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Cooperative role of c-MYC and E6/E7 from two molecular variants of human papillomavirus type 16 upon proliferation and *in vitro* transformation of primary human keratinocytes

Papel cooperativo de c-MYC e E6/E7 de duas variantes moleculares do Papilomavírus Humano tipo 16 na proliferação e transformação in vitro de queratinócitos humanos primários

Jimena Hochmann[1], Silvaneide Ferreira[2], João S. Sobrinho[2], Laura Sichero[2]

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RESUMO: As funções das oncoproteínas E6 e E7 do Papilomavírus Humano tipo 16 (HPV-16) na progressão de células epiteliais imortalizadas para tumores invasivos não são totalmente compreendidas. Aqui, estabelecemos uma nova ligação entre E6 e E7 de duas variantes moleculares de HPV-16 (AA e E-350G) e c-MYC, em relação à cooperação na promoção da transformação maligna de queratinócitos humano primários de prepúcio de recém-nascido (PHK). Nosso objetivo foi estudar os efeitos sinérgicos de E6/E7 e c-MYC na proliferação e no potencial de transformação *in vitro* de PHKs. Avaliou-se a proliferação celular através da expressão proteica do Antígeno Nuclear de Células Proliferantes (PCNA). Também avaliamos a capacidade de transformação, *in vitro,* dos PHKs através de dois ensaios complementares. Observamos que E-350G-c-MYC PHKs exibiram um aumento discreto na expressão de PCNA e formaram significativamente mais colônias tanto nos ensaios de soft-ágar quanto nos ensaios em placas de cultura de baixa adesão. No geral, concluímos que a variante E-350G co-transfectada com c-MYC pode promover a transformação celular maligna com eficiência maior do que a variante AA-c-MYC. As propriedades oncogênicas exibidas pela variante E-350G permitem entender em maior detalhe os mecanismos que podem levar à neoplasia cervical humana, dada a maior frequência de sua ocorrência na progressão de lesões precursoras de alto grau para carcinomas invasivos.

Descritores: Papillomavirus humano 16; Transformação celular viral; Genes myc; Queratinócitos; Proteínas oncogênicas.

ABSTRACT: The roles of E6 and E7 oncoproteins of Human Papillomavirus type 16 (HPV-16) in the progression of immortalized epithelial cells to invasive tumors are not fully understood. Here, we establish a novel link between E6 and E7 of two molecular variants of HPV-16 (AA and E-350G), and c-MYC, regarding the cooperation in promoting malignant transformation of primary human foreskin keratinocytes (PHK). We aimed to study the synergistic effects of E6/E7 and c-MYC upon proliferation, and the in vitro transformation potential of PHK. We evaluated cellular proliferation through the expression of the Proliferating Cell Nuclear Antigen (PCNA) protein and colony formation abilities using soft agar and low attachment plates. We observed that E-350G-c-MYC PHKs exhibited discrete higher PCNA levels and formed significantly more colonies in both soft-agar and when growth in low-adhesion culture plates. Overall, we concluded that the E-350G variant co-transfected with c-MYC might promote malignant cellular transformation with a better efficiency than the AA-c-MYC counterpart. The enhanced oncogenic properties exhibited by the E-350G-c-MYC variant offer insights into mechanisms that may operate in human cervical neoplasia, given the higher frequency of its occurrence in the progression of high-grade precursor lesions to invasive carcinomas.

Keywords: Human papillomavirus 16; Cellular transformation, viral; Genes, myc; Keratinocytes; Oncogene proteins.

1. Virology Section, Facultad de Ciencias, UDELAR, Montevideo, Uruguay. ORCID: https://orcid.org/0000-0002-0875-2333. Email: jhochmann@fcien.edu.uy.
2. Center for Translational Research in Oncology, Instituto do Cancer do Estado de São Paulo, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Brazil. ORCID: Sichero L – https://orcid.org/0000-0002-7999-5818. Email: silvaneide.ferreira@hc.fm.usp.br; simao_j@yahoo.com.br; laura.sichero@hc.fm.usp.br.

Corresponding author: Jimena Hochmann, PhD. E-mail: jhochmann@fcien.edu.uy.

