Medical Science

AIMS Medical Science, 8(1): 11–22.
DOI: 10.3934/medsci.2021002
Received: 12 September 2020
Accepted: 14 December 2020
Published: 11 January 2021

http://www.aimspress.com/journal/medicalScience

*Research article*

# Expressional correlation of Toll-like Receptor 9 (TLR9) with angiogenic factors and anti-apoptotic markers in cervical cancer cells

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**Abstract:** Cervical cancer is one of the most frequently occurring oncological malignancies which in turn is attributed with the chronic persistence of human papillomavirus (HPV). Recent explorations into cancer immunobiology have elucidated that innate immune response are important for proliferation of cancerous cells. Toll-like receptors (TLRs), belonging to the pattern recognition receptors (PRRs) family of innate immune receptors have been reported for their expression in various cancers, where they aid cancer cell proliferation along with tumor development. The present study focuses on investigating TLR9 mRNA within HPV18$^{+}$ HeLa (cervical cancer cells) and its correlation with angiogenic factors and anti-apoptotic markers. The expression of TLR9 mRNA was substantially higher in HeLa cancer cells in comparison with normal kidney cells. MTT assay demonstrated that treatment with CpG ODN significantly increased the proliferation in dose-dependent manner. Augmented levels of cell percentage within G2/M phase was also recorded post CpG ODN treatment within HeLa cells. qRT-PCR analysis also elucidated elevated levels of Bcl-2, Bcl-$X_L$, COX-2, VEGF, and TLR9 within HeLa cells. Thus, to conclude, our study established that HeLa cells have increased TLR9 mRNA expression concomitant with elevated COX-2, Bcl-2, Bcl-$X_L$ and VEGF. These results indicate toward potential applicability of targeting TLR9 as a therapeutical intervention for the treatment and management of this pernicious malignancy.

**Keywords:** Toll-like receptors; apoptosis; cervical cancer; human papillinoma virus

## 1. Introduction

Globally, Cervical Cancer (CC) represents a dominant reason for cancer-related deaths in women with a speculated incidence rate of 528,000 [1,2]. Chronic infection with subtypes of High risk human papillomavirus (HPV) predominantly HPV16 & HPV18 are associated with approximately 99% of CC cases and thus remains to be a critical risk factor modulating the advancement of invasive cervical carcinomas [3,4]. Incidences and mortality rates of CC are reportedly higher in Central and Eastern Europe, sub-Saharan Africa, Latin America, Southeast Asia, and the Caribbean regions [5]. The major factor associated with such delayed diagnosis is the inappropriate management of screening programs, which itself is accountable for more than 1/5th of global CC burden. Consequently, CC is regarded as the second most customary cancer among women having an average age group of 15–44 years [6]. The primary interventional approach against early stages of CC involves the use of radiation treatment, cytotoxic chemotherapy and surgery [5,7]. However, effective therapeutical modalities for treatment of patients at advanced CC stages are still limited which in turn necessitates the development of novel and effective therapeutical regimes against this dreaded oncological manifestation among women [8].

The innate immune system is the primary line of defense against the potential extrinsic and/or intrinsic antigens. This functional attribute of innate immune components in terms of recognizing foreign or intrinsic stress signals relies upon the existence of pathogen recognition receptors (PRRs) that mediates the identification of pathogen and/or danger associated molecular patterns (P-and/or D-AMPs). Among various other PRRs, the family of Toll-like receptors (TLRs) are structurally characterized as single trans-membranous receptors that mediate the crucial role of not only recognizing extrinsic and/or intrinsic P-and/or D-AMPs but are also central in bridging the innate arm of immunity with its adaptive counterpart [9,10].

