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Original Research Article

Deciphering the impact of genetic variation of the TLR9 gene in cervical cancer pathogenesis

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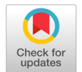

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ABSTRACT

Aims: 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. The present study aimed to identify the association of toll like receptor 9 (TLR9) (2848C>T; rs352140) single neucleotide polymorphism (SNP) with cervical cancer in East Indian women.
Materials and Methods: Study subjects comprised 71 women with histologically proven cervical cancer, and 100 women with benign cervical lesions. The case group included HPV 16 +ve subjects with malignancy. Among subjects with benign lesions, 43 were HPV 16 +ve (intermediate group) and 57 were HPV –ve (control group). TLR9 genotyping was performed using PCR RFLP. TLR9 expression was analyzed using real time PCR.
Results: For the C2848T polymorphism of TLR9, the TT genotype and the T allele frequency were significantly increased in the case group compared to those in the control group and the intermediate group.
Conclusion: Our study suggests that the TLR9 C2848T polymorphism may be a genetic risk factor for cervical cancer and the progression from HPV infection to cervical cancer.

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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 6.6% of all female cancer. Mortality from cervical cancer in India is 4.2million, and 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. Persistent infection by oncogenic Human Papilloma Virus (HPV) types is associated with most premalignant and malignant cervical epithelial lesions. Of the 100 known HPV genotypes, at least 15 are oncogenic. HPV 16 and 18 cause approximately 70% of all cervical cancers worldwide.[2]

The host immune response plays a crucial role in the progression or regression of hrHPV infection of the cervix. An effective immune response clears the infection, while a compromised immune response often promotes it into higher grade cervical dysplasia. The HPV oncoproteins E6 and E7 promote cervical carcinogenesis by disturbing apoptosis, the cell cycle, and adaptive immune surveillance.

It takes 15 to 20 years for cervical cancer to develop in women with normal immune systems compared to only 5 to 10 years in women with weakened immune systems.

The common sexually transmitted high-risk human papillomavirus (Hr HPV) is a necessary factor for cervical cancer.[3] Most newly acquired HPV infections are transient, but a small fraction of persistent infections progress to

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cervical cancer.[4,5]

Pathogens may be identified by pattern recognition receptors (PRRs) such as the Toll-like receptor family (TLRs). TLRs recognize exogenous pathogen-associated molecular patterns (PAMPs) and play an elementary role in the innate immune response.

Since TLRs regulate inflammatory responses, dysregulated signaling circuits within TLRs could adversely affect inflammatory processes and promote carcinogenesis.[6] TLR polymorphism can modify susceptibility to cancer and influence therapeutic outcomes.

Several studies have been conducted to investigate the association of TLR polymorphisms with cancer. A 22-bp nucleotide deletion (- 196 to - 174 del) in the promoter of the TLR2 gene and the D299G TLR4 polymorphisms were found to have a significantly higher frequency in breast cancer patients compared to healthy controls.[7] TLR5 rs5744174 C carriers (TC + CC) had an increased risk of gastric cancer and Helicobacter pylori infection.[8] TLR3 (rs11721827) was associated with rectal cancer, and TLR3 (rs3775292) and TLR4 (rs11536898) were associated with colon cancer[8]. The - 196 to - 174 del TLR2 and T399I TLR4 polymorphisms have been associated with an increased risk of prostate and cervical cancer in a North Indian population, whereas the TLR3 (c.1377C/T) [rs3775290] and TLR9 (G2848A) mutations showed no correlation.[9–11] Our study aimed to investigate whether the TLR9 - C2848T polymorphism is a genetic risk factor for cervical cancer in the East Indian population

Our study aimed to investigate whether the TLR9 - C2848T polymorphism is a genetic risk factor for cervical cancer in the East Indian population

2. Materials and Methods

A case control study was conducted on Indian women of the state of West Bengal, India from October 2015 to April 2017. The study population included 171 women: the case group (HPV +ve with malignancy) comprising 71 women and the nonmalignant group comprising 100 women, of which 43 belonged to the intermediate group (HPV infection +ve) and 57 belonged to the control group (HPV infection –ve). Women with a history of chronic/ 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) were included in the study. Women with a history of recent childbirth or miscarriage (within the last 4 months), prior treatment for cervical malignancy, and menstruating, pregnant, and unmarried women were excluded from the study. A questionnaire was used to collect information from the subjects on history, demographic data, life style, and reproductive factors. All samples were collected from the subjects after they provided informed consent.

