

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

THE EFFECT OF HUMAN PAPILLOMAVIRUS 16 E6 AND E7
ONCOPROTEINS ON THE TRANSCRIPTIONAL REGULATION OF
GENES INVOLVED IN KERATINOCYTE DIFFERENTIATION

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INTRODUCTION

In the last few decades, one of the central discoveries in studying malignant malformations was to demonstrate the etiologic role of human papillomavirus (HPV) in the development of cervical cancer. Epidemiological and case studies using molecular biological techniques demonstrated in different countries that HPV DNA can be detected in 90-100 % of samples from cervical cancer patients. The most studied and most common high-risk oncogenic HPV type is HPV 16, this type is found in 50 % of cervical cancer cases.

HPVs have a circular double-stranded DNA genome of about 8 kbp length. The virions are 55 nm in size and not enveloped. Their genome contains an early (E1-E7), a late (L1, L2) and a control region (long control region, LCR). Over 170 types belonging to the Papillomaviridae family have been described until now, and newer types are persistently identified.

The HPVs infect the proliferating basal cells of epithelium, cutaneous and mucosal types have been described according to their tropism. Cutaneous types (HPV 1, 2, 3, 10) cause common warts, and some types (HPV 5, 8) cause lesions in patients with the hereditary disease epidermodysplasia verruciformis. Low and high-risk oncogenic types can be distinguished among types infecting the mucous membranes. Low risk types (HPV 6, 11, 42) are mainly found in benign genital lesions (condyloma acuminatum) or in low grade cervical dysplasias. HPV 16, 18 and other high-risk types (HPV 31, 33, 35 etc) occur in high grade cervical intraepithelial neoplasias (CIN) and are causally linked to the development of cervical cancer .

The E6 and E7 oncoproteins of high-risk HPVs are responsible for the transforming activity of the virus. High-risk HPV E6 induces the degradation of the p53 tumour suppressor protein through the ubiquitin-proteosome pathway. High-risk HPV E7 is able to bind to the pRB (retinoblastoma) tumor suppressor protein, and by releasing the E2F factors, induce the progression of the cell cycle. The two oncoproteins of HPV regulate several other viral and cellular factors.

The structural component of the multilayer epithelium present in highest amount is the keratinocyte. After the basal cells divide, a portion of cells pass over morphological and biochemical changes in the upper epithelial layers. During keratinocyte differentiation, the activity of genes involved in the process (involucrin (IVL), loricrin (LOR), keratins (KRT), transglutaminases (TGM), S100 calcium-binding proteins (S100), small proline-rich proteins (SPRR) and desmosomal proteins: desmocollin (DSC), desmoglein (DSG)) is increased and a

number of structural proteins are synthesized. The process is strictly regulated in time and space, the final result is the dead layer of skin, the stratum corneum.

The life cycle of HPV is closely related to the maturing process of the host cell. The virus reaches the basal cells of the multilayer epithelium through micro-lesions, where low level viral genome replication takes place, along with the expression of early genes, including the E6 and E7 oncogenes. In the upper part of the epidermis cells exit the cell cycle, however, as a result of HPV oncoproteins cell cycle remains active in the infected keratinocytes. The expression of late genes and production of virions take place exclusively in well differentiated epithelial layers. Papillomaviruses are non-lytic, the virions are released from the cells when the keratinocytes reach the upper layer, and peel off from the epithelium.

It is well known that the high-risk oncogenic HPVs perturb the differentiation program of the host cell. This is thought to have a role in the productive viral replication cycle, where the virus has to induce the cellular replication machinery in differentiating keratinocytes to provide replication of the viral genome. The viral E6 and E7 oncoprotein play an important role in this process. Inhibition of cellular differentiation by high-risk HPVs is even more pronounced in premalignant and malignant genital lesions, such as cervical intraepithelial neoplasia (CIN) and cervical carcinoma. In these manifestations, non-productive viral life cycle takes place, with lack of late gene expression and virion production (abortive infection).

The cellular DNA replication machinery is reactivated by the E7 oncoprotein in differentiating keratinocytes to provide a cellular environment that is permissive for the replication of the viral genome. This activity of HPV 16 E7 was shown to delay the induction of the keratinocyte differentiation markers involucrin and keratin 10 in human keratinocytes. HPV 16 E6 was shown to inhibit epithelial differentiation in organotypic cell cultures. Using a xenograft model, it was shown that infection of human keratinocytes by certain HPVs (type 11 and 59) causes altered expression of certain differentiation-associated proteins (such as LOR and SPRR), both on the mRNA and on the protein level.