TLRs are established modulators of the innate system which also play crucial role in subsequent molding of the adaptive immune response. These receptors are functionally present on a plethora of immune cells such as dendritic cells, macrophages or natural killer (NK) cells which perceives the pathogenic intruders and results in activation of various innate and adaptive immune mechanisms which eventually results in microbicidal and anti-cancerous effects [11]. TLR9 is a member of intracellular TLR family involved in recognition of non-methylated cytosine-phosphateguanosine sequences (CG repeats) found commonly within microbial genome. Synthesized homologs of microbial cytosine-guanosine repeats are short single-strand of synthetic DNA referred as CpG DNA or unmethylated cytosine-phosphate-guanine (CpG) dinucleotides and are responsible to synthetically mimicking microbial CG DNA to activate TLRs mediated downstream signaling [12]. Among these synthetic TLR agonists, certain CpG ODNs have elucidated for their competent anti-cancerous activity in respective disease models [13].

Advancements in TLRs mediated research has evidently established that being an integral innate immune mediator these are also associated with chronic hepatological [14], neurological disorders [15] as well as with infectious diseases such as Leishmaniasis [16]. The involvement of TLRs in various disorders may be due to the fact that its expression is not only restricted to immune cells but is also expressed on several other cell types including melanocytes, keratinocytes as well as cancer cells. along with tumorigenesis through promoting chronic inflammation within the tumor environment [17]. Recent studies have established that TLR9 is an active mediator involved in advancement of cervical carcinogenesis and thereby further substantiating its potential of being an

important indicator for malignant transformation of cells with squamous origin [18]. During homeostasis, apoptosis plays a central role and represents a well-organized process governed by numerous pro- and anti-apoptotic proteins, specifically belonging to the Bcl-2 protein family. These proteins are extensively elaborated for their abnormal expression in multiple carcinomas and have also been suggested for their plausible role in resistance to chemotherapy and radiation [19]. Bcl-2 family proteins are the molecular key factors of apoptosis and their aberrant expression is pivotal for the development of oncological manifestations [20]. Thus, Bcl-2 proteins are considered to be the propitious therapeutic targets in the development of effective cancer therapies targeted towards the prevention and treatment of numerous carcinomas. In cancer biology, angiogenesis is regarded as an extremely important biological event responsible for primary cancer growth and metastasis. Vascular endothelial growth factor (VEGF) is a significant biomarker responsible for tumor angiogenesis. It has been substantiated that the over expression of VEGF is associated with various carcinomas including CC [21]. Furthermore, Cyclooxygenase-2 (COX-2) is also involved in tumorigenesis and cancer progression. Increased expression of COX-2 has been elucidated in epithelial cancer cells such as colon, gastric, lung, hepatic, prostate, breast and CCs [22]. Thus the aim of the prsent study was to ascertain expressional levels of TLR9 within HeLa cells and subsequently explore the interrelation between expression of TLR9 and apoptotic indicators, namely BCL-$X_L$ and Bcl-2 along with well angiogenic markers (VEGF & COX-2) in human CC cells.

## 2. Material and methods

### *2.1. Chemicals*

DMEM high glucose, MEM (E), fetal bovine serum (FBS), HiPurATM Total RNA Miniprep Purification Kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals used during the study were purchased from Himedia India, Ltd., Mumbai, India. CpG ODN 2216 with phosphorothioate (*) stabilized backbone was acquired from Integrated DNA Technologies (USA). Propidium iodide (PI) solution and Verso cDNA synthesis kit, and DyNAmoColorFlash SYBR Green qPCR Kit was purchased from Sigma (St. Louis, MO, USA) and ThermoScientific, USA, respectively.

### *2.2. Cell culture and conditions*

Human kidney and cervical cell lines, namely HeLa and HEK293 were acquired from National Center for Cell Science (NCCS), Pune, India. These cells were maintained in DMEM-high glucose, and MEM (E) supplied additionally with 10% FBS and 1X 1% antibiotic-antimycotic solution. The cells were incubated in humidified incubator supplied with 5% $CO_2$ at 37 °C. Cells were passaged through trypsinization on attaining >90% confluency roughly twice a week. Prior to experiments, cells were seeded after counting on hemocytometer where trypan blue was used to differentiate the dead cells. Cells without any treatment of CpG-ODN served as control group. For flow cytometric estimations, a minimum of 10,000 events were aanalyzed during each individual experiment repeated thrice. The flow cytometer used for evaluation was FACS Calibur (BD, San Jose, CA).

