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Human papillomavirus 16 infection alters the Toll-like receptors and downstream signaling cascade: A plausible early event in cervical squamous cell carcinoma development

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HIGHLIGHTS

- Expression of Toll-like receptor genes and downstream signaling pathway was studied in cancer cervix vis-a-vis normal cervix.
- The study was performed on freshly obtained human cervical tissues.
- Universal downregulation of TLR genes & downstream signaling pathway was observed in cervical cancer.
- Downregulation of TLR & downstream signaling pathway points to a dampened immune response.
- Low expression of TLR7 was further associated with poor overall patient survival.

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ABSTRACT

Objective. Toll-like receptors constitute an important component of innate immune mechanism. HPV is a known etiological factor of cervical cancer and is known to interfere with the expression of TLRs and downstream signaling pathway. It remains poorly understood whether HPV modulates the expression of TLRs. Hence, understanding HPV mediated immune alterations might aid in identifying novel therapeutic targets. The aim was to study the relative gene expression of TLRs & downstream signaling pathway in cervical carcinoma.

Methods. Cervical squamous cell carcinoma (CSCC) and normal cervical tissues were obtained. Subsequent to HPV genotyping, mRNA expression profiling using PCR Array was performed. Protein expression of relevant genes with western blot was studied. Levels of cytokines in cervicovaginal washes were estimated using a Luminex multiplex platform.

Results. All cases of cervical cancer were HR-HPV positive and predominant subtype was HPV16 (71.1%). Significant TLR4 upregulation and TLR2,7 downregulation were observed in HR-HPV infected cervix. TLR4,7 demonstrated low expression in CSCC. Molecules from cancer allied pathways; RELA, AKT, CDKN2A, and MDM2 demonstrated upregulation in CSCC. Protein expression data corroborated with gene expression profile. A diminished level of Th1 cytokines TNF-α, IFN-ɣ, IL-17, and IL-12 was observed in CSCC. Significantly increased levels of IL-1β, IL-6 and IL-2 were detected in HR-HPV infected cervix. Kaplan Meier curve demonstrated high TLR4 and low TLR7 expression was associated with poor prognosis.

Conclusion. The study demonstrates the HPV mediated dampening of the innate immune response in CSCC and provides support for exploring potential TLR2, 7 agonists as an adjunct therapy in CSCC patients.

1. Introduction

Cervical cancer is the most common gynecological malignancy among women worldwide, particularly in developing countries. Role of high-risk HPV in causing cervical cancer is undebatable. Interestingly, only a small percentage of women having persistent HPV infection undergo cervical carcinogenesis [1], implying the contribution of interplay between the host immune system and HPV in its clearance or

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persistence. Intriguingly, HPV related cancers are known to evade the immune surveillance, although the exact mechanism needs exploration.

Toll-like receptors being key players of the innate immune system, recognize conserved pathogen-associated molecular patterns (PAMPs) on microbes. The Interaction between TLRs and HPV is well documented in the literature. The early HPV oncogenic proteins E5, E6, and E7 are known to impair the presentation of antigen and alter innate and adaptive immune responses [2]. In addition to toll-like receptors, cytokines produced as a result of downstream signaling, regulate the expression of key genes which aid the requisite tumor milieu [3]. There is documentation of involvement of HR-HPV16 subtype in transcriptional repression of TLRs enabling their immune escape [4]. However, there is limited literature available on the exploration of the gene expression repertoire of Toll-like receptors as well as the study of downstream signaling pathways in high-risk HPV infection and cervical cancer. Furthermore, it would be interesting to understand the receptor-regulated gene expression mechanism and the outcome in high-risk HPV infection.

The present study attempts to investigate the differential expression of toll-like receptors and downstream signaling molecules in HR-HPV infected cervix and carcinoma cervix. We hypothesize that altered expression of TLRs and downstream signaling molecules have a role to play in cervical carcinogenesis. Our results highlight the alteration of TLRs in HPV infected cervix which could be an early event necessary for viral persistence, which progressively down-regulate the TLR expression in cervical carcinoma.

2. Materials and methods

2.1. Sample collection

This study was conducted at the Department of Immunopathology and Obstetrics & Gynecology, Post Graduate Institute of Medical Education & Research, Chandigarh, India. It was approved by the Institutional Ethical Committee and informed consent from participants was obtained as per the guidelines.

One thirty nine clinically normal cervical tissue samples were available from hysterectomy specimens of women, who underwent surgery for non-neoplastic causes including abnormal Uterine bleeding (AUB) and uterine fibroids. Cervical histopathology confirmed their non-neoplastic pathology. Tissue from the suspected growth on cervix was obtained from women attending the outpatient department of Obstetrics & Gynecology. Subsequently, confirmed cases of cervical squamous cell carcinoma were enrolled.

Cervicovaginal lavage was collected from carcinoma and control cervix subjects at the time of biopsy and hysterectomy respectively by flushing the cervix with 10 ml of 1× PBS (phosphate buffer saline). Subsequently, the fluid that pooled was centrifuged. The supernatant was subjected to cytokine estimation.

2.2. Genomic DNA extraction and HPV typing

DNA was extracted from cervical tissues with commercially available DNA extraction kit (Pure Link® Genomic DNA mini kit, Invitrogen, U.S.A). DNA with an A260/280 ratio of 1.8 qualified for HPV typing. The quality was assessed by checking the expression of beta-actin gene by polymerase chain reaction.

Screening of HPV subtypes was performed using HPV Genoarray Diagnostic kit (Hybribio Ltd., Hong Kong, China). It is a dot blot hybridization-based kit that simultaneously detects 15 high-risk and 6 low-risk HPV types. Polymerase Chain Reaction (PCR) was used to amplify specific HPV DNA present in the template using L1 consensus primers. The 25 μl reaction mixture comprised of 23.25 μl master mix, 0.75 μl Taq polymerase, and 1 μl DNA template. Thermocycling conditions included initial denaturation at 95 °C for 9 min, followed by denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5mins. Further flow through hybridization-based technology allows binding of amplified DNA amplicons to specific HPV probes located on the membrane. Results were interpreted based on the location of colored precipitate against specific HPV probes on the membrane. Two internal controls were incorporated onto hybrimembrane: 1. Biotin control: for detection of accurate enzyme conjugate reaction 2. Internal control: to monitor successful PCR amplification without any inhibitor.

2.3. RNA extraction and cDNA synthesis

RNA was extracted from approximately 100 mg of cervical tissues using Trizol reagent (Thermofisher Scientific U.S.A.). Three thousand nano-grams of RNA samples with absorbance A260/280 ratio of 2.0 were converted to cDNA (complementary DNA) using RT2 first strand kit, Qiagen (U.S.A).

2.4. PCR Array for relative gene expression by real-time PCR

The cDNA was subjected to customized RT2 Profiler PCR Array (Qiagen, U.S.A) for studying the expression of different target genes. KEGG (Kyoto Encyclopedia of Genes and Genomes) was used as a source of pathway information. The expression profiling of 42 short-listed genes (Supplementary Table S1) was studied containing the primer assays of interest namely;

Toll-like receptors: TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 TLRdownstream molecules: MYD88, TICAM1, TICAM2, IRF3, IRF7, IRAK4, JUN Negative regulator of the TLR signaling pathway: TOLLIP

NF-κB pathway: RELA, NFKB1, TNF

MAPK pathway: MAPK14, MAPK8

PI3-AKT pathway: RAC1, PIK3CA, AKT1, PTEN, mTOR, Nos3, MDM2, TP53

Anti-apoptotic genes: BCL2, MYC, CCND1, CDKN2A

Apoptotic pathway: FADD, CASP3, CASP8

Cytokine genes IL-10, IL-17A, IFNA1, IFNG, IL-1β

Housekeeping genes (HPRT1, GAPDH, 18SrRNA) and inbuilt quality controls (RTC control, HGDC control and PPC) were included to check the PCR efficiency. The cycle conditions are described in the supplementary information (S2A).

