



RESEARCH ARTICLE

**REVISED** **Absence of toll-like receptor 9 Pro99Leu polymorphism in cervical cancer [version 2; referees: 2 approved, 1 not approved]**

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**Abstract**

**Background:** Toll-like receptor 9 (TLR9) plays a key role in the elimination of viral pathogens by recognising their CpG DNA. Polymorphisms in the *TLR9* gene may influence their recognition and subsequent elimination. Therefore, the present study was designed to elucidate the role of a rare unexplored *TLR9* gene polymorphism C296T/ Pro99Leu (rs5743844) in cervical cancer susceptibility among Indian women.

**Methods:** The genotyping of *TLR9* Pro99Leu polymorphism in 110 cervical cancer patients and 141 healthy controls was performed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP).

**Results:** The genotype frequency detected in both cervical cancer and control populations was 1.0 (CC), 0.0 (CT) and 0.0 (TT); while the allele frequency was found to be 1.0 (C) and 0.0 (T).

**Conclusions:** The present study demonstrates no involvement of *TLR9* C296T/ Pro99Leu polymorphism in cervical cancer susceptibility and supports minor allele frequency (MAF) (0.0002) status of the same as no nucleotide variation was detected in any of the study subjects.

**Keywords**

Cervical cancer, TLR9, Polymorphism, Genotypic frequency, Susceptibility

**Open Peer Review**

Referee Status: ✓ ✗ ✓

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	1	2	3
<b>REVISED</b>	<span style="color: green;">✓</span>	<span style="color: red;">✗</span>	<span style="color: green;">✓</span>
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**Author roles:** **Chauhan A:** Formal Analysis, Investigation, Methodology, Visualization, Writing – Original Draft Preparation; **Pandey N:** Formal Analysis, Investigation, Writing – Review & Editing; **Raithatha N:** Investigation, Resources, Writing – Review & Editing; **Patel P:** Investigation, Resources, Writing – Review & Editing; **Desai A:** Investigation, Resources, Writing – Review & Editing; **Jain N:** Conceptualization, Funding Acquisition, Project Administration, Supervision, Validation, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

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*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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**REVISED Amendments from Version 1**

This version has been modified based on the reservations given by Dr. Gopeshwar Narayan and Prof. B. C. Das. The previous version of this article did not include the power calculation. In this version, we have calculated the statistical power based on the global minor allele frequency of the polymorphism under study and have drawn appropriate conclusions. The reason for the selection of *TLR9* gene for this study has been mentioned in the Introduction as well as in the Discussion.

See referee reports

**Introduction**

Cervical cancer is the fourth-most common cancer among women globally and second leading cause of cancer-related deaths in Indian women<sup>1</sup>. Although persistent infection of high-risk human papillomavirus (hrHPV) is considered as the chief causative agent of cervical cancer, variations in host genetic make-up does influence the risk of acquiring HPV infection, and susceptibility to cervical carcinogenesis<sup>2-4</sup>. In this context, variations in Toll-like receptor (*TLR*) genes, that play a crucial role in activating immune response by identifying pathogen-associated molecular patterns (PAMPs), have drawn significant attention, as single nucleotide polymorphisms (SNPs) in *TLR* genes have been shown to alter susceptibility to many infections and human diseases including cancer<sup>5-8</sup>.

Ten functional *TLR* genes are known in humans, the products of which recognizes specific PAMPs<sup>9</sup>. *TLR1*, *TLR2*, *TLR4*, *TLR5* and *TLR6* which are located on the cell surface of immune cells are known to recognize triacyl lipopeptides, peptidoglycans, lipopolysaccharides, flagellin and diacyl lipopeptides respectively that belong to bacterial cell wall or virus particles<sup>10,11</sup>. On the other hand *TLR3*, *TLR7*, *TLR8* and *TLR9* are located in the endosomal compartments, wherein *TLR3* recognizes single as well as double stranded viral RNA, *TLR7* recognizes ssRNA from viruses while the *TLR9* gene product recognizes bacterial and viral DNA motifs including HPV16 CpG motifs<sup>5,10-12</sup>. Frequently analysed *TLR9* SNPs G2848A and -1486 T/C have been suggested to alter cervical cancer susceptibility<sup>13-16</sup>, but no report is available elucidating the role of *TLR9* Pro99Leu polymorphism in cancer. Although *TLR9* Pro99Leu is a rare population SNP with a global minor allele frequency (MAF) of 0.0002 as reported in the single nucleotide polymorphism database (dbSNP), *in-vitro* analysis has revealed its significant role in DNA ligand hyporesponsiveness<sup>17</sup>. Considering the fact

that cervical cancer is largely caused by hrHPV infection and *TLR9* has the ability to respond to viral DNA, while other *TLRs* do not, the present study was designed to elucidate the association of the *TLR9* Pro99Leu polymorphism with cervical cancer.