*2.3. MTT assay*

The effect of CpG-ODN on HeLa cell line was assessed using MTT toxicity assay with slight modifications [23]. Briefly, $5 \times 10^3$ HeLa cells were seeded in each of a 96-well plate. The plate was then left undisturbed for 24 h at 37 °C for adherence. Thereafter, HeLa cells were treated with differential CG ODN dosages ranging from 0–2.0 µg/ml and incubated for additional 24 h in stated conditions. At the end of incubation, MTT dye (5 mg/ml; 10 µl) was added in each well, followed by 3 h incubation at 37 °C. The characteristic formazan crystal formed were subsequently solubilized using DMSO (100 µl/well) before recording the absorbance of wells at 570 nm on microplate reader (Bio-Rad, USA).

*2.4. Estimation of cell percentage in different phases of cell cycle*

CpG-ODN 2216 treated HeLa cells were subjected to cell cycle analysis, which employed DNA interpolating propidium iodide (PI) fluorochrome as used previously [24]. HeLa cells ($5 \times 10^5$ cells) were cultured in a 6 well plate for 24 hours at 37 °C and 5% $CO_2$ and treated with CpG-ODN (0–2.0 µg/ml) for the next 24 hours. Subsequently, HeLa cells were detached and washed by centrifugation with (2200 g: 5 min) and treated with 200 µg/ml of RNase A (30 min; 37 °C). Thereafter, 70% chilled methanol was employed to fix HeLa (overnight incubation; −20°C). Eventually, cells fixed HeLa cells were stained with 25 µg/ml of PI (1 h; 37 °C. Cell cycle analysis was performed using a flow cytometer.

*2.5. Quantitative Real-time PCR assay*

HeLa cells were seeded in a 6 well plate ($10^6$ cells/well) and left for overnight undisturbed in a humidified atmosphere (5% $CO_2$; 37 °C). Post-treatment with CpG ODN 2216 at earlier stated concentration or control cells were additionally incubated for another 24 h. Thereafter, HiPurATM Total RNA Miniprep Purification Kit (Himedia, India) was used for total RNA extraction from HeLa cells treated with or without TLR9 agonist as per manufacturer's protocol. Initially, 2 µg of total extracted RNA was used to synthesize the first cDNA strand through cDNA synthesis kit (Thermo Scientific, USA). Quantitative PCR was performed using qPCR Kit (DyNAmoColorFlash SYBR Green; Thermo Scientific, USA) as per the manufacturer's protocols and ABI-7500 real-time PCR machine (Applied Biosystems). List of primers used during the qRT-PCR study is given in Table 1. Expressional data obtained for target mRNA was normalized to that of comparator samples and subsequently expressed using $^{\Delta\Delta}C_T$ method {2 − [(Ct of gene of interest) − (Ct of internal control)]}.

*2.6. Statistical analysis*

Data reported herewith illustrates mean ± SEM of three individual sets of experiments performed in triplicates. These results were further analyzed through column analysis using one-way ANOVA followed by Dunnett's multiple comparison post-test and unpaired two-tailed student t-test through GraphPad Prism software (Version 5.0). The difference in p-value by <0.05 was considered significant between different groups. Significance: *$p < 0.05$ and **$p < 0.01$.

