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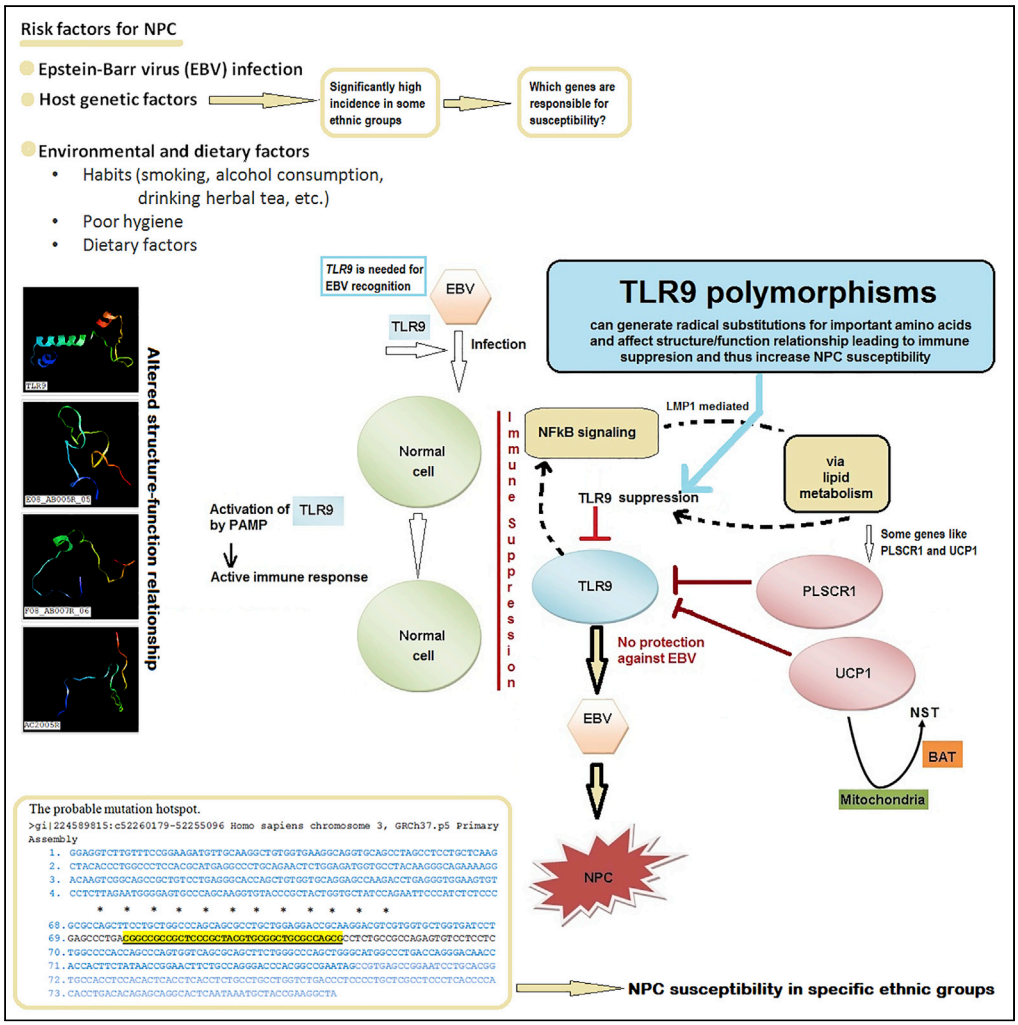

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HIGHLIGHTS

EBV-associated nasopharyngeal carcinoma (NPC) is endemic in Mongoloids

TLR9 is essential for virus recognition and immunity against cancers

Polymorphisms are common in TLR9 protein in EBV-infected Mongoloids with NPC

NPC susceptibility prediction by TLR9 mutation screening in people for prevention

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TLR9 Polymorphisms Might Contribute to the Ethnicity Bias for EBV-Infected Nasopharyngeal Carcinoma

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SUMMARY

Nasopharyngeal carcinoma (NPC) is a rare malignancy in most parts of the world, but is endemic in some ethnic groups. The association of NPC with the Epstein-Barr virus (EBV) is firmly established; however, the mechanism is still unclear. *TLR9* is well known for its essential role in viral pathogen recognition and activation of innate immunity. Here, we report a set of *TLR9* polymorphisms in the TIR-2 domain of the TLR9 protein collected from the EBV-infected NPC samples from northeast Indian populations sharing the aforesaid ethnicity. The occurrence of mutations is significantly high in these samples as we found a p value of <0.0001 at a significance level of 0.05. These might play an important role for the lack of function of *TLR9* and thus for the higher occurrence of EBV-mediated NPC in such ethnic groups.

