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In vivo HPV 16 E5 mRNA: Expression pattern in patients with squamous intra-epithelial lesions of the cervix

Laura Lorenzon[a,*], Francesca Mazzetta[b], Aldo Venuti[c], Antonio Frega[d], Maria Rosaria Torrisi[b], Deborah French[b]

[a] *Surgical and Medical Department of Clinical Sciences, Biomedical Technologies and Translational Medicine, Faculty of Medicine and Psychology, University of Rome "La Sapienza", Italy*
[b] *Department of Clinical and Molecular Medicine, Faculty of Medicine and Psychology, University of Rome "La Sapienza", Italy*
[c] *Laboratory of Virology, Regina Elena Cancer Institute of Rome, Italy*
[d] *Department of Woman's Health and Territorial Medicine, Sant'Andrea Hospital, Faculty of Medicine and Psychology, University of Rome "La Sapienza", Italy*

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ABSTRACT

Background: Human Papillomavirus (HPV) type 16 E5 is a small protein, which is reported to display transforming activity *in vitro* and in animal studies. The E5 transcriptional activity, however, has been rarely reported *in vivo* in literature.
Objectives: (a) To detect the E5 transcripts *in vivo* in a population of HPV 16 positive patients with abnormal cytology and (b) to correlate the level of expression to the degree of the cytological lesion.
Study design and methods: 250 cytological samples of HPV positive women were obtained and tested for the E6/E7 mRNA expression. Patients were selected if HPV 16 only mRNA positive and with a cytology consistent with low-grade/high-grade squamous intra-epithelial (LSIL/HSIL) lesions. Selected patients were tested for the E5 transcripts by reverse RT PCR, comparing the expression level *in vivo* with a transfected HPV 16 E5 HaCaT cell line.
Results: 27 HPV 16 E6/E7 mRNA positive LSIL/HSIL patients were selected. 13 out of 17 LSIL patients were tested positive for the E5 mRNA, showing an ample range of positivity. In the HSIL group 7 out of 10 patients were tested positive, displaying lower and more homogeneous levels of expression if compared with the transfected cells.
Conclusion: The HPV 16 E5 transcripts levels showed a broad distribution *in vivo*; the discrepancy was wider in LSIL patients, with HSIL patients displaying a more homogeneous profile. However, because of the limited number of patients, we could not draw a firm conclusion about the correlation between the E5 expression and the disease progression.

1. Background

According with the National Comprehensive Cancer Network (NCCN),[1] cervical cancer is a major world health problem for women. In 2002, it was the third most common cancer in women worldwide,[2,3] 78% of cases occurring in the developing countries, where cervical cancer is the second most frequent cause of cancer death in women. Almost all of these cancers are related to high risk (HR) HPV cervical infection.

Viral gene expression leads to the expression of six nonstructural viral regulatory proteins (E1, E2, E4, E5, E6 and E7) and two structural viral proteins (L1, L2). E5, E6 and E7 are the viral oncogenes and their expression induces immortalization and transformation; E6 and E7 inactivates respectively p53 and Rb.[4]

HPV DNA can be present in host cells in either episomal or integrated form. Integration of the HPV DNA into the host cell genome has been identified as a critical step for cancer promotion and progression.[5] However *in vivo* studies demonstrated that an integrated, episomal or mixed HPV genome could be detected in invasive cancers and pre-cancerous lesions.[5,6] The viral integration in host genome usually leads to a disruption of the E2 gene and causes a loss of the HPV 16 E5 expression.[6,7]

HPV 16 E5 is a small protein, localized to the intracellular membranes, including the Golgi apparatus and the endoplasmic reticulum.[8]

Studies from HPV 16 E5 has been reported causing anchorage independence and tumorigenic transformation of murine

Abbreviations: HPV, Human Papillomavirus; LSIL, low grade squamous intra-epithelial lesion; HSIL, high grade squamous intra-epithelial lesion; RT PCR, real time PCR.

* Corresponding author at: Surgical and Medical Department of Clinical Sciences, Biomedical Technologies and Translational Medicine, Faculty of Medicine and Psychology, University of Rome "La Sapienza", Sant'Andrea Hospital, via di Grottarossa 1035-1039, 00189 Rome, Italy. Tel.: +39 06 33775504; fax: +39 06 33775257.
E-mail address: laura.lorenzon@uniroma1.it (L. Lorenzon).

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fibroblasts and epidermal keratinocytes[9,10]; moreover HPV 16 E5 seems to cooperate with E7 in inducing proliferation of primary rodent cells, and an acute expression of the HPV 16 E5 protein stimulates cellular DNA synthesis in primary human keratinocytes.[11–14]

There are evidences suggesting that receptor tyrosine kinase signalling plays an important role in transformation by the HPV 16 E5 protein and most studies indicate the epidermal growth factor (EGF) receptor, is involved in the HPV E5-related transformation. In this model of interaction, the E5 protein binds and inhibits the vacuolar ATPase, resulting in an impaired endosome acidification and thus a minor EGF receptor degradation, leading to an increased recycling of the receptor to cell surface and enhanced receptor signalling.[13,15] Finally in transgenic mouse models, the HPV 16 E5 expression in the skin produces epithelial hyperproliferation with spontaneous tumor formation, whereas in oestrogen-treated mice, the expression of E5 alone can induce cancer, suggesting a role of E5 as a full oncogene.[16]

Moreover *in vitro* studies documented that the HPV 16 E5 protein down-regulates the major histocompatibility (MHC) class I antigen due to the interaction of its first hydrophobic domain with the heavy chain of HLA-I, thus highlighting that E5 is able to interfere with the clearance of the virus by the host immune system.[17,18]

The E5 transcriptional activity has been rarely reported *in vivo* in literature.