Several studies reported that the expression of genes involved in keratinocyte differentiation changes in the presence of HPV oncogenes in cervical cancer cells and in vitro cell cultures. However, it is not known whether the HPV oncoproteins have effects on the promoters of differentiation-regulated genes or exert their effects post-transcriptionally. In our study, we examined the effects of HPV 16 E6 and E7 oncoproteins on the expression of genes involved in differentiation, and studied the mechanism of their effect. Initially, we examined the expression and the promoter activity of involucrin, an important marker of differentiation, in the presence of HPV 16 E6 and E7 oncoproteins. In the second part of our work we studied

the effect of HPV 16 oncoproteins on the expression of several other differentiation-associated genes, on the activity of their regulatory region and on the activity of some transcription factor (such as activator protein 1(AP-1) and CCAAT enhancer binding protein (C/EBP)) important in the regulation of keratinocyte differentiation.

AIMS

In my PhD work, I studied the effect of HPV 16 oncoproteins on the expression and transcriptional regulation of genes associated with keratinocyte differentiation. The purpose of this study was to:

- Investigate the effect of HPV 16 E6, E7 or E6/E7 oncogenes on the mRNA and protein level of involucrin in human keratinocytes.
- Study the effect of HPV 16 oncogenes on the activity of involucrin promoter and localize this effect on the regulatory region.
- In HPV 16 E6, E7 or E6/E7 expressing keratinocytes, investigate expression of cellular genes, mainly genes important in keratinocyte differentiation, in microarray analysis.
- Validate the microarray results and study the expression of other, well-known keratinocyte differentiation genes with real-time PCR.
- Study the promoter activity of some differentiation-associated genes in the presence of HPV oncogenes in transient transfection assays.
- Investigate the effect of HPV 16 E6 and E7 oncogenes on the activity of some transcription factors, which are important regulators of the differentiation process.

MATERIALS AND METHODS

Cell culture and retroviral transduction

Primary human foreskin keratinocytes (HFK) were cultured in Defined Keratinocyte-Serum Free Medium containing < 0.1 mM calcium. PA317-LXSN, -16E6, -16E7, and -16E6E7 recombinant retrovirus producing cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum. Primary keratinocytes were infected with culture supernatants from PA317 cell lines and selected in media containing geneticin. The infected HFK cells are: HFK-LXSN (cells transduced by control retrovirus vector), HFK-16E6, HFK-16E7 or HFK-16E6E7. These cell lines were either left untreated or induced to differentiate by culturing in DMEM (containing 1.8 mM calcium and 10% foetal calf serum) for 24 h.

Microarray processing

Total RNA was isolated from each transduced keratinocyte cell line in three biological replicates using RNeasy Mini Kit. RNA integrity was checked on Agilent Bioanalyzer 2100. RNA samples with >9.0 RNA integrity number (RIN) values were used in the further experiments. Global expression pattern was analyzed on Affymetrix GeneChip Human Gene 1.0 ST arrays. 200 ng of total RNA samples were used in the amplifying and labelling reaction. Samples were hybridized at 45 degrees Celsius for 16 hours and then standard washing protocol was performed using Affymetrix GeneChip Fluidics Station 450. The arrays were scanned on GeneChip Scanner 7G. RNA labelling and hybridization were processed by Szilárd Póliska (UD-GenoMed Medical Genomic Technologies Ltd, Debrecen, Hungary).

Microarray data analysis

Microarray data were analyzed by GeneSpring 12 GX software. Affymetrix CEL files were normalized by Robust Multichip Analysis (RMA) algorithm and median normalization. Expressed genes were determined by filtering out the lowest 20 percentile of genes based on raw signal intensity, then genes which did not show at least 2-fold change difference compared to the median were also filtered out, and the statistical analysis was performed on the remaining 3293 genes. We used one-way ANOVA test with Tukey post hoc test. Statistically significant difference was considered at $p < 0.05$ and fold change cut off value was 1.5. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA) software.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from proliferating or differentiating transduced cells by using TRI reagent. The High Capacity cDNA Reverse Transcription Kit was used to prepare cDNA. The PCR reaction was performed with GoTaq DNA polymerase according to the manufacturer's protocol. The primer pairs amplified the HPV16 E6, E7 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNAs.

Real-time RT-PCR

The real-time PCR was performed on the 7500 Real Time PCR System using TaqMan Gene Expression Master Mix and Assays according to the manufacturer's recommendations. In Relative Quantification (RQ), GAPDH was used as endogenous control and the RQ value of proliferating cells transduced by LXSN was set to 1, and other values are shown relative to this. The comparative Ct method was used to obtain the RQ values with standard deviation and confidence intervals (7500 System SDS Software, version 1.4). Each PCR reaction was performed in triplicate at least three times.