INTRODUCTION

High-risk Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 y 68) are the main etiological agent of cervical cancer. HPV-16 is the most prevalent type and is responsible for about 500,000 new cancer cases and over 250,000 cancer deaths worldwide every year. HPV variants arise in consequence of cumulative nucleotide mutations in key positions of the viral genome and differ in nucleotide sequence by no more than 2% in coding regions and until 5% in the non-coding region[1]. Data obtained from 301 HPV-16 positive cervical samples from 25 different geographical regions in the world revealed that HPV-16 has evolved along five major branches, two being present mainly in Africa, one Asian-American, one Asian and one in Europe[2,3]. The E6/E7 oncogenes of high-risk HPVs are both necessary and sufficient to immortalize Primary Human Foreskin Keratinocytes (PHKs), and their expression is required for the continued proliferation of HPV-positive cervical cancer cells[4,5]. However, these immortalized cells are not tumorigenic in mice, which means that addition. Cellular events are required for the complete transformation of human epithelial cells. Several observations suggest that cooperative signaling of host cellular factors, along with E6 and E7, are essential in promoting entire tumor progression *in vivo*[6].

One of the most studied human oncogenes is *RAS* which expression leads the immortalized human epithelial cells to a fully transformed phenotype with all the hallmarks of cancerous cells[7,8]. It has also been shown that c-MYC can replace Notch1 in cooperating with HPV-16 E6 and E7 in the transformation of human epithelial cells since c-MYC is a direct t.arget of CBF1-dependent signaling triggered by activated Notch1[9].

Although exacerbated levels of c-MYC are observed in many cases of cervical cancer, the function of c-MYC in the process of HPV-mediated transformation is unclear. E6 is believed to directly transactivate the c-MYC promoter in H358 (bronchio.alveolar carcinoma/non-small cell lung cancer) and C33a (cervical cancer HPV negative) cells[10]. On contrast, other reports suggest that E6 controls the degradation of c-MYC in a neuroblastoma cell line that expresses high levels of the human N-MYC protein[11]. In addition, it was observed that c-MYC associates with the HPV-16 E6 protein *in vivo* to transactivate the hTERT promoter in PHKs[12]. As seen, the mechanisms through E6 leads to transformation can be cell-type specific.

c-MYC belongs to the myc family of genes (MYC, MYCN, MYCL), is located on chromosome 8q24.1, has three exons and encodes a nuclear protein of 62 kDa that holds transcription factor activity and responds to different cell signaling pathways which are responsive to cellular growth factors[13]. The c-MYC gene is frequently activated in human cancers through several mechanisms, such as mutations[14,15,16], or even, as a result of the inactivation of key controllers of the cell cycle, such as p53[13].

In this study, we analyzed the putative function of c-MYC in HPV-mediated transformation of PHKs as an important partner of E6 and E7 proteins of two molecular variants of HPV-16: Asian-American (AA) and European with the 350G substitution (E-350G). Here we demonstrated that cells co-transduced with E-350G and c-MYC presented an enhanced *in vitro* transformation potential in comparison to AA and c-MYC co-expressing cells.

MATERIAL AND METHODS

Transduction of c-MYC in primary human keratinocytes (PHK), co-transduced or not with E6/E7 of two HPV-16 molecular variants (AA and E-350G)

Pools of primary newborn foreskin human keratinocytes (PHK) were purchased from Clonetics, NJ, USA (cat n. 00192906, lot n. 0000252415, certificate of analysis provided by the supplier), and maintained in keratinocyte serum-free medium (KSFM) supplemented with 5ng/mL epidermal growth factor (EGF) and 50µg/mL bovine p.ituitary extract (BPE) (Invitrogen, CA, USA). PHKs immortalized by E6 and E7 from different molecular variants of HPV-16 were obtained as previously described[17,18]. HPV-immortalized cells used in this study were tested internally for HPV DNA status, viral RNA expression patterns, and E6/E7 protein expression levels. HPV transduced cells were continuously sub-cultured 1:6 when 80–90% confluence was reached and considered immortalized after 30 passages[19]. PHKs immortalized with two distinct HPV-16 variants were used throughout this study: E-350G (E6:L83V), and AA (E6:Q14H/H78Y/L83AA). We infected primary and high passage (›p100) E6/E7 PHKs w.ith the lentiviral pCDH-puro-c-MYC plasmid (Addgene # 46970). Briefly, 10µg of this plasmid was co-transfected with three plasmids encoding the lentiviral proteins Gag, Pol, Env in HEK293T cells using a calcium phosphate protocol. Infectious particles were used to infect HPV-immortalized and normal PHKs using a MOI of 5 in presence of 8 µg/mL of polybrene.

