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RESEARCH ARTICLE

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The papillomavirus *E5* gene does not affect *EGFR* transcription and overall survival in cervical cancer

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Abstract

Introduction: The human papillomavirus (HPV) *E5* gene encodes a small and highly hydrophobic oncoprotein that affects immune evasion, cell proliferation, loss of apoptotic capacity and angiogenesis in tumors. E5 shows an affinity for biological membranes and was associated with an increase of epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) signaling through the accumulation of EGFR in cellular membranes. Due to the frequent integration of the HPV genome into the host cell genome, *E5* is frequently not transcribed in cervical tumors.

Aim: In this study we looked forward to verifying whether the potential expression of E5 protein in human papillomavirus 16 positive (HPV16$^+$) and human papillomavirus 18 positive (HPV18$^+$) cervical tumors was associated with levels of *EGFR* and vascular endothelial growth factor A (*VEGFA*) transcription and with patients overall survival.

Results: Association between the presence of *E5* transcripts and viral genome disruption was observed for HPV16$^+$ and HPV18$^+$ tumors. Association was not observed between tumors potentially capable of translating E5 and *EGFR* or *VEGFA* transcriptional levels. Similarly, the capability of translating E5 and overall survival in patients with HPV16$^+$ squamous cell carcinoma tumors stage ≥ IB2 were not associated.

Conclusion: The likely presence of E5 transcripts was neither associated to a higher activity of the EGFR-VEGFA pathway nor to the overall survival of patients with HPV16$^+$ squamous cell carcinoma in stages ≥ IB2.

KEYWORDS

cervical cancer, E5 oncogene, epidermal growth factor receptor, HPV16, HPV18, human papillomavirus, vascular endothelial growth factor

1 | INTRODUCTION

Cervical cancer is the fourth most common cancer worldwide and the fourth leading cause of death associated with cancer in women.[1] In Brazil, its incidence is ranked number three among women (excluding non-melanoma skin cancer), with 5920 deaths reported in 2016 and accounting for 16 370 newly expected cases for 2019 (https://mortalidade.inca.gov.br/MortalidadeWeb/).[2]

Human papillomavirus (family *Papillomaviridae*, subfamily *Firstpapillomavirinae*, Genus *Alphapapillomavirus*) infection is considered as an indispensable factor, albeit not sufficient, for developing cervical cancer.[3] Currently, 226 human papillomavirus (HPV) genotypes have been described to date (https://www.hpvcenter.se/human_reference_clones/),[4] while 15 HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) have been associated with high risk for cervical cancer (HR-HPV).[5] HPV16 and

HPV18 are the most frequently, worldwide-detected genotypes in these tumors, and are present in approximately 67% of all cases.[6]

The function of *E6* and *E7* HPV genes has been extensively studied. They encode the main, essential HPV oncoproteins for promoting cancer development; E6 accounting for TP53 degradation, and E7 for inhibiting pRb and E2F interaction which lead to cell cycle progression.[7,8] Although not extensively studied, the *E5* gene has also been considered to be an oncogene.[9] It encodes a small hydrophobic protein of approximately 83 amino acids (for HPV16) in a hexameric configuration forming ion channel pores,[10,11] which is detected in the membranes of the Golgi apparatus and endoplasmic reticulum.[12] E5 is mainly operative during the early stages of HPV infection, associated with immune evasion, inhibition of apoptosis and increasing cell proliferation.[13] During the initial phases of HPV infection, immune evasion is an important step favoring HPV persistence and progression throughout its productive cycle.[14] The E5 protein decreases the immune response and apoptosis by retaining the major histocompatibility complex in the golgi apparatus[15,16] and inhibiting the Fas ligand and the tumor necrosis factor-related apoptosis-inducing ligand pathways.[17] E5 also promotes cell proliferation by repressing P21 [18] and stimulating the extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway through epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) mitogen-signaling.[19] Moreover, in cervical cancer cell lines, E5 was found to upregulate the expression of vascular endothelial growth factor A (*VEGFA*) through the EGFR/MAPK/ERK pathway.[20] Briggs et al[21] proposed that E5 maintained mitotic-signaling by avoiding EGFR degradation through inhibition of V-ATPAses and endosome acidification. However, this proposition was challenged by Suprynowicz et al[22] who associated enhanced EGF/EGFR signaling with inhibition of endosome trafficking and maturation.