2.1. Sample collection

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

2.1.1. 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 with PCR, using L1 consensus primers: MYO11 and MYO9 (Figure 1) L1 negative samples were reamplified with nested GP 5/6 primers for further HPV screening. 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. (Figure 2).

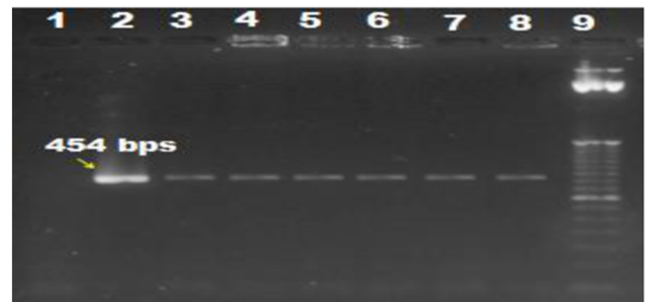

Fig. 1: Gel doc image of sample with L1 primers
Lane 1 – negative control, Lane 2 – positive control, lane 9 – 50bp marker, Lanes 3 – 8 test samples. All show positive bands at 450bp, thus all are HPV +ve.

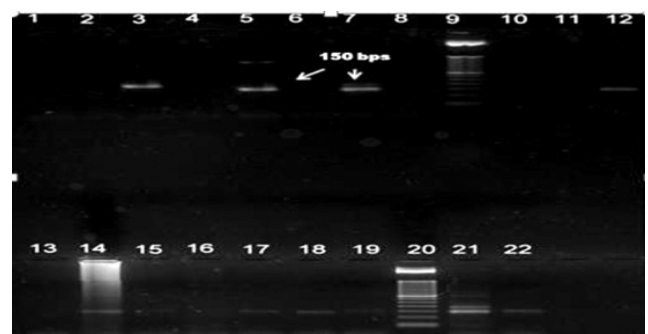

Fig. 2: Gel doc image of samples with GP5/6 primers Lane 1- negative control; lanes 2 to 8, test samples; lane 9 - 50bp ladder; lanes- 10 to 19 test samples and lane 21, 22-positive controls (cell line DNA Caski, SiHa)

Table 1: Demographic and clinical table

Characteristics	Cases n= 71	Intermediates n=43	Controls n=57
		Mean +/-SD	
Age at diagnosis	52.15 ± 10.02	50.19 ± 9.29	47.75 ± 10.12
Age at First Childbirth	18.41 ± 2.31	20.09 ±2.09	20.37 ± 2.06
Age at menopause	46.14 ± 5.11	44.66 ± 4.12	46.45 ± 3.76
Parity	4.76+/-1.89	4.02 +/- 1.87	3.80+/- 1.93
Illiterate	40 (56.34%)	10 (23.26%)	13 (22.81%)
premenopause	13 (18.31%)	13 (30.23%)	29 (50.88)%
postmenopause	58 (81.69%)	30 (69.77%)	28 (49.12%)
smokers	14 (19.72%)	6 (13.95%)	18 (31.58%)
nonsmokers	44 (61.97%)	24 (55.81%)	10 (17.54%)
Tumour Squamous cell cancer			
Stage 1	0		
Stage 2	7 (9.9%)		
Stage 3	62 (87.3%)		
Stage 4	2 (2.8%)		
Histological Grading			
Well differentiated	13 (17.57%)		
Moderately differentiated	8 (10.81%)		
Poorly differentiated	50 (71.62%)		

2.1.2. *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. (Figures 3 and 4)

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 and HPV 16 +ve were classified into the case group, non-malignant HPV-ve samples were classified into the control group and non-malignant HPV 16 +ve samples were classified into the intermediate group.