Proliferation assay

Cell lines (PHK, AA, AA-c-MYC, E-350G, and E-350G-c-MYC) were harvested by trypsin digestion and seeded into 6-well cell culture plates (1×10^4 cells/well) at day 0. Cells were dissociated from wells with 0.25% trypsin and counted every other day manually using a hemocytometer under a microscope. Cell growth curves were obtained from live cell numbers during six days.

Clonogenic assay

Primary cells, PHKs transducing c-MYC, or PHKs co-transducing E6/E7 of two variants of HPV-16 and

c-MYC (E-350G-c-MYC and AA-c-MYC) were seeded at low de.nsity (300 cells) in six wells plates. After two weeks with medium changed every other day, cells were stained with 0.01% crystal violet, and colonies were counted macroscopically. Experiments were performed at least in triplicates three times independently. Colonies were counted by using the VisionWorks™LS Image Acquisition and Analysis software (UVP, Jena, Germany).

Soft agar assay

One of the hallmarks of transformed cells consists on the ability to grow independent of substrate adhesion. The anchorage-independent condition can be recreated *in vitro* using a semi-solid medium. For this assay, 7,000 cells were mixed with 0.3% low melting point (LMP) agarose in KSFM medium and seeded in 24 wells plates on top of a 1% LMP agarose bed. After 20 days, colonies were stained using 1mg/mL MTT (Sigma, MI, USA), and observed macroscopically to visualize the number and size of the colonies formed. Experiments were conducted three times in six replicates.

Immunoblot

Cells pellets were washed with ice-cold phosphate-buffered saline (PBS), centrifuged, and protein lysates extracted by incubation on ice for 30 min with RIPA buffer (20 mM Tris–HCl, pH=7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) containing complete protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Eighty micrograms of protein extracts were loaded onto 10–15% SDS-polyacrylamide gels, electrophoresed, and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). The primary antibodies were used at the following dilutions: anti-cMYC (ab69987, 1:250), anti-PCNA (ab29, 1:1000), and anti-tubulin (1:5,000, OT-9026, Sigma, MI, USA). Anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (GE Healthcare Buckinghamshire, UK) were used at a dilution of 1:5000. Proteins were visualized using the ECL Plus Western Blotting detection system (GE Healthcare, Buckinghamshire, UK) in a ImageQuant LAS 4000 equipment (GE Healthcare, Buckinghamshire, UK). Protein levels were quantified using the ImageQuant TL software (GE Healthcare, Buckinghamshire, UK).

Low attachment assay

We seeded 50,000 cells on low attachment six well plates with 5mL of KSFM. Every three days 500µL of KSFM were added to each well. After thirteen days, colo. nies were photographed using the EVOS FL Auto Imaging System AMAFD 1000 (Life Technologies, MA, USA), and 25 photos were obtained for each well. Experiments were conducted three times in triplicates.

Statistical analysis

Mean values and standard deviation (SD) are shown. Whenever appropriate, data was analyzed by unpaired two-tailed Student´s t-test using GraphPad Prism Version 7, and ANOVA for repeated measures to compare proliferation rates using SPSS v.25 for Windows. Significance level was 5% for all hypothesis.

RESULTS

Characterization of PHKs co-transduced with E6/E7 of two HPV-16 variants and c-MYC

We initially transduced c-MYC in HPV-16 immortalized keratinocytes. As expected, PHKs co-infected with AA-c-MYC or E-350G-c-MYC exhibited higher levels of c-MYC expression relative to PHKs transduced only with E6/E7 of HPV-16 or normal PHKs (Figure 1a,b). However, the increment in cMYC expression was statistically significant solely for PHKs transduced with E-350G-c-MYC.