The *E5* gene is early expressed during the viral productive cycle[23] and throughout neoplastic progression of cervical lesions when the viral genome frequently integrates into the cell genome following cleavage inside the *E1* or *E2* viral genes, leading to a subsequent abrogation or a low level of *E5*[24] transcription. However, cervical tumors carrying episomal viral genome have been frequently reported,[24-28] while the presence of E5 protein was detected by mass spectrometry in the CasKi cervical cancer cell line carrying multiple copies of integrated HPV16 DNA into the host genome.[29]

In view of the E5 role on carcinogenesis, the presence of this protein might promote angiogenesis in cervical cancer through the EGFR/VEGFA pathway which would affect the outcome of patients with invasive cervical cancer (CC). In this study, we verified, through detection of *E5* messenger RNA (mRNA), whether the likely presence of E5 was associated with (i) the transcriptional level of *EGFR* and *VEGFA* in HPV16$^+$ and HPV18$^+$ cervical tumors, and (ii) the overall survival of patients with HPV16$^+$ tumors.

2 | MATERIALS AND METHODS

2.1 | Samples and DNA/RNA isolation

A cohort of 360 patients with HPV16$^+$ or HPV18$^+$ invasive cervical cancer, enrolled at diagnosis from August 2011 to August 2013, was initially selected from previously studied group of patients attended at Instituto Nacional de Câncer (INCA-Brazil).[30] Biopsies were collected before treatment and HPV genotypes were previously identified by De Almeida et al.[30] Sixty-seven of these 360 patients could not be included because biopsy samples, used in previous studies, were no longer available. All procedures were approved by the Ethics Committee of Instituto Nacional de Câncer (protocol CAAE 53398416.0.0000.5274) and all patients signed an informed consent.

DNA and RNA were isolated from the same biopsy fragment of each patient with Qiagen Allprep DNA/RNA mini kit, following the manufacturer's recommendations. Following isolation, DNA and RNA were stored at − 25°C and −80°C, respectively.

2.2 | Disruption of *E1* or *E2* genes

The physical status of the viral genome (episomal or integrated) was determined by polymerase chain reaction (PCR) with primer sets for amplifying HPV16/HPV18 *E1* and *E2* genes.[31] Amplification of HPV16 and HPV18 *E1* and *E2* was carried out with eight and nine primer pairs, respectively (see Table S1). Following amplification, products were submitted to electrophoresis in 1.5% ultrapure agarose gels. Absence of at least one amplicon indicated disruption of the viral genome and integration while presence of all amplicons indicated presence of an episomal viral genome or, alternatively, integration following disruption outside *E1/E2* or tandem integration.[31]

PCR was carried out in 25 μL mixtures containing 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer, and 1 U of Platinum *Taq* DNA Polymerase (Thermo Fisher Scientific, Brazil). PCR conditions were: 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, annealing temperature for 30 seconds (see Table S1), extension at 72°C for 40 seconds, and a final extension at 72°C for 5 minutes.

2.3 | cDNA synthesis

Total RNA was treated with DNase I (RQ1 RNase-Free DNase; Promega) following the manufacturer's recommendations. cDNA syntheses were carried out with DNase I treated RNA (1-500 ng/μL) using the SuperScript II RT Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

A glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) fragment was amplified to check DNase treatment and cDNA synthesis with a primer pair designed for targeting both DNA and cDNA. PCR was carried out in 50 μL mixtures containing 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer (see Table S1), and 1 U of Platinum Taq DNA Polymerase (Thermo Fisher Scientific). PCR conditions were: 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, annealing temperature at 54°C for 30 seconds, extension at 72°C for 40 seconds, with a final extension at 72°C for 5 minutes. PCR products were submitted to electrophoresis in

1.5% ultrapure agarose gels. Amplicons were expected to be observed for cDNA samples but missing for DNase-treated RNA.

