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Original Research Article

Impact of TLR9 gene polymorphism on cervical HPV infection and its progression to cervical cancer

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ABSTRACT

Introduction: Cervical carcinogenesis is a multi-step process associated with refractory infection by high-risk human papillomavirus (HPV) types. Only a minority of HPV infected women develop cervical intraepithelial neoplasia (CIN) or cervical cancer, indicating that HPV infection is not the sole risk factor to induce cervical cancer.. Single nucleotide polymorphisms are the most common form of genetic variants in human genome, some of which can influence the susceptibility to human diseases including cancer. TLR9 gene polymorphisms appear to have considerable role in disease susceptibility, including HPV induced cervical cancer.

Aim: The present study aims to identify the role of TLR9 C2848T (rs352140) gene polymorphism in cervical cancer susceptibility in East Indian women.

Materials and Methods: Our study was a case – control study. Study subjects comprised 33 women with histologically proven cervical cancer, and 24 women with benign cervical lesions. The case group included HPV 16 +ve subjects with malignancy. Among the benign group, 15 were HPV16 +ve (intermediate group) and 9 were HPV –ve (control group).. HPV status and HPV type was confirmed by PCR based method, using specific primers. TLR9 genotyping was performed using PCR RFLP. TLR9 expression was analyzed across all categories of samples using real time PCR.

Results: Our study showed increased expression of TLR9 in malignant group, compared to the control group. TLR9 expression was also increased in the HPV +ve nonmalignant group, compared to HPV –ve controls. Correlating TT, CT, CC genotype with TLR9 expression analysis across malignant group and control group, there was increased expression of TLR9 among TT genotype and CT genotype compared to CC genotype.

Conclusion: Our study showed that TLR9 C2248T polymorphism causes upregulation of TLR9 expression among cervical cancer patients. Our studies suggest that the TLR9 C2848T (rs352140) polymorphism may be a risk factor of cervical cancer in East Indian women.

1. Introduction

Cervical cancer is the fourth most frequent cancer in women worldwide with 570,000 new cases in 2018 representing 6.6% of all female cancers. Approximately 90% of deaths from cervical cancer occurred in low- and middle-income countries. In India, about 60,078 cervical cancer deaths occur annually (estimates for 2018), responsible for 7.5% of all female mortality from cancer. Cervical cancer ranks as the second leading cause of female cancer deaths in India in the 15 to 44 years age group.[1]

Cervical carcinogenesis is a multi-step process associated with refractory infection by high-risk human papillomavirus (HPV) types.[2,3] This includes the transformation of normal cervical epithelium to cervical intraepithelial neoplasia (CIN), which progresses to invasive cervical carcinoma of the 200 HPV types known till date, fifteen (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82)

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considered as high-risk types are associated with cervical cancer and precancerous lesions.[4,5]

Almost every sexually active individual will acquire at least one high-risk HPV infection during their lifetime. Fortunately, the majority of HPV infections are eradicated by the host immune system within 1-2 years of acquisition, and only <1% of infected people develop HPV mediated cancers.

Persistent infection with an oncogenic HPV type (especially HPV-16 and HPV-18) is the most important risk factor for progression to high-grade dysplasia and invasive cancer.[6] Studies using quantitative type-specific PCR for high-risk HPV and low-risk HPV have shown that HPV-16 can attain very high viral loads compared to the other types.[7] The integration of the high-risk HPV (hrHPV) DNA results in the constitutive expression of its oncogenes E6 and E7. HPV E6 oncoprotein binds to the tumor suppressor protein p53 and directs its ubiquitin-mediated proteolytic degradation whereas E7 binds to and inactivates another cellular tumor suppressor protein Rb, thereby interfering with the cell cycle control, consequently oncogenic growth.[8]

Protective immunity results from the interplay of nonspecific innate immunity and antigen-specific adaptive immunity. The innate immune system senses " danger " *via* signals from molecules that would normally not be found in the human body, such as damaged tissue, repetitive surface structures of bacterial cell walls, or DNA sequences containing typical viral sequence motives. These structures are recognized by pattern recognition receptors, such as e.g. Toll-like receptors (TLRs). Sentinel cells, such as dendritic cells (DCs) or Langerhans cells (LCs) in the skin and mucosa, continuously screen the environment and – if triggered - coordinate innate immune effectors and the initiation of an adaptive immune response.