INTRODUCTION

Nasopharynx is a box-like chamber near the base of the skull and covers the upper region of the throat behind the nose. Carcinoma of the nasopharynx, or nasopharyngeal carcinoma (NPC), starts at the mucosal epithelium of the nasopharynx and in the minor salivary glands present there (Chan et al., 2005; Wee et al., 2010; Wei and Sham, 2005; American Cancer Society, 2016). It may occur at any age and occurs much more frequently in the Chinese and Southeast Asian populations, also known as *mongoloid populations*. NPC accounts for about 0.7% of all cancers in a global perspective, but in endemic populations, it occurs in 0.02%–0.03% males and about 0.01%–0.015% females, and the commonest form is (UC) undifferentiated carcinoma. It is endemic among Chinese (Cancer Incidence in Five Continents, 1987), Maghrebian Arabs in North Africa and Algeria (Cancer Occurrence in Developing Countries, 1986), and the Eskimos in the Arctic (Cancer Incidence in Five Continents, 1982). NPC is uncommon in most regions in India except for the Northeast (NE) region of the Indian subcontinent (Bhatia and Singh, 1981). The NE region in India is well known by a majority of Tibeto-Burman languages and is supposed to be populated by people migrated from East Asia, also bringing the nasopharyngeal cancer with them (Kataki et al., 2011). In non-endemic regions like North America and Europe, this incidence is much lower (about 0.001%, including both genders) and other forms of NPC are seen.

The risk factors of NPC include genetic factors, diet and other environmental factors, and infection with human papillomavirus (HPV) or Epstein-Barr virus (EBV) (http://www.cancer.org/cancer/nasopharyngealcancer/detailedguide/nasopharyngeal-cancer-risk-factors). It is well known that the populations with higher incidences of NPC follow a few interesting dietary habits and their lifestyles maintain poor hygiene. Most people from these populations consume some kinds of preserved foods, for example, the Chinese consume salt-preserved foods, the NE Indian Nagas consume smoke-preserved food, etc. With regard to the lifestyles, all these susceptible populations live in poor hygienic conditions, e.g., staying long time in smoky environment, inhaling various toxic fumes like that of formaldehyde, habits of smoking, etc. The association of NPC with EBV was firmly established in as early as 1973 (Xu et al., 2012). Yet, the role for the virus in the pathogenesis of NPC is still unclear. Almost all cases are EBV positive, irrespective of geographical origin. Although controversial, EBV has been classified as a group I carcinogen by the International Agency for Research on Cancer (IARC), among other reasons, because of its association with NPC (IARC Monographs on the Evaluation of

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Continued

Carcinogenic Risks to Humans, Vol 70, IARC press, Lyon, France, International Agency for Research on Cancer, 1997). EBV, a human gammaherpesvirus, was first detected in African patients with Burkitt lymphoma and infects B cells and epithelial cells (Epstein et al., 1964). This virus is very well known for causing infectious mononucleosis (glandular fever) and various types of cancers including Burkitt lymphoma, Hodgkin lymphoma, NPC, and gastric carcinoma (Klein et al., 2007; Young et al., 2016). After initial infection, the EBV nuclear antigens (EBNAs) are expressed; but eventually different latency programs of EBV give rise to different sets of expressed viral antigens (Alfieri et al., 1991; Halder et al., 2009; Houldcroft and Kellam, 2015). EBNA-2 is one of the first genes expressed upon infection of B cells and is essential for establishment of latent infection and cell growth transformation. So this antigen is used widely for the detection of EBV infection in patients.