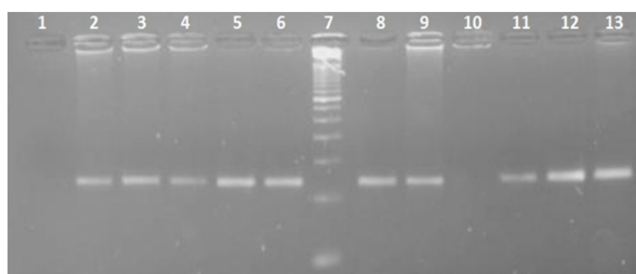

Fig. 3: Gel doc image of samples with HPV 16 primers
Lane 1- negative control; lanes 2 to 6 test samples; lane 7- 50bp ladder; lane 8 to 11- test samples; lane 12,13 positive controls (HPV16 +ve cell line DNAs- SiHa, Caski).

2.1.3. *Detection of genetic polymorphism of TLR9 gene rs352140 (C/T)*

DNA samples were amplified and a 360-bp amplicon, corresponding to TLR9 promoter region was obtained.(Figure 5) The PCR fragments were digested by endonuclease BST UI (CG/CG) (New England BioLabs,

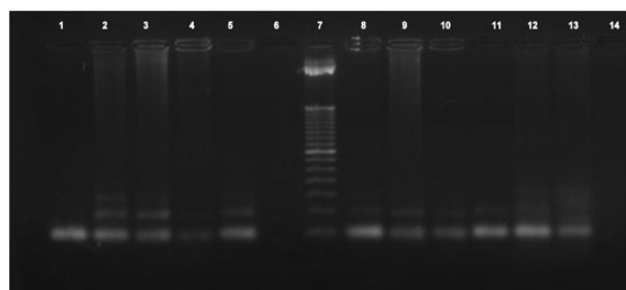

Fig. 4: Gel doc image of samples with HPV 18 primers
Lane 1- negative control; lanes 2 to 6 test samples; lane 7- 50bp ladder; lane 8 to 12- test samples; lane 13 positive control (HPV 18 +ve cell line DNA-HeLa)

Ipswich, USA). The TLR9 C allele was cleaved into 227 and 133-bp fragments. The TLR9 T allele remained uncut. DNA fragments were separated by gel electrophoresis. A single band indicated the homozygous TT genotype, double bands indicated homozygous CC genotype, and triple bands indicated heterozygous CT genotype (Figure 6).

2.2. *Statistical analysis*

The number and percentage of patients were compared across the groups using Pearson's Chi Square test for Independence of Attributes (software: SPSS version 20). The changes in expression levels were tested for statistical significance using non-parametric test (Mann-Whitney U Test). (SPSS version 20). $P < 0.05$ was considered as statistically significant.

Table 2: All primers that were used

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

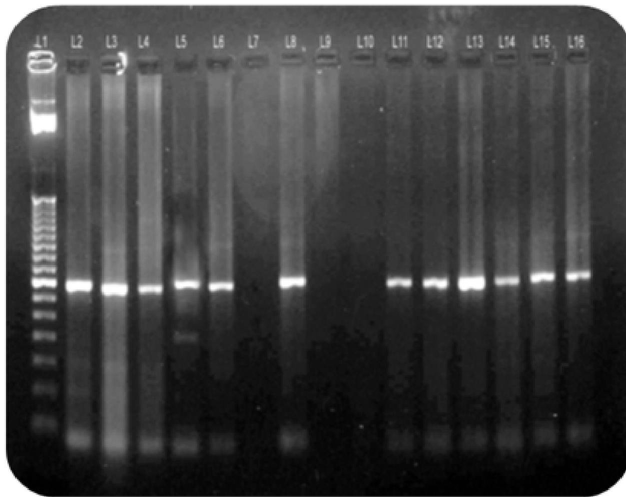

Fig. 5: Gel doc image of samples with TLR9 primers
Lane 1- 50bp ladder Lane 2-6, 8,11-16 – samples with PCR amplification present; lanes 7,9,10 samples with PCR amplification absent