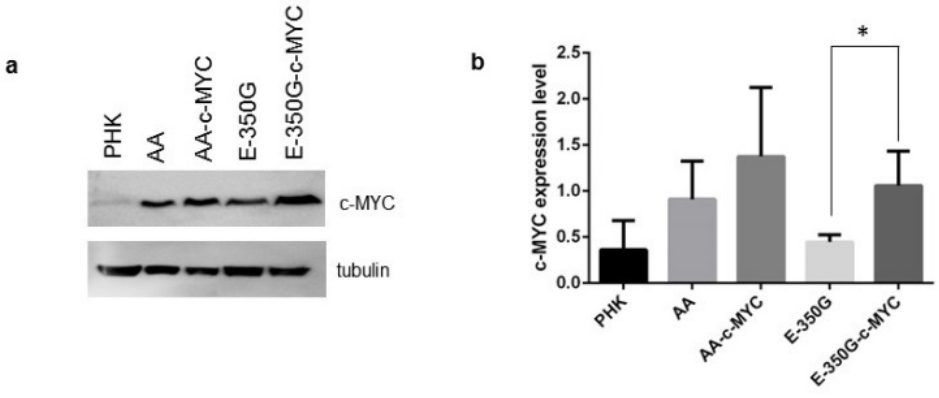

Figure 1. Characterization of PHKs immortalized by E6/E7 of two HPV-16 variants co-transduced with c-MYC. (A) Total protein extracts were submitted to SDS-PAGE followed by immunobloting using antibody against c-MYC. One representative experiment of two independent assays is shown. (B) Densitometric analysis of c-MYC protein expression of parental cells and HPV-16 immortalized PHKs co-transduced not with c-MYC. Values were normalized to tubulin expression levels, and averages ± SDs from two independent experiments are shown

E6/E7 HPV-16 immortalized PHKs transduced with c-MYC do not significantly differ in their proliferative potential

We inquired whether c-MYC increases the proliferation rate of HPV-16 immortalized PHKs. Our results showed that PHKs co-transduced with the E-350G variant and c-MYC presented a discrete but not statistically significant increment in PCNA expression levels relative to that of AA-c-MYC PHKs (Figure 2a,b).Furthermore, we evaluated the growth rate of these cells over a period of 6 days in culture and observed no significant differences among cells immortalized by the different HPV-16 variants and those co-transduced with HPV-16 and c-MYC. Taken together, our data shows that independently of the variant analyzed, HPV-16 immortalized PHKs transduced with c-MYC do not attain differences regarding proliferation.