**Methods****Biological specimens**

Biopsies from 110 cervical cancer patients and cervical smears from 141 healthy volunteers were collected from Shree Krishna Hospital, Anand; Sir Sayajirao General Hospital, Vadodara; and GMERS Hospital, Ahmedabad, India. The samples were collected from 2012 to 2017. The cancer biopsies and healthy cervical smears were histopathologically and cytologically confirmed. The clinical staging of cervical cancer samples was done as per The International Federation of Gynecology and Obstetrics (FIGO) guidelines.

**DNA isolation and genetic analysis**

DNA was isolated from cervical cancer biopsies and cervical smears by standard phenol-chloroform extraction method<sup>18</sup>. In the case of a low number of cervical cells, a spin-column based DNA isolation kit (Macherey-Nagel, Germany; Cat# 740952.50) was utilized as per manufacturer's instructions. The quality and quantity of DNA was determined using ethidium bromide-stained 1% agarose gel on GelDoc system (BioRad, USA) as well as a NanoDrop 2000 (ThermoFisher, USA). The *TLR9* Pro99Leu polymorphism was detected using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method as described by Kubarenko *et al.*<sup>17</sup> Briefly, a 25µl PCR mix contained 0.1µM each of forward and reverse primer (Imperial Life Sciences, India), 0.1mM dNTP mix (Invitrogen, USA; Cat# 18427088), 2.5mM MgCl<sub>2</sub> (Vivantis, USA; Cat# RB0204), 1 unit Taq DNA polymerase (Kapabiosystems, USA; Cat# KK1015) and 100 to 150ng genomic DNA. The PCR was run on an MJ Mini thermal cycler (BioRad, USA).

Upon confirmation of 337 bp PCR product on 2% ethidium bromide-stained agarose gel, 10µl PCR product was digested with *BsiI* restriction enzyme (New England Biolabs, USA; Cat# R0555S) at 55°C overnight, separated on 12% polyacrylamide gel and analysed on a GelDoc system (BioRad, USA) for genotype identification. The details of PCR conditions and parameters for genotype consideration are mentioned in [Table 1](#) and [Table 2](#) respectively. To confirm the PCR-RFLP results, we performed Sanger sequencing of five randomly selected cervical cancer as well as control samples. All the sequencing reactions were performed on 3730xl DNA Analyzer (Applied

**Table 1. Details of *TLR9* C296T/ Pro99Leu specific PCR.**

Primer Sequence (5' – 3')	Thermal Profile	PCR Product	Visualized on
FP: GGATGTTGGTATGGCTGAGG RP: AACTGCAACTGGCTGTTCCCT	(95°C – 5') 1 (95°C – 45", 56°C – 1', 72°C – 30") 35 (72°C – 10') 1	337 bp	2% Agarose Gel

Abbreviations: *TLR9*, Toll-like receptor 9; FP, forward Primer; RP, Reverse Primer; PCR, Polymerase Chain Reaction; bp, base pairs

**Table 2. Parameters for genotypes consideration of *TLR9* C296T/ Pro99Leu polymorphism.**

Enzyme	Digested Products (bp)	Genotype
<i>BsiI</i>	166 + 136 + 35	CC (Pro/Pro)
	201 + 166 + 136 + 35	CT (Pro/Leu)
	201 + 136	TT (Leu/Leu)

Biosystems, USA) using BigDye™ Terminator v3.1 kit (Applied Biosystems, USA; Cat# 4337454) as per manufacturer's instructions. The 10µl sequencing reaction was comprised of 7.0µl BigDye™ Terminator v3.1 Ready Reaction Mix, 10pmol forward primer and 50ng PCR product. The sequencing results were analyzed on [Sequencing Analysis Software](#) version 5.3.1 (Applied Biosystems, USA).