HPV has evolved to evade human immune detection in multiple ways to establish an infection and maintain a persistent life cycle that leads to viral reproduction. This persistence is the greatest risk factor for the development of HPV-mediated invasive malignancies. The most important HPV immune evasion mechanism is to become invisible to the host immune system by not triggering any danger signals, such as cytolysis, cytopathic cell death, or inflammation. Suppression of the interferon response, resistance to immune-mediated apoptosis, and down-regulation of adhesion molecules for APCs, and active MHC class I down-regulation and impaired antigen presentation also play a critical role in HPV immune evasion.

Toll-like receptors (TLRs), a family of evolutionarily conserved pathogen recognition receptors, are emerging as key players in the pathophysiology of a host of human diseases, including cancer. *TLR9* recognizes unmethylated CpG motifs present in bacteria and viruses. Human B cells can be activated by stimulation of TLR9 and results in innate immune responses in preclinical tumor models and in patients.[9,10]

Single nucleotide polymorphisms are the most common form of genetic variants in human genome, some of which can influence the susceptibility to human diseases including cancer. *TLR* 9 gene polymorphisms appear to have considerable role in disease susceptibility, including cancers.. The present study aims to identify the role of *TLR* 9 C2848T (rs352140) gene polymorphism in cervical cancer susceptibility in East Indian women.

2. Aims

To understand the role of innate immune response on the development of HPV related cervical cancers.

3. Objectives

1. To estimate the prevalence of HPV16/18 infections in various categories of cervical samples (grouped according to histopathology, presence/absence of HPV infection).
2. To evaluate whether TLR9 expression is deregulated among the Cancer Cervix cases compared to non malignant controls.
3. To determine whether the polymorphism such as rs352140 (C/T), within the TLR9 promoter, is associated with the risk of cervical cancer and to identify whether the significantly overrepresented genotype among cervical cancer compared to controls, correlate with TLR9 deregulation as well.

4. Materials and Methods

4.1. Recruitment of subjects

This is a case - control study, conducted in the period from October 2016 to April 2017. The study was conducted, on East Indian women of West Bengal. Study sample included 57 women; case group comprised 33 wom en, control group comprised 24 women, of which 15 were found to have HPV infection and 9 were HPV – ve. Women with history of chronic or recurrent pruritus vulvae and leucorrhoea, persistent abnormal vaginal bleeding (like post coital, post menopausal bleeding or menorrhagia) and persistent cervical lesions (cervical hypertrophy, erosion, ulceration, cervical growth noted after speculum examination of cervix) were included in the study. Women with history of recent childbirth, miscarriage/abortions (within previous 4 months), menstruation at the time of visit, prior treatment for cervical malignancy, pregnant & unmarried women were excluded from the study. A questionnaire was used to collect information from patients on clinical history, demographic data, life style and reproductive factors. Intervention was per speculum

cervical examination, with cervical smear or cervical punch biopsy. Clinical examination findings and histopathological report of cervical biopsy samples, (noted from hospital records) were recorded in the questionnaire form.

4.2. Ethical consent

All samples were collected from the study participants with informed consent approved by the Institutional Ethical Committee.

4.3. Sample collection

Ecto-cervical and endo-cervical tissue samples were collected from subjects for cytopathological examination.

4.4. Detection of HPV positivity

DNA was isolated from all cervical tissue samples using the QIAamp DNA mini kit according to the manufacturer's protocol. All samples were screened for the presence of HPV infection by PCR, using L1 consensus primers: MYO11 and MYO9. L1 negative samples were reamplified with nested GP 5/6 primers for further HPV screening. The amplified PCR products were subject to electrophoresis on a 2% agarose gel and amplified bands (150bp) were visualized under UV light after staining with ethidium bromide. The primer sequences are shown in Table 1. The samples that were negative for both the primers were considered to be HPV -ve. The samples that were positive for either primer were considered to be HPV +ve.

