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Determination of the Changes in Human Leukocytes Inflammatory Genes Expression after Infection with Human Papillomavirus

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ABSTRACT

Keratinocytes are the natural target cells for infection by humanpapillomaviruses (HPVs), most of which cause benign epithelial hyperplasias (warts). HPVs, cause lesions that can progress to carcinomas. Inflammatory genes such as those coding for cytokines, chemokines and other genes are induced in response to a viral infection. HPV effect on host cellular responses is critically important to explore the molecular mechanisms of viral-host interaction. The aims of this study were to detect changes in the mRNA expression in a panel of 84 inflammatory genes in the leukocyte of three HPV infected patients using real time-PCR array technology. Nine inflammatory genes showed a significant gene up regulation ranging between 16.897 mean fold change for Interleukin-1 alpha and 353.804mean fold change for Interferon-alpha2 gene. The up regulation of mRNA of nine geneswith high significance ($P > 0.05$) might suggest that the host-viral interaction is a cellular response against viral effect. In conclusion,this study revealed several alterations of many Leukocyte`s inflammatory gene expressions as a response against HPV. The common up regulated genes after HPV infections stimulate the activity of different immunological functional pathways represented by cytokine-cytokine receptor interaction, NOD-like receptor signaling pathway, and Toll-like receptor signaling pathway.

Keywords: Human Papillomavirus, gene expression, Real-Time-PCR Array, immuno Antimicrobial activity, methanolic extract, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Proteus mirabilis, Cucrbitapepo.

التغيرات في التعبير الجيني للجينات الالتهابية لكريات الدم البيضاء في الانسان بعد الاصابة بفايروسات الحليمات البشرية

الملخص

تعد الخلايا الكيراتينية الجلدية الهدف النهائي لفايروسات الحليمات البشرية والتي تسبب معظمها التضخم الكمي الطلائي (الثأليل الجلدية) والذي قـد يتطـور الـى حـالات سـرطانية. الجينـات الالتهابيـة مثـل السايتوكايناتوالكيموكاينات وجينات التهابية اخرى تستحث الخلايا المناعية ضـد الاصـابات الفيروسـية. ان

دراسة تأثير إصابة الفيروسات الحليمية على الاستجابة المناعية الجزيئية تعد مهمة جدا لتحديد الميكانيكيـات الجزيئية التي تربط العلاقة بين الفايروس والمضيف. تمت دراسة التغيرات فـي التعبيـر الجينـي لأربعـة وثمانين جيناً التهابياً- مناعياً لخلايا الدم البيضاء من ثلاثة مرضى مصابين بفيـروس الحليمـات البـشرية وباستخدام تقنية جزيئية حديثة وهي Real time PCR-Array. استجابت خلايا الدم البيضاء تجاه الفـايروس بزيادة نسخ الحامض النووي الرايبوزي لتسعة جينات التهابية وبمعـدل تـضاعفي يتـراوح بـين 16.897 للسايتوكاين نوع IL1A و 353.804 للسايتوكاين نوع IFNA2 وبمستوى معنوية ($P > 0.05$) وهذا يـدل على ان التفاعل بين الفايروس والخلية هو تفاعل استجابة خلوية ضد الفايروس. نستنتج من هذه الدراسـة ان الاصابة بفايروس الحليمات البشرية يحدث تغيراً في التعبير الجيني والذي يؤثر علـى مـسارات التهابيـة- مناعية مهمة جدا داخل المضيف مثل مسار تفاعل السايتوكاين-مستقبل السايتوكاين ومسار تحفيـز انتحـار الخلية المصابة بالفايروس ومسار انتاج الانترفيرون المضاد للإصابات الفيروسية.

INTRODUCTION

Human papillomaviruses (HPVs) cause benign epithelial hyperplasias (warts) and stimulatethe recruitment of the efficient antiviral response that is centered on the release of type 1 interferon (See and Wark, 2008). Effective communication between the cells of the immune system is essential for the proper functions of a whole complex network. Cells secrete cytokines that mediate signals between cells. These signals lead to alterations in the gene expression by the target cell (Green, 2000). Up-regulated and down-regulated genes provide insights into functional responses of both host and pathogen (Baqai *et al*., 2009; Penelope *et al*., 2004). Detection of these pathogens and determination of their effects on gene expression in white blood cells is very important forinitiating antiviral therapy, avoiding unnecessary antimicrobialtherapy, preventing nosocomial spread, decreasing the durationof hospital stays, and reducing management costs (Henrickson, 2005). Only few studies examined the effect of viral infections on human gene expression using advanced techniques. Expression microarray analysis is a powerful method to determine global profiles of genesin cells and tissues under a variety of complex biological conditions (Clewley, 2004). Recent methodological improvements in experimental design and data analysis permit the adaptation of this method to more complex biological systems (Silver *et al.,* 2008; Penelope *et al*., 2004). *In vivo* studies of viral infections on human cells using molecular assays (mostly RT-q PCR and Microarrays) are highly sensitive and specific **(**Connolly**,** 2004**).** Several genes whose expression profiles were altered in response to virus infection have been identified by microarray analysis (Samuel**,** 2001). From the previous studies it is clear that most of them investigate the viral effect on the cellular gene expression profile *in vitro* cell lines (Schena, 2004). This *in vitro* model of study did not give precise results compared with *in vivo* studies since results of gene expression in all of them studies gave completely different results from *in vivo* model studies (David, 2008; Penelope *et al*., 2004). The reason for these variations in results of gene expression in the two models is the inability to provide the suitable conditions for virus and host cells in *in vitro* studies. Development of microarrays facilitated the screening of viral pathogens from

broad viral families (Uttamchandani, 2009). Systematic studies of gene expression patterns using cDNA microarrays provide a powerful approach to molecular dissection of cells and tissues by comparing expression levels of tens of thousands of genes at a time (Tanaka, 2004; Bone *et al*., 2005). Real-time-reverse transcription PCR-array (RT-PCR-array) (SA Bioscience Company) is the most sensitive and reliable method for gene expression analysis (Montgomery and Daum, 2009). Its wide dynamic range makes RT-PCR the preferred choice for the simultaneous quantification of both rare and abundant genes in the same sample (Campeau *et al*., 2009 ; Myskiw *et al*., 2009). The reverse transcriptase PCR-array takes advantage of real-time PCR performance and combines it with the ability of microarrays to detect the expression of many genes simultaneously and with high accuracy due to the presence of internal control genes that are used for normalization with the sample genes (Sundararaj *et al*., 2009). RT-PCR-arrays are designed to analyze a panel of genes related to a disease state or biological pathway (Chittur *et al*., 2009 ; Aubin *et al*., 2007).

