Differential mRNA Expression of IRAK1, TRAF6, KAL1 and GAN in Response to HPV16 Infection

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Abstract:

Human papillomavirus type 16 (HPV16) is one of the major disease inducing papillomavirus and also the cause of 99.7% of the cases of cervical cancer. Previous studies have indicated that HPV infection can induce cell morphological changes as well as the activation of Toll-like receptor (TLR) signaling via MyD88-dependent pathway. This project tested mRNA expression levels of 4 genes, IRAK1, TRAF6, TAL1 and GAN, which participate importantly in either TLR signaling and the communication between cell and its external matrix. After 4 hours of infection, human adult keratinocytes cells (HaCaT cells) did not show expression difference of IRAK1 and TRAF6 on mRNA level, whereas KAL1 mRNA increased about 50% and GAN mRNA decreased for 20%.

Introduction:

HPVs are members of the papillomavirus family that has the capacity of infecting humans. HPVs establish productive infections only in skin keratinocytes or mucous membranes. Infection of HPVs may lead to different kinds of disease such as skin

warts, oral papillomas and genital cancers. Among different genotypes of HPVs, HPV16 is one of the “high-risk” types which contribute to nearly all cases of invasive cervical cancer. It is a non-enveloped double stranded DNA virus which encodes 8 genes.

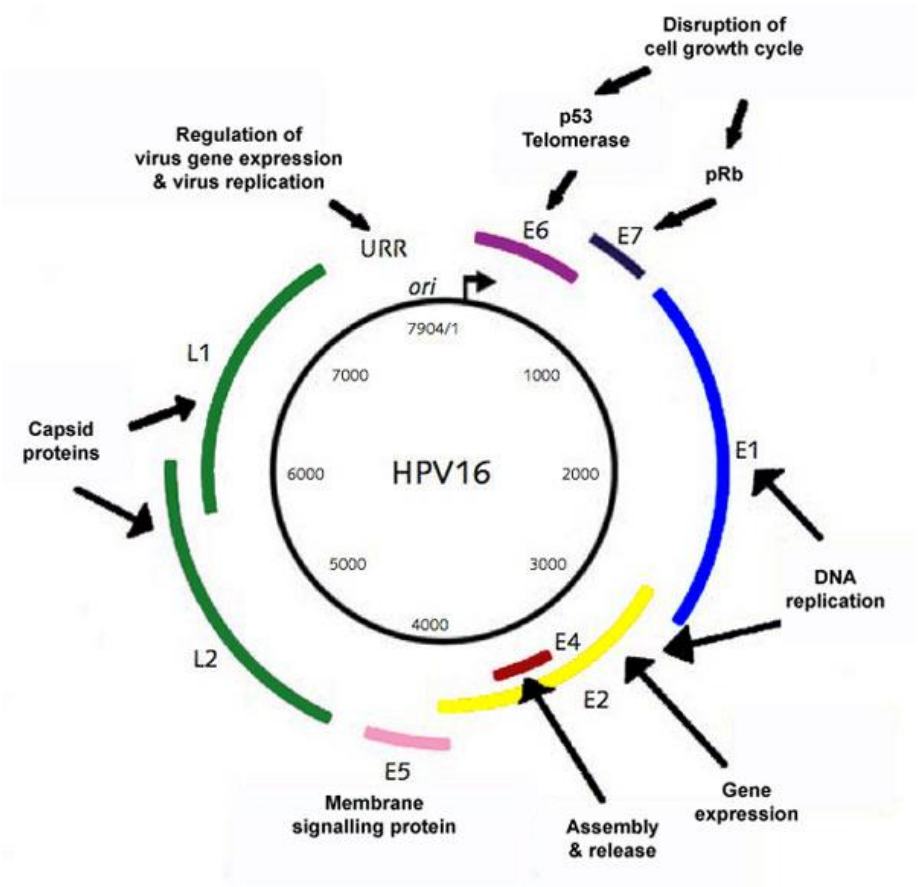

Fig. I Genome structure of HPV16.

Studies have demonstrated that Toll-like receptor (TLR) signaling participates in the innate and adaptive immune response induced upon HPV16 infection. Both TLR9 and TLR4 bind HPV16 particles as their ligands and activate downstream reactions, including activation and translocation of NFκB via MyD88-dependent pathway [2].