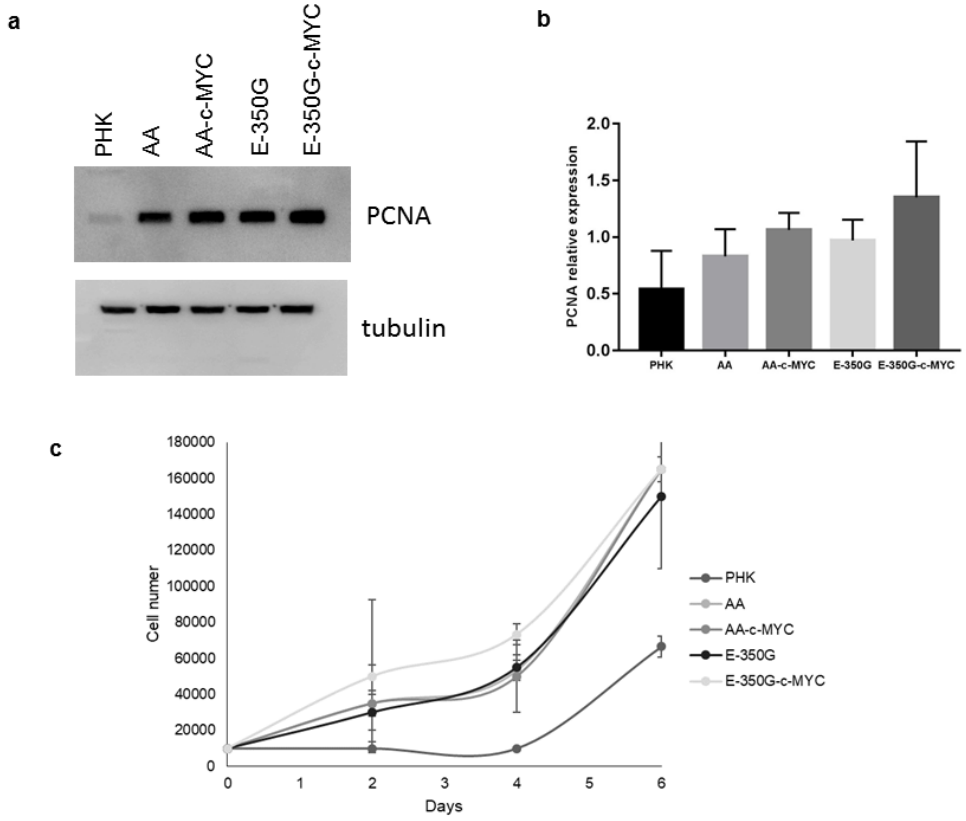

Figure 2. Proliferation ability of PHKs immortalized by E6/E7 of two HPV-16 variants co-transduced with c-MYC. (a) Expression profile of the PCNA protein. Total protein extracts from parental and immortalized PHKs co-transduced or not with c-MYC, were submitted to SDS-PAGE followed by immunoblotting using an antibody against the proliferating cell nuclear antigen (PCNA). One representative experiment of two independent assays is shown. (b) Densitometric analysis of PCNA levels in parental cells and HPV-16 immortalized PHKs co-transduced or not with c-MYC. Values were normalized to tubulin expression levels, and averages ±SDs of two independent experiments are shown. (c) Cell growth curves over a six days period of normal, and HPV-16 immortalized PHKs transduced or not with c-MYC. Cell numbers were accessed manually with a hemocytometer under a microscope. One representative experiment of two independent assays is shown

Colony formation and transformation potentials of immortalized human keratinocytes co-transduced or not with c-MYC

The cooperative role of c-MYC and E6/E7 from two HPV-16 variants upon *in vitro* transformation was evaluated using three different approaches: We initially carried out clonogenic assays by plating cells in low density and allowing colonies to grow for two weeks. Our results showed that both variants co-transfected with c-MYC formed significantely more colonies when compared to PHKs. In addition, PHKs co-transduced with the E-350G variant and c-MYC formed even more colonies when compared to PHKs co-transduced with the AA variant and c-MYC (Figure 3a,b). However, no significant differences were found regarding the colony diameter (Figure 3c). Next, cells were plated in semi-solid medium and we observed that PHKs co-transduced with the E-350G variant and c-MYC were also able to produce a significantly higher number of colonies relative to the AA-c-MYC variant counterpart (Figure 4 a and b). Finally, our data was further corroborated using an assay that mimics 3D growth conditions, showing a more efficiency in the number of colonies formed by E-350G-c-MYC variant (Figure 4 c and D). In sum, our results point towards a higher *in vitro* transformation potential for the HPV-16 E-350G variant in cooperation with c-MYC.