Western blot

Protein extract from proliferating or differentiating transduced HFK cells were prepared in RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Cells were scraped, incubated on ice, and after centrifugation the protein concentration of the supernatants was measured by Bradford protein assay. Ten µg of protein extracts was electrophoresed through 10 % polyacrilamide gels (SDS-PAGE) and electroblotted into nitrocellulose membrane. Membranes were blocked in Tris buffered saline with Tween 20 (TBST) containing 5% non-fat dry milk. The blots were incubated with primary antibodies (anti-involucrin, anti-p53 and anti-actin) diluted 1:2000 in 5% non-fat dry milk in TBST. After washing in TBST, the membrane was incubated with secondary antibodies conjugated with horseradish peroxidase. Antibody complexes were visualized using the SuperSignal West Pico Chemiluminescent Substrate and exposed to X-ray films. The amounts of proteins were quantitatively determined by densitometry using Gel Doc 2000 gel documentation system and the Quantity One (version 4.0.3) software. Protein levels of IVL and p53 were normalized to actin levels and shown relative to control vector (LXSN) transduced cells. Each reaction was performed in triplicate at least three times.

Plasmid constructs

The luciferase reporter vector pGL3-IVL contains a 3.7 kb fragment of the human IVL gene regulatory region in front of the luciferase gene. The other fragments of IVL promoter were created by PCR using pGL3-IVL as template and cloned into pGL3-Basic between the *XhoI* and *HindIII* restriction sites resulting pGL3-IVL-2418, pGL3-IVL-1809, pGL3-IVL-744 and pGL3-IVL-272. Amplifications were performed with GeneAmp High Fidelity System according to the manufacturer's protocol. The resulting clones were verified by sequencing (UD-GenoMed Medical Genomic Technologies Ltd, Debrecen, Hungary).

The p53-Luc, pAP-1-Luc and pC/EBP-Luc reporter plasmids contain several copies of the p53, AP-1 and C/EBP binding site and luciferase gene. The pAdE2Luc reporter construct contains adenovirus E2 promoter. The pcDNA-16E6 and pcDNA-16E7 expression vectors were described previously by our workgroup. The *Renilla* luciferase reporter vectors contain the promoter regions of keratin 4 (KRT4), keratin 5 (KRT5), small prolin-rich protein 1A (SPRR1A), S100 calcium binding protein A8 (S100A8) and desmocollin 1 (DSC1) genes.

Transient transfection

Primary human keratinocytes (within 3-6 passages) were plated on 6-well plates at approximately 70-80% confluence. The cells were co-transfected by 0.5 µg of reporter plasmids (pGL3-IVL, pGL3-IVL-2418, pGL3-IVL-1809, pGL3-IVL-744; pGL3-IVL-272 p53-Luc, pAP-1-Luc, pC/EBP-Luc, pAdE2Luc, KRT4, KRT5, SPRR1A, S100A8 or DSC1) along with 0.25 µg of expression vectors (pcDNA) encoding HPV 16 E6 and/or E7 genes using Effectene transfection reagent. After incubation for 5 h at 37 °C the medium was changed to keratinocytoma medium. Twenty-four hours after transfection, HFKs were either left untreated or induced to differentiate in DMEM (containing 1.8 mM calcium and 10% foetal calf serum for 24 h). The cells were washed with PBS (phosphate buffered saline) 48 h after transfection and lysed in appropriate lysis buffer. The luciferase activity of the cell extracts was measured by Luciferase Assay System using a Berthold luminometer. Bradford protein assay was used to standardize for the protein concentration of the cell extracts. Each transfection experiment was performed independently at least three times. To analyse the results of luciferase tests, mean and SEM (standard error of mean) of standardized luciferase values (from at least 3 independent experiments) were calculated using the 2-sample t-test or the ratio t-test (a paired t-test performed after logarithmic transformation of standardized luciferase values). Significance was accepted at $p < 0.05$.

RESULTS

Effects of human papillomavirus 16 oncoproteins on the expression of involucrin in human keratinocytes

Effects of HPV 16 oncogenes on the expression of selected cellular genes involved in keratinocyte differentiation

To study the effects of HPV oncogenes on the expression of cellular genes, human foreskin keratinocyte (HFK) cells were transduced by recombinant retroviruses carrying either the control vector (LXSN) or vectors encoding HPV16 E6 or E7 or both oncogenes. The presence of functionally active HPV oncoproteins was confirmed by demonstrating their effect on the level of the cellular p53 oncoprotein and telomerase reverse transcriptase mRNA. Real-time RT-PCR assays were used to examine the effects of the HPV oncogenes on the expression of the squamous differentiation marker TGM1 and one of its major substrate IVL in HPV oncogene transduced cells. As expected, induction of differentiation of HFK cells by serum and increased calcium resulted in highly increased levels of both IVL and TGM1 mRNA. In proliferating and differentiating cells, both E6 and E7 had significant inhibiting effect on IVL mRNA levels. We found a very strong down-regulation of IVL mRNA in cells expressing both HPV oncoproteins compared to vector control transduced cells, in proliferating and differentiating cells. In proliferating cells, HPV 16 E6 and E7 oncogenes together had only moderate inhibitory effect on the endogenous mRNA level of TGM1, while in differentiating cells, the HPV oncogenes had no significant effect on TGM1 mRNA expression.

