors, HPV was detected in squamous cell carcinoma (39,39%), followed by adenocarcinoma (33,33%) and small cell carcinoma (18,18%) and large cell carcinoma (9,1%). The presence of the E6 and E7 oncoproteins was detected by immunohistochemical stain technique in 28/33 samples and 25/33 samples, respectively, and E5, E6 and E7 was quantified in 10/19, 2/19 and 4/19 fresh samples, respectively. PCNA, p21 e p16 also showed differential expression between HPV+ and HPV- samples, and correlation to the oncoproteins expression. Conclusions: Our results suggest that HPV is not only present but also altering key human genes involved in tumor formation.

Keywords: Lung carcinoma. Human Papillomavirus. Oncoproteins. carcinogenesis.

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LISTA DE ABREVIATURAS E SIGLAS

ADA3	Histona acetiltransferases
AP-1	Proteína ativadora 1
Bak	Gene Bcl-2 antagonista homologo
Bax	Gene Bcl-2 associado x
Bcl-2	Proteína de célula B de linfoma 2
CDK	Kinase dependente de ciclina
c-Myc	Proteína proto-oncogene Myc
E(1 a 8)	Gene de expressão precoce 1 a 8
E2F	Fator de transcrição E2
E6AP	Proteína ubiquitina ligase E3A associada a proteína E6
EGFR	Receptor de fator de crescimento endotelial
EMT	Transição Epitelial- Mesenquimal
ERC-55	Proteína ligadora de cálcio
FAD	Dessaturase de ácido graxo
FAS	Receptor de superfície de morte celular
FFPE	Amostras fixadas em formalina e parafinadas
HDAC	Histona desacetilase
HPV	*papiloma vírus humano*
hTERT	Trascriptase reversa da telomerase humana
IL	Interleucinas

L1	Gene de expressão tardia 1
L2	Gene de expressão tardia 2
LCR	Região longa de controle
MMP	Metaloproteinases de matriz
MS	Ministério da saúde do Brasil
NSCLC	câncer de pulmão de células não pequenas
p16	Proteína codificada pelo gene CDKN2A
p16INK4	Gene que codifica um inibidor de kinase 4
PCR	Reação em cadeia de polimerase
PCNA	Proliferating Cell Nuclear Antigen
p21	Proteína inibidora de kinase dependente de ciclina
p27	Proteína inibidora de kinase dependente de ciclina
P300/CBP	Proteína ligadora de CREB
p53	Gene que codifica uma fosfoproteína nuclear
pRb	Proteína do Retinoblastoma
SCLC	câncer de pulmão de pequenas células
SP1	Proteína de especificidade 1
STK11/ LKB1	Gene serina/treonina kinase 11
SUS	Sistema único de saúde
VEGF	Fator de crescimento endotelial vascular

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1 INTRODUÇÃO

O papiloma vírus humano (HPV) é um agente infeccioso envolvido com o desenvolvimento de tumores. Seu estreito relacionamento com o câncer cervical e por ser uma doença sexualmente transmissível, provoca grande preocupação no âmbito da saúde pública brasileira. Ao longo dos últimos 20 anos, o HPV mostrou estar associado com outros tipos de câncer, ano-genitais e não ano-genitais, comprovando a importância da necessidade de métodos de prevenção e tratamento eficazes para combater a infecção.

Entre os tumores onde já foi encontrado o HPV, temos o câncer de pulmão. O Estado de Pernambuco lidera as estatísticas de incidência de câncer de pulmão na região Nordeste, correspondendo a 21% do total de casos dessa doença na região e o câncer pulmonar ocupa a quarta posição dentre os tipos de câncer mais frequentes no estado. Em torno de 40% dos casos de câncer pulmonar tem fatores etiológicos diversos e muitas vezes desconhecidos, e por isso é necessária uma maior compreensão do desenvolvimento e progressão do câncer de pulmão, e se sua formação está relacionada HPV.

Para solucionar a questão acima apresentada, é importante a implantação de estudos que visem a detecção do HPV em amostras de pacientes com este tipo de câncer e a avaliação do possível efeito do vírus na célula hospedeira.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Detectar a presença e atividade do HPV através da sua influência nas vias de proliferação celular em pacientes com câncer de pulmão.

1.1.2 Objetivos específicos

- Detectar o DNA do HPV nas amostras de câncer de pulmão;

- Em casos HPV-positivo, identificar os tipos de HPVs que estão presentes;

- Avaliar a expressão dos oncogenes virais E5, E6 e E7.

- Avaliar a expressão de genes-alvos humanos das vias de proliferação celular: p16, p21 e PCNA.

- Correlacionar as expressões relativas dos oncogenes E5, E6 e E7 com os genes humanos p16, p21 e PCNA.

2 REVISÃO DE LITERATURA

2.1 CÂNCER DE PULMÃO

O câncer de pulmão é uma das principais causas de morte por câncer no mundo (BRAY *et al.*, 2013), apresentando aumento de 2% por ano na sua incidência. No Brasil, a estimativa para 2018 foi de 31.270 casos, sendo 18.740 homens e 12.530 mulheres (INCA, 2019).

Quanto à histologia, o câncer de pulmão está categorizado em dois grandes grupos: câncer de pulmão de pequenas células (SCLC) e câncer de pulmão de células não pequenas (NSCLC). O SCLC está presente em 20% dos casos de câncer de pulmão, enquanto o câncer de pulmão de células não pequenas compreende de 70 a 80% dos casos. O NSCLC é ainda subdivido em três grupos: carcinoma de células escamosas, adenocarcinoma e carcinoma de células grandes (TRAVIS et al., 2015).

A exposição à fumaça do tabaco por longos períodos é a causa mais comum do câncer pulmonar. Entretanto, entre 15 e 50% dos casos de câncer de pulmão são diagnosticados em pacientes não fumantes (KAWAGUCHI et al., 2010; TOROK et al., 2011; YANG, 2011).

Câncer de pulmão em não fumantes é considerado uma doença com características clínicas, moleculares e genéticas específicas e distintas daquela causada pelo cigarro, pois, tais tumores têm uma maior proporção de alterações genicas do que aqueles desenvolvidos em fumantes (THU et al., 2012; YANO et al., 2008). Até o momento, a etiologia desses tumores não está elucidada. No entanto, alguns fatores têm sido sugeridos como possíveis causadores dessa doença, como: poluição do ar, exposição a metais, a radiação, ao gás radão, inalação de óleo de cozinha, fatores genéticos e infecciosos, como infecção virais (AMABILE et al., 2009; CHENG et al., 2001; MATAKIDOU et al., 2005; SILVERMAN et al., 2012; XUE et al., 2013; YU et al., 2011).

O câncer de pulmão e sua possível relação com a infecção pelo HPV ainda é bem controversa e polêmica no meio acadêmico. Em 1979 foram encontradas semelhanças citológicas e histológicas entre amostras de escarro de pacientes com lesões brônquicas e a infecção por HPV no colo do útero, com visualização de

coilócitos (RUBEL; REYNOLDS, 1979). Entre 1979 e 1980, Syrjänen (1979) também observou alterações histológicas no epitélio brônquico que eram semelhantes às alterações presentes no trato genital infectado por HPV. Tal observação o levou a sugerir que a infecção por HPV estaria envolvida no desenvolvimento de lesões pulmonares.

2.2 INFECÇÃO POR HPV E CÂNCER

O HPV está associado a diversos tipos de tumores, mas inicialmente ficou conhecido pela sua atuação no câncer cervical (ZUR HAUSEN, 2002). Atualmente, já foi apontado o seu envolvimento com os tumores de ovário, mama, cabeça e pescoço e outros (COPPOCK; LEE, 2016; FREITAS; COIMBRA; LEITÃO, 2014).

Várias evidências experimentais sustentam a estreita correlação entre a infecção por alguns tipos específicos de HPV e a etiologia do câncer, em especial o carcinoma cervical, para o qual o HPV 16 está presente em 60-70% dos casos (BERNARD *et al.*, 2010). No Nordeste os tipos de HPV mais prevalentes em lesões cervicais, além do HPV 16, são o HPV 31, HPV 58, HPV 18 e HPV 33, em ordem de frequência (GURGEL *et al.*, 2015).

Em 1983 foi descrito a primeira associação entre o HPV e um câncer não genital, o câncer de cabeça e pescoço (SYRJÄNEN *et al.*, 1983). Dos tipos inclusos no câncer de cabeça e pescoço, o câncer de orofaringe se destaca pela alta quantidade de casos associados a infecção pelo HPV (Verma, Simone e Lin, 2018) chegando a até 90.9% de frequência (MAMMAS *et al.*, 2011). O HPV foi considerado um dos principais fatores etiológicos do carcinoma de células escamosas orofaringeais, além do álcool e tabaco, estando o HPV 16 presente em aproximadamente 95% dos casos de câncer de orofaringe que são positivos para o vírus (BERMAN; SCHILLER, 2017).

Além da orofaringe, o HPV tem sido detectado também em câncer de laringe, chegando a até 50% de frequência em alguns casos, e com o HPV 16 sendo o mais presente. Junto a ele temos o câncer de esôfago, onde a taxa de frequência do HPV

chegou em até 82%, porém em 28 de 55 estudos analisados, a frequência foi de 0% a 20% (MAMMAS *et al.*, 2011).

Saindo dos tumores de cabeça e pescoço, podemos encontrar o HPV também no câncer de mama. Em 15 de 18 estudos avaliados, o HPV estava presente e chegou a uma frequência de 74% (MAMMAS *et al.*, 2011). Porém, o papel do vírus na formação ou progressão do câncer de mama ainda não está bem elucidado.

Mesmo com tantos estudos mostrando a presença do HPV nos diversos tipos de câncer, o papel dele como agente etiológico dos tumores só foi 100% comprovado no câncer cervical. Deixando muitas questões que ainda precisam ser respondidas para o completo entendimento da atuação desse vírus nos diversos processos carcinogênicos.

Desde então, vários outros pesquisadores mostraram que o HPV estava presente em tecidos pulmonares cancerígenos (tabela 1) e poderia ser um fator de risco para este tipo de câncer (KLEIN; AMIN; KOTB; PETERSEN, 2009; SHIKOVA *et al.*, 2017; SYRJÄNEN, 2012). Entretanto, outros trabalhos não encontraram o HPV no câncer de pulmão ou apesar de terem encontrado, levantaram a hipótese de uma relação meramente oportunista e casual, onde o HPV estaria presente mas não estaria causando ou aumentando o câncer pulmonar (COISSARD *et al.*, 2005; COLOMBARA *et al.*, 2016).

Tabela 1- Trabalhos que identificaram HPV em amostras pulmonares

Ano	autor	País	Tipo de HPV	Tipo de amostra	Técnica de detecção
1989	(SYRJÄNEN *et al.*, 1989)	Finlândia	6 e 16	Tecido parafinado	ISH
1990	(KULSKI *et al.*, 1990)	Austrália	6, 11, 16, 18	Tecido parafinado	ISH
1995	(KINOSHITA *et al.*, 1995)	Japão	18	Tecido fresco congelado	PCR/DBH/ISH
1995	(TAUB *et al.*, 1995)	Finlândia	6, 11, 16, 18, 31, 33	Tecido parafinado	PCR
1996	(HIRAYASU *et al.*, 1996)	Japão	6, 16, 18	Tecido parafinado	PCR
1998	(TANAKA *et al.*, 1998)	Japão	6, 11, 16, 18	Tecido parafinado	PCR
1999	(HIROSHIMA *et al.*, 1999)	Japão	16	Tecido parafinado	PCR
2001	(CHENG *et al.*, 2001)	China	16, 18	Tecido fresco	PCR/ISH
2002	(NAGAO *et al.*, 2002)	Japão	6,11,16, 18	Tecido parafinado	PCR
2003	(CHIOU *et al.*, 2003)	Taiwan	16, 18	sangue	PCR
2005	(JAIN *et al.*, 2005)	Índia	18	Tecido parafinado e Sangue	PCR
2005	(COISSARD *et al.*, 2005)	França	16	Tecido fresco congelado	PCR

2007	(BRANCA *et al.*, 2007)	Italia	16, 18, 31	Tecido parafinado/tecido fresco	PCR
2006	(CASTILLO *et al.*, 2006)	colombia, méxico e peru	16, 18, 33	Tecido parafinado	PCR
2007	(NADJI *et al.*, 2007)	iran	16, 18, 6, 11, 26, 31	Tecido parafinado	PCR
2007	(AGUAYO *et al.*, 2007)	Chile	6, 16, 18, 31, 45	Tecido parafinado	PCR/SB
2008	(GIULIANO *et al.*, 2008)	Italia	16, 18, 31, 53	Tecido parafinado/tecido fresco	PCR
2008	(CHENG, *et al.*, 2008)	China	16	Tecido fresco	ISH
2009	(HSU *et al.*, 2009)	Taiwan	16, 18	Tecido parafinado	PCR
2010	(BABA *et al.*, 2010)	Japão	16, 33, 18, 6	Tecido parafinado	PCR
2010	(ICA VRABEC BRANICA *et al.*, 2010)	Croácia	16, 18, 33	Aspirado Brôquico	PCR
2010	(PLEASANCE *et al.*, 2010)	EUA	11, 16	Tecido fresco	PCR/Seq
2011	(CARPAGNANO *et al.*, 2011)	Itália	16, 30, 31, 39	Ar exalado condensado/ Escovado brônquico/Tecido fresco	PCR/IHQ/ Pirosequenciamento

2011	(KOSHIOL *et al.*, 2011)	Italia	6, 18	Tecido parafinado	PCR
2011	(GOTO *et al.*, 2011)	Japão, Coreia e Taiwan	6, 11, 16, 18	Tecido parafinado	PCR
2012	(ADAMS *et al.*, 2012)	Japão	16, 58	Tecido parafinado	PCR
2012	(SYRJÄNEN, 2012)	Finlândia	16, 18	Tecido parafinado	PCR
2013	(MEHRA *et al.*, 2013)	Canadá	16, 18, 35, 52, 53	Tecido parafinado	PCR/RH
2014	(RAGIN *et al.*, 2014)	Grécia	16, 18, 31, 33, 11, 6, 59	Tecido parafinado	qPCR
2015	(ZHAI; DING; SHI, 2015)	-	16, 18	Tecido parafinado/ tecido fresco	Meta-análise
2016	(ILAHI *et al.*, 2016)	Paquistão	16, 18	Tecido parafinado	PCR
2017	(SHIKOVA *et al.*, 2017)	Bulgária	16, 18	Tecido parafinado/ tecido fresco	PCR
2018	(OLIVEIRA *et al.*, 2018)	Brasil	16,18	Parafinado	PCR

DBH hibridização dot blot, *ISH* hibridização *in situ*, *SB* Southern blot, *seq* sequenciamento

O DNA do HPV foi detectado, na maior parte dos casos, no tecido de biopsia tumoral pulmonar, mas também em tecido sanguíneo, em aspirado brônquico e ar exalado condensado de pacientes com câncer de pulmão (CARPAGNANO *et al.*,

2011; CHIOU *et al.*, 2003; ICA VRABEC BRANICA *et al.*, 2010). Dentre os tipos de HPV mais prevalentes no câncer de pulmão, estão HPV 16, 18, 31, 33, 6 e 11.

Corroborando com a presença do HPV encontrado em tecidos pulmonares, alguns trabalhos mostraram possíveis mecanismos utilizados pelas oncoproteínas E6 e E7 do HPV para induzir o desenvolvimento do câncer de pulmão (BUONOMO *et al.*, 2011; CHENG *et al.*, 2007; LIU *et al.*, 2005; SUNG; LEE, 2013; TUNG *et al.*, 2013; WU *et al.*, 2011).

2.3 PAPILLOMA VÍRUS HUMANO

O HPV é um vírus cujo material genético é o DNA e que é responsável por causar alterações benignas e malignas, dependendo do tipo de HPV envolvido na infecção (MARUR *et al.*, 2010). Existem mais de 200 tipos de HPVs, que estão divididos em HPV de alto risco, como os HPVs 16, 18, 31, 45; e de baixo risco, como os HPVs 6, 11, 40, 70, 72 (CUBIE, 2013). Essa classificação está relacionada com o risco que a infecção tem de levar a formação de câncer.