**Table 1.** List of primers used during the qRT-PCR study as described earlier [24–30].

| S. No | Target gene | Forward sequence | Reverse sequence |
|---|---|---|---|
| 1. | GAPDH | AGA AGG CTG GGG CTC ATT TA | AGG GGC CAT CCA CAG TCT TC |
| 2. | TLR9 | CAT GCC CTG CGC TTC CTA TTC | AGC TTG CGC AGC TGT GTT AGG |
| 3. | Bcl-2 | GAT TGT GGC CTT CTT TGAG | CAA ACT GAG CAG AGT CTT C |
| 4. | Bcl-xL | CAG AGC TTT GAA CAG GTA G | GCT CTC GGG TGC TGT ATT G |
| 5. | VEGF | AAC CAT GAA CTT TCT GCT CTC | GTG ATT TTC TGG CTT TGT TC |
| 6. | COX-2 | TAA ACT GCG CCT TTT CAA GG | GTG ATA CTT TCT GTA CTG CG |

## 3. Results

### *3.1. Comparison of TLR9 expression levels between HeLa cervical cancer cells and human embryonic kidney cells (HEK293)*

TLR9 mRNA expression levels within HPV18$^{+}$ HeLa cells and normal human embryonic kidney cells were analyzed using qRT PCR analysis escalated levels of TLR9 mRNA was observed as compared to the normal kidney cells. The fold change in the mRNA expression of TLR9 was found to be 4.23 ± 2.29 within HeLa cells as compared to that of HEK293 cells (Figure 1). Knowing the fact that HeLa cells have persistent of HPV18$^{+}$ genome, therefore the expression level of TLR9 mRNA was found to be substantially higher in these cells.

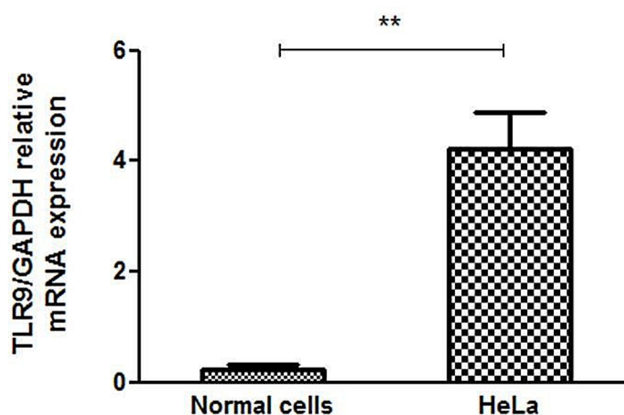


**Figure 1.** Comparison of expression level of TLR9 mRNA in normal HEK293 and HeLa cervical cancer cells. Fold change in the expression level of mRNA relative to that of control were analyzed by qRT-PCR. Fold change in expression of target genes were normalized to GAPDH mRNA used as internal control. The mRNA levels were examined by RT-PCR analysis. Data shown is mean ± S.E.M of three individual experiments performed thrice. Data analyzed by unpaired two-tailed student t test where ** indicate significant difference at $p < 0.01$ from the untreated control cells.

*3.2. TLR9 agonist-mediated HeLa cells proliferation*

To evaluate CpG ODN 2216 mediated proliferative effects on HeLa (HPV18$^+$) if any, MTT assay was performed with various concentrations of CpG (0–2.0µg/ml) for 24h. As shown in Figure 2A, dose-course effects of CpG ODN 2216 were observed in HeLa cells at 1.5 µM (0.6 ± 0.07) and 2 µM (0.81 ± 0.06) as absorbance value (570 nm) increases with an increase in the dose of CpG ODN (Figure 2A).

*3.3. Cell cycle distribution post TLR9 agonist treatment within HeLa cells*

Flow cytometry mediated cell cycle estimation to delineate the percentage of the cell population in different phases of cell cycle post TLR9 agonist treatment on HeLa cells was performed. Briefly, cells were analyzed through flow cytometric analysis after treatment with TLR9 agonist (0, 0.5, 1.0, 1.5 and 2.0 µg/ml) and incubated (24 h) followed subsequently by PI stain. As observed in Figure 2B, the distribution of cells within the control sample (without any treatment) was 58.3 ± 3.74% (G0/G1 phase) and 27.05 ± 1.36% (G2/M phase). Low doses of CpG ODN does not exhibit any marked significant difference in the proliferation of cells at G0/G1 and G2/M cell cycle phases. However, at higher doses of CpG ODN such as 1.5 µM (54.42 ± 1.06%) and 2 µM (62.42 ± 1.85%), substantial escalation within the population of HeLa cells at G2/M phase was recorded (Figure 2B). Thus, our results exhibited that CpG ODN treatment increases the % cells at G2/M phase, implicating an augmentation in the proliferation of cervical cancer cells.