4.5. Detection of HPV type 16 and 18

HPV +ve samples were typed by specific primers homologous to the E6 region of HPV-16 and 18. HPV 18 +ve were few, so the study was concentrated on HPV 16 +ve and HPV 16 –ve samples. Samples which were histopathologically confirmed squamous cell carcinoma were classified as the case group, non-malignant HPV-ve samples were classified as the control group and non-malignant HPV 16 +ve samples were classified as the intermediate group. Detection of genetic polymorphism of *TLR9* gene rs352140 (C/T)

PCR amplification of DNA samples followed by RFLP (restriction fragment length polymorphisms).

4.6. PCR amplification of DNA samples

PCR amplification of DNA samples was done using 100ng of DNA. TLR9 gene (promoter region) was amplified. (Figure 1)

4.7. Performing RFLP (restriction fragment length polymorphisms)

The PCR fragments were digested by endonuclease BST UI (CG/CG) New England BioLabs, (Ipswich, USA). TLR9 C allele cleaved into 227 & 133bp fragments. TLR9 T allele remained uncut. DNA fragments were separated by gel electrophoresis on 2% agarose gel, visualized by ETBr staining. If one band was visualized, no allele was cut, the homozypous TT genotype was inferred; if two bands were seen, then homozygous CC genotype was inferred; if three bands were seen, then heterozygote CT genotype was inferred. (Figure 2)

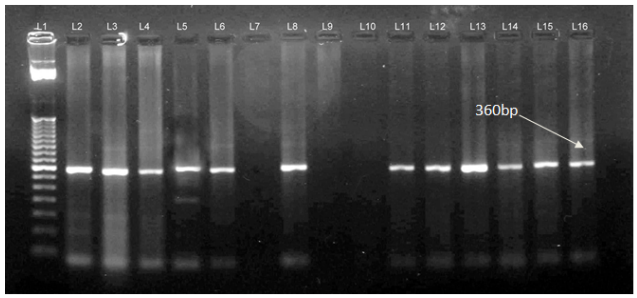


Fig. 1: Gel doc image showing PCR amplification of DNA samples for TLR9 gene
Lane1- 50bp ladder, lane 7- negative control. Lane 2-6 and Lane 8 – 16 – test samples

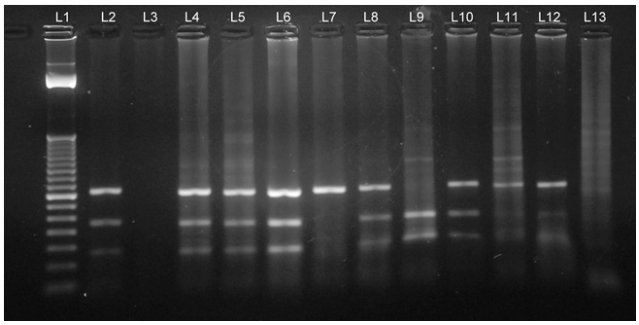


Fig. 2: Gel doc images of RFLP showing different genotypes of TLR9 C2248T polymorphism

Lane 1 – 50bp ladder, Lane 7- single band- TT genotype, Lane 9 - double band - CC genotype, lanes 2,4, 5, 6, 8, 10, 11, 12 – triple band – CT genotype

4.8. Detection of TLR9 gene expressions by real time PCR based detection

1. Tissue homogenization
2. RNA isolation from tissue samples
3. Quantitation of RNA samples and
4. cDNA preparation

Table 1: List of primers used with amplicon length

Target Name	Primer sequence	Product length
L1	Forward primer : 5' GCM CAG GGW CAT AAT AAY CC-3' Reverse primer: 5'- CGT CCM ARR GGA WAC TGA TC-3'	454bps
GP5/6	Forward primer: 5'TTG GTT ACT GTG GTA GAT ACT AC-3' Reverse primer 5' GGA AAA TAA ACT GAT AAT CAT ATT C3'	150bps
HPV16	Forward primer 5'TCA AAA GCC ACT GTG TCC TG 3' Reverse primer 5' CGT GTT CTT GAT GAT CTG CA 3'	116bps
Hpv18	Forward primer 5' ACC TTA ATG AAA AAC CAC GA 3' Reverse primer 5' CGT CGT TGG AGT CGT TCC TG 3'	100bps
TLR9	Forward Primer: 5' GCA GCA CCT CAA CTT CAC C 3' Reverse Primer: 5' GGC TGT GGA TGT TGT TGT GG 3'.	360bp