### Statistical analysis

Statistical analysis was performed on [GraphPad Prism](#) version 5.00 for Windows (GraphPad Software, USA). Age of patients and controls were compared using two-sided Student's t-test. Due to the presence of single genotype across all the samples no additional statistical association was performed. The power of the study was calculated using [Online Sample Size Estimator](#).

## Results

### Demographic and clinical characteristics

The average age of cervical cancer patients (52.43±11.78 years) and controls (51.8±11.35 years) was comparable without any

statistically significant difference ( $p=0.668$ ). Histopathologic analysis revealed all the cervical cancer cases to be of squamous cell carcinoma type. According to FIGO analysis, 9 (8.2%), 39 (35.5%), 55 (50%) and 7 (6.3%) patients belonged to Stage I, II, III and IV respectively. In the absence of polymorphic allele in the present population we calculated the power of the study based on the global minor allele frequency (0.0002) which was found to be 3.6%.

### *TLR9* Pro99Leu polymorphism

PCR amplification revealed the presence of a single intact band of 337 bp ([Figure 1](#); [Dataset 1](#)<sup>19</sup>). A single genotype CC (Pro/Pro) was detected across all the sample types ([Table 3](#); [Dataset 2](#)<sup>20</sup>) which was evident by the presence of 166 bp, 136 bp and 35 bp DNA bands after RFLP assay ([Figure 2](#); [Dataset 3](#)<sup>21</sup>). Sanger sequencing of the randomly selected PCR products corroborated with RFLP results ([Figure 3](#); [Dataset 4](#)<sup>22</sup>).

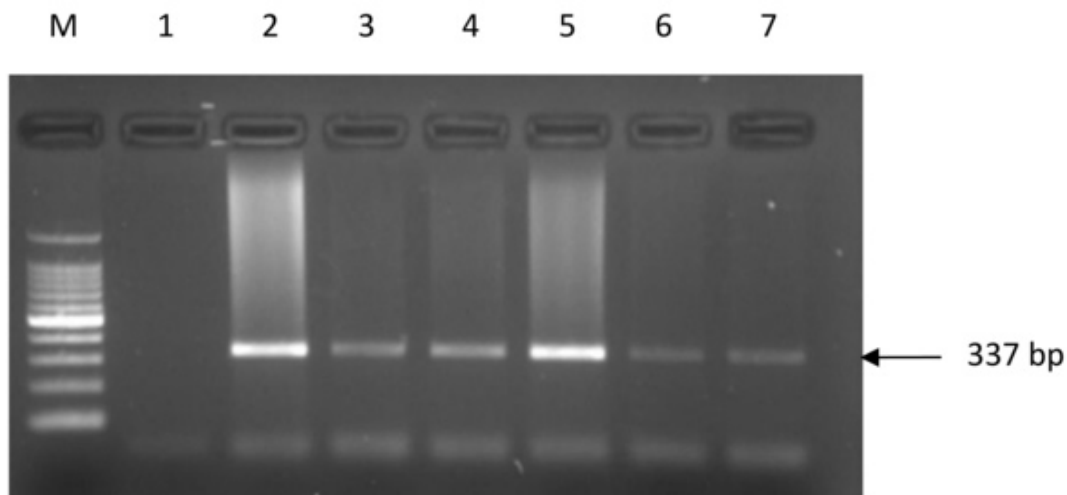
**Dataset 1. Raw agarose gel images of PCR amplification of *TLR9* gene segment for C296T/ Pro99Leu polymorphism from 50 samples consisting of 26 controls and 24 cervical cancer cases**

<http://dx.doi.org/10.5256/f1000research.14840.d203405>

[Figure 1](#) is a representative picture of the same.