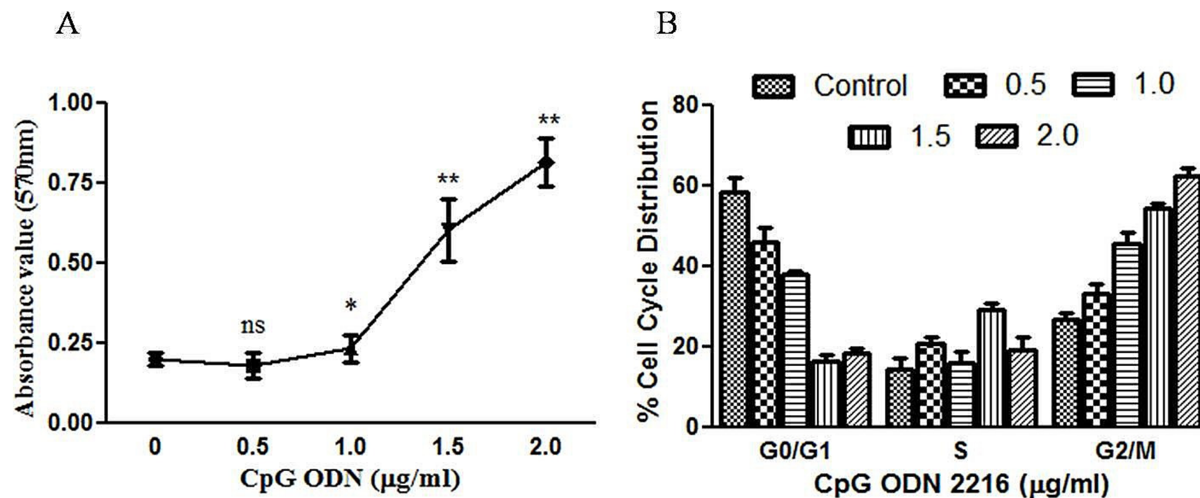


**Figure 2.** Effect of CpG-ODN treatment on the proliferation of HeLa cervical cancer cells. (A) CpG-ODN treatment for 24 h enhanced the proliferation of cervical cancer cells in a dose-dependent manner via MTT assay. (B) CpG-ODN treatment increases the population of HeLa cervical cancer cells at G2/M phase as determined by flow cytometry. Data shown is mean ± S.E.M of three individual experiments performed thrice. Data analyzed by one way ANOVA with Dunnett post test where ** indicate significant difference at $p < 0.01$ from the untreated control cells.

*3.4. TLR9 agonist increased the expression levels of angiogenic markers in HeLa cells*

Investigations into the role of angiogenic markers (VEGF & COX-2) in HeLa cells post CpG-ODN treatment qRT-PCR was used to analyze the expression levels of angiogenic markers in HeLa cells. As evident in Figure 3B, fold change within COX-2 mRNA expression were 1.06 ± 0.08, 1.56 ± 0.38, 2.81 ± 0.10, 4.34 ± 0.21, respectively at different CpG ODN 2216 doses, whereas fold change in the expression levels of VEGF mRNA were 0.98 ± 0.20, 1.74 ± 0.13, 3.12 ± 0.26, 4.76 ± 0.11, at the indicated doses of 0.5, 1.0, 1.5 and 2.0 µg/ml TLR9 agonist CpG ODN within HeLa cells (Figure 3A). Thus, the COX-2 and VEGF expressional levels were found to be substantially inflated, correlating positively with the concentration of TLR9 agonist.