5. Estimation of TLR9 gene expression

4.9. Homogentisate preparation

The sample tissues was minced using sterile mortar and pestle. RLT buffer was added to the 1.5ml tubes and minced tissue was stored in RLT (lysis buffer) mix for overnight storage at -80ºc.

4.10. RNA isolation from tissue samples

RNA isolation was done using the RNeasy Mini kit, following the instructions of the manufacturer.

4.11. Quantitation of RNA samples

RNA was estimated by the Qubit RNA Broad Range (BR) Assay Kit.

4.12. CDNA preparation

cDNA was prepared using random hexamer primers, as well as oligo –dT primers and reverse transcriptase enzyme (Fermentas). All cDNA samples were stored at -80°C for long term usage in small aliquots.

4.13. Estimation of TLR9 gene expression – by RT PCR (real time PCR)

2 samples of 0.5μl cDNA were taken in 2 PCR tubes, of which 1 was diluted 5 times with milliQ water. Syber green mix was prepared according to manufacturer's instruction, using the following primers FP: 5' TGG GAA GGG ACC TCG AG

RP: 5 ' CAG GGT AGG AAG GCA GGC A3'

A 96 well plate was used – each well was loaded with cDNA 0.5μL (in replicate - undiluted and 5X diluted) and SYBR Green (Thermo Fisher Scientific) mix (4.5μl), which comprised syber green (2X) - 2.5 μl, 100ng/ml of primers, each of volume 0.05μl. Each well was carefully marked by sample number. After loading, the plate was sealed with paraffin film. The plate was centrifuged at 2000 rpm (pulse centrifuge), then loaded in thermocycler, setting the programme of TLR9 real time PCR in 3 stages- Stage I (denaturation) at 95°C for 5 min., Stage II of 40 cycles with denaturation at 95°C (30sec), annealing at 60°C (30sec) and elongation at 72°C (60sec) and a Stage III of dissociation - 95°C (15sec.) followed by 60°C (15sec) followed by 95° C (15sec).

(Figures 3 and 5) The (melting temperature) Tm of *TLR9* gene = 86°C, Tm of *GADPH* gene = 79.7°C.

4.14. Statistical analysis

Categorical variables were expressed as Number of patients and percentage of patients and compared across the groups using Pearson's Chi Square test for Independence of Attributes. The software used was SPSS version 20. Kolmogorov Smirnov Test (K-S TEST), was performed to determine whether the continuous variables in each sample categories followed normal distribution. The continuous variables did not follow normal distribution. Thus, Mann-Whitney U Test (SPSS version 20) was used to compare the median values between two different groups. An alpha level of 5% has been taken, i.e. if any p value is less than 0.05 it has been considered as significant.

5. Results

Samples belonging to case group (33), control group (9) and intermediates (15) were analysed. The TLR9 mRNA expression level was quantified by syber green assay using real time PCR. The real time PCR data, corresponding to TLR9 expression and GAPDH expression was represented as C_T values, where C_T defines the threshold cycle of PCR at which the amplified product is first detected. The delta C_T value of TLR9 mRNA expression was obtained from the difference in C_T value of TLR9 mRNA expression and C_T value of GAPDH (housekeeping gene).

All the cDNA samples showed the presence of GAPDH housekeeping gene (quality control). The mean C_T value of GAPDH did not differ significantly between case, (mean= 18.33+/- 1.78) intermediate (mean = 19.99 +/- 1.78) and control samples (mean = 19.57 +/- 0.03), suggesting a similar amount of input DNA for all 3 categories.