O genoma do HPV é dividido em uma Região longa de controle (LCR), uma região que codifica as proteínas de expressão precoce (E1 a E7) e uma região que codifica as proteínas de expressão tardia (L1 e L2) (McBride, 2008) (Figura 1). Dentre essas regiões, a responsável pela expressão das proteínas precoces é a mais envolvida num evento importante que ocorre durante a infecção viral, que é a integração do genoma do vírus ao genoma do hospedeiro. Essa integração resulta na perda de uma parte do genoma, E2 e parte de E5, o que leva a um aumento da expressão de E6 e E7, pois E2 tem uma função de controle na expressão dessas proteínas (BROECK; VANDEN, 2013).

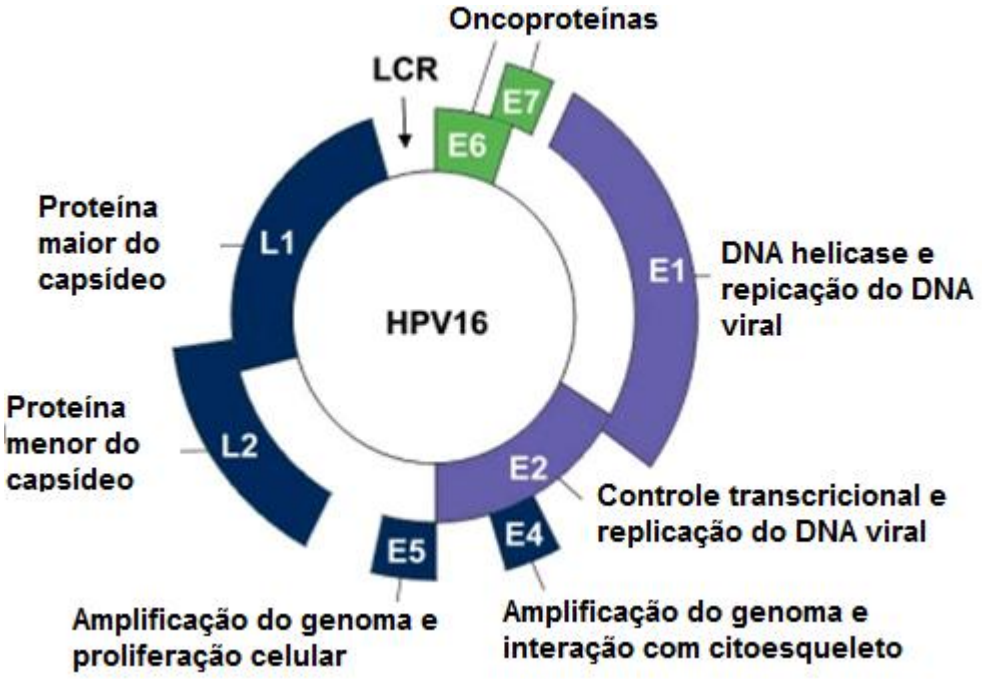

Figura 1 - Genoma do HPV. Regiões gênicas divididas em Região tardia (L1 e L2) e região precoce (E1-E7), devido ao momento de atividade das respectivas proteínas codificadas (D'ABRAMO, 2011)

E5, E6 e E7 são as proteínas virais conhecidas como oncoproteínas, pois são responsáveis por alterar o ciclo celular, a proliferação, a apoptose e o sistema imune do hospedeiro (ARREGUI, 2012). E6 e E7 tem suas atividades já bem estabelecidas e estudadas, tanto no câncer cervical como em outros tipos de câncer associados a infecção pelo HPV. E6 atua em alvos como p53, E6AP ligase, p300/CBP, histona acetiltransferases (ADA3), AP-1, Bak, Bax, FAD, procaspase 8, ERC-55 e paxillin (BROECK; VANDEN, 2013). Já a oncoproteína E7 tem atividade comprovada em alvos como pRb, ciclina A/CDK2, ciclina E/CDK2, p27 e p21 (NGUYEN; MÜNGER, 2008).

A oncoproteína ainda menos estudada é a E5, mas que mesmo assim, já possui algumas atividades delimitadas. Ela atua em alvos como EGFR, bcl-2, BAX, FAS e calnexina, relacionados com diferenciação celular, sobrevivência e proliferação. Além disso, ela aumenta a atividade carcinogênica das outras duas proteínas, E6 e E7, quando estão todas expressas (DIMAIO; PETTI 2013; GRABOWSKA; RIEMER 2012).

Foi verificado que diferentes tipos de HPV codificam diferentes variantes das oncoproteínas, e isso caracteriza sua capacidade de causar ou não alterações (com mais ou menos intensidades) (BRAVO; ALONSO 2004). Esse fato pode explicar o

porquê do HPV 16 e o HPV 18 serem os mais frequentes em diferentes tipos de câncer e em diversas partes do mundo.

2.4 ONCOPROTEÍNAS VIRAIS E5, E6 E E7

Nas últimas décadas estudos mostraram que além de presentes nos tumores pulmonares, o HPV também está ativo e expressando seus oncogenes em alguns casos. Quando expressos, desregulam a expressão de diversos genes do hospedeiro. E6 e E7 foram capazes de aumentar a progressão tumoral pulmonar através da super expressão de fatores angiogenicos, como metaloproteinases (MMP)-2, MMP-9, interleucina (IL)-6, IL-8, e IL-17 (CHANG *et al.*, 2010; CHENG, *et al.*, 2008; SHIAU *et al.*, 2013). Além disso, as mesmas oncoproteínas também promoveram a angiogênese tumoral pelo aumento de expressão do fator de crescimento endotelial vascular (VEGF) e do fator 1α induzido por hipóxia (HIF-1α) (LI *et al.*, 2011).

Além de interferir na angiogênese, as oncoproteínas atuam também alterando fatores de transcrição envolvidos na Transição Epitelial- Mesenquimal (EMT), evento crucial para a invasão e metástase tumoral (DAUPHIN *et al.*, 2013). Nesse processo ocorre o remodelamento do citoesqueleto, perda da adesão célula-célula e polaridade celular, além de diminuição de marcadores epiteliais (ex.: E-caderina e citoqueratina) e aumento de marcadores mesenquimais (ex.:N-caderina e vimetina). E6 e E7 levaram a super expressão de fatores de transcrição e consequente indução de EMT em cultura de células tumorais de pulmão (ZHANG *et al.*, 2017).

Outros fatores envolvidos no crescimento, migração e invasão celular também são alterados pelas oncoproteínas. Yang JH e colaboradores mostraram que E6 levou a super expressão da transcriptase reversa de telomerase humana (hTERT) através da inibição do gene serina/treonina kinase 11(STK11) e consequente aumento da expressão da proteína 1 de especificidade (SP1) (YANG *et al.*, 2017). Em situação normal, STK11 também conhecido como LKB1, é um gene supressor de tumor que além de atuar em outros alvos, diminui a expressão e atividade de SP1, uma família de fatores de transcrição que levam ao crescimento e migração celular. No câncer,

incluindo o câncer de pulmão, SKT11 está inibido e o fator de transcrição SP1 está super expresso e aumentando a expressão de hTERT (CHA *et al.*, 2014; TSAI *et al.*, 2015). E6 também aumenta a expressão de hTERT através da inibição de p53. Para a ativação da transcrição de hTERT é necessária a ligação em seu promotor dos fatores de transcrição SP1 e c-Myc, e este último só tem sua expressão ativada quando p53 está inibido (CHENG; WU *et al.*, 2008; JAMES; LEE; KLINGELHUTZ, 2006; KYO *et al.*, 2008; LIU *et al.*, 2005).

A formação do complexo entre E7 e a proteína supressora retinoblastoma (pRb), é um dos principais papeis dessa oncoproteína. Sob condições normais, pRb tem afinidade pelo fator de transcrição E2F, formando assim, o complexo pRb/E2F que recruta as histonas desacetilases (HDAC) e com isso, a expressão dos genes envolvidos na proliferação celular é suprimida. Quando os genes de proliferação celular precisam ser ativados, a fosforilação de pRb por CDKs impede a associação de pRb com HDAC e E2F, o que deixa E2F livre para que seja feita a ativação. A fosforilação de pRb é inibida por quatro proteínas INK, como p16, cujo gene (CDKN2A) é regulado por pRb. Dessa forma, a atividade das proteínas INK, pRb e CDK fazem parte da via de controle da progressão do ciclo celular da fase G1 para a fase S. Na infecção por HPV, a proteína E7 se liga à pRb provocando a dissociação do complexo pRb/E2F/HDAC. Tal dissociação, deixa livre HDAC e E2F para ativarem a transcrição dos genes de proliferação celular e inativar os genes supressores de tumor, como o CDKN2A, através das hipermetilação do promotor e remodelação da cromatina (FINZER *et al.*, 2001; SHERR; MCCORMICK, 2002). A super expressão de p16 atualmente é indicativo de infecção por HPV em tecido cervical e funciona como um biomarcador para triagem de lesões cervicais pré-cancerosas. Entretanto, em alguns casos excepcionais, p16 pode estar diminuído ou ausente em lesões e câncer cervical assim como pode estar positivo em tecidos normais (LI *et al.*, 2019).

Existem mais alguns alvos que foram estudados em linhagens celulares tumorais de pulmão, e que também sofrem influência de E6 e/ou E7 (KIM *et al.*, 2017; WANG *et al.*, 2017). Porém algo que chama mais a atenção é que das oncoproteínas, E5 ainda não foi estudada quanto sua ação no câncer de pulmão.

Sabe-se que E5 tem ação isolada e em conjunto com as outras oncoproteínas na formação do câncer cervical. Ela atua em vias como a angiogenica, imune, comunicação celular, apoptose e outras, em prol da imortalização das células (DIMAIO; PETTI, 2013). Um importante alvo de E5, e que é ao mesmo tempo crítico para o câncer pulmonar, é o Receptor do fator de crescimento endotelial (EGFR). E5 inibe a degradação de EGFR, aumentando seu nível intracelular e altera a atividade de moléculas-chave envolvidas da cascata de ativação deste receptor. No final, a atuação de E5 leva a síntese alterada de citocinas, aumento da proliferação celular e angiogênese, com consequente progressão tumoral (FREITAS, *et al.*, 2017).

2.4.1 P16^{INK4A}

O gene INK4A/ CDKN2A expressa um inibidor de ciclina dependente de kinase (CKI), a proteína p16, que em condições normais leva a parada do ciclo celular e inibição da proliferação (MISSAOUI *et al.*, 2018). Ao ser ativada, p16 se liga a CDK 4 e 6 inibindo a fosforilação de pRB e a passagem de G1 para a fase S do ciclo celular. Em condições de estresse como instabilidade genica, p16 impede a divisão celular e caso sua atividade seja continua, a célula é levada a morte programada (LAPAK; BURD, 2014).

Em diversos tipos tumorais, p16 é suprimido através de deleções, metilações ou inibição pós-transcricional e pós-traducional (ROCCO; SIDRANSKY, 2001). Por conta da atuação de E7 em pRb, E2F fica livre e pode estimular a transcrição do gene INK4A levando a super expressão de p16. Ao mesmo tempo E6 também inibe a expressão de p16 por meio de deleções e metilações no gene. Mesmo assim, p16 se apresenta super expresso no câncer cervical (e em outros tipos de câncer) mas sua atividade final de parada do ciclo celular não é exercida (ZUR HAUSEN, 2002).

A expressão aumentada de p16 é um marcador de malignidade e está associada a infecção por HPV no câncer cervical (KURSHUMLIU; THORNS; GASHI-LUCI, 2009). Entretanto, em alguns tumores p16 foi visto com expressão diminuída por conta de metilação no promotor do gene, inclusive no câncer de pulmão de não-pequenas células (SUN *et al.*, 2004; VAISSIÈRE *et al.*, 2009).

2.4.2 Antígeno nuclear de proliferação celular (PCNA)

PCNA é uma molécula que atua em diversos processos celulares e em todas as fases do ciclo celular. Através de interações com DNA polimerases ela atua favorecendo a replicação do DNA; através de interações com DNMT1, HDAC1 e p300 ela atua na reformulação da cromatina e controle da expressão gênica; atua também no reparo do DNA através das proteínas Msh3/Msh6 ; e através de $p21^{CIP1/WAF1}$ faz o controle do ciclo celular (TAN *et al.*, 2012). Em vários tipos de câncer como câncer de mama, fígado, próstata, cólon e cabeça e pescoço o PCNA está super expresso e tem sido estudado como marcador para prognostico de agressividade e também como alvo terapêutico (CHANDRASHEKAR *et al.*, 2015; HAMZAOUI *et al.*, 2015; HU *et al.*, 2017; ZHOU *et al.*, 2018).

Uma análise *in silico* de banco de dados de microarray revelou alguns genes diferencialmente expressos no câncer de pulmão de células pequenas. Dentre os genes alterados, PCNA foi encontrado com expressão aumentada em amostras de SCLC nos dois bancos que foram usados(WEN *et al.*, 2018). Há mais de 20 anos, este gene vem sendo estudado (BRAVO; CELIS, 1980) e apontado como biomarcador para alterações cancerígenas, porém não existem estudos até o nosso conhecimento, da análise de PCNA em amostras pulmonares com infecção pelo HPV.

No câncer cervical, a super expressão do PCNA foi associada ao grau de diferenciação celular da lesão, a metástase, ao grau de invasão tumoral e a infecção pelo HPV (LUO *et al.*, 2017).

Foi visto que a oncoproteína E6 é capaz de alterar a expressão e atividade de PCNA através de várias vias diferentes. Através da ativação do *cervical carcinoma expressed PCNA regulatory (*CCEPR), um RNA longo não codificante (lncRNA), E6 consegue aumentar a atividade de PCNA. Os lncRNAs são capazes de se ligar a proteínas, DNA ou RNA e proteger seu alvo, estabilizando sua estrutura (Long *et al.*, 2017). Foi sugerido que o CCEPR se liga ao RNAm do PCNA e o estabiliza, assim aumentando a proliferação celular no câncer cervical (YANG *et al.*, 2015), entretanto, em outro trabalho não foi confirmada correlação entre CCEPR e PCNA, apenas confirmada a atuação de E6 e E6/E7 no aumento da proliferação celular (não

necessariamente via PCNA) (SHARMA; MUNGER, 2018). Além disso, anteriormente aos trabalhos citados, foi visto que E7 estimulou a proliferação e replicação do DNA através do aumento de PCNA com objetivo de estimular a maquinaria celular de replicação para replicar o DNA viral (ROMAN; MUNGER, 2013a).

E6 também pode atuar na expressão de PCNA através da inativação de p53. Ao inativar p53, E6 leva a instabilidade no DNA por não reparo de danos, a continuação do ciclo celular e inibição da apoptose mediada por p53 (POL; VANDE; KLINGELHUTZ, 2013). Consequentemente, a diminuição de p53 leva a proliferação celular e aumento de PCNA (CAI; SUN; LI, 2014; QIN *et al.*, 2016).

2.4.3 p21

P21 é um inibidor de ciclina dependente de quinase (CKI), responsável pela saída da fase S do ciclo celular para a diferenciação celular (NGUYEN; MÜNGER, 2008). Nos primeiros estudos, p21 foi colocado como supressor tumoral em diversos tipos de tumores, como cérebro, pulmão e cólon (SHAMLOO; USLUER, 2019). Sua expressão é estimulada por p53 e além de estar envolvido com o ciclo celular, ele tem ainda outras funções como senescência, de forma dependente e independente de p53. Através da atividade de p53, p21 pode levar a parada do crescimento tumoral por inibição de CDKs, PCNA, fatores de transcrição e co-ativadores (ABBAS; DUTTA, 2009).

Curiosamente, p21 também pode favorecer o crescimento e motilidade das células tumorais. Através da indução de p21, a parada do ciclo celular diminui o acúmulo de dano no DNA e assim diminuem a morte celular (VIALE *et al.*, 2009). Desta forma, p21 pode atuar como supressor tumoral ou como ongogene dependendo da sua localização celular, sendo sua localização inclusive, associada com resistência a cisplatin (sua presença no núcleo leva a sensibilidade celular a Cisplatin e sua presença no citoplasma leva a resistência celular) (KOSTER *et al.*, 2010).