To see the effects of the HPV oncogenes on the level of the IVL protein, we performed Western blot analysis on the HPV-transduced cells. Induction of differentiation by serum and high calcium resulted in increased IVL protein levels. In proliferating cells, HPV 16 E6 or E7 alone had little effect on endogenous IVL protein, while the two oncogenes together caused strong down-regulation of IVL protein level as compared to that found in vector transduced (LXSN) cells. This down-regulating effect of E6/E7 on IVL protein level was smaller but still significant in differentiating cells.

Effects of HPV 16 oncogenes on the transcriptional activity of the human involucrin promoter

Next, we aimed to determine whether the down-regulation of IVL expression by the HPV oncoproteins was caused by inhibiting the transcriptional activity of the IVL promoter. To this end, low-passage HFK cells were transiently transfected by either vector control or HPV 16 E6 and/or E7 expression vectors along with luciferase reporter constructs containing the regulatory region of the human IVL gene. The expression of functionally active HPV 16 E6 and E7 protein in the transfected HFK cells was confirmed by co-transfection of reporter constructs containing either binding sites for the p53 protein or the adenovirus E2 (AdE2) promoter containing binding site for the E2F transcription factor, respectively.

In proliferating HFK cells, HPV 16 E6 caused significant down-regulation of a reporter construct (pGL3-IVL) containing the full-length regulatory region of the human IVL gene. On the contrary, E7 alone had no significant effect on the IVL reporter construct in proliferating cells. Induction of differentiation by serum and high calcium resulted in highly increased activity of the pGL3-IVL construct in the presence of the empty expression vector pcDNA. In differentiating HFK cells, both HPV 16 E6 and E7 had a non-significant tendency to inhibit IVL promoter activity, while HPV 16 E6 and E7 together caused significant down-regulation of the IVL promoter. Taken together, these results indicate that the down-regulation of IVL promoter activity by HPV 16 E6 significantly contribute to the inhibition of endogenous IVL expression by the HPV 16 oncoproteins.

Localisation of the effects of HPV 16 oncogenes on the human involucrin promoter

In order to localise the effects of HPV 16 oncogenes on the human IVL promoter, a series of luciferase reporter vectors was constructed carrying different fragments of the promoter. First, we checked in transient transfection experiments whether these constructs responded to differentiation stimuli in HFK cells. The activity of each reporter construct was induced by differentiation of HFK cells. Next, we studied the effects of HPV 16 E6 and E7 on the transcriptional activity of the IVL reporter constructs in HFK cells. In proliferating HFK cells, HPV 16 E6 was able to down-regulate transcription from each reporter construct. E6 caused significant down-regulation of all the tested IVL reporter constructs also in differentiating HFK cells. These results indicate that most of the down-regulating activity of HPV 16 E6 on the human IVL promoter can be mapped to the proximal regulatory region of the IVL gene.

HPV 16 E7 had no significant effect on any IVL reporter construct in proliferating cells. In differentiating cells, E7 had a moderate but significant inhibiting effect on the full-length IVL reporter construct (IVL-2418), while it had no significant effect on the shorter IVL reporter plasmids.

Effects of human papillomavirus 16 oncoproteins on the expression of further genes involved in keratinocyte differentiation

Global gene expression profiling of HFK cells transduced by HPV 16 oncogenes

To explore the effects of HPV 16 E6 and E7 oncogenes on the global gene expression profile of primary HFK cells, microarray analyses were performed in HFK cells transduced by recombinant retroviruses (HFK-LXSN, HFK-16E6, HFK-16E7, HFK-16E6E7). We determined the global mRNA profile of 3 independent sets of low-passage cells transduced by virus vectors. Differentially expressed genes were determined between the four conditions by ANOVA test and then unsupervised hierarchical clustering was performed. IPA analysis revealed that these differentially expressed genes affect the regulation of cell cycle, cell death and proliferation pathways/functions. In accordance with the results of previous studies using similar methodologies, we found that the expression of several genes that are known to be involved in keratinocyte differentiation (IVL, SPRRs, S100A proteins and KRTs) were significantly down-regulated by the HPV oncoproteins.