2.4 | Detection of E5 transcripts

E5 transcripts were detected by reverse transcriptase PCR (RT-PCR) performed with HPV16- and HPV18-specific primers (Table S1). PCR was carried out in 50 µL mixtures containing 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of primers, 1 U of Platinum Taq DNA Polymerase (Thermo Fisher Scientific) and the cDNA template of each sample. PCR conditions were: 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, annealing temperature at 54°C for 30 seconds, extension at 72°C for 40 seconds, with a final extension at 72°C for 5 minutes. RT-PCR products were checked as described above. Presence of an *E5* amplicon indicated expression in a tumor biopsy.

2.5 | Quantitative PCR

To quantify *EGFR* and *VEGFA* transcripts, duplex Quantitative PCR (qPCR) was carried out with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). qPCR reactions were carried out in triplicates for each sample in final volumes of 20 µL following the instructions of the supplier with TaqMan probes for *GAPDH* (Hs02758991 used as housekeeping gene) and *EGFR* (Hs01076090) or *GAPDH* and *VEGFA* (Hs00900055). PCR was carried out in a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). Reactions with cycle thresholds (CT) above 35 cycles were excluded from analyses. Replicates with CT estimates differing >1 cycle from their remaining two replicates were excluded. Relative quantification of *EGFR* or *VEGF* expression was estimated following Pfaffl.[32]

2.6 | Statistical analysis

Fisher's and χ^2 tests were used for evaluating association between categorical variables (HPV genome status and presence of *E5* transcripts) with a significant threshold of $P < .05$. Mann-Whitney *U* and Kruskal-Wallis tests were used to compare differences in *EGFR* and *VEGFA* expression between grouped samples taking into account the capability of *E5* translation, that is: detection of *E5* transcripts and amplification of *E1/E2* genomic regions. Threshold estimates for significance were set at $P < .05$. Analyses were carried out with GraphPad Prism 6.0 (GraphPad Software, Inc).

2.7 | Overall survival analysis

Analysis of overall survival was carried out only for patients with HPV16$^+$ SCC at stages ≥ IB2, all these patients underwent chemotherapy and radiotherapy. The analysis was not carried out in patients with HPV16$^+$ ADN and HPV18$^+$ SCC or ADN because the number of patients in these groups was very small. HPV16$^+$ SCC patients that did not underwent complete chemotherapy and radiotherapy treatment (n = 69) were excluded. The remaining HPV16$^+$ SCC patients (n = 119; see Table S2 for patients' characteristics), were divided into two groups: (i) those capable of translating *E5* (*E5*$^+$, n = 54), with intact *E1/E2* and *E5* mRNA, and (ii) those incapable of translating *E5*, with *E1/E2* disruption and/or without *E5* mRNA expression (*E5*$^-$, n = 65). Overall survival estimates were obtained by follow-ups registered in medical records. Only death associated with cervical cancer was considered. Survival time was defined as the interval (in days) between dates of recruitment and death or last follow up, covering a period from July 2011 to July 2016, totalizing 60 months. Kaplan-Meier and log-rank test were used to compare overall survival. The influences of age, staging, expression of *EGFR* and *VEGFA*, and tumor grade were evaluated using the semi-parametric model of proportional hazards (Cox model). Variables were selected by univariate analysis, and those with $P < .2$ were subsequently used for multivariate analysis with significance equal to $P < .05$. Subsequently, Schoenfelder residue analysis was carried out for evaluating whether each variable was concordant with risk proportionality, viz, constant risk during the period of study. Analyses were performed with STATA 12.0R, version 3.4.2.5. Deaths of patients not associated with cervical cancer and patients with the loss to follow-up were censored.