**Dataset 2. Age, clinical stage and *TLR9* genotype status among cervical cancer patients as well as age and *TLR9* genotype status among controls**

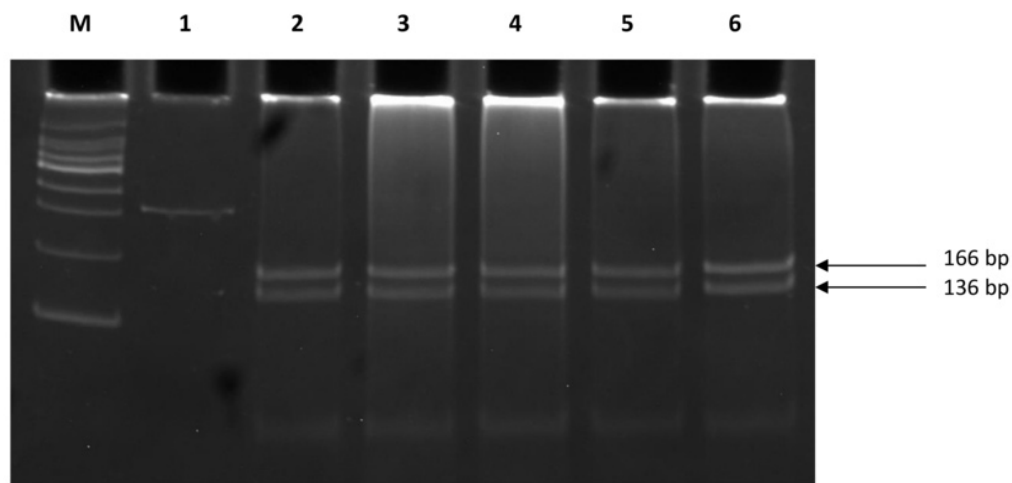
<http://dx.doi.org/10.5256/f1000research.14840.d203406>



**Figure 1. Representative PCR picture showing amplification of *TLR9* gene segment for C296T/ Pro99Leu gene polymorphism on ethidium bromide-stained 2% agarose gel.** Lane M is 100 bp molecular marker (Takara, Japan; Cat# RR820A), Lane 1 is negative control and Lanes 2–7 are tumor DNA showing PCR products of 337 bp. (Abbreviations: PCR, Polymerase Chain Reaction; *TLR9*, *Toll-like receptor 9*; bp, base pair).

**Table 3. Genotype and allele frequencies of *TLR9* C296T/ Pro99Leu polymorphism in cervical cancer patients and healthy controls.**

Genotype	Cervical Cancer n (%)	Controls n (%)
CC	110 (100.0)	141 (100.0)
CT	0 (0.0)	0 (0.0)
TT	0 (0.0)	0 (0.0)
Allele		
C	220 (100.0)	282 (100.0)
T	0 (0.0)	0 (0.0)



**Figure 2. Representative PAGE picture of RFLP results for *TLR9* C296T/ Pro99Leu polymorphism on 12% polyacrylamide gel.** Lane M is 100 bp molecular marker, Lane 1 is undigested PCR product and Lanes 2 to 6 are showing digested PCR products of 166 bp and 136 bp (35 bp band is not visible) by *BspI* enzyme representing CC genotype. (Abbreviations: PAGE, Polyacrylamide Gel Electrophoresis; RFLP, Restriction Fragment Length Polymorphism).

**Dataset 3. Raw polyacrylamide gel electrophoresis images of 27 controls and 24 cervical cancer PCR amplified products that underwent restriction fragment length polymorphism (RFLP) analysis**

<http://dx.doi.org/10.5256/f1000research.14840.d203407>

Figure 2 is a representative picture of the same.