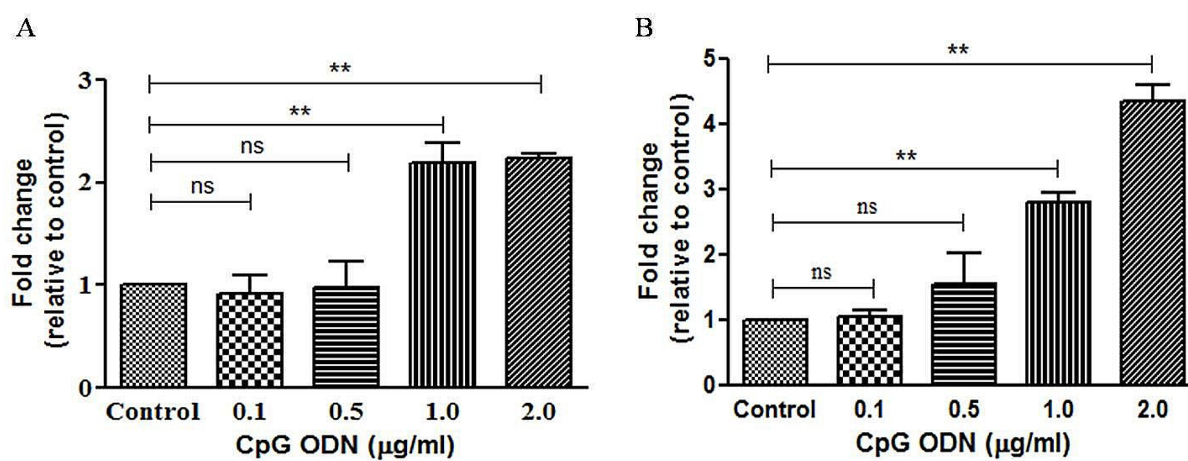


**Figure 3.** Expression of angiogenic markers in CpG ODN 2216 treated HeLa cervical cancer cells. (A) Fold change in the expression level of mRNA relative to control of (A) VEGF and (B) COX-2 in cervical cancer cells were analyzed by qRT-PCR. Fold change in expression of all target genes were normalized to GAPDH mRNA used as internal control. Data shown is mean ± S.E.M of three individual experiments performed thrice. Data analyzed by one way ANOVA with Dunnett post test where ** indicate significant difference at $p < 0.01$ from the untreated control cells.

*3.5. TLR9 agonist augmented apoptotic markers expressional levels in cervical cancer cells*

In the quest for delineating the role of apoptotic markers in HeLa cells after CpG-ODN treatment. We applied qRT-PCR to investigate the fold change within Bcl-2 family members (apoptotic factors) expressional levels in HeLa cells after treatment with TLR9 agonist CpG ODN 2216 for 24 hours. Fold change in the expression levels of Bcl-2 mRNA (Figure 4A) were 1.37 ± 0.21, 1.71 ± 0.31, 3.71 ± 0.20 and 4.67 ± 0.30, respectively, whereas fold change in the Bcl-$X_L$ mRNA expressional levels was 1.48 ± 0.21, 1.64 ± 0.30, 3.65 ± 0.17 and 4.31 ± 0.20, at the indicated doses of 0.5, 1.0, 1.5 and 2.0 µg/ml TLR9 agonist CpG ODN within HeLa cells (Figure 4B). Therefore, the expression level of Bcl-2 and Bcl-$X_L$ was significantly elevated thereby correlating positively with the concentration of TLR9 agonist.