2.5. Western blot

The immunoblot experiment was performed to deduce the protein expression of key molecules from TLR and other cancer supporting pathways. Cervical tissues (~100 mg) from both cases and controls were lysed in a defined concentration of RIPA buffer and Protease: EDTA cocktail. The quantification of total protein was completed using the Pierce BCA Protein Assay Kit (Thermo scientific, U.S.A). The detailed methodology followed is described in the supplementary information (S1B). The membrane was incubated with the following different antibodies: Mouse monoclonal antibody for TLR3 (Santa Cruz Biotechnology (SCB), California, U.S.A, SC-32232, M.wt: 112 KDa) at 1:1500 dilution, TLR7 (SCB, SC-57463, M.wt: 121 KDa) at 1:1000 dilution, Caspase 8 (SCB, SC-166320, M.wt: 39 KDa) at 1:1500 dilution, AKT (SCB, SC-5298, M.wt: 62 KDa) at 1:1000 dilution, c-JUN (SCB, SC-74543, M.wt: 37 KDa) at 1:1000 dilution, p16 (BD Biosciences, San Diego, California, U.S.A,BD-550834, M.wt:16 KDa) at 1:1500 dilution, p53 (BD Pharmingen NY -554,293, M.wt: 53 KDa), ATM (SCB, SC-23921, M.wt: 370 KDa) at 1:1500 dilution, IL-10 (SCB, SC-32815, M.wt:20 KDa) at 1:1000 dilution, rabbit polyclonal antibody for MDM2 (SCB, H-221, M.wt: 69 KDa) at 1:1500 dilution, CHK2 (SCB, SC-9064, M.wt: 63 KDa) at 1:1500 dilution, BCL2 (SCB,C21: SC-783, M.wt:29 KDa) at 1:1500

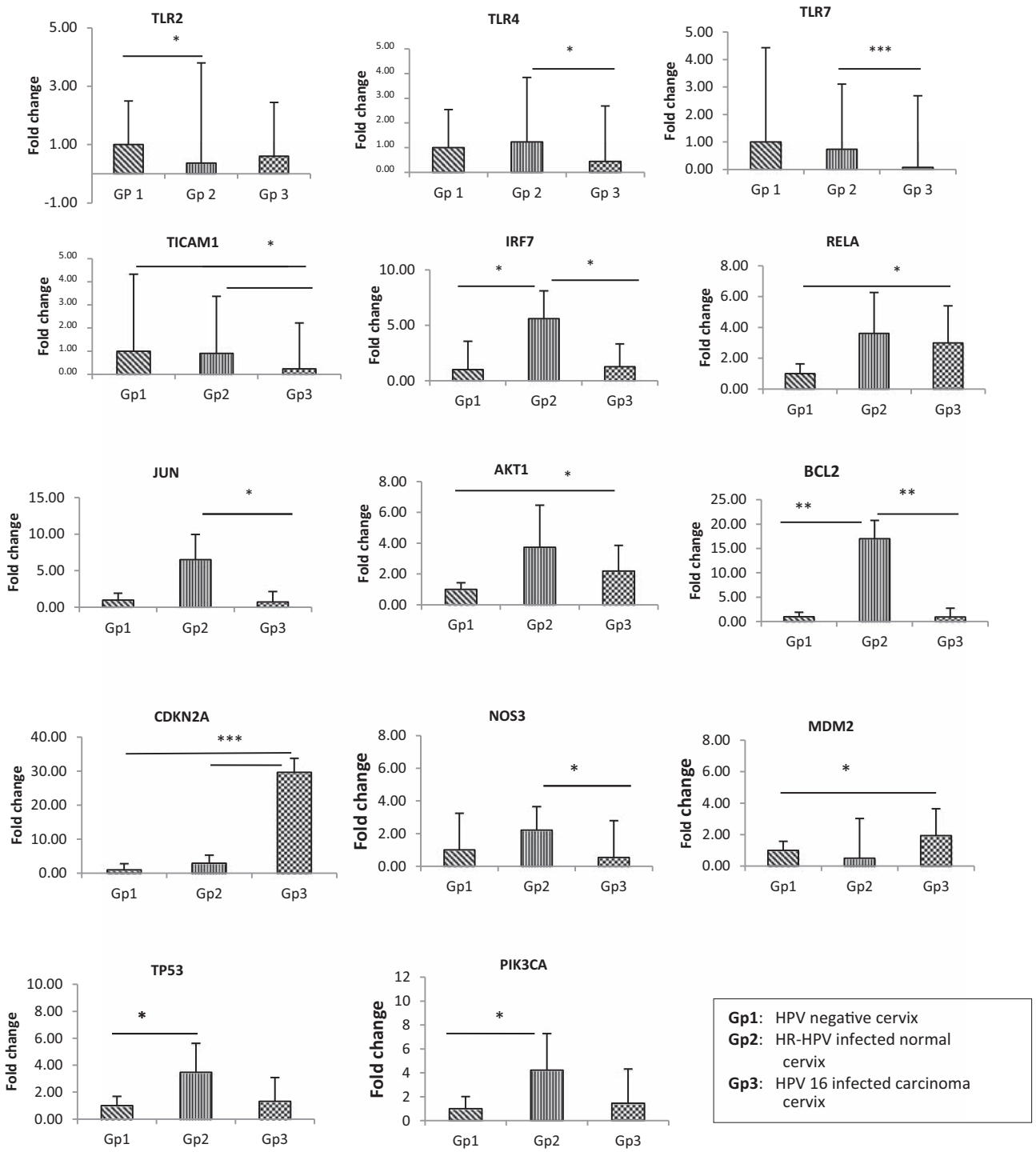


Fig. 1. Relative expression of key Toll-like receptors and the downstream signaling molecules in HPV negative normal cervix ($n = 20$, Gp1) HR-HPV infected cervix ($n = 15$, Gp2) and HPV 16 infected carcinoma cervix ($n = 30$,Gp3). Data are represented as mean fold change ± S.D. Statistical significance was determined by Kruskal Wallis and Mann Whitney (*$p \le 0.05$, **$p \le 0.001$, ***$p \le 0.000$).

dilution, and β-actin (AnaSpecInc. San Jose, CA 95131–54,590, M.wt: 42 KDa) at 1:1000 dilution.

2.6. Luminex Bio-plex assay

The level of pro-inflammatory (IL-1β, IL-6, IL-12, IL-2, IL-4, IFN-ɣ, IL-17) and anti-inflammatory (IL-10) cytokines were determined in cervicovaginal lavage specimens from HPV negative normal cervix ($n = 22$), HR-HPV infected cervix ($n = 7$) and HPV16 infected carcinoma cervix ($n = 18$) using the Luminex platform Bioplex-200 from BioRad. Magnetic beads at 1× dilution were coated to 96 well plates followed by addition of 50 μl standard dilutions and lavage sample. Incubation of 30 min was followed by addition of 1× detection antibody. Phycoerythrin labeled substrate was added and kept undisturbed for 10 min with intermittent washings using a diluted wash buffer. Analysis of the run was performed by Bio-Plex Data

Table 1
Relative gene expression of cell surface and intracellular toll-like receptors and key downstream signaling molecules in HR-HPV infected cervical tissues (n = 15) vis a vis HPV16 infected carcinoma cervix (n = 30) normalized with controls (n = 20).