However, these studies concentrate upon B lymphocytes and long-term immune responses. On the other hand, researchers have found that after a short time (10min to 30min) of infection with HPV16 psudovirus (PsV), human adult keratinocytes cells can induce internalization of PsV, morphological changes and generation of filopodias [1]. Based on these researches and findings, this project chose IRAK1, TRAF6, KAL1, and GAN genes as candidates to test their mRNA level after infecting HaCaT cells with HPV16 PsV for 30min and 4 hours as a study of the potential short time responses of different cell signaling pathway and cross-talk between extracellular matrix and cell membranes.

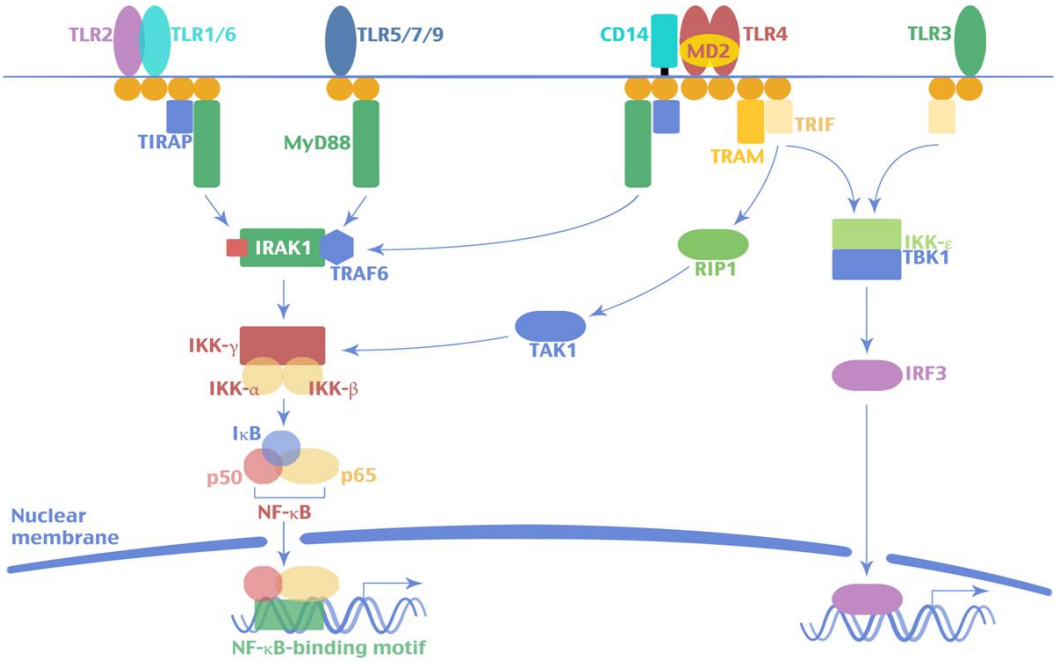

Fig. II Toll-like Receptor signaling pathway. Both TRIF-dependent and MyD88-dependent pathways are shown.

IRAK1 (Interleukin-1 receptor-associated kinase 1) and TRAF6 (TNF receptor associated factor 6) are two important proteins which participate in MyD88-dependent

pathway of TLR signaling. Ligand binding and conformational change that occurs in the dimerized Toll-like receptors (both homodimers and heterodimers are possible) recruits the adaptor protein MyD88, followed by the recruitment of IRAK1 and other cofactors. IRAK1 mediates translocation and phosphorylation of the proteion TRAF6 [3], which is a signal transducer for both TLR pathway and interlukin-1 response [8]. TRAF6 in turn polyubiquinates protein TAK1 as well as IRAK1 in order to facilitate the binding of TAK1 and IKKβ. On binding, TAK1 phosphorylates IKKβ, which then phosphorylates IκB causing its degradation and allowing NFκB to diffuse into the cell nucleus and activate transcription. Many other proteins also participate as accessory molecules or cofactors in this process that starts from ligand binding [9].

KAL1 gene encodes protein anosmin-1, which is responsible for certain precursor nerve cells' migration during embryogenesis. Deficiency of this gene results dysfunctional protein and causes incomplete development of the olfactory system. In addition, deletion or mutation of KAL1 gene can also lead to the failure of production of GnRH in some neural cells and therefore fail to incorporate into the hypothalamus. Different kinds of mutations of KAL1 gene have been identified as one of the causes of Kallmann syndrome.