MATERIALS AND METHODS

Patients

Three patients infected with HPV-warts (complete infection stage) were subjected to analysis using real time-PCR array.

Blood Samples

Blood samples five millimeter of blood were obtained from patients with HPV-warts as acute sample from and placed on ice then transported to the laboratory. Second samples of blood were taken from each patient and used as a control after warts recovery (after 6 weeks) after treatment with Montmorillite which gave 100% of HPV-warts recovery.

Real Time- PCR Array (RT-PCR Array)

The effect of viral infections on 84 inflammatory genes were investigated to determine the inflammatory gene expression profile of patient's white blood cells using the SA Bioscience kit (Campeau *et al*., 2009; Myskiw *et al*., 2009). The inflammatory genes examined in this study were the following classes:

Cytokine genes:
CD40LG (TNF-SF5). IFNA2, IL10, IL13, IL17C, IL1A, IL1B, IL1F10, IL1F5, IL1F6, IL1F7, IL1F8, IL1F9, IL22, IL5, IL9, LTA, LTB, MIF, SCYE1, SPP1, TNF.

Cytokine receptors genes:
IFNA2, IL10RA, IL10RB, IL13, IL13RA1, IL5RA, IL9, IL9R.

Chemokine genes:
C5, CCL1 (1-309), CCL11 (eotaxin)., CCL13 (mcp-4), CCL15 (MIP-1d), CCL16 (HCC-4), CCL17 (TARC), CCL18 (PARC), CCL19, CCL2 (mcp-1), CCL20 (MIP-3a). CCL21 (MIP-2), CCL23 (MPIF-1), CCL24 (MPIF-2 / eotaxin-2), CCL25 (TECK) , CCL26, CCL3 (MIP-1a), CCL4 (MIP-1b), CCL5 (RANTES), CCL7 (mcp-3), CCL8 (mcp-2), CXCL1, CXCL10 (IP-10), CXCL11 (I-TAC / IP-9), CXCL12 (SDF1), CXCL13, CXCL14, CXCL2, CXCL3, CXCL5 (ENA-78 / LIX), CXCL6 (GCP-2), CXCL9, IL8.

Chemokine receptors genes:
CCL13 (mcp-4), CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CX3CR1, IL8RA, XCR1 (CCXCR1).

Other genes involved in inflammatory response:
ABCF1, BCL6, C3, C4A, CEBPB, CRP, CARD18, IL1R1, IL1RN, IL8RB, LTB4R, TOLLIP

Extraction of RNA from patient's leukocytes

The RNAs of the 3 infected patients (acute samples) were extracted within 24 hours of sampling and stored under -87C°. Second samples after recovery were extracted within 24 hours and stored at -87C°. Each blood sample (5ml) was placed in EDTA tube and centrifuged at 2500 rpm for 17 min. The Buffy coat (containing leukocytes) wastransferred from the upper layer (300 µl) to another tube. Trizol reagent (900 µl) of Phenol-guanidin-isothiocyanate (Invitrogen, USA) was added at a ratio (1:3) to maintain the integrity of total RNA during blood cells rupturing. The tube was inverted several times 10-15 times (Diepen *et al*., 2010 ; Riny, 2005) for homogenization. Homogenized sample was incubated for 5 min at 15 – 30 ºC to permit the dissociation of nucleoprotein complexes. Chloroform (250 µl) was added and the tube was vigorously shacked by hand for 15 sec then incubated at 15 – 30 ºC for 5-7 min. Tubeswere centrifuged at 12,000 rpm for 15 min and the mixture was separated into three layers, lower-red phenol chloroform phase, an inter-phase and a colorless upper aqueous phase which contains RNA. The aqueous phase was transferred into a clean tube and500 µl of isopropanol alcohol was added. The tube was inverted 50 times and incubated at 15 – 30 ºC for 10 min, then it was centrifuged at 12,000 rpm for 10 min. A gel-like pellet (transparent) was observed. The supernatant was removed and 500 µl of 70% ethanol was added to remove any impurity. The tube was then inverted 7-10 times then centrifuged at 7,000 rpm for 5 min. Finally RNA pellet was dried for 5 min then re-suspended in 50–100 µl RNase-DNnse-free water (Invitrogen, USA). The extracted RNA was ready at this stage for production of cDNA.

RNA quantification

Spectrophotometer analysis was carried out by measuring the optical density (O.D) at λ_{260}using the following procedure: RNA sample was diluted (1:25) by mixing 4 µl RNA sample with 96 µl sterile distilled water.Spectrophotometer at $\lambda_{260}$was blanked using 1000 µl distilled water. The O.D of diluted RNA samples was checked at λ= 260 (An O.D of 1 corresponds to approximately 40 µg/ml). The concentration of RNA was calculated (µg/ml) according to the following equation: RNA concentration = 40 x 25 (dilution factor) x O.D at λ_{260}. Adequate concentration must be between 0.01 – 1.0 µg/ml. RNA quality was checked using two methods: The $O.D_{260/280}$ ratio (Ratio of RNA to protein) was checked and RNA is considered pure if the O.D ratio was between1.7 – 2.0. Lower ratio (O.D< 1.7) indicates protein contamination.

Real time PCR-Array:

The real time PCR-array takes advantage of real-time PCR performance and combines it with the ability of microarrays to detect the expression of manybiological gene lines in

human cells (Campeau *et al*., 2009; Myskiw *et al*., 2009; Adams *et al*., 2010). The gene-line in this study was the immuno-inflammatory genes (84 gene) which are tested to determine if they are up-regulated or down-regulated during infection and after recovery.