P21 pode ser ativado/inibido por diversas vias. Através da ativação da via PI3K-Akt ocorre a inibição de p21 e consequente aumento da proliferação celular. Essa via é estimulada pelas oncoproteínas E5 e E7 do HPV (FREITAS *et al.*, 2017). Esta

mesma via também estimula MDM2 que inibe p53 e essa inibição acaba por não estimular p21 e a célula continua proliferando, por não sofrer parada do ciclo celular nem morte programada. Outras duas vias também inibem a ação de p21, c-Myc e HDAC, esta última via como vimos anteriormente, é estimulada pela oncoproteína E7 do HPV via pRb (SHAMLOO; USLUER, 2019). Devido a suas funções, foi visto HPVs de alto e baixo risco carcinogênico atuam inibindo a ação de p21 para manter a célula em estágio de proliferação celular (SHAMLOO; USLUER, 2019).

Como mostrado acima, o HPV e suas oncoproteínas estão envolvidos em diversas vias que levam a promoção e/ou desenvolvimento do câncer, inclusive verificado no câncer de pulmão. Até o momento, apenas mais 1 trabalho foi feito no sudeste do brasil, avaliando a relação entre o HPV e o câncer de pulmão na população brasileira (SILVA *et al.*, 2019). Desta forma, é necessário o desenvolvimento de trabalhos nesse sentido para que possam ser traçadas estratégias adequadas de diagnóstico, prognóstico e tratamentos voltados para o câncer pulmonar.

3 METODOLOGIA

Objeto de estudo

Foram coletadas 63 amostras do Hospital uiversitário Oswaldo Cruz, inlcuindo amostras parafinadas e frescas. Para todas as coletas foi aplicado um formulário de conentimento. O presente estudo foi aprovaod pelo comitê de ética da Universidade Federal de Pernambuco (CAE 06396812.0.3001.5192).

Extração de material genético

O DNA e RNA das amostras foram extraídos atraves de kit específico QIAmp DNA FFPE tissue kit (QIAGEN), Dneasy Blood and Tissue kits (QIAGEN) e RNeasy tissue kit (QIAGEN), de acordo com as instruções do fabricante. O detalhamento consta nos resultados e artigo publicado (Apêndice A).

Detecção, genotipagem do HPV e expressão gênica

Foi realizada uma PCR convencional para detecção da presença do HPV nas amostras, atraves da amplificação da região L1 do vírus. Foi usado primers consenso e degenerados MY09/MY11 e GP5/GP6 (SILVA *et al.*, 2009).

Em seguida, as amostras positivas foram genotipadas atraves de sequanciamento pelo ABI PRISM Big dyeTM Terminator Cycle sequencing v 3.1 ready reaction kit (Applied Biosystems).

Todos os experimentos de expressão gênica das oncoproteínas E5, E6 e E7 e dos genes humanos p16, p21, PCNA, EEF1A1 e ACTB foram feitos por PCR quantitativa em tempo real no Rotor gene (QIAGEN). Logo em seguida foi feita a análise extatistica aplicando o teste de Shapiro-Wilk para avaliar a normalidade dos dados. Após, foram usados os testes Kruskal-Wallis, comparação de Dunn’s, Mann-Whitney para comparação dos alvos e o Spearman para avaliação da correlação.

4 RESULTADOS

4.1 THE EFFECT OF HPV INFECTION IN LUNG CANCER GENES EXPRESSION

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ABSTRACT

Purpose: Evaluate if the presence of Human Papillomavirus (HPV) and the expression of viral oncoproteins in lung cancer biopsies is changing the expression of p16, p21 and PCNA, key human genes involved in cell proliferation and tumor development. In cervical cancer the p16 alteration is a biomarker of HPV infection and p21 and PCNA genes disruption is also closely related to tumor development. *Methods*: The detection of HPV was performed by PCR, followed by genotype The expression of p16, p21 and PCNA was detected by qPCR in both fresh and paraffin-embedded from 63 patients, and the expression of E5, E6 and E6 were quantified by qPCR in 19 fresh samples. *Results:* HPV was found to be present in 47 of the 63 samples. E5, E6 and E7 were quantified in fresh samples and correlated with the expression of PCNA, p21 e p16. All targets also showed differential expression pattern between HPV+ and HPV- samples and correlation to E5, E6 and E7 expression. Conclusions: Our results suggest that the Brazilian lung tumor patients have high prevalence of HPV and the virus is not only present but also altering key human genes involved in tumor formation.

KEYWORDS: Lung carcinoma; *Human Papillomavirus;* oncoproteins; PCNA; p21, p16.

INTRODUCTION

It is well known the relation between the *Human papillomavirus* (HPV) infection and cervical tumor development, however other types of tumors have been associated to the action of HPV (COPPOCK; LEE, 2016; RAGIN *et al.*, 2014). The virus can cause disruption of several cell pathways through its oncoproteins E5, E6 and E7 expression. One of these pathways is the cell cycle, leading to cell proliferations and stop of cell differentiation (ARREGUI, 2012).

Since the HPV requires the host cell DNA replication machinery to its progeny production, the virus must ensure that the S-phase of cell cycle continues and to do so several key molecules suffers alteration. The Cyclin-dependent kinase inhibitor (CKI) p21 and p16 are two of the disrupted factors (KURSHUMLIU; THORNS; GASHI-LUCI, 2009; NGUYEN; MÜNGER, 2008) as well as the proliferating cell nuclear antigen (PCNA) in order to maintain the cell in constant proliferation..

The p16 gene (INK4A/ CDKN2A) in normal conditions is activated to cause G1 phase arrest through binding with cyclin-dependent kinase (CDK4 and CDK6) and inhibition of retinoblastoma protein (pRB) phosphorylation (MISSAOUI *et al.*, 2018). If there is any damage in DNA the p16 protein continues to be expressed until the cell is lead to senescence (LAPAK; BURD, 2014). Because of its action, p16 is inhibited in several types of cancer, including lung cancer, through methylation and deletions (ROCCO; SIDRANSKY, 2001; SUN *et al.*, 2004; VAISSIÈRE *et al.*, 2009). However, in cervical cancer and HPV infection, p16 expression is increased by the action of E7 oncoprotein, which binds to pRB and set E2F transcription factor free to activate p16 expression. Through other means, the HPV also ensures that p16 doesn’t complete its function although its levels are high (ZUR HAUSEN, 2002).

Similar mechanisms occur with p21. This gene is responsible for the transition of the S phase of cell cycle to a cell differentiation stage and for cellular senescence in a p53-dependent and independent manner. Due to that its expression was considered a tumor suppressor in several tumors like brain, lung and colon (SHAMLOO; USLUER, 2019). P21 can stop tumoral growth by p53 pathway through inhibition of CDKs, PCNA, transcription factors and co-activators (ABBAS; DUTTA, 2009). By the PI3K-AKt pathway activation, p21 is inhibited and this path is stimulated by E5 and E7

oncoproteins from HPV. The same pathways also stimulates MDM2 and inhibits p53 and consequently, inhibits p21 (FREITAS *et al.*, 2017). E7 can also decreases p21 expression through c-Myc and HDAC activation (pRB path) (SHAMLOO; USLUER, 2019).

As well as both previous genes, PCNA is also important in cancer environment. It is involved in DNA replication by interaction with DNA polymerase; gene expression control through DNMT1, HDAC1 e p300 interaction; DNA repair through Msh3/Msh6 proteins; and cell cycle control through p21 (TAN *et al.*, 2012). PCNA is overexpressed in several tumors like breast, liver, prostate, colon, lung and head and neck, and it is a prognostic marker of aggressiveness as well as a therapeutic target (CHANDRASHEKAR *et al.*, 2015; HAMZAOUI *et al.*, 2015; HU *et al.*, 2017; WEN *et al.*, 2018; ZHOU *et al.*, 2018). This gene has been studied for over than 20 years and pointed as a biomarker for cancer alterations but until our knowledge there is no study about PCNA and HPV infection in lung cancer samples. In cervical cancer, PCNA overexpression was associated to differentiation of cervical lesions, metastasis, tumor invasiveness and HPV infection (LUO *et al.*, 2017).

As mentioned above, HPV and its oncoproteins are directly or indirectly involved with the alteration of p16, p21 and PCNA, but none of this was evaluated together in lung cancer samples infected by high-risk HPV. We aimed to analyze if the HPV and its oncoproteins are causing the genes disruption or at least helping to increase their alteration in lung cancer.

MATERIALS AND METHODS

Study Subject

All the clinical samples were obtained from patients of the pneumology sector of the Oswaldo Cruz University Hospital, located in the Campus of Pernambuco University, in Pernambuco, Brazil. This study enrolled 63 lung biopsies, 44 archived paraffin embedded (FFPE) and 19 fresh samples. The mean age of the patients was 55 years (range 11-81ys). At the time of enrollment, the patient’s consent was obtained. The study protocol was approved by the Federal University of Pernambuco ethics committee (register number 06396812.0.3001.5192).

Extraction of DNA

The DNA was extracted and deparaffinized from the sections, using the QIAamp DNA FFPE tissue kit (QIAGEN) and xilol (30min at 65°C), respectively. Fresh biopsies (25-100mg) were macerated and homogenized using liquid nitrogen and 1ml de Trizol (Invitrogen). Then, DNA was purified using DNeasy Blood & Tissue Kits (Qiagen) The DNA was quantified by spectrophotometry (nanodrop LITE - Thermo Scientific). The quality control of the DNA samples was accomplished by checking for the successful amplification of human beta-globin gene by PCR technique (*polymerase chain reaction),* using the primers 5'-ACACAACTGTGTTCACTA-3' e 5'GGTGAACGTGGATGAAGTTG-3' (Baldez da Silva *et al.*, 2009). Meticulous care was taken to free the samples of any contamination (appropriate individual protection equipment, extensive cleaning of the work area and analyst, UV light irradiation to avoid double-stranded DNA contamination, few samples manipulated at a time, negative control for each extraction and all work was performed inside the laminar flow cabin).

HPV Detection

The HPV detection was performed by PCR assay using two sets of consensus primers, MY09/MY11 (forward: 5'- CGTCCMARRGGAWACTGATC-3' and reverse: 5'-GCMCAGGGWCATAAYAATGG-3`) and GP5/GP6 (forward: 5'-TTTGTTACTGTGGTAAG-3' and reverse: 5'- GAAAAATAAACTGTAAATCA-3')(Roda Husman, De *et al.*, 1995), which amplify a region in the highly conserved L1 gene. The PCRs reaction mixture contained 2,0 µL of sample (contained 100 ng of DNA), 20pmoles of each oligonucleotides, 0.1mM of each dNTP, 1,0 µM of each primer and 1,0 U Taq DNA polymerase (Qiagen) in a total volume of 25µL of reaction buffer (50 mMKCl, 10mM Tris HCl and 0,1%Triton X-100). The following "hot start" protocol was used for the amplification of the gene: initial denaturation at 95° C for 5 min; subsequent 35 cycles at 95° C for 30 seconds, 55° C for 40 seconds and 72° C for 45 seconds and final extension at 72°C for 5 min. The PCR products were detected

through electrophoresis on 2,0% agarose gel. The same protocol mentioned above to avoid contamination was used, including negative controls for the PCR.

HPV genotyping

All the HPV-positive samples were subjected to genotyping using ABI PRISM Big DyeTM Terminator Cycle Sequencing v 3.1 Ready Reaction kit (Applied Biosystems). To compare de DNA sequences the BLAST program available at http://blast.ncbi.nlm.nih.gov/Blast.cgi was used.

RNA extraction and cDNA synthesis

Total RNA extraction was extracted and deparaffinized from the sections, using the RNeasy tissue kit (QIAGEN) and xilol (30min at 65°C), respectively. Fresh biopsies (25-100mg) were macerated and homogenized using liquid nitrogen and 1ml de Trizol (Invitrogen). Quality of the RNA was assured by a NanoDrop 2000 Spectrophotometer (ThermoScientific Wilmington, USA) and electrophoresis on a 1% agarose gel (Bustin, 2010; Conceicao Gomes Leitao, Da *et al.*, 2014; Rueda-Martínez *et al.*, 2014). Next, 500 ng of purified RNA of adequate quality (an OD260/280 from 1.8 to 2.1 and intact rRNA subunits - 28S and 18S) was used to synthesize cDNA by means of FIREScript RT cDNA Synthesis KIT (Solis BioDyne) following the manufacturers instruction. For each sample a negative control RT reaction (no Reverse Transcriptase enzyme) was prepared.

Primers: design and efficiency estimation for qPCR

Primers for detection of E5, E5 and E7 from HPVs detection were designed through CLCbio Main Workbench software version 5.7.1 (QUIAGEN) as follow: E5 HPV-16 (F:ACTGGCTGCTTTTTGCTTTG and R:GACACAGACAAAAGCAGCGG); E5 HPV-18 (F:CGCTTTTGCCATCTGTCTGT and R:ACACAAATACCAATACCCATGC) ; E6 HPV-16 (F:TGCAATGTTTCAGGACCC and R: CATAACTGTGTGGTAACTTTCTGGG) ;E6 HPV-18 (F:CTCTACTTGTCATCCGTCTGT and R:ACGCATATACCGATACTCATGC) ; E7 HPV-16 (F: AGCTCAGAGGAGGAGGATGA and R: GAGACCAGATGGGGCACACA)

; E7 HPV18 (F:ATATCCTGCTATCAGTCTGT and R:ACGCGAATACCTTATATCCATGC). For the target human genes, the primers were: p21 (F: ATGTCCGTCAGAACCCATGC and R: CCATTAGCG CATCACAGTCG); p16 (F: ACATCCCCGATTGAAAGAACC and R: ATGAAA ACTACGAAAGCGGGG); PCNA (F: AGACTTTCCTCCTTCCCGC and R: AGT GCCTCCAACACCTTCT). The Reference genes used for relative quantification were EEF1A1 (F: GTTGCGGTGGGTGTCATCA and R: GAGTGGGGTGGC AGGTAT) and ACTB (F: AAGAGAGGCATCCTCACCCT and R: TACATGGCTGGGGTGTTGAA), previously validated in lung tissues (ZHAN *et al.*, 2014). Primer pairs efficiency were evaluated by serial dilution of 10 potency and was used an actual cDNA of a positive lung sample to exemplify the real assay condition.

Real-time qPCR

All viral oncogenes and human genes were quantified using QuantiTect SYBR Green PCR kit (Qiagen) and the amplification performed by Rotor Gene 6000 thermocycler (Qiagen, Hilden, Germany). The cycling conditions were: 95°C for 12 minutes for polymerase activation, 94°C for 15 seconds for denaturation, 60 seconds of annealing temperature (see table 1), 35 cycles, and 72°C for 60 seconds for extension. This way, the geometric mean of EEF1A and ACTB reference genes was used to calculate the relative expression of all targets (LIVAK; SCHMITTGEN, 2001).

In order to detect and quantify E5, E6 and E7 from the FFPE samples we performed a nested PCR based on two previous works (TAWE *et al.*, 2018; TRAN *et al.*, 2014). The First was a conventional PCR as follows: 95°C for 5 minutes, 15 cycles of 94°C for 15 seconds and 60 seconds of annealing (table 1), and 72°C for 60 seconds. Then, with 1ul of the PCR product we performed the Real-time PCR following the condition mentioned on the previous paragraph.

Every qPCR reaction was performed in duplicate for each sample (NOLAN; HANDS; BUSTIN, 2006). Additionally, no template controls were added to detect contamination.

Table 1 – Annealing temperatures used for qPCRs.

Gene	Annealing temperature
E5 HPV-16	60°C
E5 HPV-18	58°C
E6 hPV-16	58°C
E6 HPV-18	53°C
E7 HPV -16	56°C
E7 HPV-18	58°C
p21	60°C
P16	60°C
PCNA	60°C
EEF1A1	60°C
ACTB	60°C

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 8.3.0) software. Shapiro-Wilk test was made to determine if the data has or has not a Gaussian distribution. Kruskal-Wallis test, Dunn's comparison test and Mann-Whitney test were conducted to compare the expression levels of the genes in different types of lung cancer and in the presence and absence of HPV infection. Correlation between the gene's expression was evaluated by the Spearman correlation test. P-values lower than 0.05 were considered statistically significant.

RESULTS

HPV detection and sample characterization

Sixty-three patients were included in this study. The mean age of the patients was 55 years and the median were 59 years, with a range of 11 to 81 years old.