3 | RESULTS

A total of 246 HPV16$^+$ patients and 47 HPV18$^+$ patients were studied. Clinical profiles are described in Table 1.

3.1 | Disruption of *E1/E2* genes and presence of *E5* transcript

E1/E2 disruption was detected in 49.59% (122 of 246) of HPV16$^+$ patients, while presence of *E5* transcript (*E5*$^+$) was detected in 60.56% (149 of 246). *E5*$^+$ samples were more frequently detected with intact *E1/E2* (88.70%; 110 of 124) while patients with *E1/E2* disruption (n = 122) were more frequently *E5*$^-$ (68.03%; 83 of 122). A significant association was found between *E1/E2* disruption and absence of *E5* transcripts in HPV16$^+$ patients (χ^2 test; $P < .001$) (Table 2). In HPV18$^+$ patients, 72.34% (34 of 47) of tumors showed *E1/E2* disruption and presence of *E5* transcript in 48.93% (26 of 47). A significant association was found between *E1/E2* disruption and lack of *E5* transcripts in HPV18$^+$ tumors (χ^2 test; $P < .002$) (Table 2).

3.2 | *EGFR* and *VEGFA* expression

To evaluate the influence of *E5* on *EGFR* and *VEGFA* transcription, samples were allocated in two groups with respect to capability of E5 translation. Tumors presumably incapable of E5 translation (136 HPV16$^+$ and 35 HPV18$^+$) showed *E1/E2* disruption despite the presence of an *E5* transcript or intact *E1/E2* without *E5* transcripts. Tumors capable of E5 translation (110 HPV16$^+$ and 12 HPV18$^+$)

TABLE 1 Characterization of the 293 patients analyzed in respect to age at the diagnosis, tumor histological type, tumor grade, and FIGO stage

Patient clinical information	HPV 16 (n = 246)	HPV18 (n = 47)
Patient age at diagnosis		
Mean ± SD	48 ± 13.05	47.73 ± 11.01
Median	48	46
Tumor histologic types		
SCC	201 (81.7%)	30 (63.8%)
ADC	33 (13.4%)	16 (34.0%)
Others[a]	12 (4.9%)	1 (2.1%)
Tumor grade		
G1	10 (4.1%)	6 (12.8%)
G2	160 (65.0%)	25 (53.2%)
G3	47 (19.0%)	12 (25.5%)
No information	29 (11.8%)	4 (8.5%)
FIGO stages		
IB1≤[b]	18 (7.3%)	5 (10.6%)
IB2	30 (12.2%)	3 (6.4%)
II	93 (37.8%)	16 (34.0%)
III	90 (36.6%)	21 (44.7%)
IV	15 (6.1%)	2 (4.2%)

ADC, adenocarcinoma; FIGO, Fédération Internationale de Gynécologie et d'Obstétrique staging system; SCC, squamous cell carcinoma; SD, standard deviation.

[a]Others = carcinoma, clear cell carcinoma, and adenosquamous carcinoma.

[b]IA1, IA2, and IB1 = patients with tumor stages submitted to surgical treatment.

presented intact *E1/E2* genes and *E5* transcripts. Comparisons between these two groups within HPV genotypes did not show differences with respect to relative *EGFR* or *VEGFA* expression (Figure 1A-D).

In view that a higher EGFR expression had been previously reported in squamous cell carcinoma,[33,34] similar analyses were carried out for histological types (SCC or ADN). These did not show differences of *EGFR* and *VEGF* expression respective to the capability of *E5* translation in SCC and ADN (Figure 1E-H). However, a significant difference was found between SCC and ADN when comparing the relative expression of *EGFR* in HPV16$^+$ tumors (Figure 1E). In HPV18$^+$ tumors, these comparisons did not show significant differences (Figure 1F).