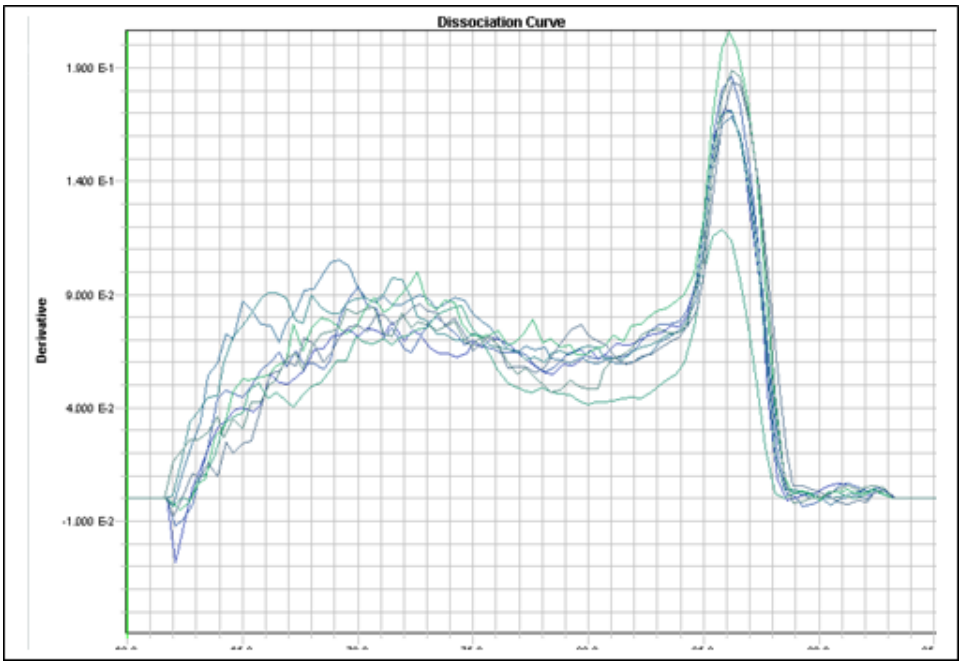


Fig. 3: TLR 9 mRNA expression dissociation curve

The above graph shows at 86°C, there is maximum dissociation of TLR9mRNA. Thus melting temp(Tm) is 86°C

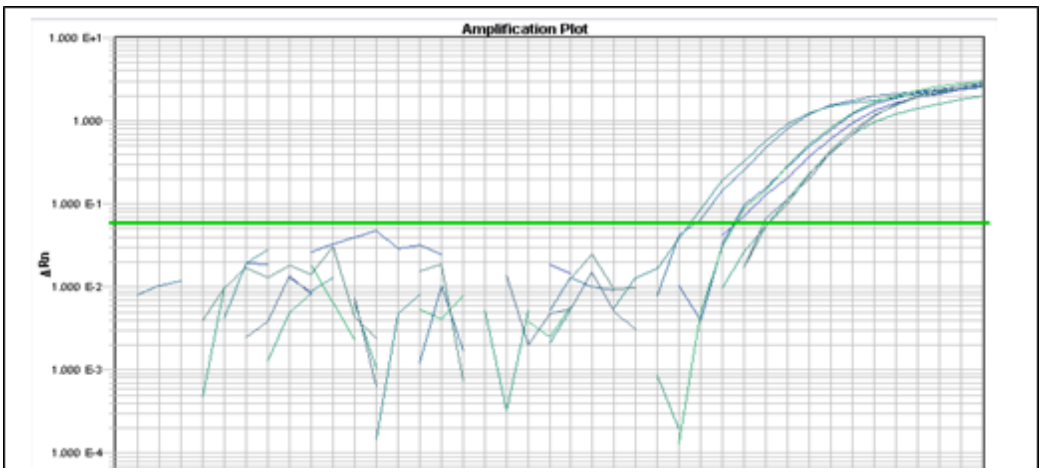


Fig. 4: TLR 9 mRNA amplification curve

Above graph shows amplification of TLR9 mRNA with corresponding C_T values (26.5.28.5 30)

The delta C_T values of TLR9 mRNA expression were compared between cases (n= 33) and controls (n= 15) but not found to be significantly different (p value 0.134). Similar analysis was carried out between case and intermediate samples, again no significant difference in their delta C_T value of TLR9 mRNA expression was noted ((p value 0.857). Analysis between intermediate and control values of delta C_T TLR9 mRNA showed no significant difference (p value 0.3734). The median values of delta C_T values of TLR9 mRNA expression are shown in Graph 1.