Anosmin-1 is an extracellular matrix-associated protein which plays multifunctional roles in neuronal development, migration and organogenesis. [4] Previous studies have demonstrated that KAL1 gene expression is cancer regulated and TGF-β can induce gene expression of KAL1 and secretion of anosmin-1 as well as that anosmin-1 can protect cancer cells from apoptosis and increase their mobility

[5]. Furthermore, anomsin-1 can directly bind to fibroblast growth factor receptor 1 (FGFR1) with high affinity and enhance the activity of this signaling pathway. [6,10]

GAN gene encodes a protein named Gigaxonin which is responsible for the disease of giant axonal neuropathy (GAN). Gigaxonin is a member of the cytoskeletal BTB / kelch (Broad-Complex, Tramtrack and Bric a brac) repeat family (Kelch repeats are predicted to form a beta-propeller shape.) and functions in neurofilament architecture and is mutated in giant axonal neuropathy. Previous research has indicated that gigaxonin may play a crucial role in the cross-talk between the intermediate filaments and the membrane network. [7].

The object of this study is to identify differential mRNA expression of IRAK1, TRAF6, KAL1 and GAN. Data of this project indicated that after infection of HPV16 PsVs for 30 minutes and 4 hours, IRAK1 and TRAF6 did not show changes in their mRNA levels whereas the mRNA of KAL1 increased for about 50% and GAN mRNA decreased by 20%. These findings may indicate that cells need longer time to induce TLR activity and that HPV16 infection may introduce changes of cell mobility and migration in a short time period.

Materials and Method:

Cell culture and HPV16 PsVs infection:

HaCaT cell line and HPV16 PsV were provided by Dr. Meneses at Fordham University.

HaCaT cells were plated in a 6-well plate. After 24 hours of 37℃ incubation.

Cells were then treated with serum free medium for 1 hour on ice. Then HPV16 PsVs were added to cells and bound in serum free medium for another hour on ice. Unbounded PsVs were removed by washing 2 times with appropriate medium. Cultures were then incubated at 37℃ for 30min and 4hr, respectively.

RNA extraction:

RNAs were extracted with RNeasy kit following the manufacturer's instruction.

Primers design and RT-PCR:

RT-PCR primers were designed to locate in different exons to exclude amplification from potential DNA in RNA samples. Actin β mRNA level was also tested as an quantitative control. Annealing temperatures and cycle numbers of amplification used in RT-PCR were adjusted based on pre-experiments.

Primer sequences, locations, and product sizes are listed in the following table:

Genes	Primer Sequences	Locations	Product Sizes (basepairs)
IRAK1	Forward: 5'- CATTGTGGACTTTGCTGGCTAC -3' Reverse: 5'- GGCTGTCCTGATGTAGAAACTG -3'	Exon7 Exon8	191
TRAF6	Forward: 5'- TGGCATTACGAGAAGCAGTG -3' Reverse: 5'- GTTCCATCTTGTGCAAACAACC -3'	Exon2 Exon3	210
KAL1	Forward: 5'- CTGTGTTGAAAGCTGCGAAG -3' Reverse: 5'- GTACCACATAGATCACAGGCTC -3'	Exon4 Exon5	193
GAN	Forward: 5'- TGTGGAGATAGATGGGATGC -3' Reverse:5'- CTGGGTCCTGGGATCATAAC -3'	Exon7 Exon8	184
ActB	Forward: 5'- GATCCACTGGCATGGTGATGGACT -3' Reverse: 5'- CCGCTCATTGCCAATGGTGAT -3'	Exon4 Exon4	323

Tab.1 Primer sequences, locations and product sizes of IRAK1, TRAF6, KAL1, GNA and ActB.

Data analysis and bar graph generating:

More than 3 times of experiments were done and gel pictures were scanned. Software ImageJ was used to count the pixel numbers in gel pictures of 3 experiments. Data collected were normalized to pixel numbers of ActB mRNA band.

Result:

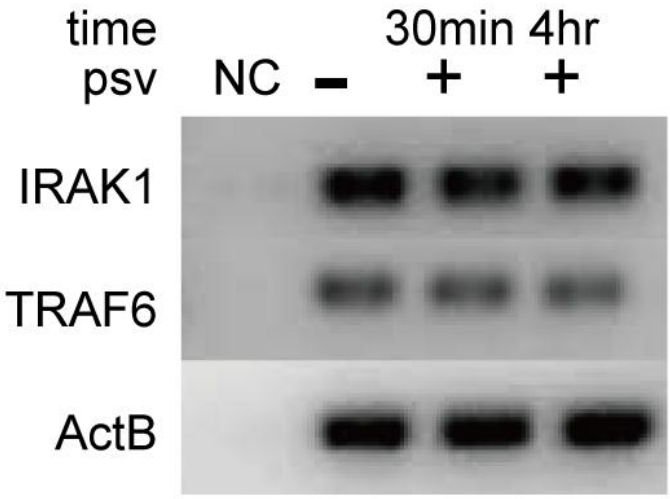

Fig. 1: Transcription of IRAK1 and TRAF6 do not respond to HPV16 PsV infection. HaCaT cells were plated in a 6-well plate. After 24 hours, HPV16 psv were added to cells and bound for another hour on ice. Unbounded PsV were removed by washing 2 times with appropriate media. Cultures were then incubated at 37℃ for 30min and 4hr, respectively. RNAs were extracted with RNeasy kit. RT-PCR primers were designed to locate in different exons to exclude amplification from potential DNA. Actinβ mRNA level was also tested as an quantitative control. A: 20ng RNA templates were used in the RT-PCR reaction. Templates were amplified for 35 cycles with IRAK1 and TRAF6. ActB was amplified for 25 cycles.

Using the RNA templates extracted from HaCaT cells infected by HPV16 PsVs for 30min and 4hr, from the RT-PCR result, no significant change on mRNA level of neither IRAK1 nor TRAF6 was observed. Similar assay was used to test KAL1 and

GAN mRNA expression level.

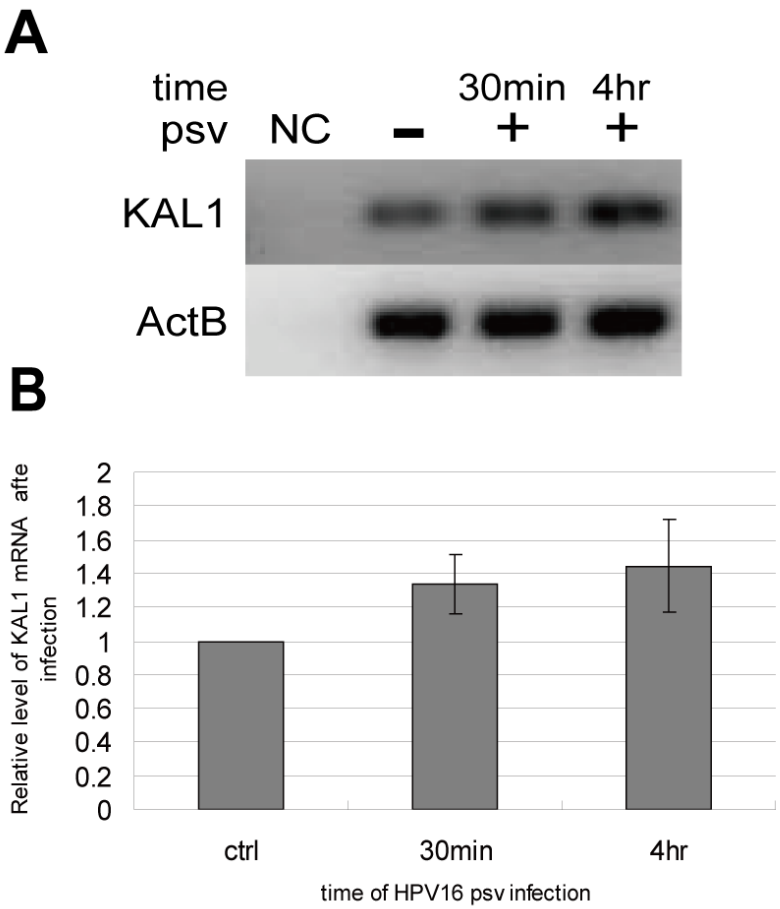

Fig. 2: KAL1 mRNA expression increased after 30min and 4hr infection of HPV16 PsV. Cells are treated and infected same with Fig. 1. **A**: 20ng RNA templates were used in the RT-PCR reaction. Templates were amplified for 35 cycles with KAL1. ActB was amplified for 25 cycles. **B**: generated based on repeatable results, values are tested by ImageJ, KAL1 mRNA level was normalized to ActB mRNA. N=3.

After both 30min and 4hr infection, KAL1 mRNA expression increased. For the 4hr sample, an increase of about 50% was observed. At the same time, GAN gene showed a decrease by 20% on its mRNA expression level.