Genomic DNA elimination mixture:

The isolated RNA was treated with DNase:
For each RNA sample, the following contents were mixed in a sterile PCR tube:

Total RNA	25.0 ng to 5.0 µg
GE(5X gDNA Elimination Buffer)	2.0µl
RNase-free H2Oto a final volume of …	10.0µl

The same amount of total RNA in this reaction was used for every sample starting with 0.5 or 1.0 µg of total RNA for 96-well plate formats. The contents were mixed gently with a pipette followed by brief centrifugation. The mixture was incubated at 42 °C for 5 min and chilled on ice immediately for at least one minute.

cDNA synthesis

The cDNA was prepared from RNA samples (control and acute sample).The reverse transcriptase (RT) mixture was prepared as follows:

RT mixture	1 reaction	2reaction	4 reaction
BC3(5X RT Buffer 3) contains dNTPs	4 µl	8µl	16 µl
Primer (P2 - Oligo-dt primer & external Control Mix)	1 µl	2 µl	4 µl
RE3(Rev.T Enzyme Mix 3)	2 µl	4 µl	8 µl
RNase-free H2O	3 µl	6µl	12µl
Final Volume	10 µl	20 µl	40 µl

The RT mixture (10µl) was added to each 10µl genomic DNA elimination mixture and the contents were mixed well but gently with a pipette, incubated at 42 °C for exactly15 min then the reaction was stopped immediately by heating at 95 °C for 5 minutes. DdH2O (91µl) was added to each 20µl of cDNA synthesis reaction then the contents were mixed well. The first strand cDNA synthesis reaction was placed on ice until the next step or stored overnight at -20 °C (Hideshima *et al*., 2009).

Addition of cDNA to RT-qPCR Master Mix

The cDNAwas added to RT-qPCR Master Mix (Master mixes contain SYBER Green and reference dye). Then the master mix was used to prepare the experimental mixture as follows:

The following components were mixed in a 5-ml tube

2X SABiosciences RT- qPCR Master Mix;- 1350 µl
Contains; syber green, Taq-DAN
polymerase,

forward and reverse primers and dNTPs.	102 µl
Diluted First Strand cDNA Synthesis reaction	
ddH2O 1248 µl	2700 µl
Total Volume	

Loading the 96-Well PCR-Array

The mixture was aliquot across the PCR-arrays, each PCR-array profiles the expression of 84 pathway-specific geneplus 12 wells for controls. The experimental mixture (cDNA and RT-qPCR Master Mix) was added (25µl) to each well of the PCR-array.

Real-Time PCR Detection

Seal the RT-PCR-array carefully but tightly with the optical thin-wall 8-cap strips. Be sure that no bubbles appear in any of the wells of the RT- PCR-array. To remove bubbles, the plate was tapped gently on the bench top or the plate was centrifuged briefly. The plate was placed on ice while setting up the RT-PCR cycling as mentioned below. One plate is placed in real-time thermal cycler.RT-PCR-arrays containing experimental mixture may be stored at -20 °C wrapped in aluminum foil for up to one week until ready to run. The appropriate program was entered and run using the Real-Time instrument software.

Performing Thermal Cycling

The real-time amplification data was collected using software instrument to get two profiles (for control sample and acute sample). The program was a two-step cycling used for Bio-Rad-I Cycler.

Cycles	Repeat	Duration	Temperature
1	1	10 minutes[1]	95 °C
2	40	15 seconds	95 °C
		1 minute[2]	60 °C

1- The 10-minute step at 95 °C was required to activate the Hot-Start DNA polymerase.
2- SYBR-Green fluorescence was detected and recorded from every well during the annealing step of each cycle.

Calculation of the Threshold Cycle (Ct) for each well

Using the instrument's software the threshold cycle (Ct) was calculated for each well. The baseline and threshold values were determined. The baseline was determined at the two cycles before that gave the earliest visible amplification, usually around (Fig. 1). The threshold value was determined within the lower one-third part of the linear phase of the amplification plot (Fig. 2). The same thresholds are used across all PCR-array runs in the same analysis. If the RNA sample quality is adequately controlled, the cycling program is executed properly, and the thresholds is defined correctly. Then the value of threshold cycle of positive PCR control (C_t^{PPC}) should be 20 ± 2 across all of PCR-arrays or samples. The resulting threshold cycle (Ct) values for all wells are analyzed using a Blank Excel Spread-sheet SA Bioscience Data Analysis Template Excel File (Campeau *et al*., 2009; Myskiw *et al*., 2009).

Data Analysis: $\Delta\Delta C_t$ Method

According to the PCR-array Data Analysis Web Portal change all Ct values reported as greater than 35 were considered as negative calls. The threshold cycle values of the control wells are examined as mentioned in instructions of RT-PCR Array SABioscience kit.
The ΔCt for each gene was normalized for the house keeping genes (Beta-2-microglobulin, Hypoxanthine phosphoribosyltransferase 1, Ribosomal protein L13a, Glyceraldehyde-3-phosphate dehydrogenase, and Actin, beta) in each plate as follows:

$$\Delta Ct = C_t^{GOI} - C_t^{AVG\ HKG}$$

The ΔΔCtwas calculated for each gene across two PCR-arrays (or groups) as follows:

ΔΔCt = ΔCt (group 2) - ΔCt (group 1).

Where group 1 is the control sample and group 2 is the experimental sample (acute sample)**.** The fold-change was calculated for each gene from group 1 to group 2 as 2 ^ (-ΔΔCt). If the fold-change is greater than 1, then the result may be reported as a fold up regulation. If the fold-change was less than 1, then the negative inverse of the result may be reported as a fold down-regulation.