3.3 | Overall survival

Analysis of overall survival of patients with HPV16$^+$ SCC in stages ≥ IB2 was carried out, with comparisons between patients capable and incapable of *E5* translation. These two groups did not show significant differences with univariate analysis (Figure 2). When considering additional patient profiles (age, histological type, tumor staging and tumor grade), only tumor staging was independently associated with overall survival (Table S3).

TABLE 2 Association between human papillomavirus (HPV) genome disruption (intact or disrupted) and the presence or absence of E5 transcript

HPV type	Presence of E5 transcript	Intact E1/E2	Disrupted E1/E2	*P* value (χ^2)
HPV16	E5$^+$	110	39	$P = 8.92 \times 10^{-21}$
	E5$^-$	14	83	
HPV18	E5$^+$	12	14	$P = .002$
	E5$^-$	1	20	

4 | DISCUSSION

Several studies investigated the oncogenic role of *E5* in cell lines transfected with plasmids expressing this gene. These showed that E5 inhibited EGFR degradation and induced angiogenesis as well as cell transformation, and interfered in the intracellular transport of HLA proteins.[15,16,20,35,36]

In this study, we analyzed the effect of *E5* expression on *EGFR* and *VEGFA* transcription and the overall survival of patients with invasive cervical cancer. A major limitation of the present study resulted from the lack of commercially available antibodies for detecting E5, which prevented us from demonstrating the unequivocal presence of this protein. An alternative strategy was therefore used by proxy on behalf of E5, based on the presence of *E5* mRNA and intactness of *E1* and *E2* coding regions. In this study, we detected *E5* mRNA in several cervical tumors, and showed an association between the presence of *E5* mRNA and non-disruption of the HPV genome as well as between absence of *E5* mRNA and disruption. However, a similar pattern of *E1/E2* amplification resulting from the presence of episomal DNA can also occur in three other situations: (i) a concomitant presence of episomal and integrated viral DNA, (ii) in tandem integration of viral DNA, and (iii) out of *E1/E2* disruption in the viral genome. On the other hand, tumor samples without *E5* transcripts or with *E1/E2* disruption coexisting with E5 mRNAs would not translate the E5 protein. This was why categorization in two groups with respect to the capacity of translating *E5* was appropriate for investigating the role of the E5 protein in tumors.

The likely presence of E5 and the level of *EGFR* and *VEGFA* transcription in HPV16$^+$ and HPV18$^+$ cervical tumors were not significantly associated; only the association between the *EGFR* mRNA level and histological type in HPV16$^+$ tumors could be demonstrated. A higher EGFR expression in SCC respective to ADN was previously reported in cervical carcinomas[33] and herein observed at the transcriptional level in HPV16$^+$ tumors. Our data suggested that this association was restricted to HPV16$^+$ tumors but not for HPV18$^+$ histological types.

Analysis of overall survival did not show differences between patients with HPV16$^+$ SCC in stages ≥ IB2 with respect to the capability of *E5* translation. This finding, together with the absence of association between the capability of *E5* translation and level of *EGFR* transcripts, suggested that *E5* exerted a trivial influence in cancer outcome in stages ≥ IB2. Ramqvist et al[37] found a similar result for overall survival in patients with tonsillar or base of tongue HPV16$^+$ cancers (based on paraffin-embedded biopsy samples) when detecting *E5* mRNA expression. Nevertheless, the presence of E5 might influence early stages of disease (precursor lesions), facilitating