A total of 33 from 44 paraffin-embedded samples were positive for high risk HPV (hrHPV+) at base line screening by conventional PCR. In the genotype step, HPV 16 was the most frequent, representing 81,81% (27/33) of the positive samples, while the HPV 18 was 18,19% (6/33). The 19 fresh samples were also analyzed separately, and 5 samples were positive for hrHPV in the first screening. 80% (4/5) positive for HPV 16 and 20% (1/5) positive for HPV 18.

The higher prevalence of HPV was in squamous cell carcinoma (39,39%), followed by adenocarcinoma (33,33%) and small cell carcinoma (18,18%) and large cell carcinoma (9,1%), including all samples.

HPV Oncoproteins detection

Unfortunately, perhaps due to viral load or viral DNA degradation, we were not able to properly quantify the oncoproteins in FFPE samples, so we used the HPV detection results to evaluate the expression profile of human genes in HPV + and HPV- sample groups.

On the other hand, we analyzed the expression of E5, E6 and E7 oncogenes in all HPV+ and HPV- fresh samples (19 samples) (Table 3). 12/19 were positive for E5, 2/19 were positive for E6 oncogene and E7 was detected in 5/19 samples, and all five were previously negative for HPV by the conventional PCR results (table 2). Based on the detection of the oncoproteins, we separate again the fresh samples in positive and negative for HPV infection (5 HPV – and 14 HPV +), and the prevalence of HPV in all types of tumor in both fresh and FFPE samples (table 3).

Table 2. Oncoproteins mRNA expression in fresh biopsies compared with previous results of HPV detection by PCR.

Oncogenes	HPV POSITIVE (MY)	HPV NEGATIVE (MY)
E5	4	4
E6	1	
E7		1
E5 + E7		3
E5 + E6 + E7		1

Table 3. Histopathological classification and HPV type through DNA sequencing.

Tumor type	Subtype	HPV 16	HPV 18
Non-small cell carcinoma	Squamous cell carcinoma	14 (36,84%)	2 (5,26%)
	Adenocarcinoma	11 (28,95%)	2 (5,26%)
	Large cell carcinoma	1 (2,63%)	2 (5,26%)
Small cell carcinoma		5 (13,16%)	1 (2,63%)
		38 total positive samples	

Individual human genes expression: p16, p21 and PCNA.

p16 expression was analyzed in all fresh and FFPE samples. It was positive in 7/19 fresh samples, 5 were HPV+ and 2 were HPV -. The expression was higher in HPV + samples.

In FFPE samples we were able to perform a more extensive analysis due to the number of samples. p16 was positive for all 33 HPV+ and for 10 of HPV- FFPE samples, and the medians were almost the same between the groups. When placed together, all the fresh and FFPE samples still no statistical difference was found for p16 expression between HPV+ and HPV- groups. p16 was positive in 50/63 samples, 38/50 in HPV+ and 12/50 in HPV- samples. Almost no difference was found between the groups.

The p16 expression also varied from the different types of tumors. It showed higher expression in Squamous cell carcinoma, followed by large cell carcinoma, Small cell carcinoma and Adenocarcinoma (Figure 1).

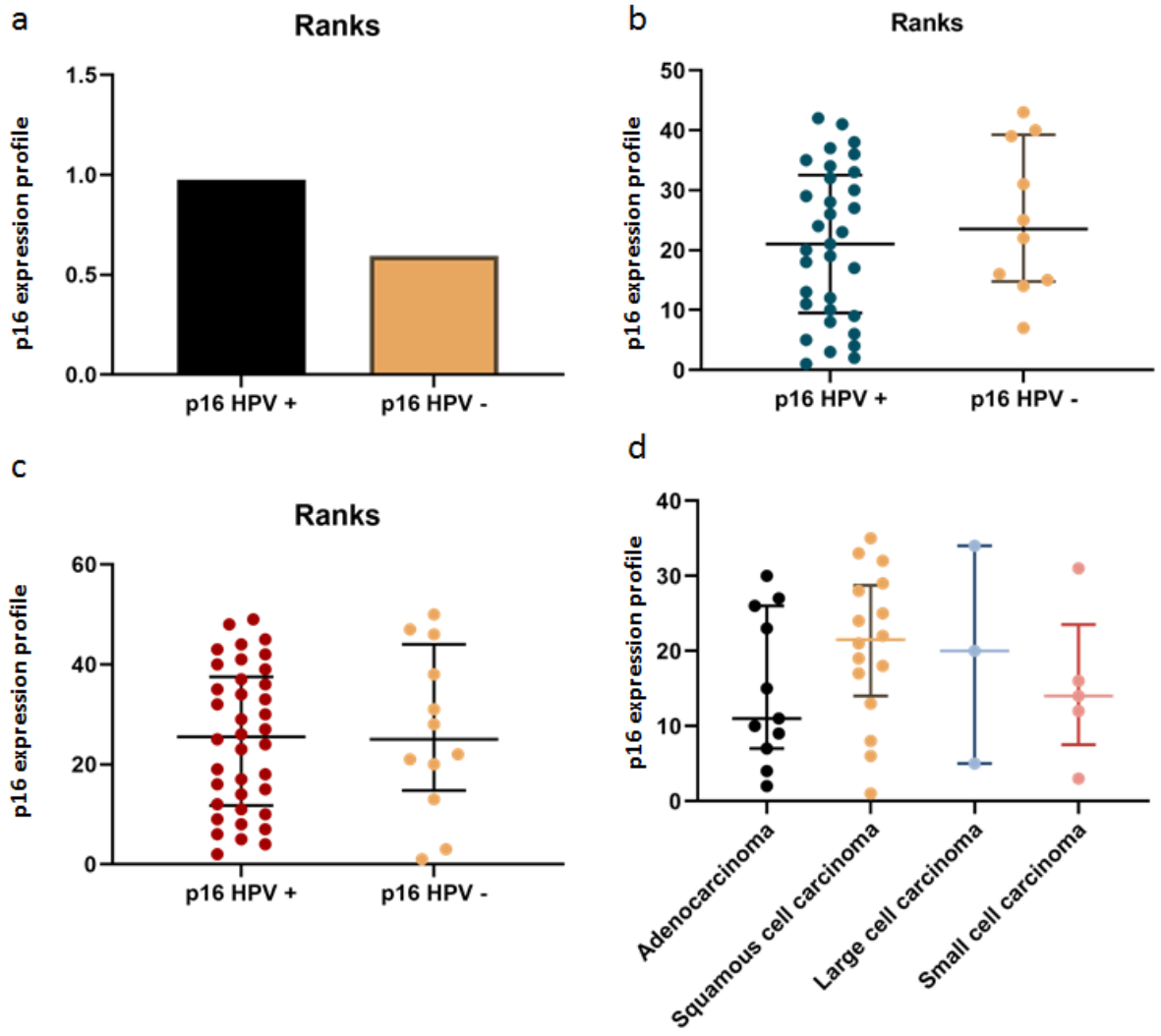

Figure 1 - p16 expression profile. (a) p16 expression in HPV+ fresh samples. Mann-Whitney test was performed to compare the differences between both groups. No statistical significance was found for p<0,05 and the medians were 0,9752 for HPV + and 0,5960 for HPV -; (b) p16 expression in HPV+ and HPV- FFPE samples. Ranks Plot from Mann-Whitney U test with median and interquartile range. The medians were 45,20 for HPV+ and 46,81 for HPV- and no statistical significance was found for p<0,05; (c) p16 expression in HPV+ and HPV- FFPE and fresh samples. The medians were 41,32 for HPV+ and 39,16 for HPV- and no statistical difference was found for p<0,05; (d) Fig. 4 p16 expression difference in all types of tumors – median and interquartile rank analysis by Kruskal-Wallis test. The medians of p16 expression was: Adenocarcinoma 32,33; Squamous cell carcinoma 53,10; Large cell carcinoma 50,01; Small cell carcinoma 42,92. No significant statistical difference was found for p<0,05.

p21 expression was obtained from 16 fresh samples, 12/16 were HPV+ and 4/16 were HPV-, but the HPV- samples presented higher p21 expression median than HPV+. In turn, p21 was detected in all FFPE samples. 33/44 were HPV+ and 11/44 were HPV-. As well as in fresh samples, the p21 expression median was higher in HPV- than HPV+ FFPE samples. When analyzed together, fresh and FFPE samples, 45/60 were HPV+ and 15/60 HPV-, and p21 still presented higher median rate in HPV – group.

As well as p16, p21 analysis in FFPE samples were more extensive due to the number of samples. The expression profile was analyzed among the tumor types, and the median expression was higher in squamous cell carcinoma, followed by Adenocarcinoma, Small cell carcinoma and Large cell carcinoma, but no significant difference was found for $p<0,05$ (Figure 2).

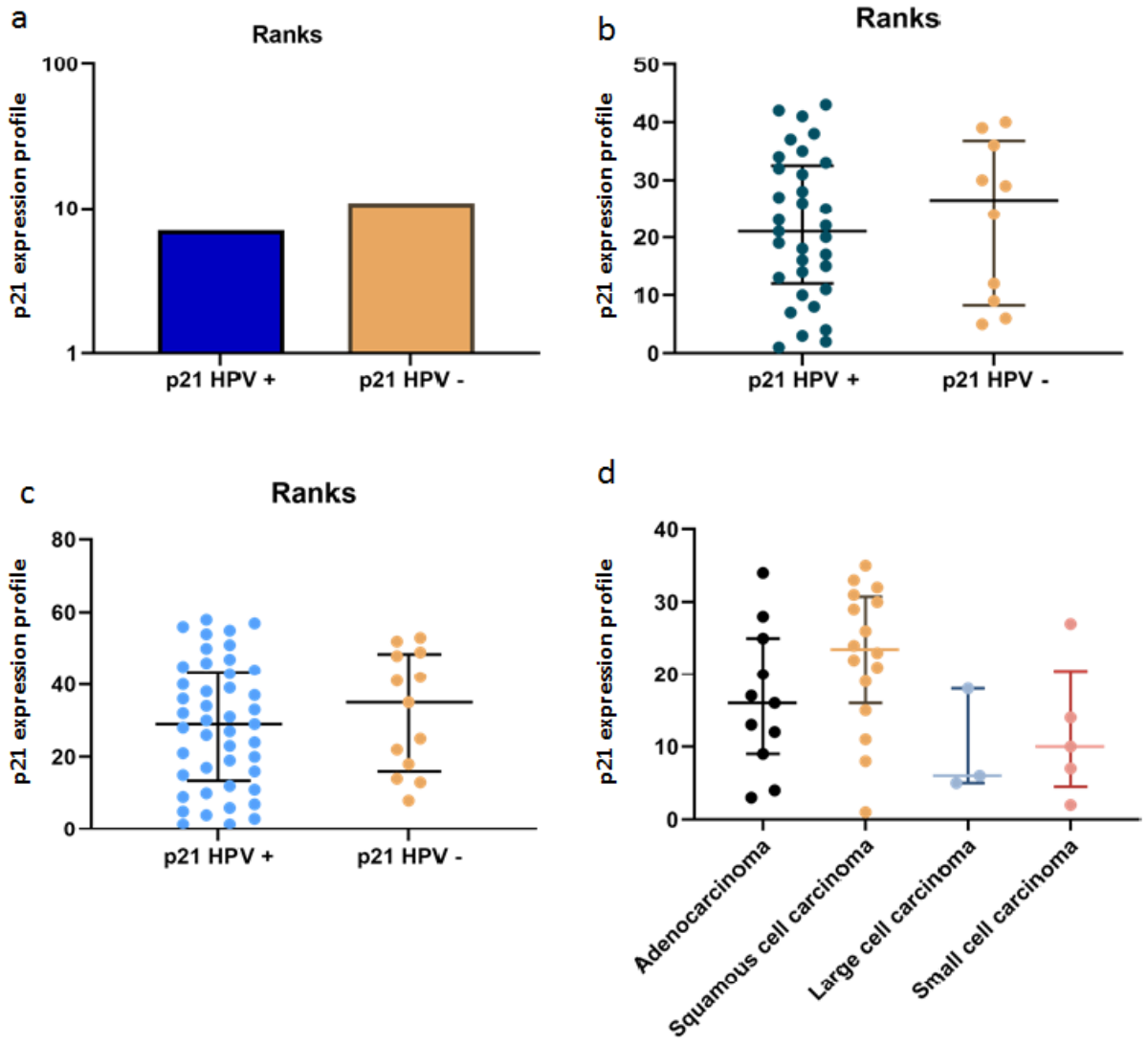

Figure 2 - (a) p21 expression in HPV+ and HPV- fresh samples. Ranks Plot from Mann-Whitney test with median and interquartile range. The expression medians were 0,835 for HPV+ and 5,120 for HPV- and no statistical significance was found for p<0,05; (b) p21 expression in HPV+ and HPV- FFPE samples. Ranks Plot from Mann-Whitney test with median and interquartile range. The expression medians were 6,703 for HPV+ and 8,553 for HPV- and no statistical significance was found for p<0,05; (c) p21 expression in HPV+ and HPV- in FFPE and fresh samples. The expression medians were 6,216 for HPV+ and 7,008 for HPV-. Mann-Whitney analysis was performed, and no statistical difference was found for p<0,05; (d) p21 expression profile distribution in all types of tumors – median and interquartile rank analysis by Kruskal-Wallis test. The p21 expression medians were: Adenocarcinoma 6,548; Squamous cell carcinoma 10,160; Large cell carcinoma 3,279; Small cell carcinoma 4,213. No significant difference was found for p<0,05.

The same analysis was performed for PCNA expression. PCNA expression was obtained in 15/19 fresh biopsies. 3/15 were HPV - and 12/15 were HPV +. PCNA median was just a little higher in HPV+ than HPV- samples but with no significant difference. In FFPE samples, PCNA was detected in 33/44 HPV+ and 11/44 HPV- samples, and the expression median was higher in HPV- than HPV+ samples.

In FFPE and fresh samples the PCNA expression was obtained from 59/63 samples, 45/59 HPV+ and 14/59 HPV-. The median expression was higher in HPV- samples. The PCNA expression distribution between the different tumor types showed higher expression in Squamous cell carcinoma followed by Small cell carcinoma, Large cell carcinoma and Adenocarcinoma. Even so, no significant statistical difference was found (Figure 3).