Statistical Analysis

Since each gene has 6 values (3 values form acute samples and 3 values form control samples) the difference between the two values for each gene was analyzed using the non-parametric Wilcoxon signed ranks-test to calculate the T-value (similar to t-test value) and P-value for each gene (Joshua *et al.,* 2006). The *P*-value was an important parameter for significance level that helps to establish the reliable range for ΔΔCt estimation. *P*-values were important to claim differential expression between the two Ct values (acute samples and control samples) for each gene. The *P*-values were derived from testing the null hypothesis that ΔΔCt were equal to 0.0. Therefore, a small *P*-value indicates that the ΔΔCt was significantly different from 0.0 which demonstrates a significant effect. If the $P \geq 0.05$ for each gene in acute samples versus control samples, the gene expression was significantly different among the two samples.

RESULTS AND DISCUSSION

Patients Questionnaire

Location of HPV-warts and questionnaire of patients are arranged in Table 1. All patients have not chronic diseases.

Table 1: location of HPV-warts and questionnaire of infected patients.

Patient number	Date of sampling	Address	HPV-wartsregion	Age/ year	Sex	Marital state	Job
1	8.7.08	Madaba/ Jordan	Left hand	20	Male	single	student
2	14.7.08	Amman Jordan	Left hand/ finger	34	Male	Married	laborer
3	14.10.08	Amman Jordan	Left leg /thumb	48	Female	Married	House keeper

RNA Concentration

The patients with HPV-warts (20-48 years old) were chosen for analysis. The RNA concentrations of the 3 patients ranged between 107.9 and 908.7 µg/ml and between 113.6 and 564.3 µg/ml for acute and control samples respectively. This variation in RNA concentrations belong to efficiency of RNA. The RNA quality of the 3 patients ranged from 1.8780 to 1.9100 and from 1.876 to 1.9811 foracute and control samplesrespectively (Table2).

Table2: The RNA quantity (concentration of acute and control RNA samples as measured by OD-spectrophotometer) and RNA quality (the purity of acute and control RNA samples at OD_{260} compared with protein at OD_{280} as measured by spectrophotometer).

	During infection (Acute samples)		**After recovery (Control samples)**	
Sample Number	RNA concentration OD/concentration µg/ml	OD_{260}/OD_{280} (RNA quality) (RNA purity)	RNA concentration OD/concentration µg/ml	OD_{260}/OD_{280} (RNA quality) (RNA purity)
1	107.769	1.7082	113.621	1.8780
2	489.996	1.7631	397.954	1.8991
3	908.776	1.8326	564.376	1.9100

The concentrations of 3 acute and 3 control RNA samples that are calculated by spectrophotometer (µg/ml) and then converted into RNA volume (contains 1 µg/ml) for each sample and then used in the RT-PCR Array (Table 3).

Table 3 : RNA concentrations used in the RT-PCR Array.

	During infection (Acute samples)		**After recovery (Control samples)**	
Sample Number	RNA concentration µg/ml	RNA volume (µL) used in the RT-PCR Array	RNA concentration µg/ml	RNA volume (µL) used in the RT-PCR Array
1	107.769	3.5252	113.621	2.97
2	489.996	0.6016	397.954	1.146
3	908.776	0.4382	564.376	5.224

Real time PCR –Array

Ct values

The Ct values of the 84 immuno-inflammatory genes for the 3 patients (acute samples and control samples) were obtained after RT-PCR-Array run (Bio-Rad software, USA) (Table1). Thus each patient has 84 Ct acute values and 84 Ct control values. Each Ct value represent the threshold value for each gene after amplification by RT-PCR-Array, from these Ct values the fold change for each gene was calculated using SABioscince Excel-Analysis of RT-PCR-Array. the baseline value (Fig. 1) and threshold value (Fig. 2) were

determined in all samples and this reveals the standardization differences in gene expression precisely.

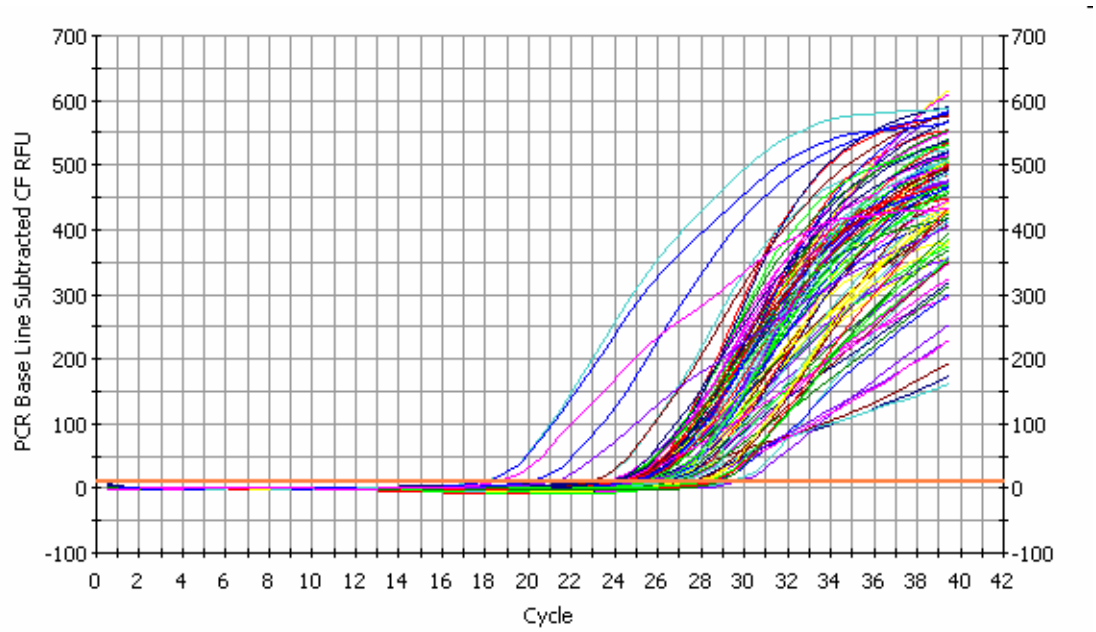

Fig. 1 : The amplification of 84 inflammatory genes and the determination of the baseline (in this plot almost at the log 10 of y axis) in all samples enhance the standardization of all samples at the same base line and this reveal the differences in gene expression precisely.