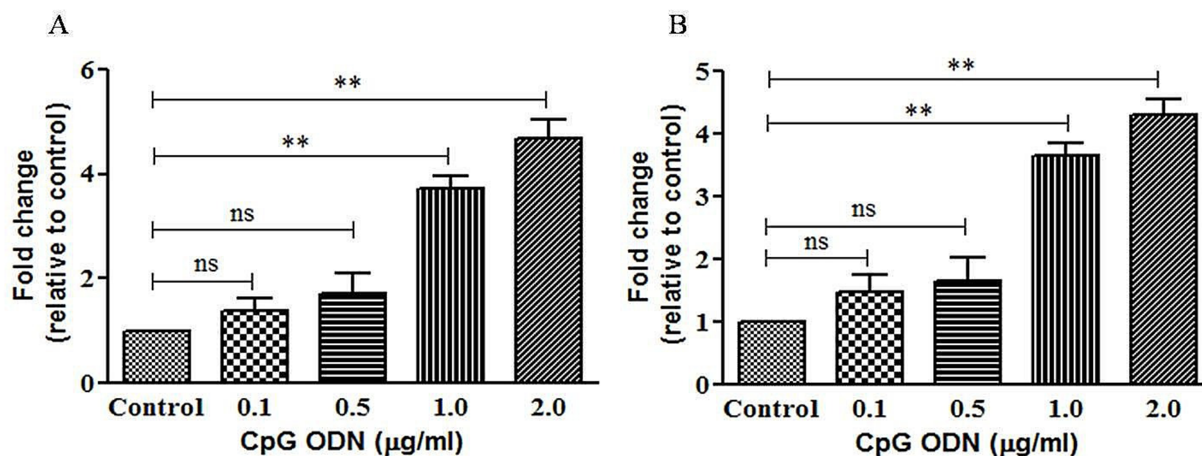


**Figure 4.** Expression of apoptotic markers in CpG-ODN treated HeLa cervical cancer cells. (A) Fold change in the expression level of mRNA relative to control of (A) Bcl-2 and (B) Bcl-$X_L$ in cervical cancer cells were analyzed by qRT-PCR. Fold change in expression of all target genes were normalized to GAPDH mRNA used as internal control. Data shown is mean ± S.E.M of three individual experiments performed thrice. Data analyzed by one way ANOVA with Dunnett post test where ** indicate significant difference at $p < 0.01$ from the untreated control cells.

## 4. Discussion

CC is regarded as one of the most frequently occurring gynecological cancers, specifically caused by the persistent infection with HPV [31]. Epidemiological evidence suggests that HPVs are the principal cause of invasive CCs. Exhaustive studies have demonstrated that high-risk HPVs are accountable for >95% of cervical carcinomas [32,33]. TLRs are regarded as critical players mediating the tumor progression through the induction of proinflammatory, anti-apoptotic, and proliferative environment, further aided by pro-fibrogenic gesticulations. Intriguingly, TLRs may even arbitrate anti-tumor signals via activating immune mediators such as dendritic cells or NK cells [34]. In recent times, signaling pathways downstream to TLR signaling pathway has appeared as propitious therapeutic intervention against various carcinomas and thus TLR agonists or stimulants are being explored.

Overexpression of TLR9 is reported in cervical lesions, where it associates itself with the histopathological grade along with the progression of CC [35]. Chronic HPV infection aided by improper clearance from within the infected tissues further augments expressional levels of TLRs and serves to be a befitting biomarker for distinguishing transformation associated with malignancies of squamous cells from the cervix [36]. Presently, the authors aimed to analyze the TLR9 expression levels of within HeLa cells followed by its subsequent effects on related anti-apoptotic markers (Bcl-2 & Bcl-$X_L$) and angiogenic factors (VEGF & COX-2) in CC. Our present investigation showed an increase in the expressional levels of TLR9 within HeLa cells, implicating that the immunity against the tumorogenic environment may be compromised due to over expression of TLRs. Furthermore, overexpression of TLR9 in HeLa cells was in accordance with the augmented level of related angiogenic factors anti-apoptotic markers.

Members of Bcl-2 family are expressed by all cell types in mammals even though they are not undergoing apoptosis [20]. Cancer cells over expresses Bcl-2 anti-apoptotic proteins, which consequently evade apoptosis and initiates carciogenesis via inhibiting the apoptotic cell death [30]. Earlier studies have demonstrated that COX-2 expression is augmented during various cancers, namely head and neck, lung, breast, and gastrointestinal malignancies, implicating that COX-2 may play a positive role in tumor progression oncological progression via regulation of related pathways such as proliferation, cell adhesion, apoptosis and or angiogenesis. VEGF is regarded as an important angiogenic factor playing pivotal role in different oncological malignancies. Furthermore, pre-clinical investigations have also exhibited that augmented COX-2 facilitates the expression of other related angiogenic factors including bFGF and VEGF within cancerous cells [37,38].