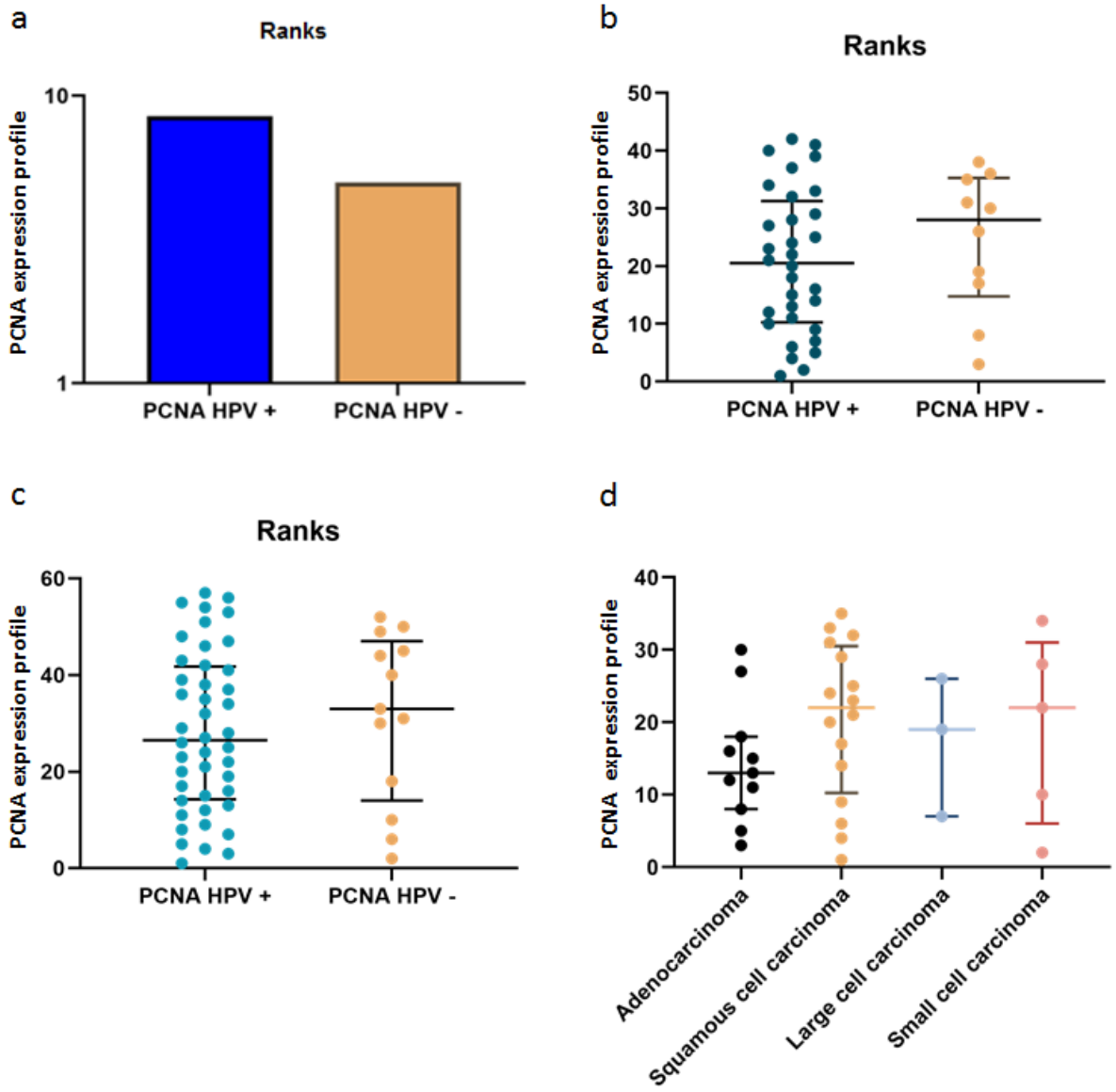

Figure 3 - (a) PCNA expression in HPV+ and HPV- fresh samples. Ranks Plot from Mann-Whitney test with median and interquartile range. No statistical significance was found for p<0,05 between the medians: 0,0807 for HPV+ and 0,0418 for HPV -; (b) PCNA expression in HPV+ and HPV- FFPE samples. Ranks Plot from Mann-Whitney test with median and interquartile range. No statistical significance was found for p<0,05 between the medians: 1,682 for HPV+ and 2,103 for HPV-. (c) PCNA expression in HPV+ and HPV- FFPE and fresh samples. The expression medians were 0,5560 for HPV+ and 1,546 for HPV-. Mann-Whitney analysis was performed, and no statistical difference was found for p<0,05. (d) PCNA expression distribution in all types of tumors – median and interquartile rank analysis by Kruskal-Wallis test. Medians of PCNA expression were: Adenocarcinoma 1,231; Squamous cell carcinoma 2,293; Large cell carcinoma 1,746; Small cell carcinoma 2,240. No significant difference was found for p<0,05.

Comparison between viral oncogenes and human genes expression

We were able to analyze the relative expression of E5, E6 and E7 in fresh samples, even the samples with small quantities. After that, we compared the expression of the viral oncogenes to p16, p21 and PCNA. There was significant statistical difference between PCNA and E6; p21 and E6; p21 and E7; E5 and p16; E6 and p16; E7 and p16 (Figure 4).

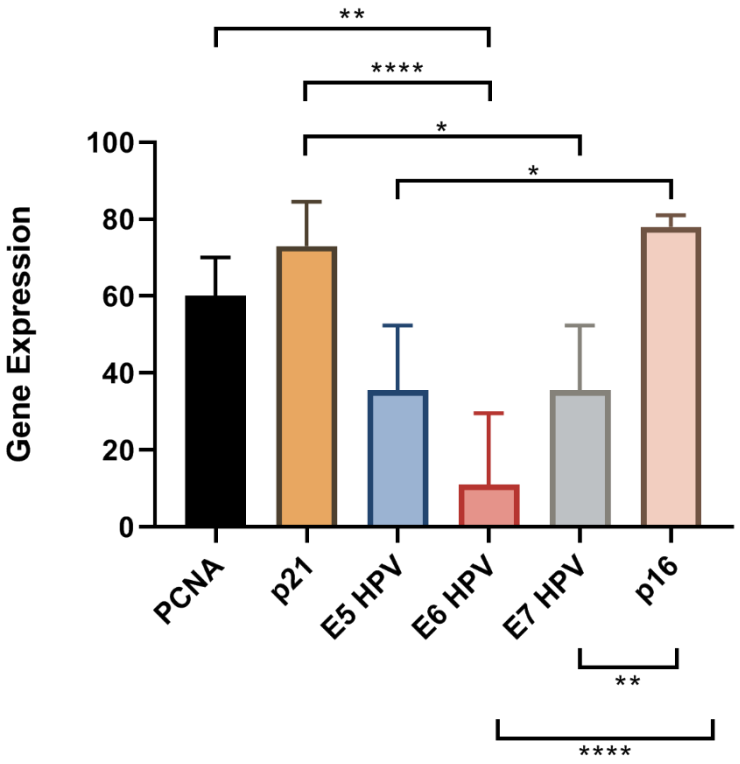

Figure 4 - Differences between the expression of viral oncogenes and human genes in fresh samples. Kruskal-Wallis test was applied with Dunn's comparison test. It was found significant expression difference between PCNA and E6; p21 and E6; p21 and E7; E5 and p16; E6 and p16; E7 and p16. P value: * <0,05; ** <0,01; *** <0,001; **** <0,0001.

In FFPE samples the viral oncogenes were not able to be detected, but we compared the human genes to each other (Figure 5) and according to HPV status (Figure 6). Significant statistical differences were found comparing p21 and p16; p21 and PCNA; p16 and PCNA; PCNA HPV + and p21 HPV +; PCNA HPV + and p16 HPV +; PCNA HPV + and p16 HPV -; PCNA HPV - and p16 HPV +; PCNA HPV - and p16

HPV -; p21 HPV + and p16 HPV +; p21 HPV + and p16 HPV -; p21 HPV - and p16 HPV +.

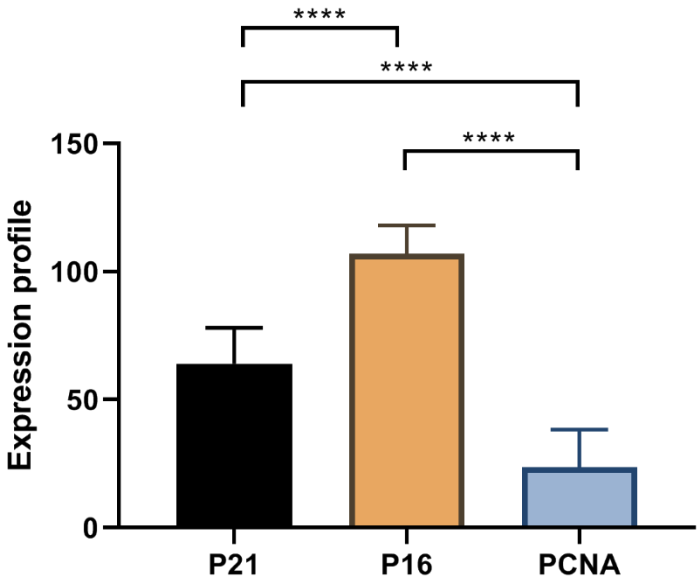

Figure 5 - Different expression pattern between p16, p21 and PCNA in FFPE samples. Significant statistical differences were found between p21 and p16; p21 and PCNA; p16 and PCNA. P value: * *<0,05; ** <0,01; *** <0,001; **** <0,0001.

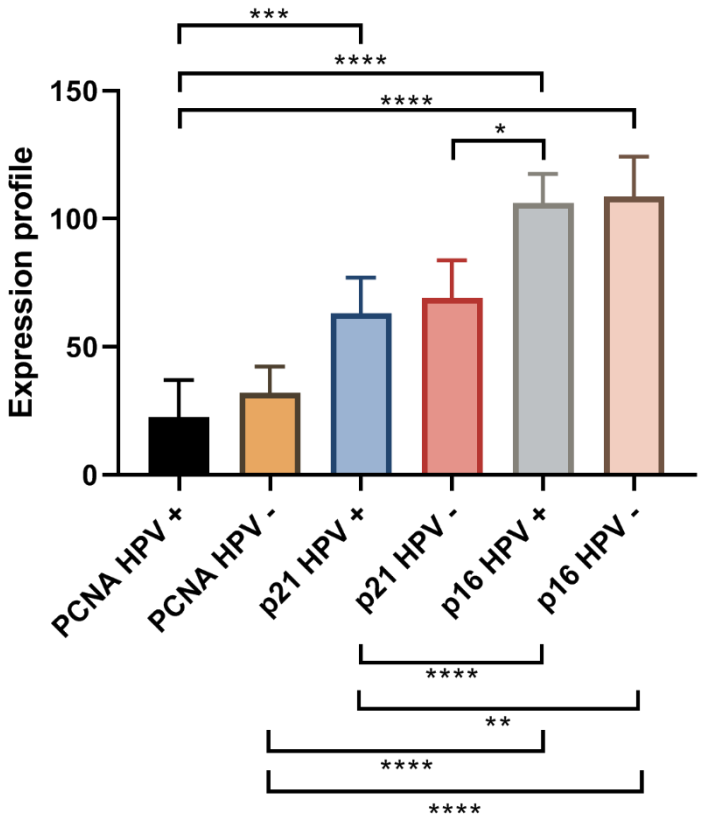

Figure 6 - Different expression pattern between p16, p21 and PCNA in HPV+ and HPV- FFPE samples. Significant statistical differences were found between PCNA HPV + vs. p21 HPV +; PCNA HPV + vs. p16 HPV +; PCNA HPV + vs. p16 HPV -; PCNA HPV - vs. p16 HPV +; PCNA HPV - vs. p16 HPV -; p21 HPV + vs. p16 HPV +; p21 HPV + vs. p16 HPV -; p21 HPV - vs. p16 HPV +. P value: * *<0,05; ** <0,01; *** <0,001; **** <0,0001.

The same statistic evaluation was done to FFPE and fresh samples together (Figure 7). All the human genes were compared according to the HPV positivity status. Significant statistical differences were found between: PCNA HPV + vs. p21 HPV +; PCNA HPV + vs. p21 HPV -; PCNA HPV + vs. p16 HPV +; PCNA HPV + vs. p16 HPV -; PCNA HPV - vs. p21 HPV +; PCNA HPV - vs. p21 HPV -; PCNA HPV - vs. p16 HPV +; PCNA HPV - vs. p16 HPV -; p21 HPV + vs. p21 HPV -; p21 HPV + vs. p16 HPV +.

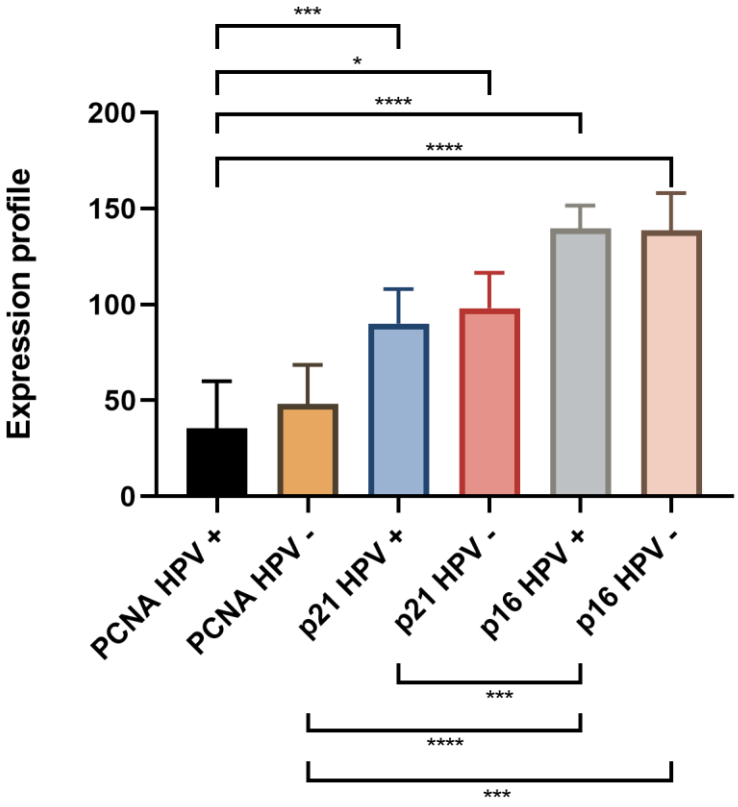

Figure 7 - Different expression pattern between p16, p21 and PCNA among HPV + and HPV- FFPE + fresh tumor samples. Significant statistical differences were found between: PCNA HPV + vs. p21 HPV +; PCNA HPV + vs. p21 HPV -; PCNA HPV + vs. p16 HPV +; PCNA HPV + vs. p16 HPV -; PCNA HPV - vs. p21 HPV +; PCNA HPV - vs. p21 HPV -; PCNA HPV - vs. p16 HPV +; PCNA HPV - vs. p16 HPV -; p21 HPV + vs. p21 HPV -; p21 HPV + vs. p16 HPV +. P value: *<0,05; ** <0,01; *** <0,001; **** <0,0001.

Correlation Analysis

All targets showed different expression patterns, and after that we verified if all targets and oncoproteins were correlated.

In fresh biopsies it was found a strong positive relation (both targets have direct relation to each other) between PCNA and E5; p21 and p16; E5 and E7. Also found a weak positive relation between PCNA and E6; PCNA and E7; PCNA and p16; E5 and E6; E5 and p16; E7 and p16. Between E6 and p21 was found a stronger negative correlation (Both targets have an inverse relation to each other) and a weak negative correlation between PCNA and p21; p21 and E5; p21 and E7; E6 and E7 (very weak negative correlation).

The statistically significant correlations were between PCNA and E5; PCNA and E7; p21 and E5; p21 and E6; p21 and p16; E5 and E7 (Figure 8).

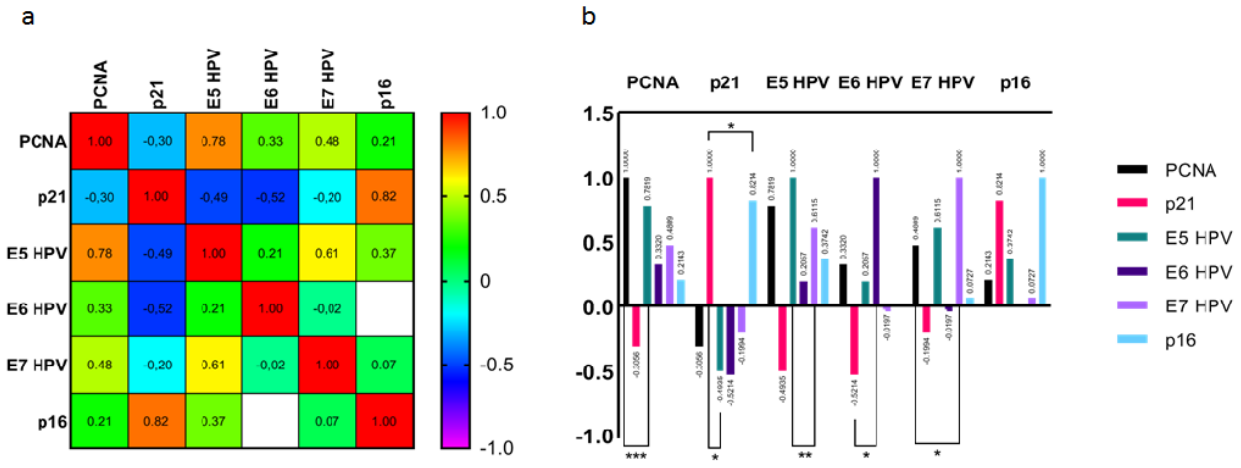

Figure 8 – Correlation analysis in fresh samples. (a) Heatmap of Spearman's correlation test between E5, E6, E7, p21, p16 and PCNA in fresh samples. r coefficient is placed inside the cells with a variation from 1 (perfect positive correlation) to -1 (perfect negative correlation); (b) Correlation between all targets in fresh samples. Each gene section shows the positive (r=1) or negative (r=-1) correlation coefficient in the bar notation of the analyzed target. P value: *<0,05; ** <0,01; *** <0,001; **** <0,0001.