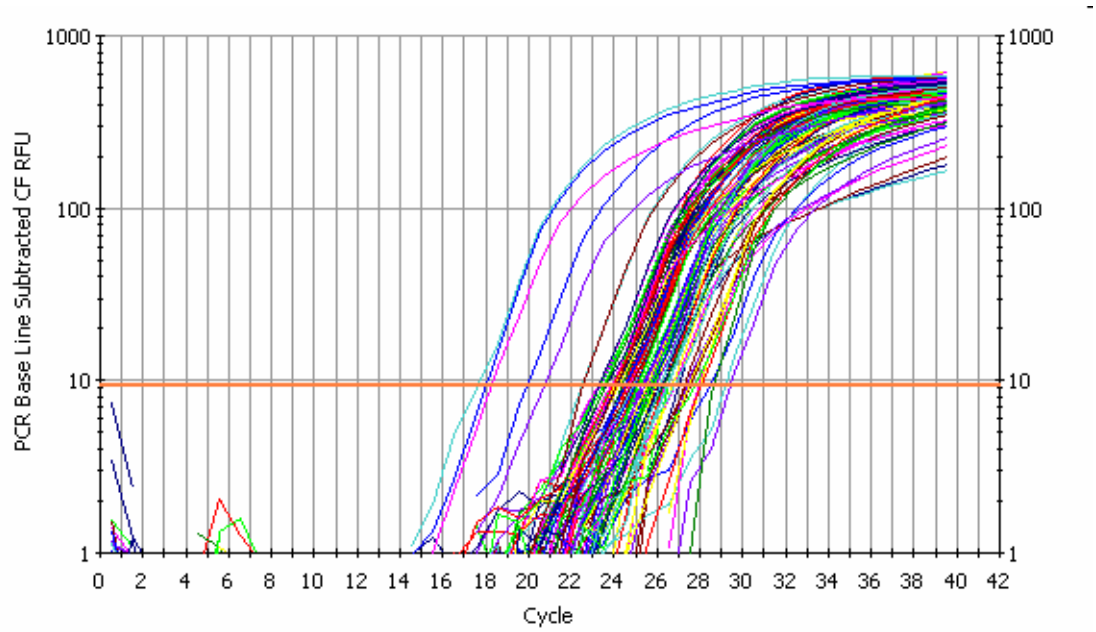

Fig. 2: The amplification of 84 inflammatory genes and the determination of the threshold value (in this plot at cycle 16) in all samples to enhance the standardization of all samples at the same threshold value and this reveals the differences in gene expression precisely.

The results of gene analysis using RT-PCR Array analysis data program (SAB ioscience) gave main alteration in 9 genes in the three patients including CCAAT/enhancer binding protein, beta (CEBPB), Interferon-alpha 2(IFNA2), Interleukin 10 (IL10), Interleukin-13 (IL13), Interleukin-1- alpha (IL1α), Interleukin-1- beta (IL1β), Interleukin-5 (IL5), Interleukin-8 (IL8), and Tumor necrosis factor (TNF) (Table 4).

Table 4: Up and Down-regulated genes in the three patients after infection with HPV.

Patients	Gene alteration	Affected genes (Fold change)								
1	Up regulation	CEBPB	IFNA2	IL10	IL13	IL1A	IL1B	IL5	IL8	TNF
	Fold change	8.6939	5.7358	4.6589	6.5887	9.3179	4.9933	4.0558	5.7358	4.3469
	Down regulation	Non								
2	Up regulation	CEBPB	IFNA2	IL10	IL13	IL1A	IL1B	IL5	IL8	TNF
	Fold change	3.2944	17.3878	1.7684	2.0279	1.434	4.0558	2.1735	2.0279	2.4967
	Down regulation	Non								
3	Up regulation	CEBPB	IFNA2	IL10	IL13	IL1A	IL1B	IL5	Mean 3.881	Mean 3.421
	Fold change	42.81	1038.29	79.89	69.55	39.94	42.81	45.88		
	Down regulation								IL8 0.034	TNF 0.110
Mean		18.662	353.804	28.77	26.05	16.897	17.286	17.36		

All altered genes were up regulated in all patients except IL8 gene and TNF gene in the third patient were down regulated (Table 4). In the first patient, the nine genes were up regulated from the 84 inflammatory genes. The fold change in the genes ranged between 4.3469 fold in TNF and 9.3179foldchange in IL1A gene (Fig. 3). On the other hand there were no any down regulated genes and all remaining genes were not changed (Fig. 4).

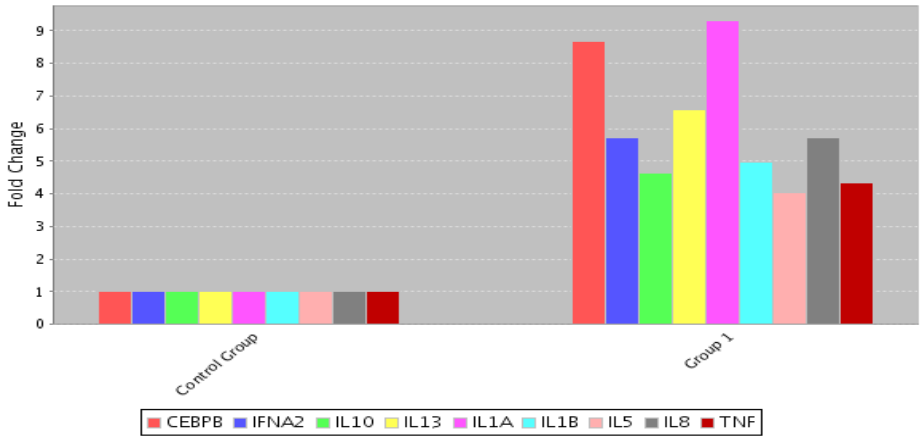

Fig. 3: The up regulated 9 (10.71%) genes after HPV infection in first patient. The most upregulated gene wasIL1A gene (9.3179fold change) and the lower upregulated genes wasTNF gene (4.3469 fold change).