Toll-like receptors (TLRs) are innate immune sensors that are integral for resisting chronic and opportunistic infections; they recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents like viruses and bacteria. TLRs are transmembrane proteins expressed on the cell surface and the endocytic compartments. *TLR9*, also called CD289, is expressed in dendritic cells, macrophages, natural killer cells, and other antigen-presenting cells of the immune system in tissues like peripheral blood, spleen, lymph node, and bone marrow. Various infections, cancers, and diseases including some autoimmune diseases, can modulate *TLR9* activation and expression. *TLR9* is activated by unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides common in microbes and starts the antiviral responses by triggering the production of antiviral cytokines such as type I interferons (IFNs). The pathway includes MyD88 and TRAF6, leading to inflammatory responses via nuclear factor (NF)-κB activation and cytokine secretion (Du et al., 2000; Takeshita et al., 2001; Doyle et al., 2007). A recent article shows that *TLR9* can recognize some other nucleotide patterns present in bacterial or viral genomes (Martínez-Campos et al., 2017). However, the *TLR9* protein is usually activated by unmethylated CpGs present in microbial DNA and moves to Golgi apparatus and lysosomes from its initial location, the endoplasmic reticulum. Then the molecule is cleaved to prevent autoimmunity and only a part of the *TLR9* original protein is actually expressed on the cell surface. Eventually the signaling pathway leads toward the production of cytokines such as IL-6 (interleukin-6), tumor necrosis factor, IFN-α, and IL-12.

Mounting evidences implicate the role of TLR-polymorphisms in susceptibilities to various infectious diseases, including human immunodeficiency virus (HIV)-1. Pine et al. investigated the impact of TLR single-nucleotide polymorphisms (SNPs) on clinical outcome in a sero-incident cohort of HIV-1-infected volunteers (Pine et al., 2009; Rahman et al., 2016; Medvedev, 2013; El-Omar et al., 2008). However, no report shows the role of *TLR9* polymorphisms in patients with NPC infected with EBV. In the current study, we tried to search the *TLR9* gene polymorphisms in patients of NE Indian populations with NPC who are EBV-positive, wherein this disease is a common problem. We found some deletions, additions, and point mutations in the *TLR9* gene of such patients, suggesting an important role of these SNPs in the patients of NE Indian region with NPC. It should be noted that NPC is endemic in this region, and the affected population follows similar characteristics as the populations of other endemic regions; thereby the findings of this study might indicate a role of *TLR9* in EBV-positive NPC with a broad spectrum including other endemic populations. We report for the first time that *TLR9* plays an important role in EBV-positive NPC development in the NE Indian population. By performing comparative genomic analyses, we found that *TLR9* is conserved on the same loci from ray-finned fishes to mammals. We specifically analyzed the domains, motifs, and interacting regions that are conserved across various species. We also analyzed the respective selection pressures imposed on these regions from their degree of conservation. These analyses uncover the hidden features of *TLR9* activities dependent on its sequence and of structure-function relationship responsible for generation of diseases.

RESULTS

The study was carried out in collaboration with a few NE Indian centers. Seventy freshly diagnosed patients with NPC along with 70 age- and sex-matched controls were registered for this study from all those centers. Routine histopathological analysis was done for each patient to confirm the diagnosis of NPC. Informed consent was obtained from each and every subject as per the guidelines of research review committee. Approval was obtained from the institutional medical ethics committees of the participating institutes for the study.

EBV Is Well Associated with NPC

One of the major problems associated with NPC is the detection of the disease properly. Cellular characteristics are confusing and poorly understood, so the disease is commonly misdiagnosed. The physical