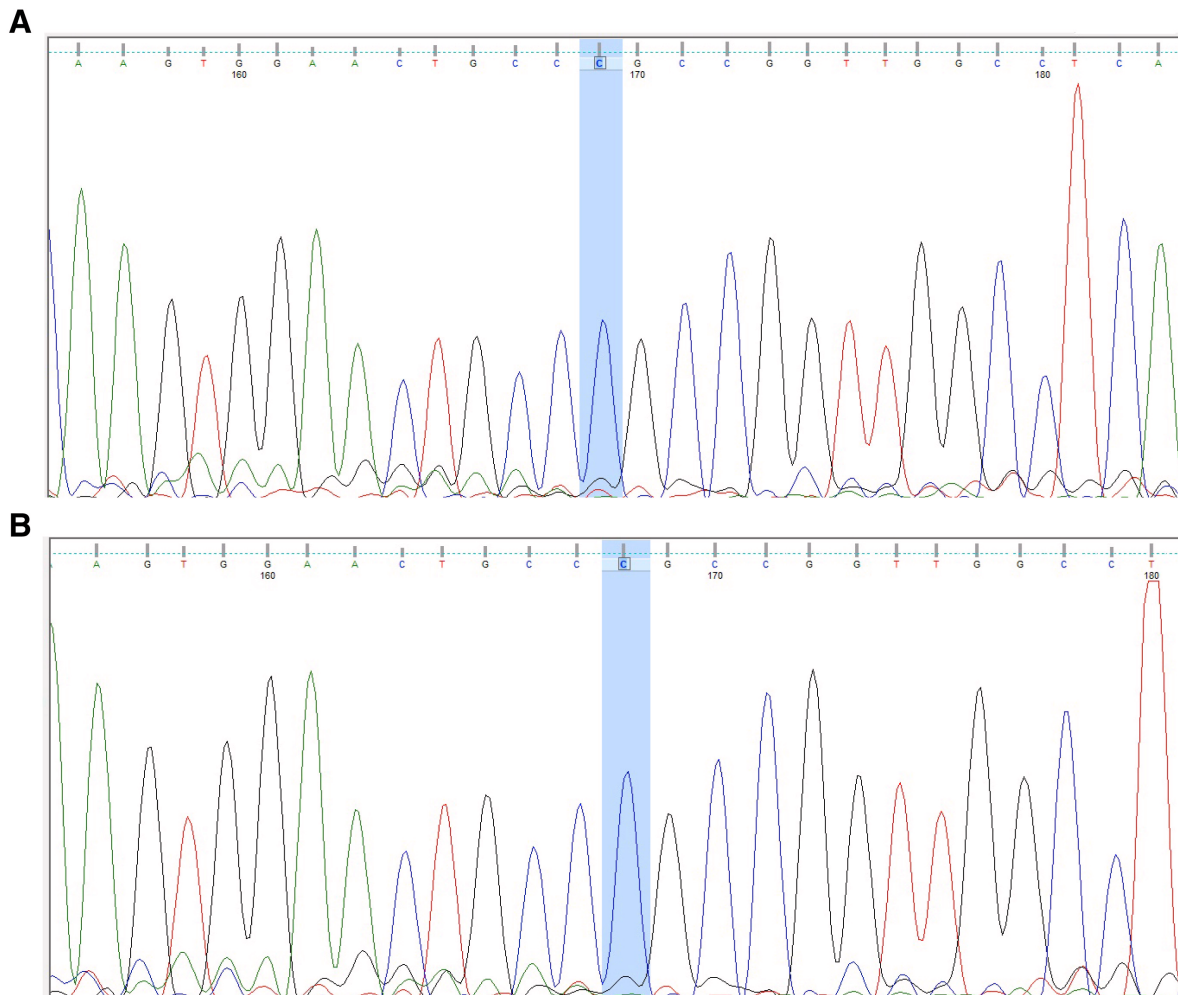
**Dataset 4. Nucleotide sequences spanning *TLR9* gene segment for C296T single nucleotide polymorphism, obtained after performing Sanger sequencing on five samples each of cervical cancer and healthy controls**

<http://dx.doi.org/10.5256/f1000research.14840.d203408>

The sequencing results confirm the restriction fragment length polymorphism (RFLP) analysis that represents single genotype CC among all the study subjects. Figure 3A and B are representative electropherograms of the *TLR9* C296T CC genotype as evident by the presence of single peak of C allele.

## Discussion

Although hrHPV infection is the primary etiological agent of cervical carcinogenesis, the role of host genetic factors, especially those associated with body immunity such as TLRs, cannot be ignored. TLR9 which recognizes CpG DNA motifs from bacteria and viruses has also been reported to recognize HPV16 CpG DNA<sup>12</sup>. Moreover, variations in the *TLR9* gene has found associations with various diseases including cancer. *TLR9* SNPs -1486 T/C and G2848A have been found to be contradictorily associated with cervical cancer risk. In Polish and Mexican populations both *TLR9* -1486 T/C and G2848A polymorphisms were suggested to be risk factors for cervical carcinogenesis<sup>13,15</sup>. In two independent studies on Chinese population, a positive association with *TLR9* G2848A SNP was detected<sup>23,24</sup> but no involvement of *TLR9* -1486 T/C was found<sup>24</sup>, however, the other study suggested -1486 T/C was not a contributory factor to cervical carcinogenesis<sup>14</sup>. From India, a single report on North Indian patients revealed a marginal role of *TLR9* G2848A polymorphism with cervical cancer risk<sup>16</sup>.