The correlation between p21, p16 and PCNA genes were also evaluated in FFPE samples. Only positive relations were obtained, and all with statistical significance (figure 9).

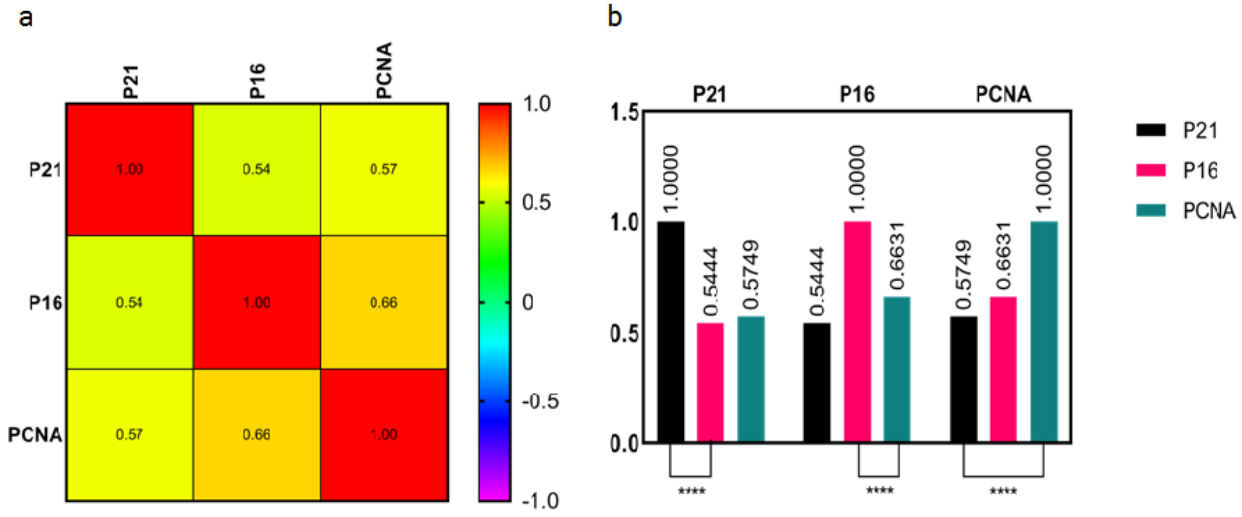

Figure 9 – Correlation analysis in FFPE samples. (a) Heatmap of Spearman's correlation test between p21, p16 and PCNA in FFPE samples. r coefficient is placed inside the cells with a variation from 1 (perfect positive correlation) to -1 (perfect negative correlation); (b) Correlation between p16, p21 and PCNA in FFPE samples. Each gene section shows the positive (r=1) or negative (r=-1) correlation coefficient in the bar notation of the analyzed target. P value: *<0,05; ** <0,01; *** <0,001; **** <0,0001.

The correlation was observed also in HPV+ and HPV- FFPE samples and was found a strong positive correlation between PCNA HPV + and p21 HPV +; PCNA HPV + and p16 HPV +; PCNA HPV - and p16 HPV -; PCNA HPV- and p21 HPV -; p21 HPV + and p16 HPV +; p21 HPV- and p16 HPV -. Besides, it was found a strong negative correlation between p21 HPV- and p16 HPV + ; and a light negative correlation between PCNA HPV+ and PCNA HPV-; PCNA HPV + and p21 HPV-; PCNA HPV+ and p16 HPV; PCNA HPV- and p21 HPV+; PCNA HPV- and p16 HPV+; p21 HPV+ and p21 HPV-; p21 HPV+ and p16 HPV-; p16 HPV+ and p16 HPV-.

The statistically significant correlation results in FFPE samples was PCNA HPV + and p21 HPV +; p16 HPV+ and p16 HPV-; p21 HPV + and p16 HPV +; p21 HPV- and p16 HPV +; PCNA HPV + and p16 HPV + (Figure 10).

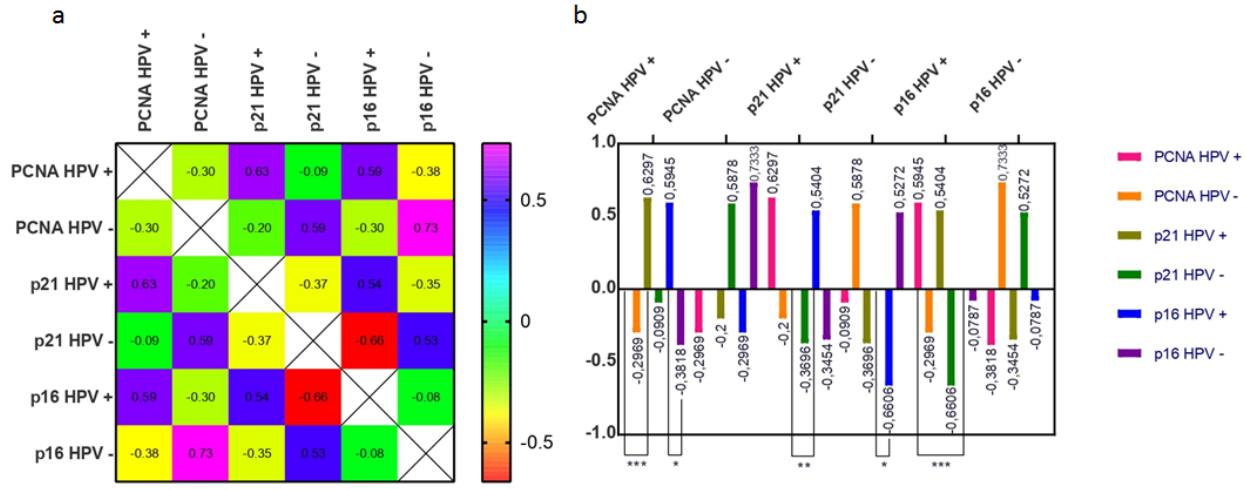

Figure 10 – Correlation analysis for HPV status in FFPE samples. (a) Heatmap of Spearman's correlation test between p21, p16 and PCNA in HPV+ and HPV – in FFPE samples. r coefficient is placed inside the cells with a variation from 1 (perfect positive correlation) to -1 (perfect negative correlation); (b) Correlation between p16, p21 and PCNA in HPV+ and HPV- in FFPE samples. Each gene section shows the positive (r=1) or negative (r=-1) correlation coefficient in the bar notation of the analyzed target. The missing bars on each section is correspond to the gene on the section name. P value: *<0,05; ** <0,01; *** <0,001; **** <0,0001.

At least we evaluated the correlation between p16, p21 and PCNA in HPV + and HPV- in all samples together (FFPE and Fresh). It was obtained a similar result to the analysis with only FFPE samples: strong positive correlation between PCNA HPV - and p16 HPV -. Light positive correlation between PCNA HPV + and p21 HPV +; PCNA HPV- and p21 HPV -; p21 HPV + and p16 HPV +; p21 HPV- and p16 HPV -; p16 HPV+ and p16 HPV-. Strong negative correlation between PCNA HPV + and p16 HPV +; p21 HPV- and p16 HPV +. Light negative correlation between PCNA HPV+ and PCNA HPV -; PCNA HPV + and p21 HPV-; PCNA HPV+ and p16 HPV-; PCNA HPV- and p21 HPV+; PCNA HPV- and p16 HPV+; p21 HPV+ and p21 HPV-; p21 HPV+ and p16 HPV- (Figure 14).

Among the results above, PCNA HPV + and p21 HPV +; p21 HPV + and p16 HPV +; PCNA HPV + and p16 HPV +; p21 HPV- and p16 HPV +; PCNA HPV- and p16 HPV- (Figure 15).

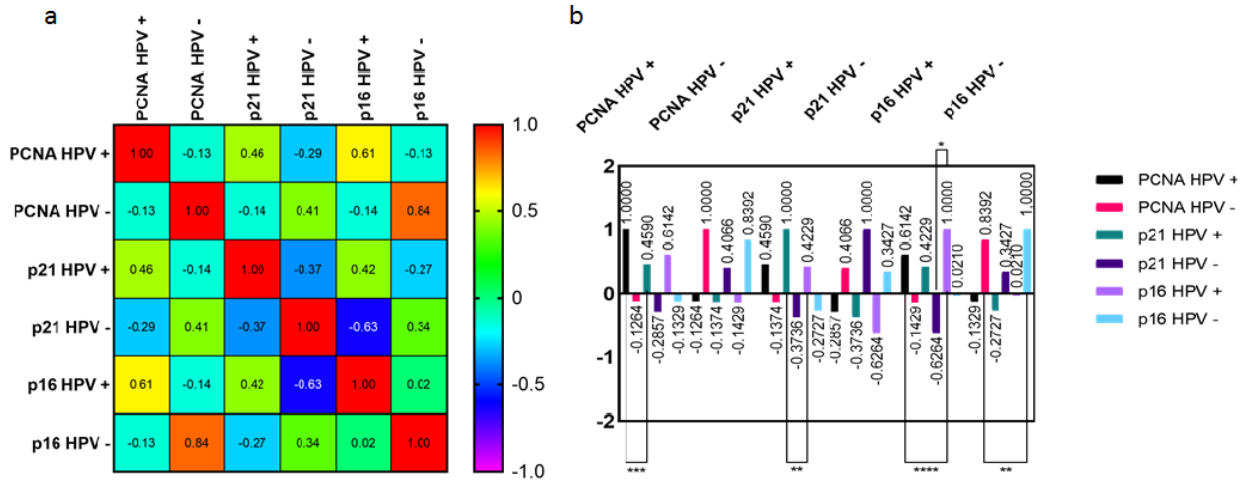

Figure 11 – Correlation analysis FFPE + fresh samples. (a) Heatmap of Spearman's correlation test between p21, p16 and PCNA in HPV+ and HPV – in FFPE and fresh samples. r coefficient is placed inside the cells with a variation from 1 (perfect positive correlation) to -1 (perfect negative correlation); (b) Correlation between p16, p21 and PCNA in HPV+ and HPV- in FFPE and fresh samples. Each gene section shows the positive (r=1) or negative (r=-1) correlation coefficient in the bar notation of the analyzed target. The missing bars on each section is correspond to the gene on the section name. P value: *<0,05; ** <0,01; *** <0,001; **** <0,0001.

DISCUSSION

In the past decade, the HPV was found in lung cancer patients and in some cases, expressing its early oncoproteins (LIU *et al.*, 2018). These oncoproteins are able to disrupt the activity of several host genes in order to maintain the host cells in constant proliferating state (ZUR HAUSEN, 2002). With this information in mind and using cervical cancer as basis for HPV infection environment, we analyzed a total of 63 samples to see if the HPV was not only present but also expressing its oncoproteins and perhaps altering the expression of p21, p16 and PCNA human genes.

In fresh lung carcinoma samples, we detected E5, E6 and E7 mRNA even in samples that were previously negative for HPV in baseline screening performed by conventional L1 region PCR amplification. This indicates that the detection of HPV should be performed at the oncogene region and not through L1 viral gene, for a more reliable detection. In fresh tissue the use of an L1 internal sequence may be a liability because this region can suffer a partial loss during viral genome integration into host DNA. That way, E6, E7 or E6/E7 gene detection may be a more secure choice.

However, in FFPE samples both amplified regions like E6/E7 and L1 sequences analysis can mislead the results due to DNA fragmentation, formalin-induced inhibition of PCR and/or cross-linking of DNA. However, both methodologies detection of small regions inside L1 and E6 or E7 is considered to be acceptable (PRAKASH *et al.*, 2016). Interestingly, E5 oncogene were the most frequent among the samples. This oncoprotein is also normally lost during the viral genome integration and is associated to early necessary actions to promote viral replication (DIMAIO; PETTI, 2013). Even so, E5 was also found in cervical cancer samples (previous unpublished work).

Because of the relation between HPV infection and p16 expression in cervical cancer, we analyzed its expression and relation to HPV presence and oncoproteins expression (KURSHUMLIU; THORNS; GASHI-LUCI, 2009). It is well described in the literature the inactivation of p16 function in several tumors, but in cervical cancer the p16 protein expression is augmented and accumulated in HPV infected cells. This accumulation is associated to E7 activity on pRb protein and the release of E2F transcription factor, which stimulates the expression of p16 gene. Perhaps due to the number of our samples, no correlation was found between E7 and p16, or between any other oncoprotein. Sill, p16 expression was higher in fresh HPV positive samples and in FFPE+fresh HPV positive samples. In FFPE it was found a significant negative correlation between the expression of p16 in HPV positive and p16 in HPV negative samples. This means that while p16 HPV+ tends to rise, p16 HPV- tends to descend.

p16 is a known marker of HPV infection and activity, therefore our results indicate that this target may has the same important value in distingue HPV infected lung cancer tissues. Furthermore, its diminished expression in non-small cell lung cancer due to methylation was associated to grater resistance to Paclitaxel-based chemotherapy, a first line of treatment of several types of cancer, including lung carcinomas (LIU *et al.*, 2019). P16 higher expression in HPV + samples rise the possibility of higher sensitivity to the chemotherapy approach.

Just like p16, p21 is also a CDK inhibitor and responsible for cell cycle arrest (NGUYEN; MÜNGER, 2008). Unexpectedly, both targets showed a positive correlation in fresh samples, FFPE samples and FFPE+fresh samples in the presence of HPV, and this means that p16 and p21 are “walking” in the same direction in a linear relation. Contradictorily our data showed diminished expression of p21 in HPV+ while p16 is

higher in those samples.

P21 presented higher expression patters in HPV negative samples in fresh, FFPE samples and FFPE+fresh samples. This gene can be stimulated or inhibited depending on which signaling path is influenced. In previous works, E5 and E7 stimulation on PI3K-Akt pathway caused inhibition of p21 (FREITAS *et al.*, 2017), also through E7 activity upon HDAC the oncoprotein led to decreased expression of p21. Besides these paths, E6 can also block p21 expression through the inhibition of p53 (SHAMLOO; USLUER, 2019). So, it makes sense that p21 is higher in HPV negative samples and the negative correlation found between p21 vs E5 and p21 vs E6. Although no statistical significance was found, p21 also showed a negative correlation to E7.

To reinforce our results, even with no statistical significance between p21 HPV+ and p21 HPV – expression, a tendency for negative correlation was found between them in FFPE and FFPE+fresh samples.

Based on the literature, the expression of p21 blocks one of the actions of PCNA which its role inside the cell is to initiate cell differentiation and stop proliferation (TAN *et al.*, 2012). Accordantly, both in FFPE and FFPE+fresh analysis p21 and PCNA showed different and correlated expression patters, however, presented positive correlation when both targets are in the presence of HPV and both in the absence of HPV infection, but was also found a negative correlation between PCNA HPV+ vs. p21 HPV – and PCNA HPV- vs. p21 HPV+. It is important to remember that even though the statistical test tell us there is a linear relation, exist a lot of other key molecules acting in the intricated network that is the cellular machinery.

PCNA plays its role in several cell processes like repair of DNA damage, chromatin remodeling, cell cycle control, DNA replication and gene expression control (QIN *et al.*, 2016). Its overexpression in various tumor types was associated to malignancy and tumor aggressiveness, and for that it has been studied as a biomarker and therapeutic target (CHANDRASHEKAR *et al.*, 2015; HAMZAOUI *et al.*, 2015; HU *et al.*, 2017; ZHOU *et al.*, 2018). Our PCNA expression results presented higher expression levels in HPV positive fresh samples, but in FFPE and FFPE+fresh samples analysis showed higher expression in HPV negative samples. This gene is already described as a possible marker for lung cancer and maybe the stage of tumors and how much they are advanced is influencing the expression in negative HPV samples. Besides, we

found positive correlation between PCNA x E5 and PCNA x E7. These results agree with previous data where E6 and E7 stimulated cell proliferation through PCNA expression (ROMAN; MUNGER, 2013B; SHARMA; MUNGER, 2018), although Sharma and Munger (2018) did not found correlation between E7 and PCNA, contrarily to our findings.

PCNA also showed positive correlation to p16. Both targets have significant differential expression pattern and have the tendency of increased expression together, and also are associated with HPV induced carcinogenesis (BRANCA *et al.*, 2007). In a recent study, p16 and PCNA protein expression was considered together as reliable high-grade cervical lesion diagnostic marker (KIM *et al.*, 2015). We can also extrapolate these results for lung cancer positive for HPV and maybe both targets can act as HPV associated lung lesion marker.