2. Objectives

We conducted a search for the E5 transcripts in a population of HPV 16 positive patients with abnormal cytology, whose transcriptional activity was testified by a positive E6/E7 mRNA test.

Primary aims of our study were: (a) to detect the HPV 16 E5 transcripts by reverse Real Time PCR comparing the level of expression *in vivo* with a transfected HPV 16 E5 HaCaT cell line, and (b) to correlate the level of expression to the degree of the cytological lesion.

3. Study design and methods

3.1. Design and patients

From April 2006 to November 2007, 250 cytological samples were obtained from patients attending the Gynaecology Day-Clinic of Sant'Andrea Hospital (second-line referral centre for cervical pathology), Faculty of Medicine and Psychology, University of Rome "La Sapienza". Pregnant women and women under treatment for invasive cancers were excluded. Written informed consent was obtained from all participants.

All the enrolled patients were previously tested positive for HR HPV DNA and referred for evaluation of the E6/E7 mRNA test by the NucliSens EasyQ HPV assay (bioMerieux), which allows the detection of a E6–E7 transcripts in a nucleic acid sequence-based amplification.[19] All the enrolled patients were tested for the HPV types 16, 18, 31, 33 and 45 E6/E7 mRNA expressions and underwent a Pap smear evaluation. Patients were selected if HPV 16 E6/E7 mRNA positive only (co-infection by more than one HPV types were excluded) and with a cytology consistent with a low grade/high grade squamous intra-epithelial lesion according with the Bethesda classification system 2001. The RNA was stored at −80 °C for the further evaluations.

According with these criteria twenty-seven HPV 16 E6/E7 mRNA positive patients with an abnormal cytology were selected.

3.2. Controls and cell lines

Five HPV cervical negative samples through genotyping (INNO-Lipa HPV Genotyping kit, Innogenetics) were selected as negative controls.

Table 1
Primers set for qRT real-time PCR.

Primer	Sequences (5′–3′)
HPV16 E5	(For-3940): CGCTGCTTTTGTCTGTGTCT
	(Rev-4085): GCGTGCATGTGTATGTATTAAAAA
β-Actin	(For-560): TCACCCACACTGTGCCCATCTACGA
	(Rev-854): CAGCGGAACCGCTCATTGCCAATGG
HPV16 E2C	(For-1244): TTGAAAGCGAAGACAGCGGG
	(Rev-3467): AGTCGTCTGTGTTTCTTCGG

E5 primers efficiency was tested using the cDNA obtained from the transfected HaCat pMSG E5 cell line, resulting as follows: $E = 98.8\%$, slope −3.364, $R\hat{2}$ 0.998. PCR conditions: 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s, followed by a melting curve starting at 55 °C.

A Human Keratinocytes cell line (HaCaT) was stably transfected with pMSG vector only (HaCaT pMSG) and pMSG containing E5 (HaCaT pMSG E5), under the control of a promoter inducible after dexamethason (Dex) treatment. Cells were cultured in a Dulbecco's modified Eagle's Medium (DMEM) plus Fetal Bovine Serum (FBS) 10%. In order to induce the E5 expression in tranfected pMSG and pMSG E5 HaCaTs, 70% confluent serum starved-cells were treated with Dex 1 μM for 24 and 48 h.[20]

Transfected HaCaT pMSG E5 cells were used as a model for mimicking the E5 expression of HPV 16-episomal infected cells.

The CaSki cell line (American Type Culture Collection, Rockville, Md.) was cultured in RPMI-1640 medium plus FBS 10%, as a standard control for evaluation of the results obtained in the transfected HaCaT pMSG E5, since the CaSki cells are reported to contain an integrated HPV 16 genome (about 600 copies per cell).[21,22]

3.3. RNA extraction (cell lines and controls)

RNAs from cell line were extracted by the use of the RNeasy Mini kit (Quiagen), otherwise RNAs from cervical cytological samples HPV negative was carried out through TRIzol Reagent (Invitrogen), both according with the manufacturer's protocol.

3.4. RNA retro-transcription

Retro-transcription was carried through iScript™ cDNA Synthetis kit (BIO-RAD), processing samples in a thermal cycler (MyMini BIO-RAD) at the following conditions: 25 °C for 5 min, 42 °C for 30 min, and then 85 °C for 5 min.

3.5. Reverse real-time PCR

Table 1 shows the set of primers employed. Reverse real-time PCR was carried out through the iCycler IQ™5 Multicolor Real Time PCR detection System (BioRad), using SYBR Green Supermix (BioRad). For evaluation of results, the gene target expression was correlated to the house-keeping expression by the use of the $\Delta\Delta C_t$ method, expressing results as $2^{-\Delta\Delta C_t}$. The ΔC_t value was obtained calculating the difference between the mean C_t value of the target and the mean C_t value of the housekeeping, correlating results with the ΔC_t obtained from the E5 expression in HaCaT pMSG E5 and CaSki cells, considered equal 1.

4. Results

Twenty-seven HPV 16 E6/E7 positive patients with an abnormal cytology were selected. Selected patients had a mean age of 33.7 years (range 18–54 years): seventeen had a Pap smear consistent with a low grade squamous intra-epithelial lesion (LSIL) and ten patients had a high grade intra-epithelial lesion (HSIL).

Fig. 1 shows the results of the E5 expression in the cell lines and *in vivo* in LSIL patients.