Genes	HPV negative cervix (n = 20) Avg. $\Delta C_T \pm SD$	HR-HPV infected cervix (n = 15) Avg. $\Delta C_T \pm SD$	HPV 16 infected cervical carcinoma (n = 30) Avg. $\Delta C_T \pm SD$	*P* value	Fold changes ($2^{-\Delta\Delta C_T}$)	
					(a)	(b)
Toll like receptor molecules (TLR1–9)						
TLR1	4.98 ± 1.67	4.40 ± 3.55	5.50 ± 2.13	0.660	1.50	0.70
*TLR2	3.28 ± 1.5	4.73 ± 3.43	4.01 ± 1.81	***0.038***	0.37	0.60
TLR3	3.01 ± 1.51	2.12 ± 2.35	3.62 ± 2.10	0.288	1.84	0.65
TLR4	3.45 ± 1.54	3.15 ± 2.62	4.62 ± 2.21	***0.026***	1.23	0.44
TLR5	3.04 ± 1.01	1.92 ± 3.81	3.56 ± 2.14	0.334	2.17	0.70
TLR6	3.91 ± 2.0	4.09 ± 3.26	4.65 ± 2.13	0.320	0.88	0.60
TLR7	1.91 ± 3.4	2.35 ± 2.37	5.64 ± 2.61	***0.000***	0.74	0.08
TLR8	5.39 ± 2.1	5.10 ± 2.57	5.75 ± 2.56	0.947	1.23	0.78
TLR9	6.40 ± 3.26	6.03 ± 3.01	6.48 ± 3.29	0.932	1.30	0.95
TLR downstream signaling molecules						
TICAM1	2.12 ± 3.33	2.26 ± 2.47	4.20 ± 1.95	***0.038***	0.90	0.24
IRF7	7.49 ± 2.57	5.01 ± 2.52	7.16 ± 2.03	***0.038***	5.60	1.26
RELA	4.20 ± 0.63	2.35 ± 2.66	2.62 ± 2.42	***0.044***	3.61	2.99
JUN	6.02 ± 0.94	3.32 ± 3.46	6.45 ± 1.40	***0.024***	6.50	0.74
Molecules from other cancer linked pathways						
*PIK3CA	3.87 ± 1.02	1.79 ± 3.06	3.32 ± 2.86	***0.031***	4.23	1.46
AKT1	4.27 ± 0.41	2.37 ± 2.74	3.14 ± 1.64	***0.027***	3.73	2.19
BCL2	5.81 ± 0.92	1.73 ± 3.77	5.92 ± 1.80	***0.002***	16.98	0.93
CDKN2A	4.31 ± 1.80	2.77 ± 2.38	−0.58 ± 4.10	***0.000***	2.91	29.67
*NOS3	5.10 ± 2.25	3.96 ± 1.44	5.99 ± 2.22	***0.029***	2.21	0.54
*MDM2	1.58 ± 0.56	1.07 ± 2.52	0.63 ± 1.72	***0.043***	0.50	1.93
TP53	4.26 ± 0.67	2.46 ± 2.16	3.85 ± 1.76	***0.025***	3.47	1.32

Bold and italics denote the values which have statistically significant p value (< 0.05) for the significantly expressed genes.
(a) Fold change value of HR-HPV infected cervix compared to HPV negative control.
(b) Fold change value of HPV16 infected carcinoma cervix compared to HPV negative control.
* Statistical significance calculated using Mann Whitney.

Manager-4. The concentration of the analytes was obtained by interpolating the intensities to 4 fold dilutions of the standards fitted into a 5-parameter logistic curve.

2.7. Statistical analysis

The data obtained were statistically analyzed using SPSS Statistics for Windows version-21. Data from the study groups were compared using Kruskal Wallis non-parametric test for more than two groups. As a part of the post hoc test, Dunn Bonferroni correction was applied for the gene groups showing a significant difference. For pair-wise comparison between the groups, Mann Whitney test was applied. Kaplan Meier method was used to calculate survival curves for the HPV16 infected carcinoma patients based on the log-rank test. Multivariate Cox-proportional Hazard model was applied on TLR expression (High or Low). The Hazard Ratio (HR) along with 95% confidence interval (C·I) was provided for covariates in the model. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

There were thirty cervical squamous cell carcinoma (CSCC) tissues and fifteen HPV positive normal cervices available for this study. The CSCC tissues which reported HPV 16 positivity were included to constitute a homogeneous group. Thirty normal cervical tissues negative for HPV constituted the control group and diagnosis included fibroid uterus (46.6%), uterine prolapse (24.4%), abnormal uterine bleeding (13.3%), endometrial hyperplasia (11.1%) and adnexal mass (4.4%).

The control and cases were age-matched with a mean age ranging from 48.76 to 54.1 years ($p = 0.179$). Most of the women with cancer cervix were illiterate (56.6%, $p = 0.000$) and did not use any contraception (96.6%, $p = 0.001$) as compared to women with the normal cervix. (Supplementary Table S3). Most of the carcinoma were categorized as stage III-B (36.66%) followed by II-B (30%), III-A & II-A (10%), I—B (6.6%), I-A & IV-B (3.33%).

3.1. HPV sub-typing in normal cervix and carcinoma cervix

We observed high risk HR-HPV16 alone as the preponderant subtype in 71.1% (47/66) of CSCC enrolled. Whereas in normal cervix 53.33% (8/15) of the cases confirmed HPV16 positivity. Other cases with normal cervix demonstrated multiple subtypes as HPV16,18,31,33 ($n = 1$), HPV16,18,35 (n = 1), HPV 11,16 (n = 1), HPV16,33 (n = 1), HPV16,31 ($n = 2$) and HPV 35 (n = 1).

3.2. Differential gene expression of Toll-like receptors and downstream signaling molecules in HR-HPV infected cervix and carcinoma cervix

3.2.1. Differential gene expression in HPV16 infected CSCC

All TLRs were universally down-regulated in CSCC with significantly low expression of extracellular TLR 4 (0.44 folds, $p = 0.026$) and intracellular TLR 7 (0.08 folds, $p = 0.000$). Subsequently, molecules downstream of TLR signaling pathway; TICAM 1 (0.24 folds, $p = 0.038$), IRF 7 (1.2 folds, p = 0.038) and JUN (0.74 folds, $p = 0.024$) demonstrated significant down-regulation. However, RELA (also known as p65 subunit of NFκB) showed significant upregulation (2.99 folds, $p = 0.017$). AKT1 demonstrated significant upregulation (2.19 folds, $p = 0.005$). Furthermore, we observed that MDM2, the principal cellular antagonist of p53 tumor suppressor gene, demonstrated significant upregulation (1.93 folds, $p = 0.04$) in CSCC compared to p53 gene expression (1.2 folds, $p = 0.219$). CDKN2A gene, a known surrogate marker for cervical cancer demonstrated significant up-regulation (29.6 folds, $p = 0.000$). Intriguingly, anti-apoptotic gene BCL2 was observed to have a significantly decreased expression (0.9 folds, $p = 0.003$) in CSCC (Fig. 1).

The relative expression data for the patient group was obtained after normalizing with the cycle threshold values (ct values) from the controls (Table 1). Delta ct values for all the cases were initially calculated by normalizing the raw ct values with GAPDH as the reference gene. Subsequently, the average delta ct values of the patient and control groups were normalized to obtain ΔΔct value (Avg. Δct patients- Avg.