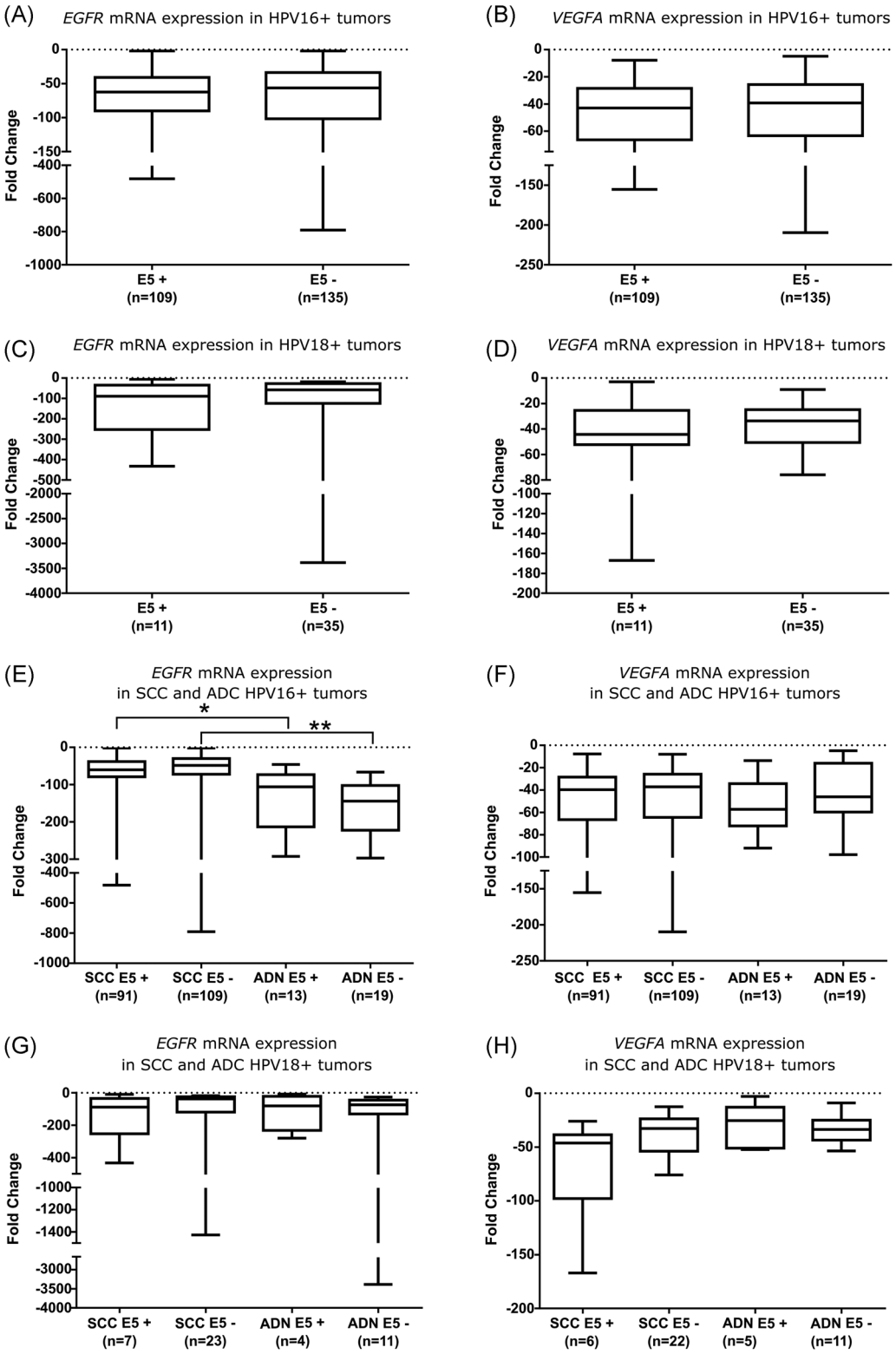

FIGURE 1 Comparison between the transcriptional level of *EGFR* and *VEGFA* between tumors capable ($E5^+$) or incapable of translating E5 ($E5^-$). A, Comparisons between $E5^+$ and $E5^-$ tumors in respect to *EGFR* mRNA level for $HPV16^+$ tumors. B, Comparisons between $E5^+$ and $E5^-$ tumors in respect to *VEGFA* mRNA level for $HPV16^+$ tumors. C, Comparisons between $E5^+$ and $E5^-$ tumors in respect to *EGFR* mRNA level for $HPV18^+$ tumors ($P = .5448$). D, Comparisons between $E5^+$ and $E5^-$ tumors with respect to *VEGFA* mRNA level for $HPV18^+$ tumors. E, Comparisons between tumors $E5^+$ and $E5^-$, of different histological types (SCC vs ADN) with respect to *EGFR* mRNA level for $HPV16^+$ tumors (*$P = .0006$)(**$P < .0001$). F, Comparisons between $E5^+$ and $E5^-$ tumors of different histological types (SCC vs ADN) with respect to *VEGFA* mRNA level for $HPV16^+$ tumors. G, Comparisons between $E5^+$ and $E5^-$ tumors of different histological types (SCC vs ADN) with respect to *EGFR* mRNA level for $HPV18^+$ tumors. H, Comparisons between $E5^+$ and $E5^-$ tumors of different histological types (SCC vs ADN), in respect to *VEGFA* mRNA level for $HPV18^+$ tumors. ADC, adenocarcinoma; EGFR, epidermal growth factor receptor; HPV, human papillomavirus; $HPV16^+$, human papillomavirus 16 positive; $HPV18^+$, human papillomavirus 18 positive; mRNA, messenger RNA; SCC, squamous cell carcinoma; VEGFA, vascular endothelial growth factor A