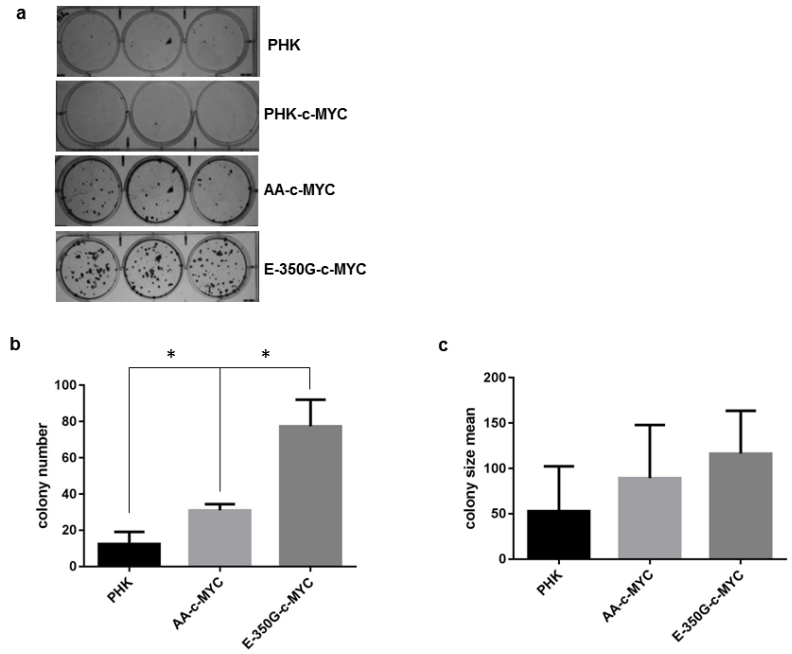

Figure 3. Colony formation potential of HPV-16 immortalized and c-MYC co-transduced cells when plated in low density. (a) For all cultures, 300 cells were seeded in triplicates and grown in six-well plates for 15 days. Colonies formed were visualized by staining with crystal violet and evaluated macroscopically. One representative experiment of three is shown. (b) and (c) Colony number and size were accessed using the the VisionWorks™LS Image Acquisition and Analysis software (UVP, Jena, Germany). Averages and ±SDs from three independent experiments are shown

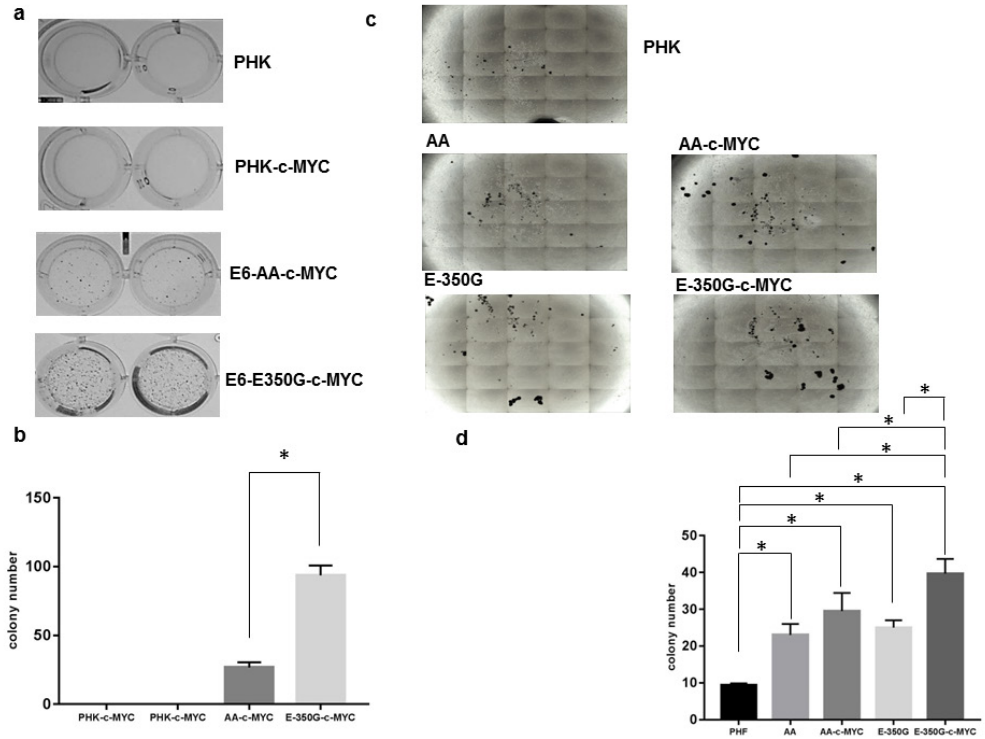

Figure 4. **Transformation potential of HPV-16 immortalized and c-MYC co-transduced cells.** a) Soft agar assay. After 15 days in culture colonies were stained with MTT and evaluated macroscopically. b) Colony number was accessed for each cell line by counting manually. Averages and ±SDs from three independent experiments are shown. c) Low attachment assay. After 15 days, 25 photographs were taken from each cell line using the EVOS equipment. d) Colony number was accessed for each cell line by counting manually. Averages and ±SDs from three independent experiments are shown