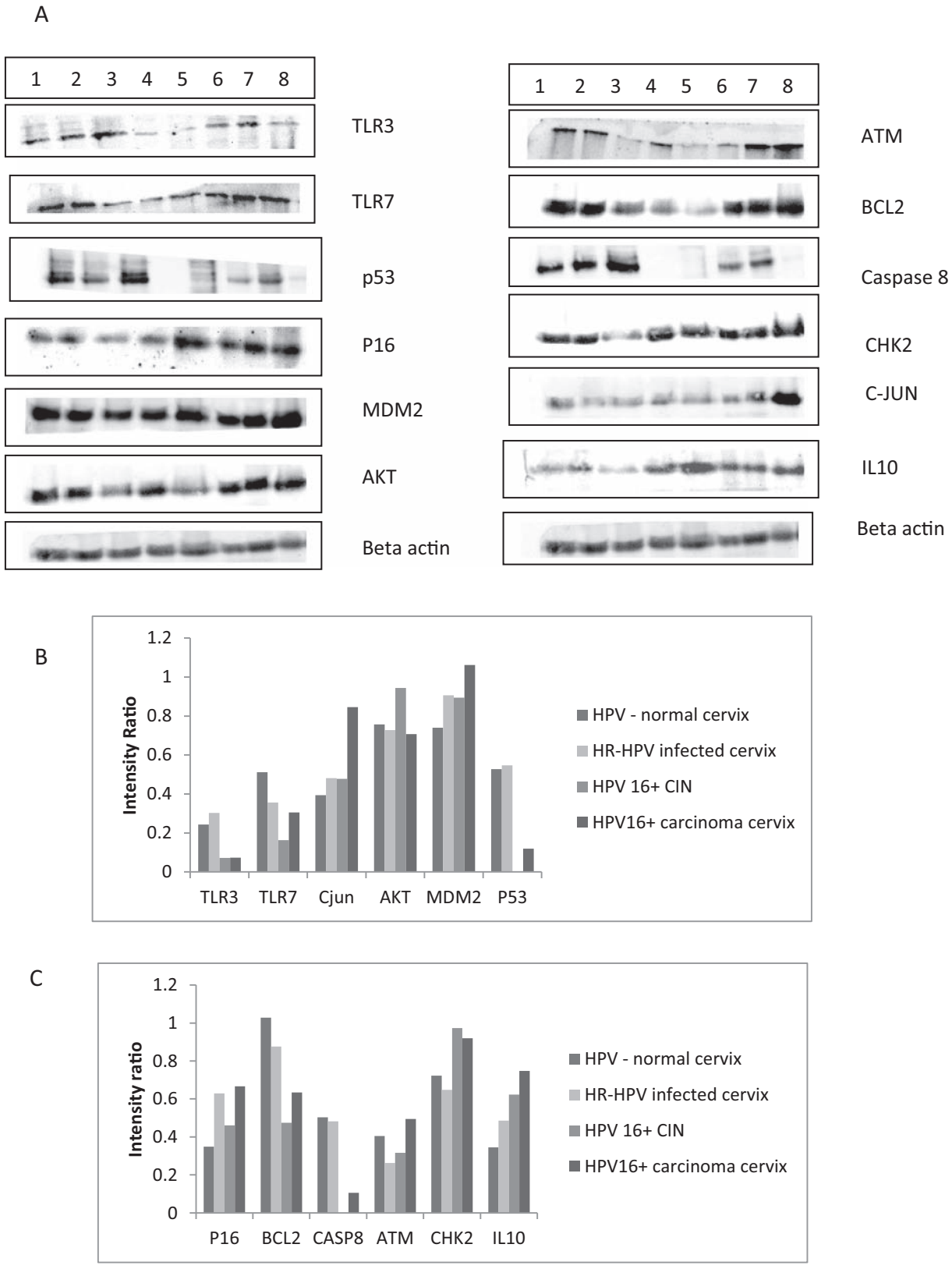


Fig. 2. A: Representative Western blot results of the TLR Pathway genes. Lane 1 and 2: HPV negative normal cervix, lane 3: HR-HPV infected cervix, lane 4: CIN–III, lane 5: HPV16 infected carcinoma cervix, lane 6 and 7: individual patients HR-HPV infected normal cervix, Lane 8: carcinoma cervix. B. & C. Column graph and relative intensities for TLR3, TLR7, MDM2, BCL2, Caspase 8, p16, p53, AKT, ATM, CHK2, IL-10 along with housekeeping gene beta-actin.