**Figure 3.** Sanger sequence electropherogram of (A) a healthy individual and (B) patient showing single peak (highlighted) of C allele of *TLR9* C296T/ Pro99Leu SNP representing CC genotype. (Abbreviations: SNP, Single Nucleotide Polymorphism).

To date, no report is available on the rare *TLR9* Pro99Leu polymorphism in cancer, which has been shown to be associated with DNA ligand hyporesponsiveness in HeLa cell lines<sup>17</sup>. Considering the fact that cervical cancer is majorly caused by hrHPV infection and the *TLR9* Pro99Leu polymorphism is associated with DNA ligand hyporesponsiveness, the present study investigated, for the first time, the role of the *TLR9* Pro99Leu polymorphism in cervical cancer susceptibility. This is also the first report to study this polymorphism in any of the cancer types globally. Our results revealed the presence of a single genotype CC (Pro/Pro) among cases and controls, demonstrating no significance of the Pro99Leu polymorphism to cervical cancer susceptibility. Similarly, no association with high-risk HPV infection, that was detected in almost 70% of the patients, was found (details of HPV infection to be published elsewhere). A complete absence of Pro99Leu in our study population corroborates with the report of Lee and group (2006) where neither controls nor lung tuberculosis and sarcoidosis patients had the *TLR9* Pro99Leu polymorphism<sup>25</sup>. Similarly, the Pro99Leu polymorphism was not detected among healthy Caucasians as

well as pneumococcal disease, bacteraemia, and leprosy patients<sup>17</sup>. Moreover, according to dbSNP, the global MAF of this polymorphism is 0.0002, and our results, albeit on a smaller cohort, do solicit its rare polymorphism status. Therefore, owing to sample size constraint, it would be inappropriate to draw a direct conclusion of the effect of above-said polymorphism on the susceptibility to cervical cancer. As the power of study calculated based on the global MAF was very low (3.6%), and to achieve a power of study of 80%, approximately 40000 samples will be required to analyze. Finally, under present scenario, a direct role of this SNP in cancer, as well as other diseases, seems a remote possibility. Nonetheless, a comprehensive analysis of a larger cohort covering a varied ethnic population globally is suggested to comprehend its role in microbial infection and/or disease susceptibility including cancer.

### Conclusion

The preliminary data obtained from the present study does not suggest a role for the *TLR9* Pro99Leu polymorphism in cervical cancer susceptibility. However, analysis on a larger

cohort worldwide may provide more insights into the frequency distribution of Pro99Leu polymorphism and reveal its influential role in various human diseases including cancer.

### Data availability

Dataset 1. Raw agarose gel images of PCR amplification of *TLR9* gene segment for C296T/ Pro99Leu polymorphism from 50 samples consisting of 26 controls and 24 cervical cancer cases. [Figure 1](https://doi.org/10.5256/f1000research.14840.d203405) is a representative picture of the same. [10.5256/f1000research.14840.d203405](https://doi.org/10.5256/f1000research.14840.d203405)<sup>19</sup>

Dataset 2. Age, clinical stage and *TLR9* genotype status among cervical cancer patients as well as age and *TLR9* genotype status among controls. [10.5256/f1000research.14840.d203406](https://doi.org/10.5256/f1000research.14840.d203406)<sup>20</sup>

Dataset 3. Raw polyacrylamide gel electrophoresis images of 27 controls and 24 cervical cancer PCR amplified products that underwent restriction fragment length polymorphism (RFLP) analysis. [Figure 2](https://doi.org/10.5256/f1000research.14840.d203407) is a representative picture of the same. [10.5256/f1000research.14840.d203407](https://doi.org/10.5256/f1000research.14840.d203407)<sup>21</sup>

Dataset 4. Nucleotide sequences spanning *TLR9* gene segment for C296T single nucleotide polymorphism, obtained after performing Sanger sequencing on five samples each of cervical cancer and healthy controls. The sequencing results confirm the restriction fragment length polymorphism (RFLP)

analysis that represents single genotype CC among all the study subjects. [Figure 3 A and B](https://doi.org/10.5256/f1000research.14840.d203408) are representative electropherograms of the *TLR9* C296T CC genotype as evident by the presence of single peak of C allele. [10.5256/f1000research.14840.d203408](https://doi.org/10.5256/f1000research.14840.d203408)<sup>22</sup>

### Ethical considerations

The research was carried out following due approval from ethics committee of all the participating institutes. Subjects were verbally informed and explained about the study, and were provided with an information sheet. Written informed consent was obtained from the subjects who agreed to enrol in the present study. Personal information of all the study subjects was kept confidential.