Effect of HPV 16 oncogenes on endogenous mRNA levels of selected keratinocyte differentiation genes

To validate gene expression data obtained by microarray analysis, we performed quantitative real-time RT-PCR (qRT-PCR) assays on a subset of genes involved in keratinocyte differentiation. Most of the genes analysed showed significantly decreased expression in the presence of the HPV 16 E6 and E7 oncogenes. Some of the genes explored (such as cornifelin (CNFN), LOR, S100A10, S100A12) were found to have significantly reduced expression in real-time RT-PCR analysis but not in microarray analysis.

Seven of the genes with altered expression by the HPV oncoproteins (in microarray analysis and/or real time RT-PCR assays) were chosen for detailed gene expression analysis by real-time RT-PCR assays. For comparison, a basal keratin gene (keratin 5, KRT5) whose

expression is known not to be altered by differentiation was also included in the analysis. As expected, KRT5 mRNA levels were not decreased by the HPV oncoproteins. On the other hand, the keratinocyte differentiation genes analysed by real-time RT-PCR (DSC1, KRT1, KRT4, KRT10, S100A8, S100A9 and SPRR1A) usually showed significantly decreased expression by the HPV oncoproteins. Taken together, our results indicate that it may be a general function of the HPV 16 E6 and E7 oncoproteins to down-regulate the expression of genes involved in the differentiation of stratified squamous epithelial cells.

Effect of HPV 16 oncogenes on the promoter activities of selected keratinocyte differentiation genes

To see the effects of the HPV oncogenes on the transcriptional activities of keratinocyte differentiation genes whose expression has been analysed by real-time RT-PCR assays, luciferase reporter assays were performed. These experiments were conducted in HFK cells transiently co-transfected by reporter vectors containing the promoters of different keratinocyte differentiation genes (DSC1, KRT4, S100A8 and SPRR1A) along with HPV 16 E6, E7 or E6 and E7 expression vectors. The promoter activities of each of these 4 genes were significantly decreased in cells transfected by the two HPV oncogenes together (16E6E7) compared to cells transfected by control expression vector. As expected, the HPV oncoproteins had little if any effect on the transcriptional activity of the KRT5 promoter. Our results suggest that the down-regulation of expression by the HPV 16 oncoproteins of several genes involved in keratinocyte differentiation is caused at least partly by inhibition of transcriptional activities of these genes.

Effect of HPV 16 oncogenes on the activities of AP-1 and C/EBP transcription factors

We supposed that the HPV 16 oncoproteins down-regulate the expression of keratinocyte differentiation genes through modifying the activities of certain transcription factors. Sequence analysis of the promoters that were shown here to be inhibited by the HPV oncoproteins revealed that each contain putative binding sites for AP-1 and C/EBP transcription factor family members. Therefore, we decided to explore if the HPV 16 oncogenes have any effect on the activities of these transcription factors. To this end, HFK cells were transfected by luciferase reporter vectors containing multiple copies of binding sites for the transcription factors AP-1 (jun/fos) or C/EBP along with HPV E6/E7 expression vectors. The results of luciferase tests showed that the HPV 16 E6 and E7 oncogenes together

had significant down-regulating effect on both C/EBP and AP-1 activity. These results suggest that the down-regulating effect of HPV 16 oncogenes on the promoters of certain keratinocyte differentiation genes may be caused by inhibiting the activities of transcription factors that are key regulators of these promoters, such as C/EBP and AP-1 family members.

DISCUSSION

In my PhD work, I studied the effect of HPV 16 E6 and E7 oncoproteins on the expression changes of differentiation-associated genes in human keratinocytes, the natural host cells of HPV. In the first part of the thesis, we examined the expression of IVL and TGM1 genes, important markers of differentiation in the presence of HPV oncoproteins. We found that the HPV 16 E6 and E7 oncoproteins caused a synergistic down-regulation of endogenous IVL mRNA and protein levels in human keratinocytes. Our finding is in accordance with previous studies performing microarray analysis on cervical cancer specimens and human keratinocytes transduced by retrovirus vectors expressing HPV oncogenes. IVL and/or other keratinocyte differentiation associated genes (such as certain keratins and small proline-rich proteins) were down-regulated by HPV oncogenes in these studies. As expected, induction of keratinocyte differentiation by serum and high calcium highly increased the endogenous mRNA levels of both IVL and TGM1 in HFK cells. Interestingly, the HPV 16 E6 and E7 oncogenes together had a very strong down-regulating effect on IVL mRNA but only a moderate effect on TGM1 mRNA. This suggests that the HPV oncogenes may have different effects on the expression of different genes involved in the differentiation of squamous epithelial cells. In proliferating cells, the E6 and E7 oncogenes seemed to have synergistic down-regulating effect on IVL mRNA. In differentiating cells, where IVL expression is highly increased, the down-regulation of IVL mRNA by the HPV oncogenes was lower but still significant. Western blot analysis showed that the joint effect of HPV 16 E6 and E7 on transcriptional down-regulation resulted in excessive decrease of IVL protein levels as well, both in proliferating and in differentiating cells. In a previous study, the expression of HPV 6 or HPV 16 E6 was shown to result in a decrease of IVL protein levels in HFK cells. We can conclude that the HPV 16 E6 and E7 oncogenes seem to down-regulate basal IVL expression and also decrease the differentiation-induced expression of the IVL gene in HFK cells.