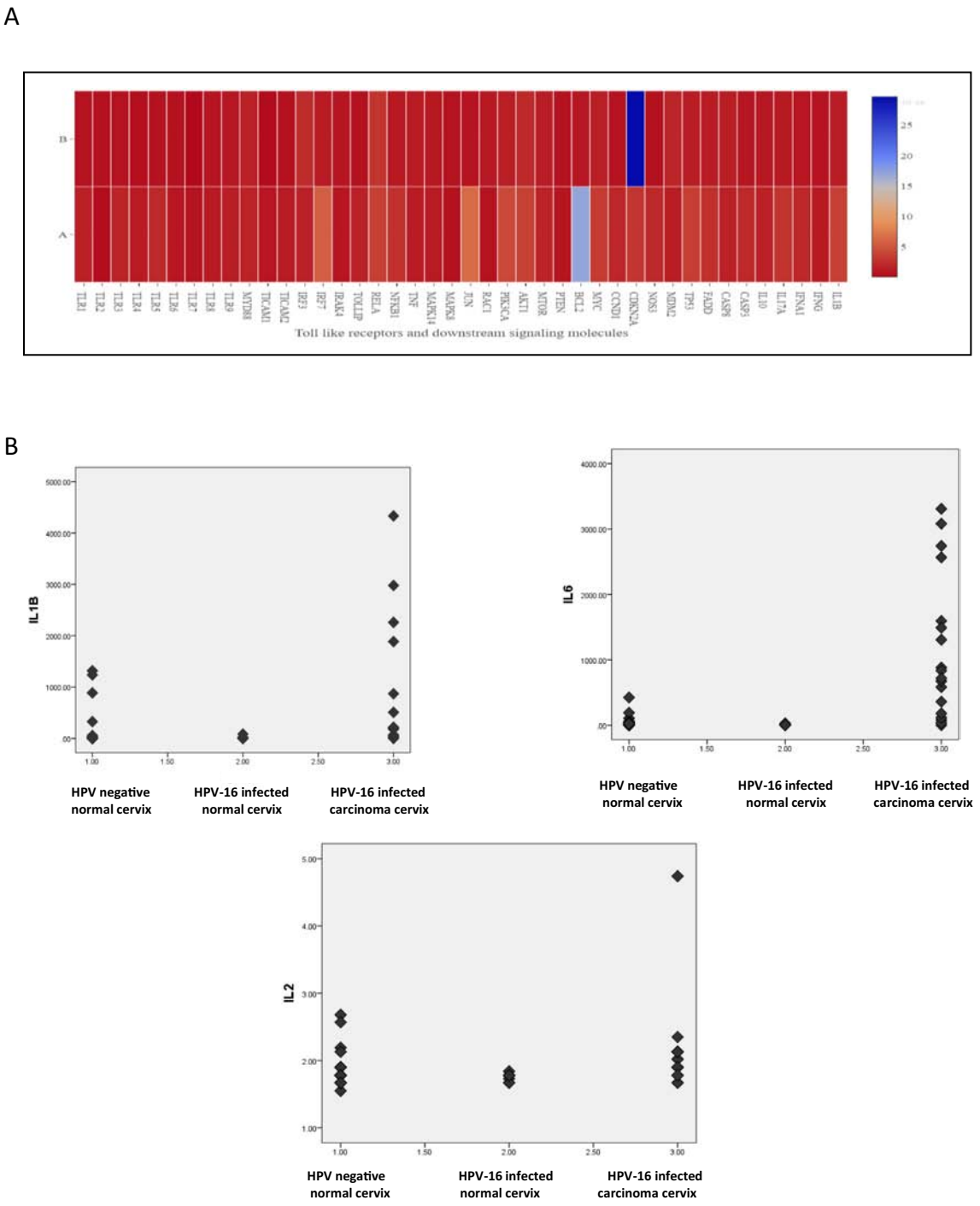


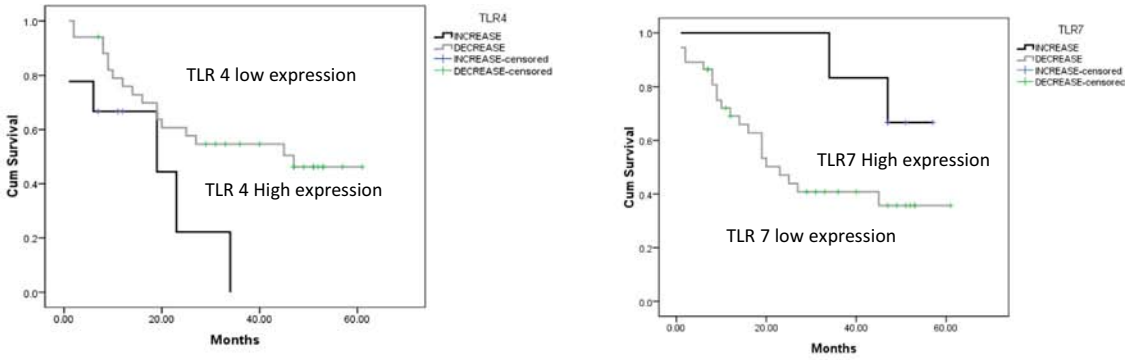
Fig. 3. A: Representative heat map for Toll-like receptor genes (TLR1–9) and the downstream signaling molecules in HR-HPV infected cervix (Group A) and HPV16 infected carcinoma cervix (Group B) generated using plotly software. The scale represents the relative fold changes of the genes; red represents down-regulation and blue represents up-regulation. B: Scatter plots for IL-6; IL-1β; IL-2 representing the secreted levels of cytokines observed in HPV negative normal cervix ($n = 22$, group 1), HR-HPV infected cervix ($n = 7$, group 2) and HPV16 infected carcinoma cervix ($n = 18$, group 3). Y-axis represents concentration in pg/ml and X-axis represents the study groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Δct controls). Fold changes of the genes were then calculated using the 2^-ΔΔct method [5].

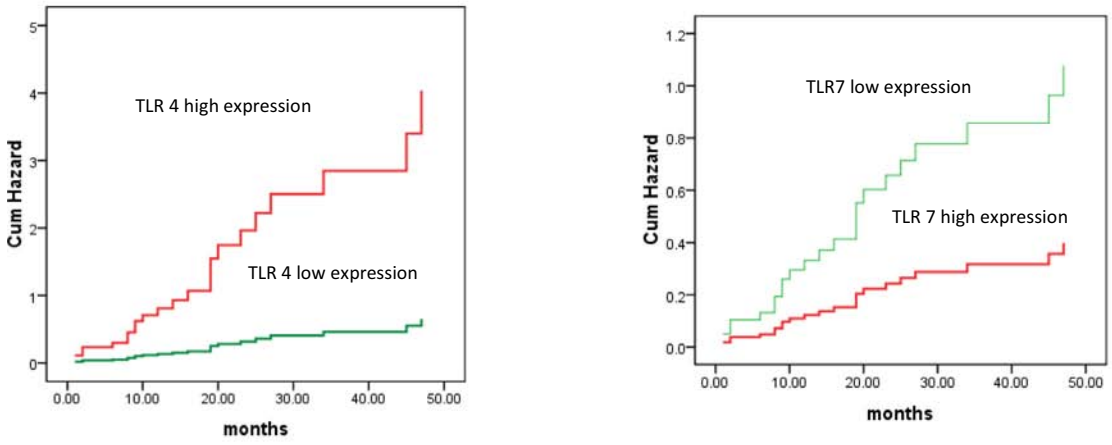
3.2.2. Differential gene expression in HR-HPV infected cervix

In contrast, in HR-HPV infected cervix TLR 4 demonstrated significant upregulation (1.23 folds, $p = 0.026$). TLR7 demonstrated downregulation (0.74 folds, $p = 0.00$) although the expression was elevated as compared to carcinoma cervix (0.08 folds, $p = 0.000$). TLR 2 reported significant down-regulation (0.37 folds, $p = 0.038$). Furthermore, TLR 1 (1.5 folds), TLR 3 (1.87 folds), TLR 5 (2.17 folds) and TLR 9 (1.3 folds) demonstrated upregulation, although it was not statistically significant ($p \geq 0.05$). Consequently, the downstream molecules IRF 7 (5.60 folds, $p = 0.026$) and JUN (6.5 folds, $p = 0.022$) confirmed significant up-regulation in HR-HPV infected cervix. Elevated expression of

A



B



C

Individual characteristics	Hazard Rate/Ratio	95 % (C.I)	P value
TLR4 expression			
High	6.192	(1.74-21.95)	0.005
Low	1		
TLR7 expression			
High	1		0.043
Low	6.468	(1.0-39.04)	

Fig. 4. A: Kaplan Meier curve demonstrating the correlation between TLR4 (A) and TLR7 (B) gene expression and carcinoma patient survival; (low TLR4 expression in $n = 34$, high TLR4 expression in $n = 9$; Low TLR7 expression in $n = 37$ and High TLR7 in $n = 6$). B: Representative Hazard function curves for TLR4 and TLR7 differential expression in women with cancer cervix. C: Multivariate Cox-regression analysis to determine the effect of independent demographic characteristics and TLR4 and TLR7 expression with patient survival.

RELA (3.61 folds, $p = 0.081$) was reported but was not statistically significant. Among the molecules from other cancer supporting pathways, PIK3CA reported significant upregulation (4.23 folds, $p = 0.031$). Increased AKT expression (3.73 folds) was observed, although it did not reach statistical significance. In contrast to CSCC, down-regulated expression of MDM2 (0.50 folds, $p = 0.537$) and significantly upregulated expression of p53 (3.47 folds, $p = 0.025$) was reported in HR-HPV infected cervix. Further, NOS3 (2.21 folds, $p = 0.026$) and anti-apoptotic gene BCL2 (16.98 folds, $p = 0.001$) demonstrated significant upregulation in HR-HPV infected cervix. The relative gene expression of 42 genes from Toll-like receptor and downstream signaling pathway observed in the three groups is depicted in supplementary Tables S4 & S5.

3.3. Differential gene expression of Toll-like receptors and downstream signaling molecules in early (I-II) vs advanced (III-IV) stage of HPV16 infected CSCC

In an attempt to understand the variation of Toll-like receptors with cancer progression, the differential gene expression between early-stage (I-II: $n = 15$) and advanced stage (III-IV: n = 15) of CSCC was studied. Intriguingly, we observed a striking downregulation of intracellular TLR7 (0.06 folds, $p > 0.05$) in the early stage. PIK3CA (2.48 folds, $p > 0.05$), AKT (2.82 folds, $p > 0.05$) and MDM2 (2.38 folds, $p = 0.345$) demonstrated upregulation in advanced stage although statistical significance was not achieved. CDKN2A reported an elevated expression (14

folds, $p = 0.151$) in an early stage that escalated to 62.96 folds ($p = 0.151$) in the advanced stage. In addition, the proto-oncogene c-MYC (2.71 folds, $p = 0.018$) demonstrated a significant upregulation in advanced stages of cancer.