The presented data helped us to better understand the HPV role in lung cancer patients as well as rise the hypothesis for PCNA, p21 and p16 use in future studies for lung cancer markers development and HPV associated diseases diagnosis, therapy and prevention, since HPV infection presented to be altering key factors in lung cancer microenvironment.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

5 CONCLUSÃO

- O DNA do HPV foi detectado em pacientes com câncer de pulmão e em alta prevalencia (74,60%).
- Os tipos de HPV presentes foram o HPV 16 e o HPV 18
- Foi detectada a expressão das oncoproteínas virais E5, E6 e E7, com E5 sendo a mais expressa e detectada em maior quantoidade de amostras.
- P16, p21 e PCNA mostraram ter diferentes perfis de expressão em amostras positivas para HPV, sugerindo uma atuação do vírus nestes genes.
- Foram encontradas correlações entre as expressões de PCNA e E5; PCNA e E7; p21 e E5; p21 e E6; p21 e p16; E5 e E7.

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APÊNDICE A – ARTIGO PUBLICADO NO JOURNAL OF CANCER RESEARCH

ORIGINAL ARTICLE – CANCER RESEARCH

Presence and activity of HPV in primary lung cancer

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Abstract

Purpose Evaluate the presence of human papillomavirus (HPV) in biopsies of Brazilian patients with lung cancer and also the expression of the E6 and E7 oncoproteins. HPV is widely known as an important condition for cervical cancer although evidence today shows it is associated with several other types of cancer and may also be involved in lung cancer development. However, there are some divergences regarding the presence and activity of HPV in lung carcinogenesis.

Methods The detection of HPV was performed by PCR, followed by genotype and immunohistochemical evaluation of E6 and E7 HPV type specific, from 63 patients.

Results HPV was found to be present in 33 of the 63 samples, and types 16 and 18 were detected with frequencies of 81% (27/33) and 19% (6/33), respectively. About the presence of the virus in different histological types of tumors, HPV was detected in squamous cell carcinoma (39.39%), followed by adenocarcinoma (33.33%) and small cell carcinoma (18.18%) and large cell carcinoma (9.1%). The presence of the E6 (antibody anti-HPV 16 and anti-HPV 18) and E7 (antibody anti-HPV 16 and anti-HPV 18) oncoproteins was detected by immunohistochemical stain technique in 28/33 samples and 25/33 samples, respectively.

Conclusions Our results suggest that the lung tumor patients have high prevalence of HPV and the virus is not only present but also active in tumor cells. Therefore, the HPV is probably playing a role in lung carcinogenesis.

Keywords Lung carcinoma · *Human Papillomavirus* · Oncoproteins · Carcinogenesis

Introduction

Lung cancer leads the mortality ranking of death by cancer for both women and men and tobacco is the most common etiological factor (Torre et al. 2015; WHO 2017). However, many non-smokers, mainly women, were also diagnosed with this condition (Clément-Duchêne et al. 2016; Huang et al. 2016). Air pollution, mutation, chromosomal aberrations, DNA methylation and viral infections are other factors involved in the development of this disease (Shurin et al. 2015; de Freitas et al. 2016). Among the viruses found in lung carcinoma, the *Human Papillomavirus* (HPV) was the most unexpected and this novelty generates controversy among researchers (Subramanian et al. 2007; Zhai et al. 2015).

Human Papilomavirus is a DNA virus (Marur et al. 2010) and is divided in two groups: high-risk HPV (hr-HPV) like HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, that can cause malignant lesions, and low-risk HPV (lr-HPV) such as HPVs 6, 11, 40, 42, 43, 44, 45, 54, 61, 70, 72, 81 which cause benign lesions (Corneanu et al. 2011; Cubie 2013). The HPV can encode viral proteins expressed early (E1–E8) and late (L1 and L2) in the infection.

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The first researcher to report the possible relationship between HPV and lung cancer was Syrjänen et al. (1989), indicating that neoplastic bronchial epithelium adjacent to an invasive squamous cell carcinoma presented morphological similarities with condylomatous lesions in the uterine cervix caused by this virus. Since then, many studies have been done and different opinions among researchers were evidenced. Some studies claimed that the relation- ship between the HPV and lung cancer is opportunistic (Coissard et al. 2005) or inexistent (Colombara et al. 2016) and others found a high prevalence of HPV in lung tumors suggesting that the virus may be a risk factor for the dis- ease evolution (Klein et al. 2009; Syrjänen 2012; Shikova et al. 2017).

In well-studied cancers related to HPV, E6 and E7 viral oncoproteins are engaged in cancer formation through interaction with key host factors (De Freitas et al. 2014). Some of these factors are involved in cell cycle control, apoptosis, immune system recognition of infected cells and other cell functions (Yuan et al. 2000; Zhang et al. 2014; de Freitas et al. 2016). Because of that, if the HPV is not only present but also active in the lung tissue, the virus must be express- ing its oncoproteins and causing cell changes.

Therefore, as showed above, the presence of HPV in lung cancer has been well-established but the association between its presence and the lung carcinogenesis has not yet been proved. A certain amount of debate over this subject goes on across the world, due to discrepancies between the results (de Freitas et al. 2016). The aim of this study is to evaluate the prevalence of HPV in lung cancer biopsies and reveal if the virus is, in fact, active and expressing its transform- ing oncoproteins. In Brazil, this is the first study conducted regarding the presence of HPV in lung tumor tissue and E6 and E7 oncoprotein expression.

Materials and methods

Study subject

All the clinical samples were obtained from patients of the pneumology sector of the Oswaldo Cruz University Hospital, located in Pernambuco, Brazil. A total of 63 paraffin-embedded lung resection specimens from 2013 to 2015 were used, and all were histologically confirmed as squamous cell carcinoma, adenocarcinoma, small cell carcinoma and large cell carcinoma. The mean age of patients was 55 years (11–81 years). At the time of enrollment, the patients' consent was obtained. The study protocol was approved by the Federal University of Pernambuco ethics committee (register number 06396812.0.3001.5192). In addition, only patients with primary lung cancer were included in this study.

Extraction of DNA

The DNA was extracted and deparaffinized from the sections, using the QIAamp DNA FFPE tissue kit (QIAGEN) and xilol (30 min at 65 °C), respectively. The DNA was quantified by spectrophotometry (nanodrop LITE—Thermo Scientific). The quality control of the DNA samples was accomplished by checking for the successful amplification of human beta-globin gene by PCR technique (polymerase chain reaction), using the primers 5′-ACACAACTGTGT TCACTA-3′ and 5′GGTGAACGTGGATGAAGTTG-3′ (Baldez da Silva et al. 2009). Meticulous care was taken to avoid any contamination (appropriate individual protection equipment, extensive cleaning of the work area and analyst, UV light irradiation to avoid double-stranded DNA contamination, few samples manipulated at a time, negative control for each extraction and performance of all work inside the laminar flow cabin).

HPV detection

The presence of HPV in all samples was evaluated by PCR assay without specifying the subtype of HPV using two sets of consensus primers, MY09/MY11 (forward: 5′-CGTCCMARRGGAWACTGATC-3′ and reverse: 5′-GCMCAGGGWCATAAYAATGG-3′) and GP5/GP6 (forward: 5′-TTTGTTACTGTGGTAAG-3′ and reverse: 5′-GAAAAATAAACTGTAAATCA-3′) (De Roda Husman et al. 1995), which amplify a region in the highly conserved L1 gene. The PCRs reaction mixture contained 2.0 µL of sample (contained 100 ng of DNA), 20 p moles of each oligonucleotides, 0.1 mM of each dNTP, 1.0 µM of each primer and 1.0 U Taq DNA polymerase (Qiagen) in a total volume of 25 µL of reaction buffer (50 mM KCl, 10 mM Tris HCl and 0.1%Triton X-100). The following "hot start" protocol was used for the amplification of the gene: initial denaturation at 95 °C for 5 min; subsequent 35 cycles at 95 °C for 30 s, 55 °C for 40 s and 72 °C for 45 s and final extension at 72 °C for 5 min. The PCR products were detected through electrophoresis on 2.0% agarose gel. The same protocol mentioned above to avoid contamination was used, including negative controls for the PCR.

HPV genotyping

All the HPV-positive samples were subjected to genotyping. Using ABI PRISM Big DyeTM Terminator Cycle Sequencing v 3.1 Ready Reaction kit (Applied Biosystems). The BLAST program available at http://blast.ncbi.nlm.nih.gov/Blast.cgi was used to compare the DNA sequences.

Immunohistochemical stain

Formalin-fixed and paraffin-embedded tumors were sliced at a thickness of 4 µm. After being deparaffinized and dehydrated, the sections were washed with tap water. The slices were incubated in PBS. Monoclonal antibody (Santa Cruz Biotechnology, Brazil) was used for HPV16/18 E6 (sc460 with 1:200 dilution) and HPV16/18 E7 (ab30731 with 1:500 dilution). The sections were washed with running water for 5 min and then incubated for 5 min in PBS. After that, they were incubated for 20 min in citrate buffer 10 nM and then kept at room temperature.

The reactivity to immunohistochemical staining was done by semi-quantitative analysis, considering region and intensity. Immunohistochemical staining intensities were based on the following scale: the sign used for negative reaction was (−). Positive reaction was classified as weak (reactivity in less than 10% of the field); moderate (reactivity between 10 and 50% of the field); and strong (reactivity above 50% of the field). All results compared to a cervical HPV-positive control and negative controls—HPV-positive cervical tissue without primary antibody and HPV-negative tissue using the whole antibody conjugate. The analysis of the region of reactivity was done by the detection of the protein in one or more sites. The analysis was registered and scanned by the ZEISS image capture system. Each slide was made with a different blade to avoid cross-contamination.

After cooled, the slide was submitted to blockade of peroxidases for 30 min at room temperature and then washed once in running water for 5 min and twice with PBS for 5 min each. After that, the slides were incubated for 2 h in a wet camera at 37 °C with primary antibody; then they were incubated with secondary antibody (ADVANCE™ HRP LINK) for 45 min at room temperature. Afterward, the slides were incubated with the tertiary antibody (ADVANCE™ HRP ENZYME) for 45 min at room temperature. DAB solution was added over sections for 3 min. At last, the section was counterstained with hematoxylin and visualized on the microscope.

Statistical analysis

Data were analyzed using the online software Social Science Statistics and the X^2 test was applied. The MedCalc software was used to odds ratio test (OR) with confidence intervals (CI) 95%. A p value < 0.05 was considered statistically significant.

Results

Sixty-three patients were included in this study. Their mean age was 55 years and the median were 59 years. They were in the 11–81 age range. Other parameters were analyzed, such as tobacco consumption, gender and alcohol ingestion (Table 1).

Overall, there was a higher prevalence of HPV in patients between the ages of 56 and 81 (54.55%) smokers or ex-smokers (51.52%), males (51.52%) and who were not alcoholic (27.27%).

The results of the histopathological examination showed a higher prevalence of HPV in squamous cell carcinoma (39.39%), followed by adenocarcinoma (33.33%) and small cell carcinoma (18.18%) and large cell carcinoma (9.1%).

A total of 33/63 samples were positive for high-risk HPV (HR-HPV+) at base line screening. In the genotyping step, HPV 16 was the most frequent, representing 81.81% (27/33) of the positive samples, while the HPV 18 was 18.19% (6/33). The clinical-demographic characteristics were classified according to the presence and absence of HPV (Table 2). Even so, there were no statistically significant differences between HPV 16 and 18 and histological types of tumors ($p = 0.455$).

Immunohistochemical staining analysis

To evaluate the presence of E6/E7-HPV-16/18 oncoproteins, immunohistochemical stain was used. A total of 84.85%of the HPV-positive lung tumors were positive using the antibody against E6 (28/33 samples) (Table 3), which was evidenced in the nucleus as well as in the cytoplasm of the tumor cells (Fig. 1) (Table 4).

The E7 oncoprotein was also evaluated, and the reactivity was observed in 75.76% (25/33 samples) of the HPV positive tumors (Table 3). As in E6 analysis, E7 was evidenced in the nucleus and in the cytoplasm (Fig. 2).

Table 1 Analysis of the clinical, biological and socio-demographic characteristics according to HPV positivity

Characteristics	HPV +	HPV −
Age		
11–55	11 (33.33%)	13 (43.33%)
56–81	18 (54.55%)	14 (46.67%)
Did not inform	4 (12.12%)	3 (10%)
Smoking		
Yes	17 (51.52%)	14 (46.66%)
No	11 (33.33%)	11 (36.67%)
Did not inform	5 (15.15%)	5 (16.67%)
Gender		
Male	17 (51.52%)	21 (70%)
Female	16 (48.48%)	09 (30%)
Alcoholism		
Yes	05 (15.15%)	08 (26.67%)
No	09 (27.27%)	10 (33.33%)
Did not inform	19 (57.58%)	12 (40%)

Table 2 Histopathological classification and HPV type

Tumor Type	Subtype	HPV 16	HPV 18
		27/33 Samples (81.81%)	6/33 Samples (18.19%)
Non-small cell carcinoma	Squamous cell carcinoma	11 (40.74%)	2 (33.33%)
	Adenocarcinoma	10 (37.04%)	1 (16.67%)
	Large cell carcinoma	1 (3.70%)	2 (33.33%)
Small cell carcinoma		5 (18.52%)	1 (16.67%)

Table 3 Histopathological characteristics in relation to HPV type and presence of E6 and E7 oncoproteins

Tumor type	Subtype	HPV 16	HPV 18	E6+ 28/33 Samples	E7+ 25/33 Samples
Non-small cell carcinoma	Squamous cell carcinoma	11 (40.74%)	2 (33.33%)	11 (39.29%)	11 (44%)
	Adenocarcinoma	10 (37.04%)	1 (16.67%)	8 (28.57%)	8 (32%)
	Large cell carcinoma	1 (3.70%)	2 (33.33%)	3 (10.71%)	2 (8%)
Small cell carcinoma		5 (18.52%)	1 (16.67%)	6 (21.43%)	4 (16%)

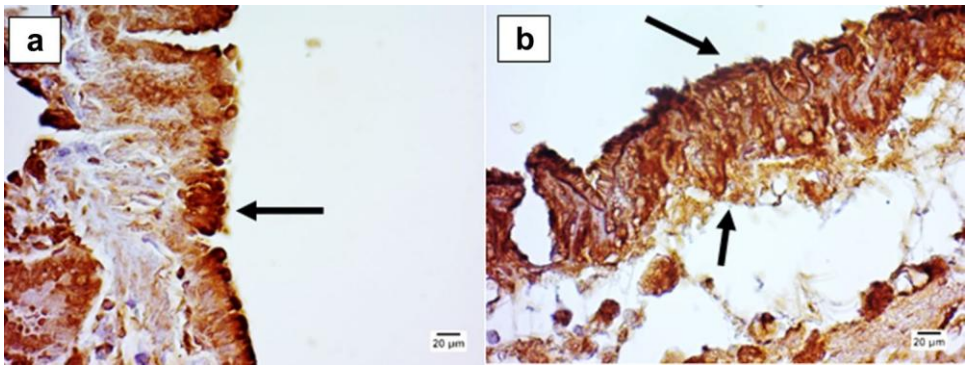

Fig. 1 Representation of the immunohistochemical stain reactivity in a lung adenocarcinoma positive for E6 oncoprotein (1:200) (E6 HPV 16/18). **a** Gland cells are showing diffuse blotting in the nucleus and in the cytoplasm, although presented more intensity in the nucleus (pointed by the arrow) (×400 magnifier glass). **b** Cilia gland cells are presenting predominant blotting in the cytoplasm and in the adjacent connective tissue (pointed by the arrow) (×400 magnifier glass)

Table 4 Classification of immunohistochemical stain of the HPV-positive samples

HPV type	Score	E6		E7	
		Nucleus	Cytoplasm	Nucleus	Cytoplasm
HPV 16	Light	12 (36.36%)	5 (15.15%)	10 (30.30%)	5 (15.15%)
	Moderate	4 (12.12%)	15 (45.45%)	5 (15.15%)	10 (30.30%)
	Strong	0 (0%)	3 (9.09%)	0 (0%)	0 (0%)
	Negative	11 (33.33%)	4 (12.12%)	12 (36.36%)	12 (36.36%)
HPV 18	Light	4 (12.12%)	0 (0%)	3 (9.09%)	0 (0%)
	Moderate	1 (3.03%)	5 (15.15%)	2 (6.06%)	4 (12.12%)
	Strong	0(0%)	0 (0%)	0 (0%)	0 (0%)
	Negative	1 (3.03%)	1 (3.03%)	1 (3.03%)	2 (6.06%)

Both negative controls were used, a cervical HPV-positive tissue slide without primary antibody and other negative control cervical tissue HPV negative. In addition, a positive control HPV positive was used and the controls worked perfectly (Fig. 3).