The expression of genes involved in keratinocyte differentiation is generally regulated on the level of transcription. Therefore, it seemed reasonable to investigate the effects of HPV 16 oncoproteins on IVL promoter activity. This approach included transfecting HFK cells by HPV 16 E6 and/or E7 expression vectors along with luciferase reporter constructs containing the whole upstream-regulatory region (URR) of the human IVL gene. A portion of the transduced cells was induced to differentiate for 24 h. In agreement with previous results, differentiation of HFK cells led to a significant increase in the transcriptional activity of the

IVL promoter. In proliferating HFK cells, HPV 16 E6, but not E7 caused a significant down-regulation of IVL promoter activity. The HPV 16 E6 and E7 oncoproteins together caused a down-regulation of IVL promoter activity in differentiating HFK cells. Taken together, these results suggest that the down-regulation of endogenous IVL mRNA and protein levels in HFK cells by the HPV 16 E6 oncoprotein is caused by inhibition of IVL promoter activity. However, it can not be ruled out that HPV 16 E6 down-regulates the expression of IVL or other differentiation-associated genes also by other mechanisms.

In order to localize the effect of the HPV oncogenes within the IVL promoter, we made luciferase reporter constructs containing different parts of the URR of the human IVL gene. The URR of the human IVL gene contains a distal regulatory region (DRR, -2473/-1953 from transcription start site) and a proximal regulatory region (PRR, -241/-7 from the transcription start site). From the 5 possible AP1 binding sites in the URR, AP1-5 (in DRR) and AP1-1 (in PRR) are essential for optimal promoter activity. AP1 factors (c-fos, fosB, Fra-1, Fra-2, c-jun, junB and junD) are expressed at specific epidermal layers and the expression pattern of these factors is thought to have a role in differentiation-regulated gene expression in keratinocytes. In our experiments, the level of inhibition by HPV 16 E6 was the highest for the construct containing the whole URR of the IVL gene, but an IVL reporter construct carrying only the PRR was still significantly inhibited by the HPV 16 E6 protein, both in proliferating and in differentiating HFK cells. This suggests that the PRR of IVL gene contains binding sites for transcription factors that are regulated by HPV 16 E6.

HPV 16 E7 had a significant inhibitory effect only on the construct containing the full-length IVL promoter (IVL 2418), and this effect was seen only in differentiating cells. This may suggest that the effect of E7 on the IVL promoter is less direct and/or less specific than that of E6. We find it conceivable that the effects of E7 seen on IVL expression (synergistic down-regulating effect with E6) and on IVL promoter (slight down-regulation only in differentiating cells) are caused not by a direct and specific interaction with the IVL promoter, but rather by recently described other mechanisms. For instance, the DEK protein was found to be transcriptionally and nucleophosmin (NPM) was found to be posttranscriptionally up-regulated by HPV 16 E7, and this was shown to have a role in the inhibition of differentiation in keratinocytes.

To continue our research, we tested the expression of other genes involved in keratinocyte differentiation in the presence of HPV 16 oncoproteins. In the next part of our work we were able to perform global gene expression profiling of human keratinocytes transduced by HPV 16 E6 and/or E7 oncogenes. We confirmed our results with real-time PCR

in the case of differentiation genes. Also in this study, some of the genes with altered expression by the HPV oncogenes (such as CNFN, KRT4, LOR, S100A10 and S100A12) could be identified by real-time PCR assays, but not by microarray analysis.

Most of the keratinocyte differentiation genes found here to be down-regulated by the HPV 16 oncogenes have been already shown to have reduced expression in cervical cancer compared to normal cervical cells. Accordingly, these genes have been shown to be down-regulated by high-risk HPV oncogenes by using in vitro cell culture model systems. Thus, it seems to be a general activity of high-risk HPV oncoproteins to down-regulate the expression of genes associated with keratinocyte differentiation. This activity of HPV oncoproteins may have a role in the productive viral life cycle. The delay of host cell differentiation is also a prominent feature of HPV-associated premalignant cervical lesions (CIN), and it can be also seen in HPV-immortalized keratinocytes grown in organotypic (raft) culture.