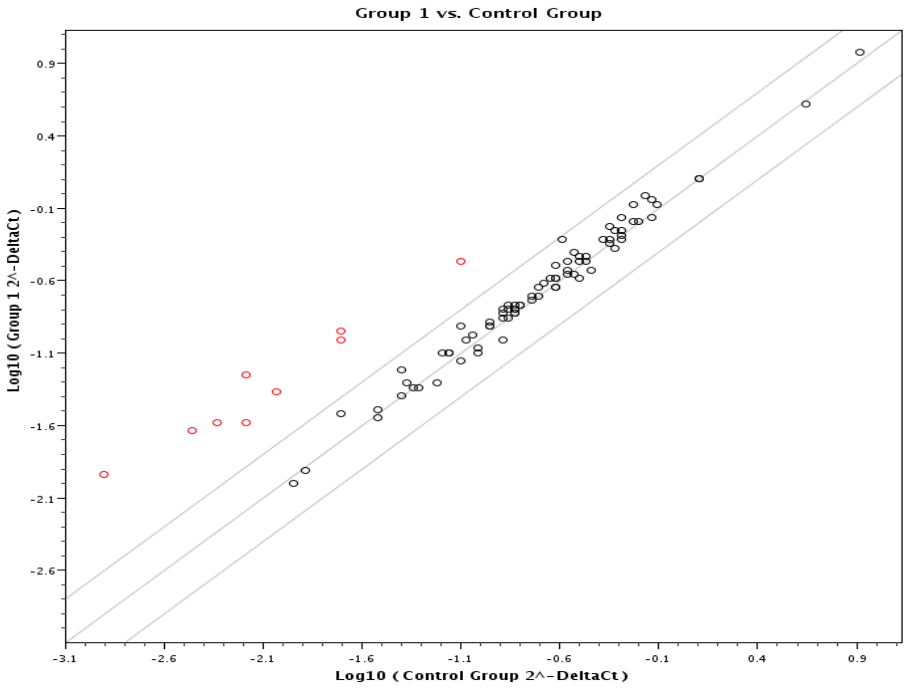

Fig. 4: Nine upregulated genes (Red color) and non altered genes (black color) in the first patients.

In the second patient all genes were up regulated with fold change ranged between 1.434 for IL10 and 17.387 for IFNA2 (Fig. 5). There wereno any down regulated genes and all remaining genes were not changed (Fig. 6).

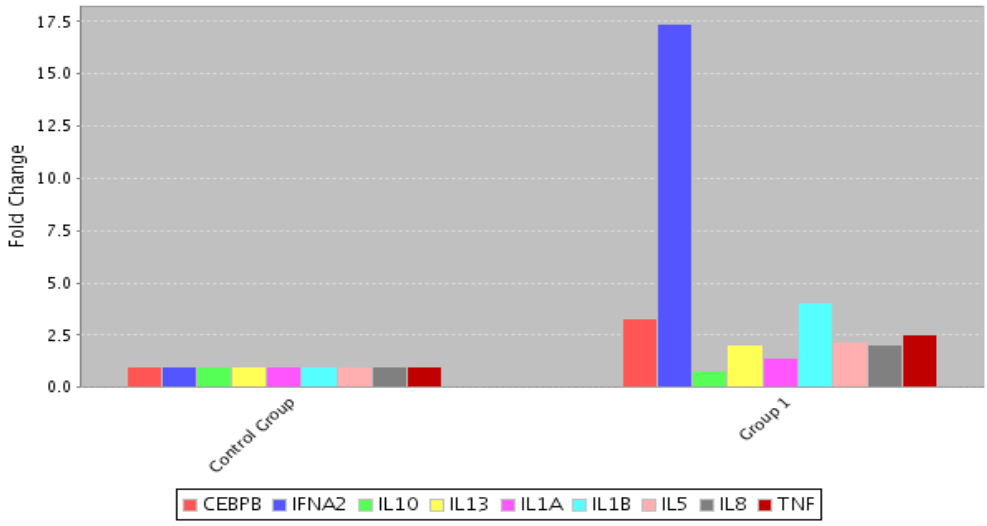

Fig. 5: The up regulated 9 (10.71%) genes after 48 h of HPV infection in seconed patient. The most upregulated gene was IFNA2 gene (17.3878 fold change) and the lower up regulated genes was IL1A gene (1.434 fold change).

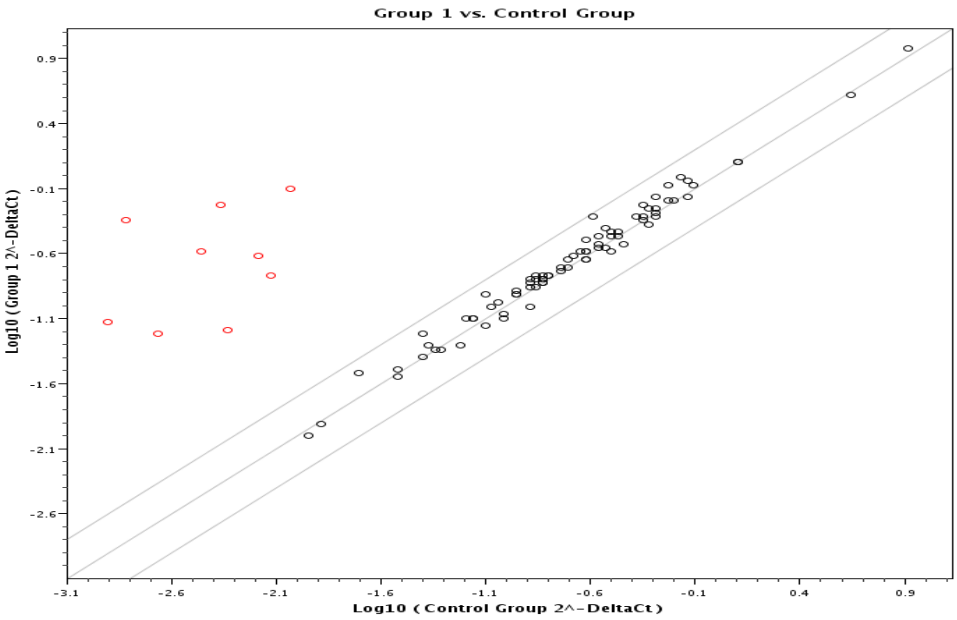

Fig. 6: Nine upregulated genes (Red color) and non altered genes (black color) in second patient.