### Grant information

The study was funded by Charotar University of Science and Technology (CHARUSAT).

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

### Acknowledgement

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# Open Peer Review

Current Referee Status:



## Version 2

Referee Report 03 October 2018

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**Gopeshwar Narayan** 

Department of Molecular and Human Genetics, Banaras Hindu University, Varanasi, India

The authors have addressed the issues raised in the previous version.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Referee Report 13 September 2018

doi:10.5256/f1000research.17000.r37741



**Balraj Mittal**

Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, India

I had already mentioned in my original review that it is useless to look for association of a monophonic allele with a disease and study has zero statistical power. The only conclusion from the present study is that *TLR9 C296T/ Pro99Leu* polymorphism appears to be monomorphic in Gujrati Indians. However, it again suffers from low sample size. The person designing such study has to understand the basic tenets of genetic epidemiology. Therefore, I do not feel that the manuscript deserves indexing.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Referee Report 04 September 2018

doi:10.5256/f1000research.17000.r37740



**Bhudev C. Das**

Dr. B. R. Ambedkar Center for Biomedical Research, University of Delhi, New Delhi, India

I have gone through the revised manuscript entitled "Absence of toll-like receptor 9 Pro99Leu polymorphism in cervical cancer" submitted by Alex Chauhan et al. The authors have revised the manuscript satisfactorily and answered all the queries therein. The English corrections that I asked for has been incorporated in the revised manuscript and I now recommend final acceptance of the manuscript for indexing of the article.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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### Version 1

Referee Report 02 August 2018

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#### Bhudev C. Das

Dr. B. R. Ambedkar Center for Biomedical Research, University of Delhi, New Delhi, India

I have gone through the manuscript entitled "Absence of toll-like receptor 9 Pro99Leu polymorphism in cervical cancer" submitted by Alex Chauhan et al. for its publication in F1000Research. The authors have studied the polymorphisms of Toll-like receptor 9 (TLR9) Pro99Leu polymorphism in cervical cancer to examine its role in cervical carcinoma in elimination of viral pathogens through recognition of CpG islands. It is suggested that polymorphism of TLR9 gene may influence the recognitions of the above DNA sequence leading to elimination of infection. The authors have used as many as 110 cervical cancer samples and normal cervical smears from 141 healthy controls and employed PCR-RFLP and sequencing methods for detection of genotype variation. The authors could not find any variation in genotypes of TLR9 hence authors concluded that the specific polymorphism C296T/Pro99Leu has no role in cervical cancer.

This is a very clean and straight forward study which could not find any link between TLR9 polymorphism and cervical cancer. The data and the figures presented including PCR and PCR-RFLP and sequencing pictures are excellent and convincing. However, there are few points which need to be clarified/corrected before the manuscript is accepted and indexed. My comments are as follows:

1. The whole study is based on only one TLR9 gene polymorphism. Authors need to very clearly justify their choice of TLR9, and not other TLRs in Introduction as well as in the Discussion.
2. It is well established that the causative agent for cervical cancer is due to infection of specific types of high risk Human papillomaviruses. Any study on cervical cancer demands for an obvious correlation/association with HPV status of the cervical cancer. If at all no HPV analysis has been done, the authors must discuss this in the Discussion.
3. There are several English and grammatical errors throughout the manuscript. Authors need to carefully re-read the manuscript and correct the manuscript. Few obvious errors are indicated here:-
  - i) Methods: 3<sup>rd</sup> line: reaction **and** restriction fragment length

- ii) Conclusion: 1<sup>st</sup> line – the present study demonstrates no involvement – (delete ‘results’), 3<sup>rd</sup> line – delete ‘worldwide’ and in 4<sup>th</sup> line – delete ‘participants’, replace it with ‘subjects’
- iii) Table 1 last column below Visualized on: write 2% Agarose gel.