As no significant difference in ΔCT values were found in TLR9 expression across categories, fold changes were used. To express the fold change in terms of $2^{-\Delta\Delta CT}$, the median delta C_T values of TLR9 mRNA expression of case, intermediate and control were used. Considering control value as calliberator, the TLR 9 expression of cases were 4.0062 folds that in the control group. Similarly TLR 9 expression of intermediates was 3.1156 folds that in the

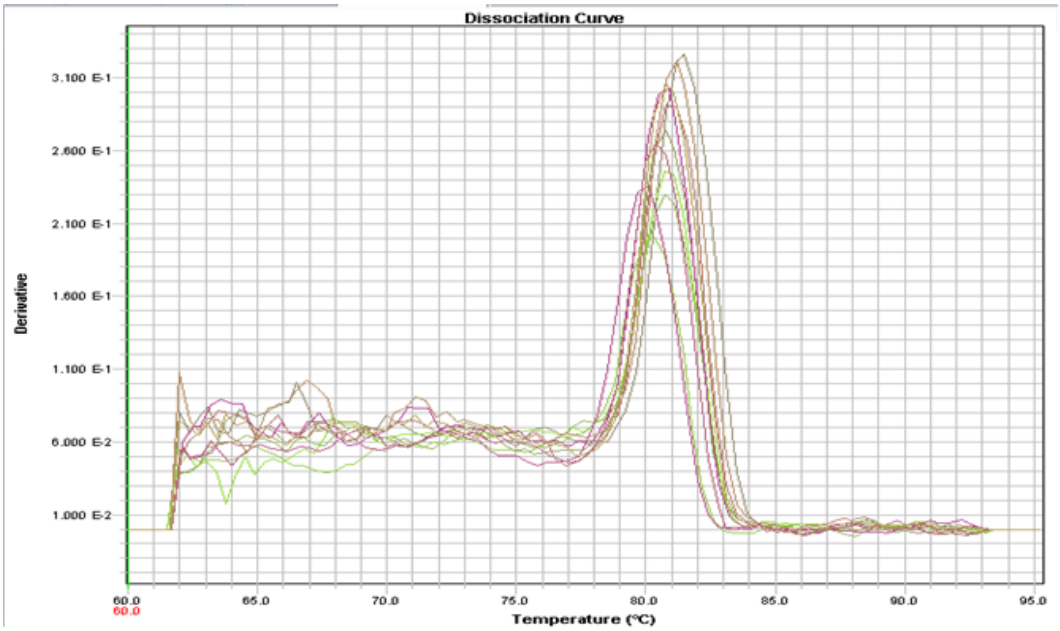


Fig. 5: GAPDH mRNA expression dissociation curve

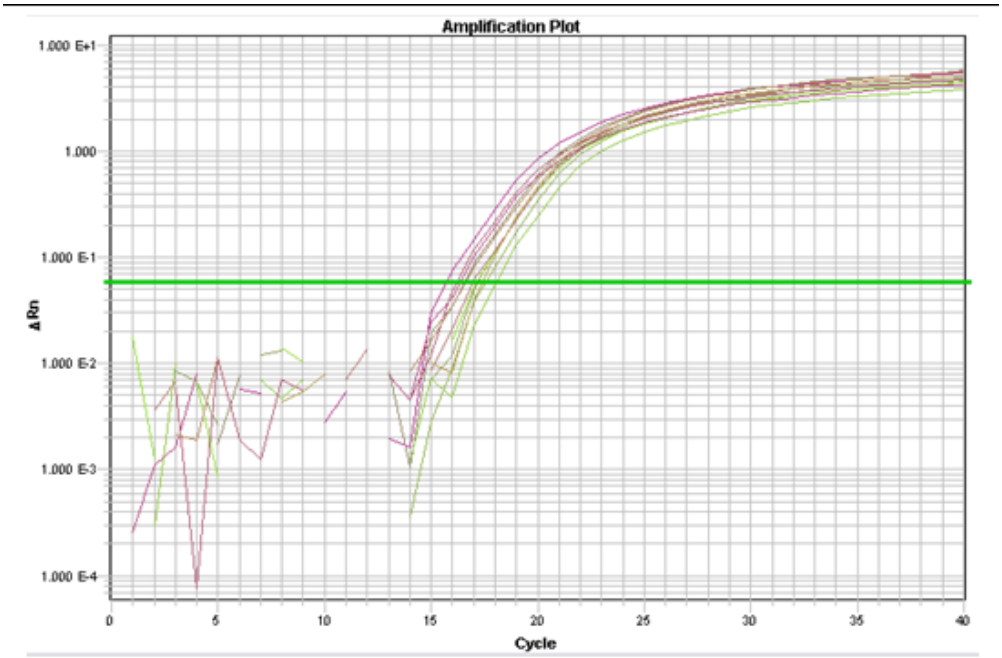
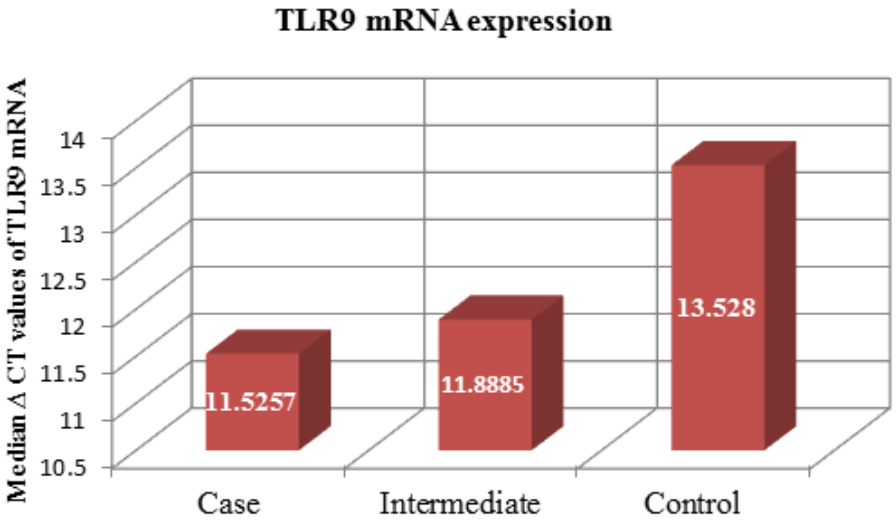


Fig. 6: GAPDH mRNA expression amplification plot: graph shows amplification of GAPDH with corresponding C_T value (17)



Graph 1: TLR9 mRNA expression analysis across all 3 categories of samples

control group and TLR 9 expression of case was 1.2907 folds that in the intermediate group. (Table 2)

TLR9 mRNA expression was studied using median ΔC_T values, comparing case and control, with respective genotypes, taking control group as calibrator. There was increased expression of TLR9 mRNA in the TT genotype among case group (1.496 fold) compared to controls. There was increased expression of TLR9 mRNA in the CT genotype among case group (3.578 fold) compared to controls. Table 3.