DISCUSSION

Expression of high-risk HPVs E6 and E7 oncoproteins is necessary for the immortalization of primary keratinocytes, a critical step for oncogenic progression, however insufficient for the complete *in vivo* transformation of these cells. Thus, the contribution of a host factor would be necessary to achieve a transformed phenotype[6]. It has been previously reported that c-MYC can replace activated Notch 1 in synergizing with HPV-16 E6 and E7 oncoproteins towards cell transformation, once c-MYC expression is regulated by Notch1[9].

In the present study, we evaluated the cooperative role between E6/E7 of two different variants of HPV-16 and c-MYC upon PHK proliferation and *in vitro* transformation using different approaches. Taken together, our data supports a higher transformation potential for the European molecular variant of HPV-16 with a nucleotide substitution in the *E6* gene (E-350G) in collaboration with c-MYC in comparison to HPV-16 AA immortalized PHKs further transduced with this cellular gene (AA-cMYC-PHK). Our results corroborate a previous study that showed that c-MYC can cooperate with HPV-16 E6 and E7 oncogenes in cell transformation since the introduction of AcN1 or c-MYC in HaCaT(transformed keratinocyte of human skin) cells expressing E6 and E7 resulted in a greater than 4-fold increase in the number of colonies formed on soft agar assays when compared to mock-transfected, or cells expressing solely HPV-16 E6/E7[9]. Furthermore, Chakrabarti et al.[20] reported that the E-350G HPV-16 variant enhances MAPK signaling and cooperative transformation with deregulated Notch1, allowing the progression of immortalized human epithelial cells to a complete transformation as accessed using *in vitro* soft agar assay.

The enhanced oncogenic potential exhibited by the E-350G variant offer insights into mechanisms that may operate in human cervical carcinogenesis. In European populations mostly all HPV-16 molecular variants detected belong to the European branch[21,22]. In Europe, the HPV-16 E-350G variant has been shown to associated with an increased risk of viral infection persistence and progression to cervical intraepithelial neoplasia 2 and 3 (CIN2 and 3) and invasive cervical cancer in some populations[21,22,23]. The fact that our data points towards a higher oncogenic l attributed to this variant in cooperation with c-MYC, may explain, at least in part, why women infected with the E-350G variant are more prone to develop cervical neoplasias. Nevertheless, it should be highlighted that not all studies conducted in different European populations showed similar results[24,25,26,27,28,29].

In conclusion, our results indicate that HPV-16 E6/E7 cooperates with c-MYC in promoting in vitro transformation of PHKs. More specifically, we further demonstrated that E-350G-c-MYC PHKs showed the highest oncogenic potential. Nevertheless, further studies are needed to elucidate the molecular mechanisms by which HPV-16 variants differ in their oncogenic potential.

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Authors' contributions: Conceived and designed the experiments: *JH, JSS, SF*, and *LS*. Performed the experiments: *JH, SF, JSS*. Analyzed the data: *JH, JSS, SF*, and *LS*. Contributed reagents/materials/analysis tools: *LS*. Wrote the paper: *JH, LS*. All authors read and approved the final manuscript.

Competing Interests: None of the other authors have conflicts of interest to report.

Ethics approval and consent to participate: not applicable

List of abbreviations: Asian-American (AA), cervical intraepithelial neoplasia (CIN), European (E), European 350G(E-350G), human papillomavirus (HPV), Primary Human Foreskin Keratinocytes (PHK), Keratinocyte serum free medium (KSFM), Proliferating Cell Nuclear Antigen protein (PCNA), Activated Notch1 (AcN1), Centromere-binding protein 1(CBF-1), epidermal growth factor (EGF), bovine pituitary extract (BPE), low melting point (LMP), MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, phosphate-buffered saline (PBS), Polyvinylidene difluoride (PVDF).

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