# HPV16 Infection Upregulates the Transcript Level of TLR9

# But not Integrins (α2, α3, αv, β1, β4 and β6)

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## Abstract

Human papillomavirus (HPV) is known for its association with cervical cancer. Integrin family of proteins are thought to mediate HPV infection. In this study expression profiles of six integrin subunit genes are investigated upon HPV16 infection in HaCaT cells by RT-PCR. TLR9 gene expression is also analyzed to use it as a marker gene for infection. The results show that all of the selected integrin subunit genes are expressed in HaCaT cells but their expression levels remain unchanged, while TLR9 is up-regulated upon HPV16 infection.

## Introduction

Human papillomavirus (HPV) is a non-enveloped, double-stranded DNA virus. HPVs carry out their life cycle in either mucosal or cutaneous epithelia. They have icosahedral capsids composed of 72 capsomeres, surrounding a circular DNA genome of ~7900bp (Miller *et al.,* 2012) (Figure 1). To date 180 HPV genotypes have been cloned from clinical lesions and grouped as, high-risk and low-risk HPVs. The high-risk HPVs are known to cause cancer and the low-risk HPVs are known to cause skin warts (Stanley MA, 2012). High-risk HPV infection accounts for approximately 5% of all cancers

worldwide and all cervical cancers are associated with high-risk HPV infection. HPV16 and HPV18 are the most cancer causing HPV types. Currently there are two HPV vaccines (Gardasil and Cervarix) available on the market and they protect against HPV16 and HPV18--although there are more than 13 types of HPVs related with cervical cancer. So, to find a more generic cure, it is essential to have a better understanding on HPV infection mechanism(s). The initial steps of infection are binding and the internalization of HPV to host cells and these mechanisms also are not clear.

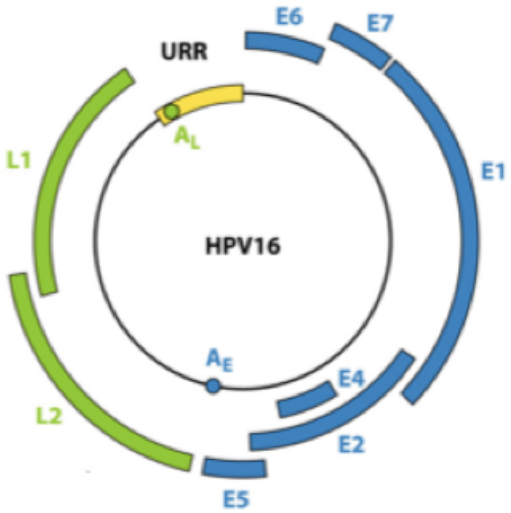


Figure 1. Genomic Organization of High-Risk HPV16 (Stanley MA, 2012)

Integrin family of proteins function as αβ heterodimers and they act as adhesion receptors that bind to extracellular matrix ligands, cell-surface ligands, and soluble ligands. They mediate proliferation, migration and differentiation as well as cell-cell interactions and attachment to extracellular matrix (Aoudjit and Vuori, 2012). At least 18α and 8β subunits have been found in humans and 24 heterodimers have been identified (Figure 2).

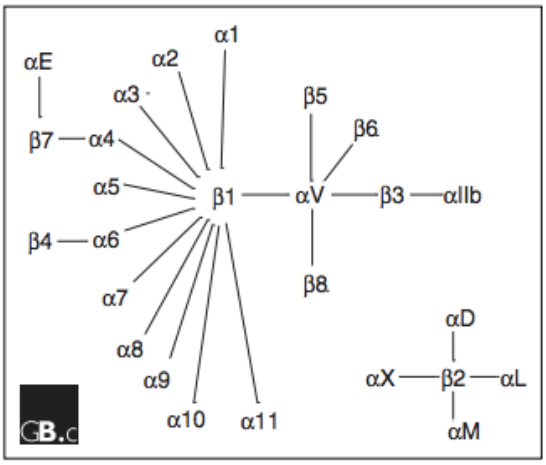


Figure 2. Human Integrin Family and Their Heterodimers (Takada *et al.,* 2007)

The expression and distribution of integrins at the cell surface is controlled by endocytosis and recycling. Recycling of integrins is especially important for migrating cells. Integrins are first endocytosed, then moved to endosomes and finally recycled back to the plasma membrane (Riggs *et al*., 2012).

Integrins can act as receptors for non-enveloped and enveloped viruses for their attachment and/or entry to host cells. Specifically, α6 integrin has been shown to act as a receptor for HPV16 and mediate infection of keratinocytes (Evander *et al.,* 1996).