4. Data set 3: delete ‘unedited’.

In summary, the manuscript may be accepted for indexing after the authors made the minor revision of the manuscript as suggested above.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 20 Aug 2018

**Alex Chauhan**, P D Patel Institute of Applied Sciences, India

Dear Prof. B. C. Das,

We thank you for approving our manuscript and appreciate your valuable suggestions. Please find below the response towards your reservations:

**Reservation 1:** The whole study is based on only one *TLR9* gene polymorphism. Authors need to very clearly justify their choice of TLR9, and not other TLRs in Introduction as well as in the Discussion.

**Response:** The choice of TLR9 is based on the fact that it recognized HPV16 DNA which is a main causative agent of cervical cancer. As suggested this has been included in the introduction as well as discussion.

**Reservation 2:** It is well established that the causative agent for cervical cancer is due to infection

of specific types of high risk Human papillomaviruses. Any study on cervical cancer demands for an obvious correlation/association with HPV status of the cervical cancer. If at all no HPV analysis has been done, the authors must discuss this in the Discussion.

**Response 2:** We do agree that cervical cancer is mainly caused by the infection of hrHPVs. We found approximately 70% of our cases with HPV positivity, that has been incorporated in the discussion. The details of HPV infection will be published elsewhere.

**Reservation 3:** There are several English and grammatical errors throughout the manuscript. Authors need to carefully re-read the manuscript and correct the manuscript. Few obvious errors are indicated here:-

i) Methods: 3rd line: reaction and restriction fragment length

ii) Conclusion: 1st line – the present study demonstrates no involvement – (delete ‘results’), 3rd line – delete ‘worldwide’ and in 4th line – delete ‘participants’, replace it with ‘subjects

iii) Table 1 last column below Visualized on: write 2% Agarose gel.

**Response 3:** The above mentioned suggestions have been incorporated.

**Reservation 4:** Data set 3: delete ‘unedited’.

**Response 4:** Deleted.

**Competing Interests:** No competing interests were disclosed.

Referee Report 23 July 2018

doi:10.5256/f1000research.16153.r35718



**Balraj Mittal**

Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, India

The basic assumption of genetic epidemiology is that two alleles of a gene are present in the population and one of the allele (usually minor) has altered frequency in cases and controls. However, if a gene is mono-morphic as is the case here for TLR9C296T, then it is non-informative and there is no point to look for its association.

Therefore, the very basis of study is wrong, even though it has been carried out technically correct.

I therefore feel that this publication will not anything the present knowledge.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

No

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Referee Report 25 June 2018

doi:[10.5256/f1000research.16153.r34771](https://doi.org/10.5256/f1000research.16153.r34771)



**Gopeshwar Narayan** 

Department of Molecular and Human Genetics, Banaras Hindu University, Varanasi, India

With the global minor allele frequency of 0.0002, the studied sample size is too small. It is interesting to observe that there is only one genotype (homozygous wild type) present in the studied cohort. The power of the study should be mentioned by the authors. The conclusion drawn from the limited data set may not reflect the real situation. I suggest the authors to first calculate the number of samples required for the study on the basis of the frequency of minor/major alleles to achieve about 80% power of study and increase the sample size accordingly.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 29 Jun 2018

**Alex Chauhan**, P D Patel Institute of Applied Sciences, India

Dear Dr. Gopeshwar Narayan,

We thank you for approving our manuscript and appreciate your valuable suggestions. Please find below the response towards your reservations:

**Reservation:** With the global minor allele frequency of 0.0002, the studied sample size is too small. It is interesting to observe that there is only one genotype (homozygous wild type) present in the studied cohort. The power of the study should be mentioned by the authors. The conclusion drawn from the limited data set may not reflect the real situation. I suggest the authors first to calculate the number of samples required for the study on the basis of the frequency of minor/major alleles to achieve about 80% power of study and increase the sample size accordingly.

**Response:** Due to the complete absence of minor allele, the power of present study cannot be calculated. However, considering the global minor allele frequency of 0.0002 of the SNP, we calculated the power of study using [Online Sample Size Estimator](#) which was found to be 3.6%. To achieve 80% power of study approximately 40000 cases and controls are required. It is presently not possible for us to collect and analyze such a larger sample size. Studies on Pro99Leu polymorphism with similar sample size and results have also been reported by Kubarenko et al., 2010 and Lee et al., 2006, which have been cited in the article.

**Competing Interests:** No competing interests were disclosed

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