There are few data on the mechanism of action of the HPV oncogenes on the expression of keratinocyte differentiation genes. In this study, we went on to see the effects of HPV 16 oncogenes on the transcriptional activities of further differentiation-associated genes (DSC1, KRT4, S100A8 and SPRR1A) which were shown to have reduced expression by the HPV oncoproteins in this study and also in previous reports. We found that the promoters of each of these genes were usually down-regulated by the HPV oncogenes. In each case, the strongest effect was found when both HPV oncoproteins were expressed. It may be speculated that the high-risk HPV E6 and E7 oncoproteins have an additive or synergistic effect on the expression of keratinocyte differentiation genes.

Usually, there was good correlation between the gene expression data obtained by real-time RT-PCR and the promoter activity data obtained by luciferase tests. However, there were also examples where the promoter activities did not correspond closely to the gene-expression data. These results may indicate that, in addition to transcriptional regulation other mechanisms (such as post-transcriptional regulation) may contribute to the down-regulation of expression of these genes by the HPV oncoproteins.

The HPV oncoproteins had no inhibitory effect on the promoter of KRT5, which is a basal keratin gene, i.e. its expression is not regulated by differentiation. Thus, it appears that the HPV 16 oncoproteins down-regulate specifically the transcription of those keratinocyte genes whose expression is regulated by differentiation. Taken together, our luciferase results indicate that the HPV 16 oncogenes have a down-regulating effect on the transcriptional activities of several keratinocyte differentiation genes, and this mechanism seems to

contribute to the reduced expression of these genes in premalignant and malignant cervical lesions compared to normal cervical epithelium.

We found that each of the promoters that were shown in this study to be down-regulated by the HPV oncoproteins have putative binding sites for AP-1 (jun/fos) and C/EBP transcription factors. These transcription factors, among others have been shown to be significant in the transcriptional regulation of keratinocyte differentiation genes and are themselves regulated by epithelial differentiation. The HPV 16 E7 protein was shown to bind to AP1 transcription factors, including c-jun, junB, junD and c-fos. Our experiments performed with reporter constructs containing binding sites for these transcription factors indicated that the HPV oncoproteins had significant inhibitory effects on the activities of these transcription factors. Therefore, it is conceivable that the HPV 16 oncoproteins down-regulate certain keratinocyte differentiation promoters through modifying the activities of AP-1 and C/EBP transcription factors. In order to prove this scenario, further experiments would be needed including promoter mutagenesis and chromatin immunoprecipitation (ChIP) assays.

Our results suggest that the HPV 16 E6 and/or E7 oncogenes may have the potential to down-regulate the expression of several keratinocyte differentiation genes at least partially by down-regulating their promoter activity. HPV replication takes place in differentiating epithelial cells, which exit the cell cycle in the absence of viral infection. The E7 oncogene is able to induce the progression of the cell cycle in differentiating keratinocytes, which is important for viral DNA replication. On the other hand, the ability of the E6 oncogene to cause a delay in the induction of epithelial differentiation may also have a role in providing a cellular environment that is favourable for HPV replication. The decreased expression of IVL and other differentiation-regulated genes by the HPV oncoproteins may have an important role in the productive virus life cycle, and probably also in virus induced carcinogenesis.

SUMMARY

The life cycle of the human papillomavirus (HPV) is closely linked to keratinocyte differentiation. The HPV 16 E6 and E7 oncoproteins have been shown to hamper the normal differentiation of keratinocytes; however, the underlying mechanisms responsible for this phenomenon are yet to be clarified. The purpose of this study was to investigate the effects of HPV16 oncoproteins on the expression of genes involved in keratinocyte differentiation.

Human foreskin keratinocytes (HFK) were transduced by LXSIN-based retrovirus vectors expressing HPV 16 E6 and E7. The differentiation of HFK cells significantly increased both the mRNA and the protein levels of involucrin (IVL). The E6 and E7 oncoproteins of HPV 16 together caused strong down-regulation of IVL mRNA and protein both in proliferating and in differentiating HFK cells. In transient transfection assays and luciferase tests, we found that HPV 16 E6 repressed IVL promoter activity in proliferating HFK cells. The inhibitory effect of HPV 16 E6 on the human IVL promoter could be localised to the proximal regulatory region (PRR) of the gene. These results suggest that the down-regulation of IVL promoter activity by HPV 16 E6 significantly contribute to the inhibition of endogenous IVL expression by the HPV 16 oncoproteins. In contrast, the down-regulation of endogenous IVL expression by HPV16 E7 is probably caused by a less direct and specific effect of E7 on the IVL promoter.