Toll-like receptors (TLRs) are the key players in innate immune response. To date 10 TLRs have been identified in humans. TLRs can recognize a wide range of pathogens and activate antigen-presenting cells to produce pro-inflammatory cytokines. This pathway eventually leads to establishment of adaptive immunity (Kaisho and Akira, 2005). Eight TLRs have been demonstrated to be involved in viral recognition and of

those, TLR9 has been shown to be down-regulated upon HPV infection (Hasan *et al.,* 2007).

In this study six integrin subunit genes (α2, α3, αv, β1, β4 and β6) and TLR9 expression profiles upon HPV infection were investigated in immortalized human keratinocyte cells (HaCaT) by reverse transcriptase PCR (RT-PCR).

## Materials and Methods

Cell Line

HaCaT cell line was kindly provided by Dr. Meneses Lab, Department of Biological Sciences, Fordham University. Cells were cultured in Dulbecco's Modified Eagle's media (DMEM) either with or without 10% Fetal Bovine Serum (FBS, DMEM-10).

Primers

Table 1. Primer sequences for RT-PCR

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| **ITGA2** | GCAGATGGACCACACTTTGA | TGTCTGTGCCCTTTTCCTCT |
| **ITGA3** | GGGATCCTGCCTAAGGTTGTCT | CCTTCTTTCTAGTTCCTTTGCTGTTG |
| **ITGAV** | TTGTTGCTACTGGCTGTTTTG | TCCCTTTCTTGTTCTTCTTGAG |
| **ITGB1** | CCTACTTCTGCACGATGTGATG | CCTTTGCTACGGTTGGTTACATT |
| **ITGB4** | CTCCACCGAGTCAGCCTTC | CGGGTAGTCCTGTGTCCTGTA |
| **ITGB6** | TCCATCTGGAGTTGGCGAAAG | TCTGTCTGCCTACACTGAGAG |
| **TLR9** | CTGCCTTCCTACCCTGTGAG | GGATGCGGTTGGAGGACAA |
| **GAPDH** | GAAGGTGAAGGTCGGAGT | GAAGATGGTGATGGGATTTC |

HPV16 Pseudovirus Infection

Pseudovirus (PsV) is also kindly provided by Dr. Meneses Lab, Department of Biological Sciences, Fordham University and produced as described by Buck *et al* (Buck *et al.,* 2004).

200,000 HaCaT cells were plated on 6-well plates and incubated at 37°C in DMEM-10 for 2 days to reach ~80% confluency. Then DMEM-10 was aspirated and cells were starved in DMEM for 1 hour on ice. After 1 hour, PsV at a multiplicity of infection (MOI) 0.15 is added to each well except the controls, as shown in Figure 3. Cells were further incubated for an additional 1 hour on ice. After incubation each well was washed 3 times with 1X PBS and cells were incubated at 37°C in DMEM-10 for either 1 hour or 4 hours.

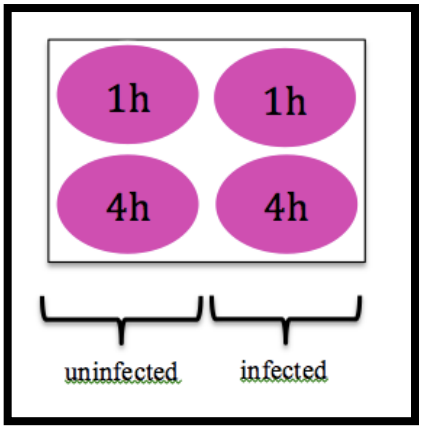


Figure 3. Setup for PsV infection of HaCaT cells

RNA Extraction

The total RNA was extracted at corresponding time points using RNeasy® Plus Mini Kit (QIAGEN), according to the manufacturer's instructions.

RT-PCR

RT-PCR was performed using QIAGEN® One-Step RT-PCR Kit following the instructions. GAPDH was used as the loading control. Twenty nanograms of RNA was amplified in 20µl RT-PCRs (4µl 5×RT buffer, 0.8µl 10mM dNTPs, 0.8 µl 10pmol/µl forward primer, 0.8µl 10pmol/µl reverse primer, 0.8µl enzyme mix, 1µl 10ng/µl RNA and 11.8µl dd$H_2O$).

| | | | |
|---|---|---|---|
| Reverse transcription: | 50 °C | 30 minutes | |
| Initial denaturation: | 95 °C | 15 minutes | |
| Denaturation: | 94 °C | 30 seconds | ITGA2-ITGA3-ITGAV-ITGB6 → 35cycles<br>ITGB1-ITGB4-GAPDH → 30cycles<br>TLR9 → 40cycles |
| Annealing: | 57 °C | 30 seconds | |
| Extension: | 72 °C | 30 seconds | |
| Final Extension: | 72 °C | 7 minutes | |

Electrophoresis

4µl of loading dye (6X) was added to each RT-PCR product. 8µl of each product was loaded to a 1% agarose gel, and electrophoresis was performed at 160V. Band intensities were visualized under UV light and documented. Ethidium bromide was used as the intercalating agent.