During our qRT-PCR analysis, enhanced TLR9 mRNA expression level was observed within HPV18$^{+}$ HeLa cells as compared with the normal HEK293 cells, where the same was found to be very low. The level of TLR9 mRNA was substantially higher due to the persistent infection of HPV18$^{+}$ genome within HeLa CC cells. Furthermore, we evaluated CpG ODN 2216 mediated effect on the proliferation of HeLa cells to determine its dose-course effects on the same. Moreover, data reported also exhibited that CpG-ODN treatment increases the % cells at G2/M phase, implicating an increased proliferation of CC cells, thereby indicating that activation of TLR9 stimulates the proliferation of HeLa cells.

We perform qRT-PCR analysis to investigate the relationship between expression levels of TLR9 and angiogenic factors (VEGF & COX-2) in HeLa cells. Our findings exhibited that the fold change in expression of VEGF and COX-2 were substantially elevated, positively related with CpG ODN concentration in a dose-dependent manner within HeLa cells. Similarly, we applied qRT-PCR analysis to explore the relationship between expression level of TLR9 and members of Bcl-2 (apoptotic factors) in HeLa cells after treatment with TLR9 agonist CpG-ODN 2216. Results from the study suggested that the expression of Bcl-$X_L$ and Bcl-2 were significantly increased, positively related with CpG ODN concentration. We hypothesized a correlation between expression of TLR9 and anti-apoptotic markers, namely Bcl-2 and Bcl-$X_L$ along with angiogenic markers (VEGF & COX-2) in HeLa CC cells. Earlier reports demonstrated that VEGF and Bcl-2 are imperatively associated in angiogenesis, a crucial attribute supporting the development of tumor. Recently, anti-apoptotic protein, namely Bcl-2 has was to be of prognostic value within classical Hodgkin lymphoma [39]. VEGF is an established regulator of tumor angiogenesis and thus has become a critical target for exploring novel anti-tumor interventions; however, its clinical potency remains to be explored [40]. Thus, targeting Bcl-2 and VEGF could be a propitious therapeutic approach in the treatment of cervical carcinoma. Human CC HeLa cells, increased TLR9, Bcl-2, and VEGF expression levels and thus could serve as a potential model to further explore mechanistic insights about TLR9 in cervical cancer. Moreover, escalated mRNA levels of VEGF, BCL-2 and COX-2 was evident within HeLa cells treated with CpG-ODN 2216, indicating the onset of angiogenesis followed by prevention of apoptosis, which may further strengthen the development of tumor.

## 5. Conclusion

Thus, to conclude, our study provides compelling testimony, suggesting that the TLR9 is over expressed within HeLa cervical cancer cells, which was consistently related with the enhanced levels of VEGF, COX-2, Bcl-$X_L$, and Bcl-2, correlating with modest prospects in various human

carcinomas. The present report exhibits that the elevated TLR9 expression within cervical cancer cells may decisively relate with tumor invasion and carcinogenesis via inhibition of TLR9 expressing immune cells in recognizing tumor/viral antigen, which consequently manipulate the immune response against tumors. Subsequent studies in relation to its role and mechanism would provide crucial evidence for improved understanding of cervical cancer pathogenesis as well as beneficial for the development of novel therapeutics. Furthermore, it would be of substantial importance to explore whether TLR9 antagonists can affect the proliferation of cervical cancer cells and in vivo tumor growth. In a nutshell, better understanding of the functional attributes of TLRs in cancer may provide the desired impetus for elaborating novel immunotherapies against cancer.

## Conflicts of interest

All authors declare that there is no conflict of interest.

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