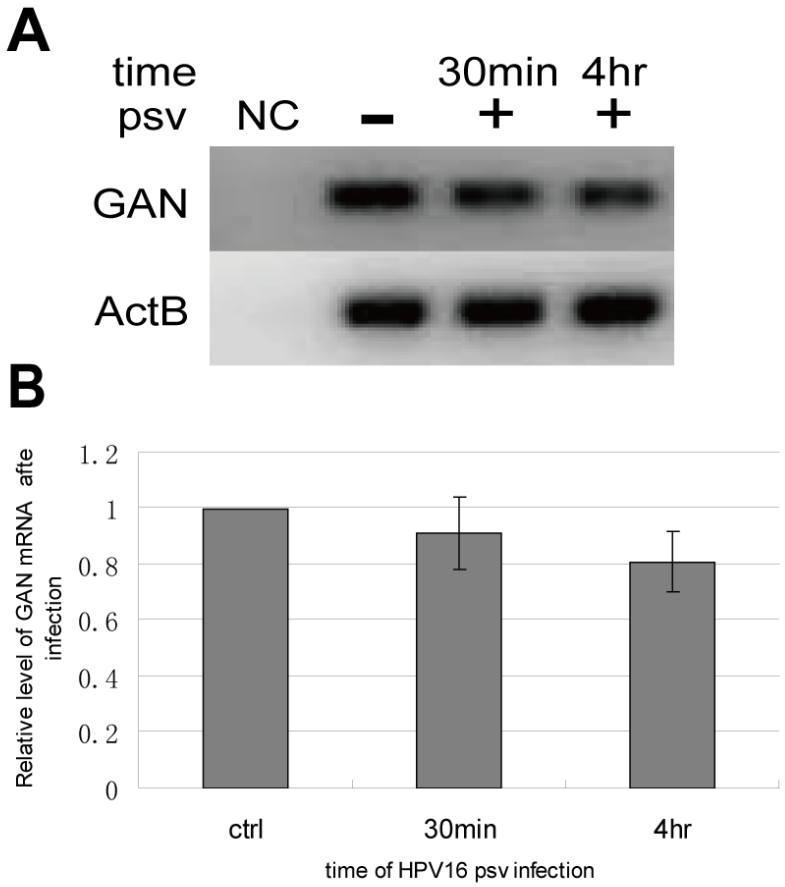

Fig. 3: A 20% decreasing of relative level of GAN transcripts can be observed after infecting HaCaT cells with HPV16 PsV for 4 hours. Cells are treated and infected same with Fig. 1. **A**: 20ng RNA templates were used in the RT-PCR reaction. Templates were amplified for 35 cycles with GAN. ActB was amplified for 25 cycles. **B**: generated based on repeatable results, values are tested by ImageJ, GAN mRNA level was normalized to ActB mRNA. n=3.

Conclusions and Discussion:

Data of this project showed that mRNA levels of IRAK1 did not respond to HPV16 PsV infection after 4 hours. This may indicate that at the very early stage of virus infection and internalization, TLR signaling keeps the normal endogenous level as cell's natural defense towards pathogens. Longer time period may be needed for the

signaling pathway to respond and the following expression of virus encoded proteins may lay more regulatory functions on the players in this pathway.

On the other hand, it is quite possible that IRAK1 and TRAF6 show increasing protein expression or their activity can be increased without overexpression of mRNA. Western Blot can be done to test the protein level of them and further experiments can be processed to test the kinase activity of IRAK1.

Interestingly, findings in this project demonstrated that KAL1 had an increase on its mRNA level as well as GAN mRNA had been down regulated. Current researches of these two genes focused on their relationships with the corresponding diseases; studies upon their correlation with virus infection, immune response and other cellular biological functions are still lacking. Changes of the mRNA level of these two genes, which were traditionally considered functioning in embryonic neuronal development, suggest that they may also participate in cell signaling and cancer development. Based on the previously published researches, KAL1 may protect cancel cells from apoptosis and increase cancel cell mobility, it is reasonable to presume that this gene has similar performance in response to virus infection. Moreover, KAL1 expression and anosmin-1 secretion were regulated by TEG-β[5]. Combinding with results of this project, it is possible that virus infection can be responded to through FGFR1 pathway and TGFF-β signaling as well as other cell rapid response.

Also, it is known that GAN encodes a Gigaxonin protein which is a member of the cytoskeletal BTB/kelch repeat family. Gigaxonin functions in neurofilament architecture and may play a crucial role in the cross-talk between the intermediate

filaments and the membrane network. Results of this project that GAN mRNA decreased by 20% in response to HPV16 infection may then indicate that GAN is used in cell response to HPV infection to function and modulate cell mobility, migration, and even reorganization of cytoskeleton.

Furthermore, KAL1 and GAN have been predicted as strong targets of miRNA-324-5p. Longer infection time and partial or whole virus genome may be needed to test miR-324-5p level in response to HPV infection. Although miRNA regulatory function in HPV16 infection upon KAL1 and GAN is largely remaining for understanding, it is reasonable to presume a new part of the functions of microRNAs that they response to pathogen invading and immune responses.

Acknowledgement:

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