PCR Product Purification and Sequencing

PCR products were purified using the QIAquick® PCR Purification Kit following the manufacturer's instructions and sent out for sequencing in order to confirm the identity of the PCR products.

## Results

RT-PCR products were confirmed by sequencing and all experiments were replicated at least three times.

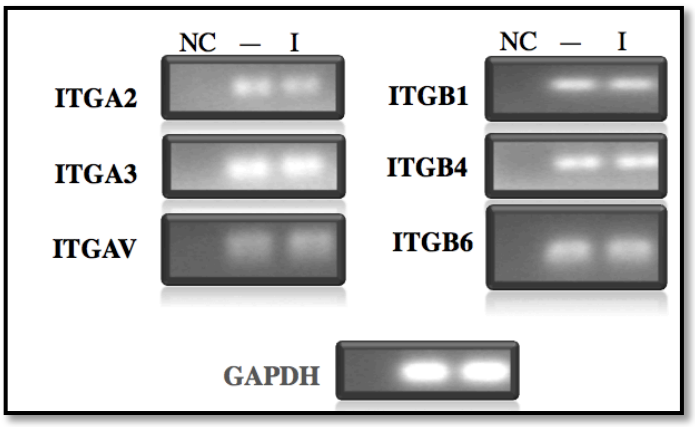


Figure 4. RT-PCR results for integrin subunit genes, 1 hour samples (NC: no template, — : uninfected, I: infected; GAPDH loading control)

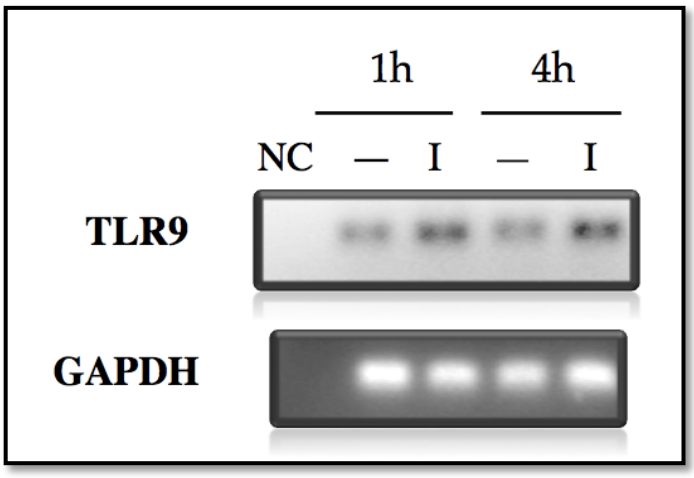


Figure 5. RT-PCR result for TLR9 gene, 1 hour and 4 hour samples (NC: no template, — : uninfected, I: infected; GAPDH loading control)

## Discussion

Integrins have been shown to act as viral receptors and α6 integrin has been shown to act as a receptor for HPV16. Upon binding to their ligand, integrins are internalized and recycle back to the cell membrane. Their internalization is thought to mediate the HPV16 infection. The cells are expected to regulate the integrin distribution on the cell surface in the presence of HPV16. For this, the cell may increase the transcript levels of HPV16 associated integrin subunits, stabilize their mRNAs and/or also can change the recycling rate.

The results show that all of the selected integrin subunit genes are expressed in HaCaT cells, but their expression levels remain unchanged after 4 hours following HPV infection, which indicates that there may be other mechanisms involved in regulation of integrin distribution, such as mRNA stabilization or increasing the recycling rate. Alternatively these results can also indicate that the selected integrin subunits are not involved in HPV16 binding and internalization.

It has been shown by others that TLR9 expression is down-regulated upon HPV16 infection. In this study, however, the results indicate an up-regulation for TLR9 upon HPV16 infection. The discrepancy might have stemmed from two reasons: First, the longest time-point in this study is 4h while it is 24h/48h in the previous studies; second, for this study PsV is used for infection, so there is no HPV16 genome surrounded by the capsid, whereas the previous studies have utilized the HPV16 genome. The down-regulation of TLR9, as shown by others, is believed to be caused by viral proteins (E6,

E7) that are produced in the cell following the infection, but since the pseudovirus used in this study lacks these genes and the time-points are not long enough for the virus to be localized in the nucleus. These results might show that the observed TLR9 up-regulation is due to a different mechanism and most likely the result of a quick immune-response of the host cell.

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