3.4. Western blot analysis

The western blot experiments were carried out to validate the gene expression findings. Additional cases of CIN-III ($n = 5$) were included from the laboratory archives. The protein intensity data was acquired by Image J software and normalized with internal control beta-actin. Thereafter, the ratios of intensities were compared. The protein expression corroborated the results of gene expression experiments. The intracellular receptors, TLR3, and TLR7 that recognizes viral ligands demonstrated a decreased protein expression in CIN-III (cervical intraepithelial neoplasia) and CSCC tissues although these were not statistically significant ($p = 0.182$ and $p = 0.487$) (Fig. 2A & B).The transcription factor AP1 (C-JUN) demonstrated increased expression in HPV 16 infected carcinoma cervix, HR-HPV infected cervix and CIN-III ($p = 0.889$).

Increased expression of AKT protein was observed in CIN-III and CSCC ($p = 0.845$). The protein expression of MDM2 was in sync with the gene expression findings. It demonstrated an upregulation in CSCC ($p = 0.914$). Consequently, we observed a down-regulation of P53 ($p > 0.05$) in CSCC as well. Further, p16INK4A protein illustrated an up-regulation in HPV infected cervix and CSCC ($p = 0.80$). The anti-apoptotic gene BCL-2 reported decreased protein expression in CIN-III and CSCC ($p = 0.839$). The expression of caspase 8, which is a primary caspase, known to get activated once the extrinsic pathway of apoptosis is triggered, was not detected in CIN-III and demonstrated low expression in CSCC ($p = 0.467$).

Furthermore, we analyzed the protein expression of key molecules activated during the double-stranded DNA damage response. The protein ATM (Ataxia telangiectasia) and its downstream CHK2 (Checkpoint kinase-2) molecule demonstrated up-regulation in CSCC although the expression was not found to be statistically significant ($p > 0.05$). This suggests an active DNA damage response in cancer cervix. To strengthen our finding of a dampened innate immune response in carcinoma cervix, the protein expression of IL-10 was studied. The immunosuppressive cytokine IL-10 demonstrated increased expression in both CIN-III and CSCC but was not statistically significant ($p = 0.796$).

3.5. Levels of pro-inflammatory and anti-inflammatory cytokines in cervicovaginal lavages

Significantly elevated levels of pro-inflammatory cytokines IL-1β (mean 786.57 pg/ml ± 1209.74) and IL-6 (mean 1142.41 pg/ml ± 1099.42) were detected in cervicovaginal fluid in CSCC ($p = 0.002$, $p = 0.000$ respectively) compared to HPV negative normal cervix and HR-HPV infected cervix (Fig. 3D, E). However, the levels of IL-2 detected were 2 pg/ml ± 0.657 ($p = 0.023$). Furthermore, the levels of IL-4, IFN-ɣ, IL-17, IL-12p, IL-10 and TNF-α (estimated with ELISA; Diaclone kit) were below the detection limits.

3.6. TLR expression and patient survival

Archival data of 56 subjects from our laboratory were included for survival analysis to assess whether there is any correlation between TLR 1–9 gene expression and 5-year overall disease-specific survival. The information for 43 subjects was available for further investigation. Kaplan Meier curves were generated for gene expression data of TLR1–9. Among all TLRs, the expression of TLR 4 correlated significantly with patient survival (Fig. 4A). We observed significantly longer survival time (mean = 38 months; 95% C·I 30–46) for patients that showed decreased TLR4 expression as compared to those subjects that demonstrated increased expression (mean = 17 months; 95% C·I 8–27); $p = 0.028$ (long rank chi-square: 4.810). The Kaplan Meier curve for TLR7 demonstrated a distinct positive correlation with patient survival, although was not statistically significant (Log-rank chi-square: 2.719; $p = 0.099$).

The multivariate cox-regression analysis identified high TLR4 and low TLR7 expression as an independent prognostic factor for cervical cancer-related deaths in affected women. We observed HR: 6.192; C·I: (1.74–21.95); $p = 0.005$ in women demonstrating higher TLR4 and HR: 6.468; C·I: (1.0–39.04); $p = 0.043$ among the ones with lower TLR7 expression in diseased cervix (Fig. 4). In addition other characteristics/factors were also studied as described in supplementary Table S6.

4. Discussion

Toll-like receptors constitute the first line of defense against the foreign invaders which usually includes micro-organisms [6]. Activated TLR signaling and other associated pathways collaborate to mount an efficient immune response. However, dysregulation at any level in signaling may compromise immune defense and surveillance against the infections. Majority of the studies on TLR expression in cervical cancer are centered around the differential expression of extracellular and intracellular receptors in low-grade vis a vis high-grade lesions. To the best of our knowledge, the profiling of Toll-like receptor transcriptome and associated downstream diverse signaling pathways in HR-HPV infected cervix vis-a-vis carcinoma cervix has not been reported yet. However, our study was limited to exploring the toll-like receptor gene profile in squamous cell carcinomas alone.

In the present study, a universal downregulation of TLRs in CSCC was reported. TLR4 demonstrated a significant ($p = 0.026$) low expression. Similar findings were observed by Yu et al., during the progression from cervical neoplasia to carcinoma cervix [7]. A study conducted by Aggarwal et al., from our center, has previously reported significantly low TLR4 (0.4 folds, $p = 0.000$) in CSCC [8] suggesting that HPV can inhibit TLR4 expression. However, our observation of elevated TLR-4 expression in HR-HPV infected cervix plausibly suggests an attempt by the infected cells to clear off the infection. The observation is in sync with the findings of Ibrahim et al., who demonstrated that increased expression of TLRs in women harboring incident HPV infection results in subsequent HPV clearance on follow up [9].

We further observed intracellular receptor, TLR7 to be significantly down-regulated in CSCC. Our results are in contrast to the findings of Hasimu et al., who demonstrated increased TLR7 expression in cervical cancer tissue [10]. However, increased expression of TLR7 is a predictor of HPV clearance in women with previous persistent infection [9,11]. Thus, TLR7 expression seems to be critical for oncogenic HPV clearance. Interestingly, our report of a significantly decreased expression of TLR2 and TLR7 in HR-HPV infected cervix and CSCC might reflect an early event in the transformation process by an incident HPV infection. We substantiate our findings associated with HPV16 infection, which is more competent in downregulating TLRs [12]. These collective observations, contemplates HPV mediated modulation of TLR7 expression, ineffectively evading the antiviral immune response [9].

In continuance to the global downregulation of TLRs, a significant downregulation of adaptor molecule TICAM1 (TRIF) points to the dampened TLR signaling. TRIF (TICAM1) is exclusively recruited by TLR3 and TLR4 [13] and its stimulation activates NFκB that dictates the production of IFN-β and other pro-inflammatory cytokines [6]. However, the role of TRIF is not widely explored in cancer. Furthermore, TLR signaling, in addition, activates interferon regulatory factors IRF7 or IRF3 that serves as an antiviral response of the host cell against viral infection [14]. Our observation of a dampened expression of IRF-7 in CSCC is well supported by some genome-wide transcription studies. The studies report HR-HPV types 16, 18 and 31 to reduce TLR3-induced cytokine expression, and type I IFN-induced interferon-stimulated gene (ISG) expression [15,16] indicating that HR-HPV

affects PRR- and type I IFN-induced signaling pathways. In contrast to CSCC, we reported a significantly upregulated expression of IRF-7 in HR-HPV infected normal cervix. TLR activation has been shown to induce IRF7 expression [17]. The current finding, therefore, encourages us to presume that a decreased IRF-7 expression in CSCC highlights diminished type-I interferon responses compared to HPV infected cervix.