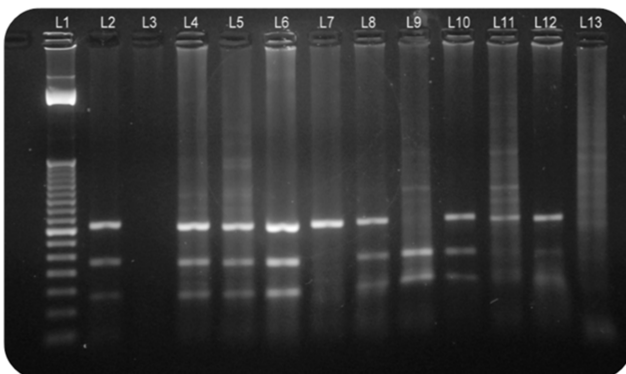

Fig. 6: RFLP of PCR product of samples Lane 1- 50bp ladder, Lane 2, 4-6, 8, 10, 12 triple band – CT genotype
Lane 7- single band- TT genotype, Lane 9 - double band - CC genotype

3. Results

The frequency distribution of the three different genotypes of TLR9 with C2848T polymorphism among the 3 groups is shown in Table 3. The control group was in Hardy Weinberg equilibrium (HWE) (P = 0.0961) with goodness of fit (χ^2= 4.68, df =2). The intermediate group was also in H WE (P = 0.1369). However, the case group was not in HWE (P = 0.0358).

There was statistically significant increased frequency of the TLR9 TT genotype in the case group (0.38) compared to the control group (0.17) (OR = 4.36, P = 0.004 Table 4) and in the case group (0.38) compared to the intermediate group (0.16) (OR = 4.7473, P = 0.0058, Table 5).

TLR9 T allele frequency was higher in the case group (0.59) than in the control group (0.40) (Table 4); this was statistically significant (OR = 2.2044, P = 0.0021). TLR9 T allele frequency was also higher in the case group (0.59) than in the intermediate group (0.39) this was statistically significant (Table 5).

TLR9 TT genotype frequency in the intermediate group (0.16) was lesser than in the control group (0.17) (OR = 0.9188, P = 0.887, not statistically significant) A lower TLR9 T allele frequency was found in the intermediate group (0.39) compared to the control group (0.40); this was not statistically significant (OR = 0.9666, P = 0.907, Table 6).

4. Discussion

In our study, the different genotypes (CC, CT, and TT) of the TLR9 gene were distributed in HWE in the intermediate and control groups but not in the case group. Analysis of the genotype of TLR9 C2848T and the minor (T) allele frequency across the case, intermediate and the control groups showed no significant difference in the TT genotype or the T allele frequency between the intermediate and the control group. However, the TT genotype and the T allele frequency were found to be significantly increased in the case group than in the control group. This indicates that the TLR9 -2848TT (rs352140) polymorphism is associated with a significantly increased risk of cervical cancer but

Table 3: Frequency distribution of genotypes ofTLR9 gene polymorphism C2848T among subjects of different cervical status

Group	CC	CT	TT	χ^2 value	P	Goodness of fit with HWE
Control n=57	21 (36.84%)	26 (45.61)	10 (17.54%)	4.6842	0.0961	In HWE
Intermediate n=43	16 (37.20%)	20 (46.51)	07 (16.28%)	3.9768	0.1369	In HWE
Cases n=71	13 (18.30%)	31 (43.66%)	27 (38.02%)	6.6619	0.0358	Not in HWE