The third patient showed up regulation of 7 genes with fold change ranged between 1038.29 and 39.94 (Fig. 7) and two genes were down regulated with 0.034 fold changes for IL8 and 0.110 fold changes for TNF (Figure 8).

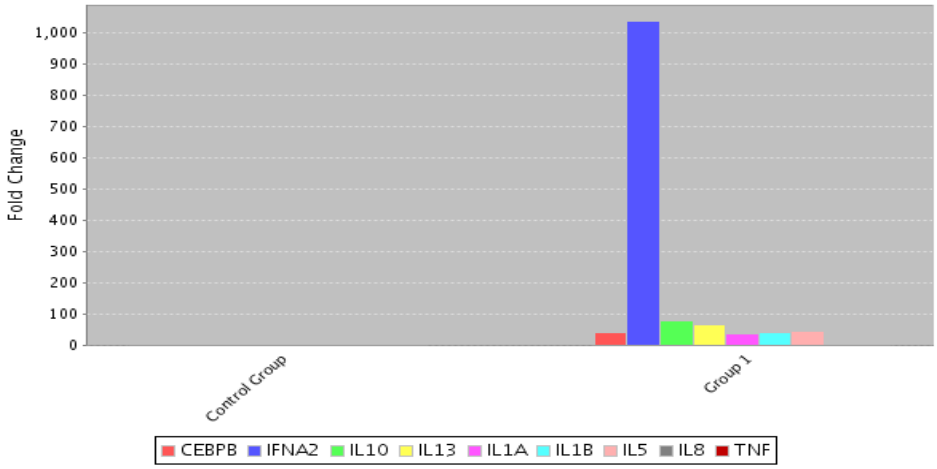

Fig. 7: The up regulated 7 (8.333 %) genes after 48 h of HPV infection in the third patient. The most up regulated gene was IFNA2 gene (1038.29 fold change) and the lower up regulated genes was IL1A gene (39.94 fold change). Two downregulated genes (2.38%). IL8 with 0.034 fold change and TNF with 0.110 fold change.

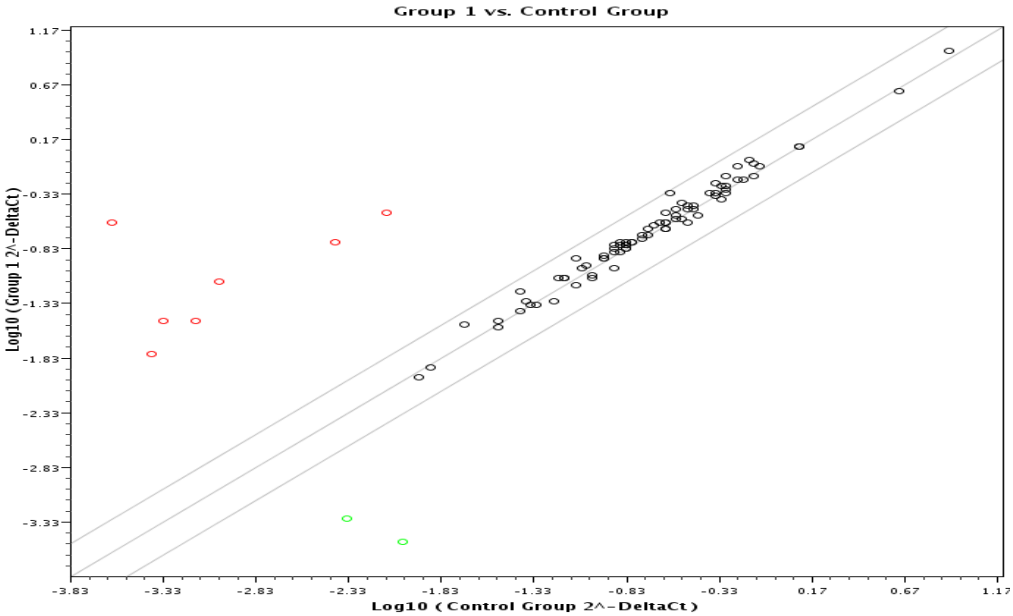

Fig. 8: Seven up regulated genes (Red color), two down regulated genes (Green color) and not changed genes (Black color) in the third patient.

The nine altered genes in this study were CEBPB, IFNA2, IL10, IL13, IL1A, IL1B, IL5, IL8, and TNF.In this study the changing in Chemokine (C-C motif) receptor 1 CCAAT/enhancer binding protein (CEBPB) gene was upregulated in all patients with mean fold change reaches 18.266. The function of this gene is activation of immune system response, cellular process, gene expression, response to stimulus (AL-Ghazal, 2010), thus HPV infection stimulates the expression of this gene to perform their functions against HPV. This results comes in agreement with previous study which showed that Overexpression of CEBPB down regulates the HPV16E6/E7 promoter and CEBPBwaspresent in cultured human foreskin keratinocytes (HFKs) andbinds HPV11 sequences. Increased expression and replicationof HPV11 DNA occur when theCEBPB is depleted in vivo. (Hao *et al*., 1996).

Interferon, alpha 2 (IFNA2) gene functions are; receptor binding, cytokine activity, interferon-alpha/beta receptor binding, protein- protein binding (AL-Ghazal, 2010). In this study IFNA2 was highly up regulated in all patients with 353.804 mean fold change and the reason for this high expression may be due to their multiple functions especially to counter the viral infection. Another study recorded that IFNA2 has antiviral effect due to their stimulation to several other cytokines (Stentela *et al*., 1994). It also act as a bridge between innate andadaptive immunity (The ofilopoulos *et al*., 2005) through the induction of IFN stimulatedgene (ISG) transcription (Sen, 2001). In addition to that IFNA2 stimulates Interferon-regulatory factors (IRF) which were involved in anti-viral defense,immune response and cell growth regulation (Barnes *et al*., 2002). More recently, cDNA micro-array analysis of gene expressionshowed that HPV16 E6 altered expression of three groups ofgenes: IFN-responsive genes, nuclear factor-kappaB-stimulated genes and cell cycle regulation genes (Nees *et al*., 2001).