Furthermore, the constitutive expression of E6 and E7 HPV oncogenes required for cervical transformation is attributed to the availability of host cell transcription factor, AP1 (activator protein 1). It can be a homodimer complex of 2 Jun proteins (c-jun, JUNB, JUND) or heterodimer of JUN and fos protein (c-fos, FOSB, Fra-1, Fra-2). We reported a significantly upregulated expression of c-JUN in HR-HPV infected cervix and a downregulated expression in CSCC. Our findings are in sync with Wilde et al., who demonstrated an upregulated expression of c-JUN in early immortalized cells and a gradual down-regulation from late HPV immortalized cell lines to carcinoma cell lines [18].

The NFκB expression plays a significant role in cancer progression [19] by inducing proliferation and immortalization. We reported an increased expression of Rela (p65) gene in HR-HPV infected cervix and CSCC despite a dampened TLR signaling in carcinoma cervix.

NFκB being a multifaceted molecule involves complex signaling pathways and cross-talks for its activation. Its expression is stimulated by external cytokine signals and intracellular pathways PIK3CA/AKT as well [20]. This provides an explanation that plausibly an exaggerated NFκB expression in normal cervix with HR-HPV16 infection is an obligation for effective clearing of the resident pathogen.

One of the TLR linked pathway that channels cell proliferation/survival includes the PI3K/AKT/mTOR pathway. Class I PI3Ks have an important role in TLR signaling [21] that further activates AKT, a serine-threonine kinase [22]. We observed a significantly up-regulated expression of AKT gene in CSCC. Literature as well documents, the activation of PI3K/AKT axis in human cancers [23,24] suggesting its contributing role in cervical carcinogenesis. In addition, HPV regulates p53 tumor suppressor protein by its proteasomal degradation [25]. Its expression is negatively regulated by MDM2 (murine double minute two) gene [26]. As expected we observed a significantly up-regulated expression of MDM2 in CSCC. In sync with our finding, Liao et al., observed overexpression of PI3K/AKT and MDM2 along with subsequent downregulation of p53 expression in cervical cancer tissues [27]. Further, significantly upregulated expression of CDKN2A in CSCC substantiate its nature as a biomarker in cervical cancer progression [28,29]. This provides evidence of viruses modulating the host cell machinery as a surrogate choice in promoting cancer progression and maintaining constitutive E7 expression.

TLR signaling culminates in the production of different cytokines. The cervicovaginal fluid from HR-HPV infected normal cervix demonstrated significantly increased secreted levels of proinflammatory cytokines. However, a significantly increased IL-6 and IL-1β levels in the CSCC contemplate their contribution to the growth of tumors [30]. In sync with our findings, Tijong et al., demonstrated higher levels of growth-enhancing IL-1β, IL-6 in cervicovaginal washes from cervical carcinoma [31]. Surprisingly, levels of crucial cytokine mediators of TH1 response TNF-α, IFN-γ, IL-10, IL-17, and IL-12 were below the detection limit in CSCC. The compromised expression of Th1 cytokines in CSCC hence reflects a hampered innate immune response.

DNA damage response (DDR) is often monitored by increased expression of ATM and CHK2 as done in the current study. ATM (kinase) detects intracellular DNA damage due to some physiological stress or pathogenic insult [32] and p53 binding protein and CHK2 that mediates cell cycle arrest [33]. However, viruses are known to have evolved strategies that impair such responses. HPV inactivates p53, hence abolishing regulation at check-point in the cell cycle that sustains constitutive cellular proliferation.

Furthermore, a high TLR4 and low TLR7 expression were associated with decreased overall survival of CSCC patients. Although there is limited literature, similar findings were demonstrated by Kim et al., and Ma et al., in ovarian and breast cancer [34,35].

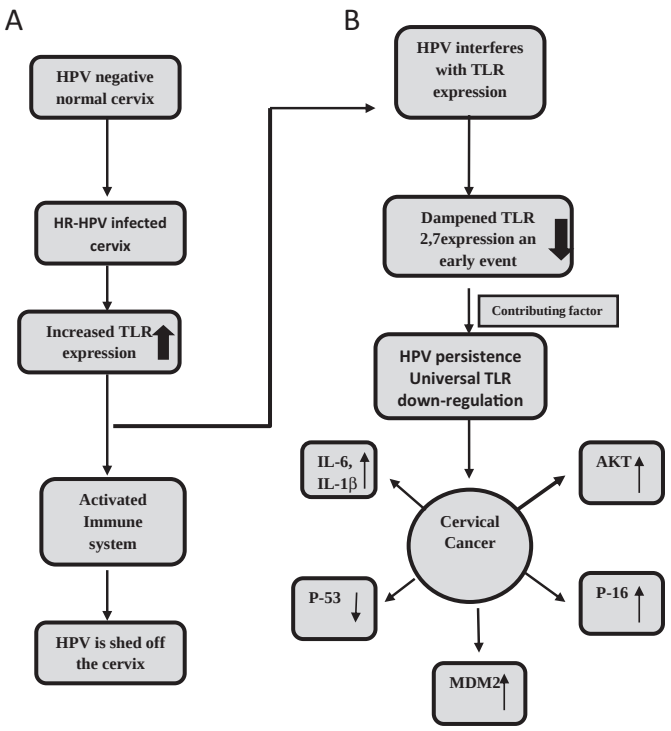


Fig. 5. Pictorial representation of the HPV mediated alteration of the Toll-like receptors and the downstream signaling pathways in HPV16 infected carcinoma cervix as a potential contributor to carcinogenesis. A: Increased TLR expression may activate the innate immune response and can result in viral clearance. B: HPV may interfere with the expression of TLRs and can evade the innate immune response, resulting in its persistence and hence modulation of additional signaling pathways to favor carcinogenesis.

The present study, therefore, explores the transcriptomics of Toll-like receptors and downstream signaling pathways in HR-HPV infected cervix and carcinoma cervix. It gives an insight to HPV assisted alteration in the expression pattern of innate immune receptors amid its preferably dormant state in the HR-HPV infected cervix to constitutively activated state in carcinoma. It is evident from our findings that down-regulation of TLR2 and 7 in HR-HPV infected cervix might be an early event towards cervical transformation. Further, HPV interferes by universally diminishing the TLR expression and downstream innate immune response in CSCC. The viral persistence thus dictates the expression of additional signaling pathways that support cancer progression (Fig. 5). Our results evoke the prospect of investigating potential TLR agonists based adjunct therapies in cervical cancer.

Author contributions

Dr. Ritu Aggarwal, Dr. Vanita Suri, and Dr. Charu have contributed intellectually by conceptualizing the issues, initializing experiments, conducting analysis and data interpretation. Dr. Ranjana Minz and Dr. Rakesh Kapoor assisted in literature review and assessing the accuracy of data.

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Declaration of Competing Interest

The authors declare no potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.07.023.

References

[1] Q. Zhou, K. Zhu, H. Cheng, Toll-like receptors in human papillomavirus infection, Arch. Immunol. Ther. Exp. 61 (2013) 203–215, https://doi.org/10.1007/s00005-013-0220-7.