Gene expression analysis performed with microarray analysis and quantitative real-time polymerase chain reaction confirmed that HPV 16 E6 and E7 oncogenes down-regulate the expression of several genes involved in keratinocyte differentiation in HPV oncogene transduced HFK cells. Furthermore, luciferase reporter assays revealed that the HPV 16 E6 and E7 oncoproteins were able to down-regulate the promoter activity of several of these genes. The regulatory region of each studied gene contains putative binding sites for AP-1 and C/EBP transcription factors, which are important regulators of keratinocyte differentiation and the HPV oncoproteins also reduced their activity in our transient transfection experiments. Our results suggest that the HPV 16 E6 and/or E7 oncogenes may have the potential to down-regulate the expression of several keratinocyte differentiation genes (such as desmocollin 1, keratin 4, S100 calcium-binding protein A8 and small proline-rich protein 1A) at least partially by down-regulating their promoter activity, maybe through modifying the activities of AP-1 and C/EBP factors. This activity of the HPV oncoproteins may have a role in the productive virus life cycle, and also in virus induced carcinogenesis.



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List of publications related to the dissertation

1. **Gyöngyösi, E.**, Szalmás, A., Ferenczi, A., Póliska, S., Kónya, J., Veress, G.: Transcriptional regulation of genes involved in keratinocyte differentiation by human papillomavirus 16 oncoproteins.
Arch. Virol. 160 (2), 389-398, 2015.
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Viol. J. 9, 36, 2012.
DOI: <http://dx.doi.org/10.1186/1743-422X-9-36>
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List of other publications

3. Hernádi, K., **Gyöngyösi, E.**, Mészáros, B., Szakács, L., Szalmás, A., Csoma, E., Mogorósi, R., Czompa, L., Veress, G., Varga, I., Márton, I.J., Kónya, J.: Elevated Tumor Necrosis Factor-alpha Expression in Periapical Lesions Infected by Epstein-Barr Virus.
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6. Hernádi, K., Csoma, E., Ádám, B., Szalmás, A., **Gyöngyösi, E.**, Veress, G., Márton, I., Kónya, J.: Association of human herpesvirus 6 subtypes with symptomatic apical periodontitis.
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DOI: <http://dx.doi.org/10.1016/j.tripleo.2011.02.007>
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Total IF of journals (all publications): 12,925

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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LIST OF PRESENTATIONS RELATED TO THE THESIS

Gyöngyösi E., Veress G.: Effect of human papillomavirus oncogenes on the expression of cellular genes involved in keratinocyte differentiation. Annual Meeting of the Hungarian Society for Microbiology, October 15-17, 2008, Hotel Helikon, Keszthely, Hungary

Gyöngyösi E., Szalmás A., Ferenczi A. Kónya J., Veress G.: Human papillomavirus oncogenes down-regulate involucrin expression. Annual Meeting of the Hungarian Society for Microbiology, October 12-15, 2010, Hotel Helikon, Keszthely, Hungary

Gyöngyösi E., Szalmás A., Ferenczi A. Kónya J., Veress G.: Effect of human papillomavirus oncoproteins on the expression of involucrin in human keratinocytes. 16th International Congress of the Hungarian Society for Microbiology, July 20-22, 2011, Budapest, Hungary

Gyöngyösi E., Szalmás A., Ferenczi A., Kónya J., Veress G.: Effects of human papillomavirus (HPV) type 16 oncoproteins on the expression of involucrin in human keratinocytes. Annual Meeting of the Hungarian Society for Microbiology, October 24-26, 2012, Hotel Helikon, Keszthely, Hungary

Gyöngyösi E., Szalmás A., Ferenczi A., Kónya J., Veress Gy.: Human papillomavirus 16 alters the expression of genes important in keratinocyte differentiation. 4th Central European Forum for Microbiology, October 16-18., 2013, Keszthely, Hungary

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LIST OF POSTERS RELATED TO THE THESIS

Gyöngyösi E., Szalmás A., Kónya J., Veress Gy.: Effect of human papillomavirus 16 E6 and E7 oncogenes on the expression of involucrin in human keratinocytes. 2nd Central European Forum for Microbiology, October 7-9, 2009, Keszthely, Hungary

Gyöngyösi E., Szalmás A., Ferenczi A., Kónya J., Veress Gy.: Keratinocyták differenciálódásában résztvevő gének expressziós változásainak vizsgálata humán papillomavírus onkoproteinek hatására. Debrecen az élettudományi kutatásokért. Május 29, 2014., Debrecen