Interleukin-10 (IL10) gene functions are activation ofimmune system, cellular process, gene expression, developmental process, and response to stimulus (AL-Ghazal, 2010). The result of this study showed that IL10 was up regulated in all patients with 28.77 mean fold change. This moderate expression in HPV patients was needed to stimulate the immune activity against HPV infection. In 1989 Vimlarani *et al*., showed that infection with HPV infection leads to increase the expression of mRNA of IL10 which provided to serum during different stages of HPV infection and play potential to be angiogenic amplifiers.Interleukin-13 (IL13) is another important cytokine that perform very important functions such as; Immune system process, cellular process, and response to stimulus (AL-Ghazal, 2010). This study revealed that IL13 was up regulated in all patients with moderate expression reached 26.05 meanfold change. Another study illustrated that HPV infection stimulate indirectly the expression of IL13 which in turn activates the production of T-helper type 2 which stimulate humoral responses against HPV infections (Paul and Seder**,** 1994).

Interleukin-1 Alpha (IL1α) gene acts as signal transducer activity receptor binding, cytokine activity, Interleukin 1 receptor binding molecular transducer activity (AL-Ghazal, 2010). In this study IL1α is up regulated in moderate rate in all patients with16.897mean fold change after HPV infection. Other study showed that high level expression of human papillomavirus (HPV) type 16 E6 and E7 on coproteins enhanced releasing of IL-1alpha from cultures of normal cervical keratinocytes (Iglesis *et al*., 1989).IL-1was also shown to has antiviral effects on HPV through down-regulation of its gene transcription. (Kyo*et al*., 1994). Interleukin-1 Beta (IL1β) gene Immune system process, metabolic process, cellular process, gene expression, developmental process, and response to stimulus (AL-Ghazal, 2010). This research recorded that IL1β was up regulated in all infected patients with moderate mRNA expression rate reached 17.286 meanfold change. This result not coincide with the study of Woodworth, and Simpson in 1993 which showed that HPV infections reduced the level of IL1β. This disagreement may be due to many reasons concerned with differences in time of infection, stage of infection, state of immune system and the methods used in both studies.

Interleukin-5 (IL5) is another cytokine that stimulates the production of T helper type 2 (Th2) cells. In this study IL5 up regulated moderately after HPV infection with expression rate reached 17.36 mean fold change. Previous research showed that IL5 expression increased in the serum of HPV patients to stimulates humoral responses and counteract the HPV infection (Paul, and Seder**,** 1994). Interleukin-8 (IL8) plays a key role in Immune system process, cellular process, viral reproduction, biological adhesion, and response to stimulus (AL-Ghazal, 2010).In addition to that IL8 acts as a potent activator and chemo-attractant for neutrophils, basophils and T cells (Oppenheim *et al*., 1991). In this study, IL8 was up regulated in the first and second patients with mean fold change reached 3.881, on the other hand IL8 was down regulated in the third patients to -0.034 fold change compared to control (1 fold change). This variation may be due to the period of infection which is in the first and second patients occurred within 3 months, thus IL8 was expressed in high rate and the level of IL8-mRNA was up regulated and reached to 3.881 fold change. Whereas it was down regulated in the third patient because the period of infection occurred within one year which means that the virus start to adapts its self in the host and suppress the expression of IL8. The result of this work is coincides with another study which showed that expression of IL-8 is down-regulated after HPV infection and the expression of HPV- genes

(E6 and E7) inhibited transcription of the IL8 promoter to a greater extent (Huang and McCance , 2002).

Tumor necrosis factor (TNF) has multifunction represented byImmune system process, cellular process, viral infection, biological adhesion, response to stimulus, and biological regulation. TNF was up regulated in the first and second patients with mean fold change reached 3.421 whereas down regulated to - 0.110 fold change in the third patient. This result not coincide with previous study which revealed that the expression of TNF-mRNA was increased during stages ofHPV infection (Santin *et al.,* 2000). Another study recorded that TNFshown to have antiviral effects on HPV through down-regulation of its gene transcription (Kyo *et al*., 1994). The interpretation for this variation in the expression of TNF may also belong to the period time of infection which was short in the first and second patients and long in the third patient and this give enough time to HPV infection in the third patient to down-regulate the expression of TNF.

Gene Ontology

Gene ontology analysis using DAVID Bioinformatics Resources (Huang *et al.,* 2009) showed the alteration of these nine genes stimulates the activity of three different immunological functional pathways represented by cytokine-cytokine receptor interaction pathway, NOD-like receptor signaling pathway, and Toll-like receptor signaling pathway (Huange *et al*., 2009). The cytokine-cytokine receptor interaction pathway stimulates many CXC-chemokines family which activate different inflammatory receptors as powerful chemotactic factors that stimulate the migration of many immune cells. The NOD-like receptor signaling pathway (nucleotide-binding oligomerization domain containing-1) enhances caspase-9 mediated apoptosis and induces nuclear factor-kB. The Toll-like receptor pathway involved in antiviral, antibacterial and anticancer activities. It belongs to interferon family (IFN beta-1 fibroblast) which mediated by JAK-STAT pathway.

CONCLUSION

It is concluded that the relationship between HPV infection and host is a host impact on virus (Cellular response) because most of altered genes were up regulated against HPV infection. The HPV infection cause a very narrow impact (Gene downregulation) on host genes represented with 2. 380% of total 84 investigated immuno-inflammatory genes. Gene ontology analysis indicates that all up regulated genes were participate with the most important human inflammatory pathways represented with Cytokine-cytokine receptor interaction pathway, NOD-like receptor signaling pathway, andToll-like receptor signaling pathway.

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