[2] D. Di Maio, J.B. Liao, Human papillomavirus and cervical cancer, Adv. Virus Res. 66 (2006) 125–159, https://doi.org/10.1016/S0065-3527(06)66003-X.

[3] C.D. Woodworth, HPV innate immunity, Front. Biosci. 7 (2002) 2058–2071 (PMID: 12165480).

[4] H.W. Herr, A. Morales, History of bacillus Calmette–Guerin and bladder cancer: an immunotherapy success story, J. Urol. 179 (2008) 53–56, https://doi.org/10.1016/j.juro.2007.08.122.

[5] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta delta C(T)) method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[6] K. Takeda, S. Akira, Toll-like receptors, Curr. Protoc. Immunol. 109 (2015) 1–10, https://doi.org/10.1002/0471142735.im1412s109.

[7] L. Yu, L. Wang, M. Li, et al., Expression of toll-like receptor 4 is down-regulated during progression of cervical neoplasia, Cancer Immunol. Immunother. 59 (2010) 1021–1028, https://doi.org/10.1007/s00262-010-0825-1.

[8] R. Aggarwal, S. Misra, C. Guleria, et al., Characterization of toll-like receptor transcriptome in squamous cell carcinoma of cervix: a case-control study, Gynecol. Oncol. 138 (2015) 358–362, https://doi.org/10.1016/j.ygyno.2015.05.029.

[9] I.I. Daud, M.E. Scott, Y. Ma, et al., Association between toll-like receptor expression and human papillomavirus type 16 persistence, Int. J. Cancer 128 (2011) 879–886, https://doi.org/10.1002/ijc.25400.

[10] A. Hasimu, L. Ge, Q.Z. Li, et al., Expressions of toll-like receptors 3, 4, 7, and 9 in cervical lesions and their correlation with HPV16 infection in Uighur women, Chin. J. Cancer 30 (2011) 344–350, https://doi.org/10.5732/cjc.010.10456.

[11] M.E. Scott, Y. Ma, S. Farhat, et al., Expression of nucleic acid-sensing toll-like receptors predicts HPV16 clearance associated with an E6-directed cell-mediated response, Int. J. Cancer 136 (2015) 2402–2408, https://doi.org/10.1002/ijc.29283.

[12] U.A. Hasan, E. Bates, F. Takeshita, et al., TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16, J. Immunol. 178 (2007) 3186–3197, https://doi.org/10.4049/jimmunol.178.5.3186.

[13] A. Chaturvedi, S.K. Pierce, How location governs toll-like receptor signaling, Traffic 10 (2009) 621–628, https://doi.org/10.1111/j.1600-0854.2009.00899.x.

[14] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (2006) 783–801, https://doi.org/10.1016/j.cell.2006.02.015.

[15] R. Karim, C. Meyers, C. Backendorf, Ludigs, et al., Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes, PLoS One 6 (2011) e17848. doi.org/https://doi.org/10.1371/journal.pone.0017848.

[16] Y.E. Chang, L.A. Laimins, Microarray analysis identifies interferon-inducible genes and Stat-1as major transcriptional targets of human papillomavirus type 31, J. Virol. 74 (2000) 4174–4182, https://doi.org/10.1128/jvi.74.9.4174-4182.2000.

[17] T. Kawai, S. Akira, Innate immune recognition of viral infection, Nat. Immunol. 7 (2006) 131–137, https://doi.org/10.1038/ni1303.

[18] J. de Wilde, J. De-Castro Arce, P.J. Snijders, et al., Alterations in AP-1 and AP-1 regulatory genes during HPV-induced carcinogenesis, Cell. Oncol. 30 (2008) 77–87, https://doi.org/10.1155/2008/279656.

[19] M. Karin, Nuclear factor-kappaB in cancer development and progression, Nature 441 (2006) 4316, https://doi.org/10.1038/nature04870.

[20] V.L. Grandage, R.E. Gale, D.C. Linch, et al., PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Mapkinase and p53 pathways, Leukemia 19 (2005) 586–594, https://doi.org/10.1038/sj.leu.2403653.

[21] K. Takeda, S. Akira, TLR signaling pathways, Semin. Immunol. 16 (2004) 3–10 (PMID: 14751757).

[22] A. Avan, R. Narayan, E. Giovannetti, et al., Role of Akt signaling in resistance to DNA targeted therapy, World J. Clin. Oncol. 7 (2016) 352, https://doi.org/10.5306/wjco.v7.i5.352.

[23] J.K. Schwarz, J.E. Payton, R. Rashmi, et al., Pathway-specific analysis of gene expression data identifies the PI3K/Akt pathway as a novel therapeutic target in cervical cancer, Clin. Cancer Res. 18 (2012) 1464–1471, https://doi.org/10.1158/1078-0432.

[24] F. Paolini, A. Carbone, M. Benevolo, et al., Human papillomaviruses, p16INK4a and Akt expression in basal cell carcinoma, J. Exp. Clin. Cancer Res. 30 (2011) 108, https://doi.org/10.1186/1756-9966-30-108.

[25] B.A. Werness, A.J. Levine, P.M. Howley, Association of human papillomavirus types 16 and 18 E6 proteins with p53, Science 248 (1990) 76–79, https://doi.org/10.1126/science.2157286.

[26] J. Momand, G.P. Zambetti, D.C. Olson, et al., The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation, Cell 69 (1992) 1237–245. https://doi.org/10.1016/0092-8674(92)90644-R.

[27] S. Liao, S. Xiao, G. Zhu, CD38 is highly expressed and affects the PI3K/Akt signaling pathway in cervical cancer, Oncol. Rep. 32 (2014) 2703–2709, https://doi.org/10.3892/or.2014.3537.

[28] I. Lesnikova, M. Lidang, S. Hamilton-Dutoit, et al., p16 as a diagnostic marker of cervical neoplasia: a tissue microarray study of 796 archival specimens, Diagn. Pathol. 4 (2009) 22, https://doi.org/10.1186/1746-1596-4-22.

[29] A. Pauck, B. Lener, M. Hoell, et al., Depletion of the cdk inhibitor p16INK4a differentially affects proliferation of established cervical carcinoma cells, J. Virol. 88 (2014) 5256–5262, https://doi.org/10.1128/JVI.03817-13.

[30] E. Boccardo, A.P. Lepique, L.L. Villa, The role of inflammation in HPV carcinogenesis, Carcinogenesis 31 (2010) 1905–1912, https://doi.org/10.1093/carcin/bgq176.

[31] M.Y. Tjiong, N. van der Vange, J.S. ter Schegget, et al., Cytokines in cervicovaginal washing fluid from patients with cervical neoplasia, Cytokine 14 (2001) 357–360, https://doi.org/10.1006/cyto.2001.0909.

[32] J. Bartkova, Z. Horejsí, K. Koed, et al., DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis, Nature 434 (2005) 864–870, https://doi.org/10.1038/nature03482.

[33] J.Y. Ahn, J.K. Schwarz, H. Piwnica-Worms, et al., Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation, Cancer Res. 60 (2000) 5934–5936 (PMID:11085506).

[34] K.H. Kim, M.S. Jo, D.S. Suh, et al., Expression and significance of the TLR4/MyD88 signaling pathway in ovarian epithelial cancers, World J. Surg. Oncol. 10 (2012) 193, https://doi.org/10.1186/1477-7819-10-193.

[35] F.J. Ma, Z.B. Liu, X. Hu, et al., Prognostic value of myeloid differentiation primary response 88 and Toll-like receptor 4 in breast cancer patients, PLoS One 9 (2014), 111639. https://doi.org/10.1371/journal.pone.0111639.