Table 4: Contribution of theTLR9 C2848T (rs352140) polymorphisms in cervical cancer Case vs. controls

	Case	Control	Odds ratio (95% CI)	P
C/C	13 (18.3%)	21 (36.84%)	Referent	
C/T	31 (43.66%)	26 (45.61%)	1.926 (0.810-4.579)	0.138
T/T	27 (38.03%)	10 (17.54%)	4.3615 (1.601-11.885)	0.004
C/T + T/T	58 (81.69%)	36 (63.16%)	2.6026 (1.161-5.833)	0.020
C	57 (40.14%)	68 (59.65%)	Referent	
T	85 (59.86%)	46 (40.35%)	2.2044 (1.334-3.644)	0.0021

Table 5: Contribution of the TLR9 C2848T (rs352140) polymorphisms in cervical cancer Case vs. Intermediate

	Case	Intermediate	Odds ratio (95% CI)	P
C/C	13,18.3%	16 (37.21%)	Referent	
C/T	31 (43.66%)	20 (46.51%)	1.9077(0.758-4.801)	0.170
T/T	27 (38.03%)	7 (16.28%)	4.7473 (1.568 – 14.370)	0.0058
C/T + T/T	58 (81.69%)	27 (62.8%)	2.6439 (1.116 – 6.264)	0.027
C	57 (40.14%)	52(60.47%)	Referent	
T	85 (59.86%)	34 (39.53%)	2.2807 (1.320 – 3.942)	0.0031

Table 6: Contribution of the TLR9 C2848 (rs352140) polymorphisms in cervical cancer Intermediate vs control

	Intermediate	Control	Odds ratio (95% CI)	P
C/C	16 (37.21%)	21 (36.84%)	Referent	
C/T	20 (46.51%)	26 (45.61%)	1.0096 (0.422 - 2.418)	0.983
T/T	7 (16.28%)	10 (17.54%)	0.9188 (0.287 – 2.944)	0.887
C/T + T/T	27 (62.8%)	36 (63.16%)	0.9844 (0.434 – 2.235)	0.970
C	52(60.47%)	68 (59.65%)	Referent	
T	34 (39.53%)	46 (40.35%)	0.9666 (0.546 – 1.712)	0.907

there is no correlation of TLR9 C2848T polymorphism with persistent HPV positivity. Our results showed an increase in the TT genotype and the T allele frequency in the case group compared to the intermediate group, indicating a positive correlation between TLR9 2848TT (rs352140) polymorphism and progression from HPV infection to cervical cancer. Studies by Chen et al. demonstrated that the TLR9 -1486 T/C (rs187084) polymorphism was associated with a significantly increased risk of cervical cancer.[10] 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 Indian population.[11] Roszak et al observed a contribution of the TLR9 -1486 T/C (rs187084) and C2848T (rs352140) polymorphisms to the risk of cervical cancer in a Polish population.[12]

Our study suggests that the TLR9 C2848T (rs352140) polymorphism may be a risk factor for cervical cancer in East Indian women.

5. Summary

Analysis of candidate gene polymorphisms has emerged as a powerful approach in understanding the genotype-disease association, which considers specific gene variants to be susceptibility factors to various cancers, including cervical cancer.

Our study reveals a positive association of the TT genotype and the T allele frequency of the TLR9 C2248T polymorphism in cervical cancer and persistent HPV 16 infection of the cervix among the East Indian population. This polymorphism can be used as a possible genetic marker in screening programs for cervical cancer.

6. Limitations and Future Prospects

The study was conducted on East Indian Population. Further study with larger sample size and from different parts of India need to be conducted for significant upregulation of TLR9 C2248T polymorphism on cervical cancer

7. Source of Support

Equipment, reagents - National Institute of Biomedical Genomics laboratory.

8. Conflict of Interest

None.

9. Ethical Clearance

The study was approved by the Institutional Ethical Committee.

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