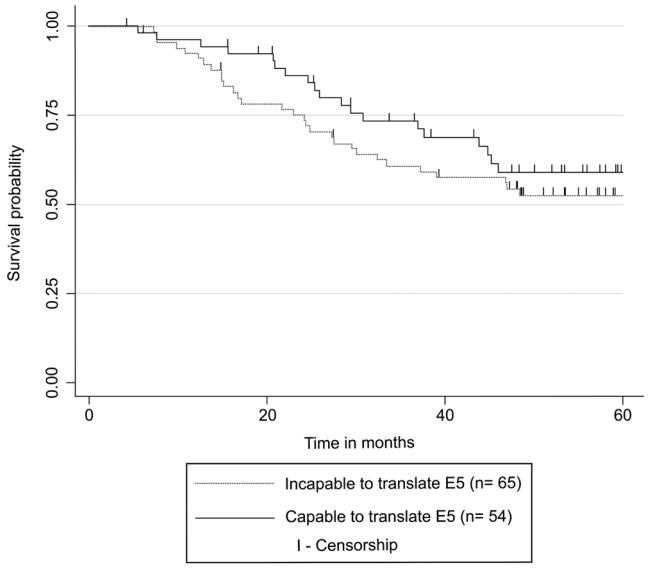

FIGURE 2 Differences in overall survival (Kaplan-Meier analysis) between patients with HPV16$^+$ tumors capable (n = 54) and incapable (n = 65) of translating E5 (E5$^+$ vs E5$^-$). No significant difference was found between groups (log-rank test, $P = .516$). Deaths not associated with cervical cancer and patients with loss to follow-up were censored. HPV16$^+$, human papillomavirus 16 positive

immunological escape,[14-16] and stimulating the EGF/EGFR pathway,[19] cell proliferation,[38] and invasion.[39]

Despite the fact that E5 does not apparently affect patient outcome this protein might be used as a therapeutic target. Some studies reported a response to therapeutic vaccines based on E5, showing that mice immunization before or after xenograft of cell lines expressing E5 prevented tumor growth or decreased tumor size, respectively.[40,41]

In conclusion, this study showed that the potential presence of E5 protein was not associated with mRNA expression of *EGFR*, *VEGFA* in HPV16$^+$ or HPV18$^+$ tumors and did not affect the overall survival of patients.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

DLB, LMA and MAMM: conceived the study. CBPC, SPF, VCV, NAC: revised the medical record. SMA-F, SPF: carried out the viral genome disruption analysis. LFLM, LMA: carried out the overall survival analysis. DLB, MAMM: wrote the manuscript. All authors revised and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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