The case samples (n=33) were further studied according to their genotype. The relative TLR9 mRNA expression of the TT, CC and CT groups were 11.5257, 11.2337 and 11.8115 respectively. The fold change was calculated between TT and CC, with CC as calliberator. TLR9 expression of TT was decreased by 1.224 folds compared to the CC group. The fold change was calculated between CC and CT, with CC as calliberator-TLR9 expression of CT was decreased by 1.2191 folds compared to the CC group. Table 4

6. Discussion

TLR9 mRNA expression was analyzed in our study, using fold change of expression values. Greater the median Δ Ct value of TLR9 mRNA, lesser will be the expression of the TLR9 gene. Our study showed increased expression of TLR9 in malignant group, compared to the control group. This indicates that TLR9 expression is upregulated in cervical cancer. TLR9 expression was also increased in the HPV +ve nonmalignant group, compared to HPV –ve controls, suggesting that TLR9 is upregulated in persistent HPV +ve infection. Our findings have been corroborated by other studies in different ethnic populations. Studies by Hasimu et al on Uighur women in China showed that expression of TLR9 can be upregulated by HPV 16 infection in CIN and in cervical squamous carcinoma cells.[11] Lee et al studied Korean women in 2007and showed increased expression of TLR9 in cervical cancer patients.[12] Hasan et al also showed increased expression of TLR9 in persistent and recurrent HPV infection;[13] these findings are similar to our findings. Chen et al investigated the TLR9 -1486T/C (rs187084), a potentially functional variant located in the promoter region, which is close to the region that interacts with HPV16 E6 and E7 oncoproteins.[14] They found a significant increase in cervical cancer risk among the Chinese women carrying this variant in the *TLR9* gene.

Hasimu et al found that the expression of TLR9 can be upregulated by HPV16 infection in CIN and in cervical squamous carcinoma cells.[11] They also suggested that TLR9 may play important roles in the development and progression of CIN and cervical carcinoma. In contrast, Pandey et al. showed that the TT genotype of TLR9 (rs352140) displayed borderline significance in increased risk for advanced cervical cancer in a North India population.[15]

Correlating TT, CT, CC genotype with TLR9 expression analysis across malignant group and control group, there was increased expression of TLR9 among TT genotype and CT genotype compared to CC genotype (Statistically insignificant, p value >0.05, perhaps can be attributed to the low sample size). Our study showed that TLR9 C2248T polymorphism causes upregulation of TLR9 expression among cervical cancer patients. Our studies suggest that the TLR9 C2848T (rs352140) polymorphism may be a risk factor of cervical cancer in East Indian women.

Table 2: Fold-changes and p-value testing for significance of the median values of TLR9 across different categories of samples

Comparison	Fold change	P value
HPV negative controls (n= 9) vs Cases (n= 33)	4.0062	0.134
HPV positive nonmalignant (n=15) vs Cases (n=33)	1.2907	0.857
HPV negative controls vs HPV positive nonmalignant	3.1156	0.373

Table 3: Fold-changes and p-value testing for significance of the median values of TLR9 using different genotypes across different categories of samples

Genotype	Case	Control (caliberator)	P value	Fold change
CC	11.2341 N=4	12.7237 N=4	0.149	CC case group vs CC control= 2.808
CT	11.8115 N=20	13.6506 N=3	0.171	CT case group vs CT control = 3.578
TT	11.5257 N= 9	12.1071 N=2	0.814	TT case group vs TT control =1.496

Table 4: Fold change with different genotypes in the case group

Comparison	Fold change
TT genotype (n= 9) vs CC genotype (n=4), CC as calliberator	1.224
CT genotype (n=20) vs CC genotype, CC as calliberator	1.219
CT +TT genotype vs CC genotype, CC as calliberator	1.470

7. Summary

Genetic variations such as single nucleotide polymorphisms (SNPs) greatly influence innate immune responses towards pathogenic challenges and disease outcome; therefore, a range of susceptibility to infections appears among people, with some of them being predisposed to certain infections while others are being protected. Several single-nucleotide polymorphisms (SNPs) within the TLR genes have been associated with altered susceptibility to infectious, inflammatory, and allergic diseases, and have been found to play a role in tumorigenesis.

Our study showed that *TLR9* gene expression was increased in the malignant group, compared to controls and intermediate (HPV +ve nonmalignant) group. There was increase of TLR9 expression in TT genotype and CT genotype individuals among malignant group compared to similar genotypes in the controls. This upregulation was not statistically significant, possibly due to small sample size. Therefore this study should be replicated in a large cohort, and among varied ethnic population.

8. Acknowledgements

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9. Conflict of interest

None.

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