Discussion

Infection by *Papilomavirus* is the main cause of benign and malign cervical lesions. Recently, this infection has been associated to some non-genital tumors such as lung cancer (Klein et al. 2009; Syrjänen 2012; Ragin et al. 2014), breast cancer (De Freitas et al. 2014),head and neck squamous cell cancer, (Coppock and Lee 2016) and others.

In our study, the DNA of hrHPV was detected in lung tumor samples. We believe that this is the first paper that shows the presence of HPV in lung tumor cells from Brazilian patients and the expression of E6 and E7 viral oncoproteins. In 52% of all samples, HPV 16 and 18 DNA were detected at a frequency of 81% and 19%, respectively. These results agree to those showed by Castillo et al. (2006) who found a higher prevalence of HPV 16 followed by HPV 18 in three Latin American countries. But differently from us, Castillo et al. (2006) also noticed the presence of HPV 33.

In agreement with the results above, both HPV 16 and 18 were also found in patients from Chile (Aguayo et al. 2007). A meta-analysis identified HPV prevalence in all continents. In fact, HPV 16 had the highest prevalence in South and Central America, followed by Europe, North America and Asia (Ragin et al. 2014). A meta-analysis performed in a Chinese cohort also revealed that HPV 16 and 18 were most prevalent in lung cancer and were associated with higher risk of lung cancer development (Xiong et al. 2017).

The most common hrHPV in cancers around the globe is HPV 16. It is the most prevalent cancer of the respira- tory tract worldwide (Ragin et al. 2014; Shikova et al. 2017) and in the majority of other HPV-related cancers (Coppock and Lee 2016; de Freitas et al. 2016; Xiong et al. 2017). However, the prevalence of HPV in lung cancer is variable. In Asia, the incidence goes from 0 to 79% (Liu et al. 1994; Hirayasu et al. 1996; Yang et al. 1998; Hiroshima et al. 1999; Lim 2009; Goto et al. 2011; Lee et al. 2016; Shikova et al. 2017), while in Europe it goes from 3.6% in Croatia to 69% in Greece(Papadopoulou et al. 1998; Branica et al. 2010; Shikova et al. 2017). In America, the number of studies about HPV and lung cancer is reduced and can be

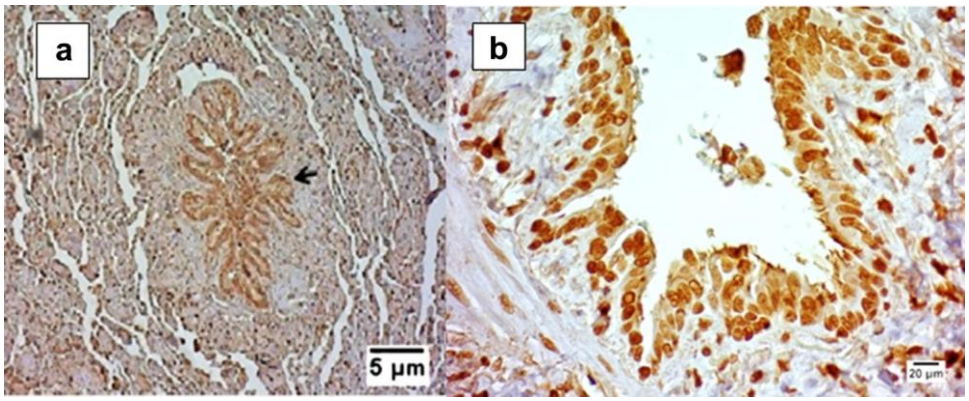

Fig. 2 Representation of the gland tissue in the lung adenocarcinoma positive for E7 oncoprotein (E7 HPV 16/18) (1:500). **a** Strong nucleus blotting followed by moderated cytoplasm blotting in all epithelial extension (pointed by the arrow) (×400 magnifier glass). **b** Weak blotting in the cytoplasm and strong E7 protein expression at the nuclear level (pointed by arrows) (×400 magnifier glass)

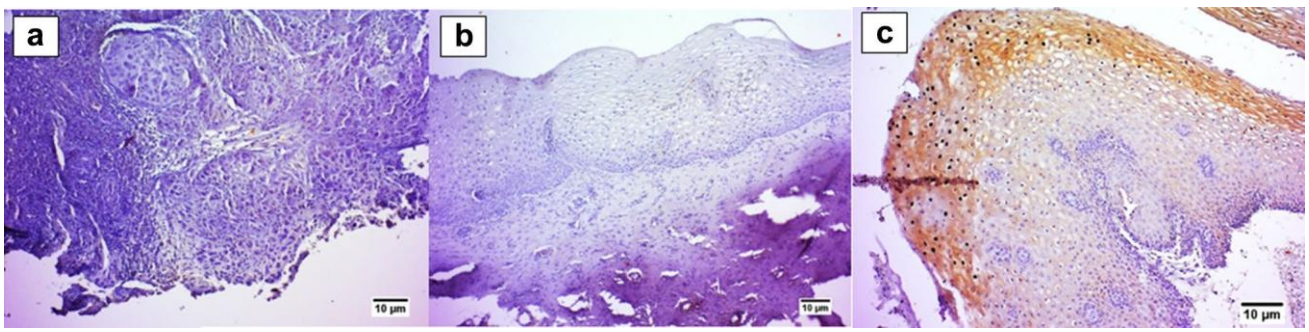

Fig. 3 Controls of the immunohistochemical stain. **a** HPV-16 and -18 (E6 and E7) negative tissue using both primary and secondary antibodies as negative control. **b** HPV-positive tissue without primary antibody for HPV-16 and 18 (E6 and E7) as a negative control. **c** HPV-16 and -18 (E6 and E7) positive tissue as positive control

classified in two types of studies: studies in North and in Latin America. In North America, the incidence goes from 0.6% in the United States to 40% in Canada (Koshiol et al. 2011; Juergens et al. 2017). Other studies were performed in North America but were directed to non-small cell lung carcinoma analysis (Mehra et al. 2013).In Latin America, tour knowledge, only two papers have so far been published. The first showed a 64.3% HPV incidence in Colombia, 7.7% in Mexico e 0% in Peru, while the other found a 29% incidence in Chile (Castillo et al. 2006; Aguayo et al. 2007).

The findings above show a wide variability of HPV's prevalence and genotypes in lung cancer (Klein et al. 2009; Colombara et al. 2016).These data differences may be related to geographic location of the studies, type of samples (fresh or paraffin samples), number of samples, HPV genome region used for genotype (L1 or E6 and E7 genes) and different HPV DNA detection techniques (in situ hybridization and PCR). For example, DNA from the fresh tissue is more preserved, less fragmented and unmodified then paraffin samples (Dubeau et al. 1986; Prakash et al. 2016). The DNA detection techniques can also change according to the researchers' choice. Most of them like to use PCR due to its sensitivity; however, the obtention of the right number of amplicons of paraffin samples is difficult. As far as we know, only one research, preferred the use of in situ hybridization technique after PCR were developed (Lim 2009).

One source for divergences between the studies could be the genome region used for the genotype of the HPV. This is an important topic to be discussed, though it rarely is. The L1 gene is a well-conserved region in the HPV genome and present in all types of HPV, due to its function in the formation of the viral capsid (Mcbride 2011). In fresh tissue, the use of an L1 internal sequence may be a liability because in the HPV genome integration process during infection, this region can suffer a partial loss. That way, E6, E7 or E6/E7 gene detection may be a more secure choice. However, in paraffin-embedded samples, long regions for analysis such as E6/E7 sequences are not indicated due to DNA fragmentation, formalin-induced inhibition of PCR and/or cross-linking of DNA. In general, both methodologies—detection of small regions inside L1 and E6 or E7 detection—seem to be successful (Prakash et al. 2016). Even so, the use of a specific primer designed for E6 or E7 from specific HPV has higher sensitivity and specificity than primer consensus for several HPV types, such as those for L1 region. This reflects on a higher prevalence of HPV in lung cancers analyzed by specific primers for each HPV (Syrjänen 2012). The information above was confirmed when even with Luminex-based assays combined with modified GP5+/6 + primers (L1 internal region alignment), low amounts of HPV 31 plasmid were not detected (Eklund et al. 2010).

Although we used the L1 region for detection of HPV, the genotyping was performed through genome sequencing. This methodology avoids mistakes, such as false negative or false positive caused by mismatch primer alignment, and it analyzes nucleotides by nucleotides and compares with the online database on NCBI.

Our findings in lung carcinoma samples are like the results found in genital cancer. In Brazil, HPV 16 is the most common type both in men and woman (Giuliano et al. 2008; Castro et al. 2013; Rodrigues et al. 2014; Bruno et al. 2014). In the uterine cervix, HPV 16 is the most frequent and in other regions, HPV 18 is the second most frequent (Fernandes et al. 2010; Lima Júnior et al. 2011). Other studies showed HPV 16 followed by HPV 31 (Baldez da Silva et al. 2009), mostly in north-east of Brazil (Chagas et al. 2015). Taking into consideration the importance of HPV 16 and 18 prevalence, two studies revealed that patients infected by HPV16 had a 6.5 risk rate of future lung cancer development (Chiou et al. 2003) and a 2.32 risk rate for infection by both HPV 16 and 18 also in lung (Hsu et al. 2009). This confirms the high carcinogenic potential of HPV 16 and shows itis more likely to be participating in lung carcinogenesis than just being present but inert.

Regarding the analysis of HPV presence associated to cancer type, human papillomavirus DNA was found in 82% of non-small cell carcinoma (40% of squamous cell carcinoma, 33% of adenocarcinoma and 9% of large cells carcinoma) and 18% in small cell carcinoma. The presence of HPV in large cell carcinoma is not commonly described in the literature since few studies found this type of cancer positive for HPV (Yousem et al. 1992; Miasko et al. 2001; Zafer et al. 2004; Jain et al. 2005; Park et al. 2007; Nadji et al. 2007). Even so, our findings corroborate the results of some works which showed a higher frequency of HPV in squamous cell carcinoma (51%) followed by adenocarcinoma (16%) (Peitsaro et al. 2002; Aguayo et al. 2007; Yu et al. 2009). Compared to other studies, our results diverge because they found higher HPV prevalence in adenocarcinoma samples than in squamous cell carcinoma (Cheng et al. 2001; Chiou et al. 2003; Hsu et al. 2009; Baba et al. 2010). The difference between these results may be due to geographic location, type of cancer prevalence in the location, ethnicity and other variables.

Additionally, some papers revealed that HPV 16 was more frequent than HPV 18 (Fei 2006) in both, squamous cell carcinoma (45.18%) (Zhai et al. 2015) (43.1%) (Yu et al. 2009) and adenocarcinoma (22.78%) (Zhai et al. 2015). A similar result was found for our patients. The frequency of HPV 16 was 41% in squamous cell carcinoma, 37% in adenocarcinoma, 18% in small cell carcinoma and 4% in large cell carcinoma. When analyzing HPV 18, the frequency was 33% in both squamous carcinoma and large cell carcinoma, and 17% in both adenocarcinoma and small cell carcinoma. Even with no statistical significance between HPV prevalence and tumor type, our samples showed a diversity of

histological cancer classification which provides a general view of HPV prevalence in different types of lung cancer. Most of the studies only showed HPV prevalence in adenocarcinoma and squamous cell carcinoma (Ragin et al. 2014).

To evaluate the viral activity, the samples were tested for the presence of E6 and E7 oncoprotein expression by immunohistochemical stain. The immune staining was observed in the nucleus as well as in the cytoplasm for both oncoproteins. This result suggests that the HPV is active and probably causing cell transformation. Therefore, the presence of these oncoproteins suggests that HPV is not in its latent form in the lung tissue but actively expressing its genes and probably causing cell alterations, due to what is already consolidated in other types of HPV-related cancer (Tian et al. 2018; Liu et al. 2018). A different result from ours was found in a recent study in Greece, which also analyzed HPV presence and E6/E7 expression in lung cancer (Argyri et al. 2017). Sixty-seven lung cancer samples were analyzed and showed 3% of prevalence of HPV (2/67 samples) and no E6/E7 expression. This result shows the high divergence between data around the globe and suggests that geographic location and ethnicity may cause more interference in HPV lung infection than we thought.

HPV is present in our samples of lung cancers as well as in several types of tumors, such as breast (Wang et al. 2014), colorectal (Lee et al. 2001), skin (Iannacone et al. 2013) and others. This virus is adapted to cervical tissue and why it infects other body regions and how it gets there are questions still unanswered. However, there are some hypotheses. One possible explanation for HPV infection in unconventional body areas is variations in the DNA virus as an adaptive strategy; an event called speciation (Burk et al. 2013). Studies showed that variation in the oncogenic potential and infection persistence and progression are related to changes in the L1, E6, E7 and LCR viral regions (Chagas et al. 2011; Gurgel et al. 2013). These genetic alterations interfere in the viral gene expression, in the interaction between the viral proteins and the host proteins and in the viral recognition by the host immune system (Gurgel et al. 2013). Still, more research about genetic variants needs to be performed to better understand the relationship between HPV and its host. Another probable explanation for lung infection by HPV is the similarity between the respiratory tract histology and the cervical histology. The epithelium in the pharynx is composed of columnar cells, while the larynx and the trachea are composed of squamous cells. This histological transition (metaplasia) is called squamous columnar junction (SCJ) and is like the SCJ in the cervix, which is the preferential HPV enter pathway and may facilitate the establishment of HPV infection in the lung tissue. Corroborating with this hypothesis, our squamous cell carcinoma samples showed the highest prevalence of HPV. Besides that, the respiratory tract SCJ can occur by natural causes or by induction. Tobacco smoking is one of the main causes of SCJ formation (Schamberger et al. 2015; Argyri et al. 2017). Moreover, tobacco also reduces the columnar ciliated cells and its destruction facilitates the entrance of pathogenic agents (Kashima et al. 1993; Syrjänen 2002; Schamberger et al. 2015).In fact, the prevalence of HPV was higher in our patients who were smokers or had been smokers in the past (60%) than in no smokers. However, as in other studies, no significant association was found between HPV positivity and the use of tobacco(Guo et al. 2017).

About the hypothesis of how HPV gets to the lung, two are the most accepted. The first says the virus enters through the oral cavity and goes cell-by-cell until it reaches the lung. Studies of oral and pharynx cancer showed that sexual behavior constitutes a significant risk factor for HPV infection, especially for patients who have anal–oral contact (OR = 4.6) (Smith et al. 2004).The second hypothesis says that HPV travels from the genital area through the blood until the lung cells. Several types of HPV (6, 11, 16, 18) were detected in blood cells (Pao et al. 1991; Bodaghi et al. 2005; Chen et al. 2009), plasma (Dong et al. 2002), serum (Liu et al. 2001) and arterial blood from umbilical cord (Rombaldi et al. 2008). Besides, the analysis of healthy blood donors detected the presence of HPV 16 in 15.8% of the samples (Bodaghi et al. 2005). Previous works attributed the presence of HPV in blood to the presence of cervical cancer (Tsai et al. 2005; Ho et al. 2005; Pao et al. 2014); however, as seen, the presence of HPV in blood is independent of cervical infection. In corroboration with this hypothesis, a study showed that patients with non-small cell lung cancer presented high HPV prevalence in the blood (Chiou et al. 2003) which makes us believe that blood is a suitable pathway for virus propagation.

No matter how HPV gets to the lung, our results show that the DNA of HPV is not only present in lung cancer patients but also active and expressing viral oncoproteins. Although further studies on this matter are needed, the data here presented raises questions about possible changes in the prophylactic and therapeutic measures adopted by medicine in the fight against this great challenge called lung cancer.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all participants included in the study.

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