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symptoms include lump(s) in the neck, hearing loss, recurrent ear infection, stuffiness, headache, blurred vision, nosebleeds, etc. (Figure S1B). Although some tests like physical examinations, endoscopic nasopharyngeal examinations, and computed tomographic imaging, or magnetic resonance imaging are done for the diagnosis of the disease, confirmation by biopsy is still considered as the "gold standard" (Li and Zong, 2014; Li et al., 2012; Wang et al., 2012). In 2005, the updated version of World Health Organization classification of NPC describes three types: keratinizing squamous cell carcinoma (KSCC), non-keratinizing carcinoma (NKC), and basaloid squamous cell carcinoma (Chan et al., 2005). The first variant is invasive; squamous differentiation with intercellular bridges and/or keratinization is present in most part of the tumor(s). Association of EBV in this type is low. The second type of NPC or the non-keratinizing type of NPC is subdivided into two varieties: the differentiated type (NKDC) and the undifferentiated type (UC), of which the latter is more prevalent in the high-incidence regions. Overall, this undifferentiated variety is the major type of NPC seen throughout the world. Here, the cells have vesicular nuclei, large nucleoli, and less distinct cell borders. These make the cell clusters look syncytial and overlapping. A huge amount of lymphocytes and plasma cells are found in these cell clusters, thus the epithelial nature of the tumor(s) is lost. The differentiated variety has clear cellular stratification and is uncommon in the high-incidence regions. The third type or the basaloid squamous cell carcinoma of the nasopharynx is very rare and harbors closely packed small cells with hyperchromatic nuclei without nucleoli and a small amount of cytoplasm. Stages differ by the depth of invasion of the soft tissue and bony structures at and near the nasopharynx, degree of presence of affected cranial nerves, and the involvement of local and regional lymph nodes of the head and neck (Wei et al., 2011). We have performed histopathological examinations of the biopsy samples taken from the patients and confirmed NPC in all of them. The tumor samples were stained with H&E and were examined microscopically, showing evidences for both keratinizing and non-keratinizing types, but not the basaloid type. All types are compared with the photomicrograph showing normal nasopharyngeal tissue lined by respiratory-type pseudo-stratified columnar epithelium, the non-keratinizing type (Figure 1A). The undifferentiated types show evidence of a little keratinization accompanied by inflammatory infiltrate rich in lymphocytes and composed of tumor cells arranged in a diffuse pattern inside nests surrounded by fibrous tissue. The non-keratinizing type shows tumor cells with mixed inflammatory infiltrate, predominantly lymphocytes. The keratinizing types show distinct evidence of keratinization. After confirmation by histological examinations, these samples were further used for genetic studies.

EBV and HPV both have been reported to be associated with NPC. After initial infection, the EBNAs are expressed, EBNA-2 being the first of those (Lung et al., 2014). EBNA-2 is essential for establishment of latent infection and cell growth transformation (Cohen et al., 1989; Hammerschmidt and Sugden, 1989), whereas EBNA-1 is known to function for the lytic phase with its ability to bind DNA in a site-specific manner (Frappier and O'Donnell, 1991). EBNA-1 is a stable homodimer that recognizes a DNA-binding site 16 bp in length (Baer et al., 1984). Soon after the EBNA-2 expression, the EBV latent membrane proteins 1 and 2 (LMP1 and LMP2) are expressed. For the determination of association of EBV infection in NE Indian patients with NPC, we have examined the presence of EBNA-1 and EBNA-2 genes in the blood samples of patients with NPC and controls. After PCR amplification, all those samples showed the presence of EBNA-1 or EBNA-2 (Figure 1B, only a few representatives are shown).

Different Types of Mutations Are Identified in *TLR9*

Various signaling pathways have been reported for their contributions in development and pathogenesis of NPC (Chan, 1980; Tulalamba and Janvilisri, 2012). As there is cumulative evidence showing the possible role of *TLR9* in the pathogenesis of NPC, we hypothesized that some SNPs/mutations of the *TLR9* gene might be associated with susceptibility toward and/or the severity of NPC in the NE Indian population representing a distinct ethnic group. PCRs were performed with the DNA samples isolated from blood samples of patients and controls (all primers are listed in Table S3). For the control group, blood samples were collected from healthy age- and sex-matched volunteers from same ethnic background. The PCR-amplified DNA samples were purified and sequenced. These sequences were compared with a standard genomic sequence of *TLR9* obtained from the published database of National Center for Biotechnology Information (NCBI) database (Database: >gi|74054321|gb|AAZ95520.1| *TLR9* [*Homo sapiens*]). Mutation screening in those sequences was performed by Finch TV software (https://digitalworldbiology.com/FinchTV). In addition, we have checked the mutations by direct sequencing and using the ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalo/), and all the sequences were authenticated by checking in human BLAST with the help of NCBI (www.ncbi.nlm.nih.gov). We have found some mutations in exon 2 of *TLR9* gene

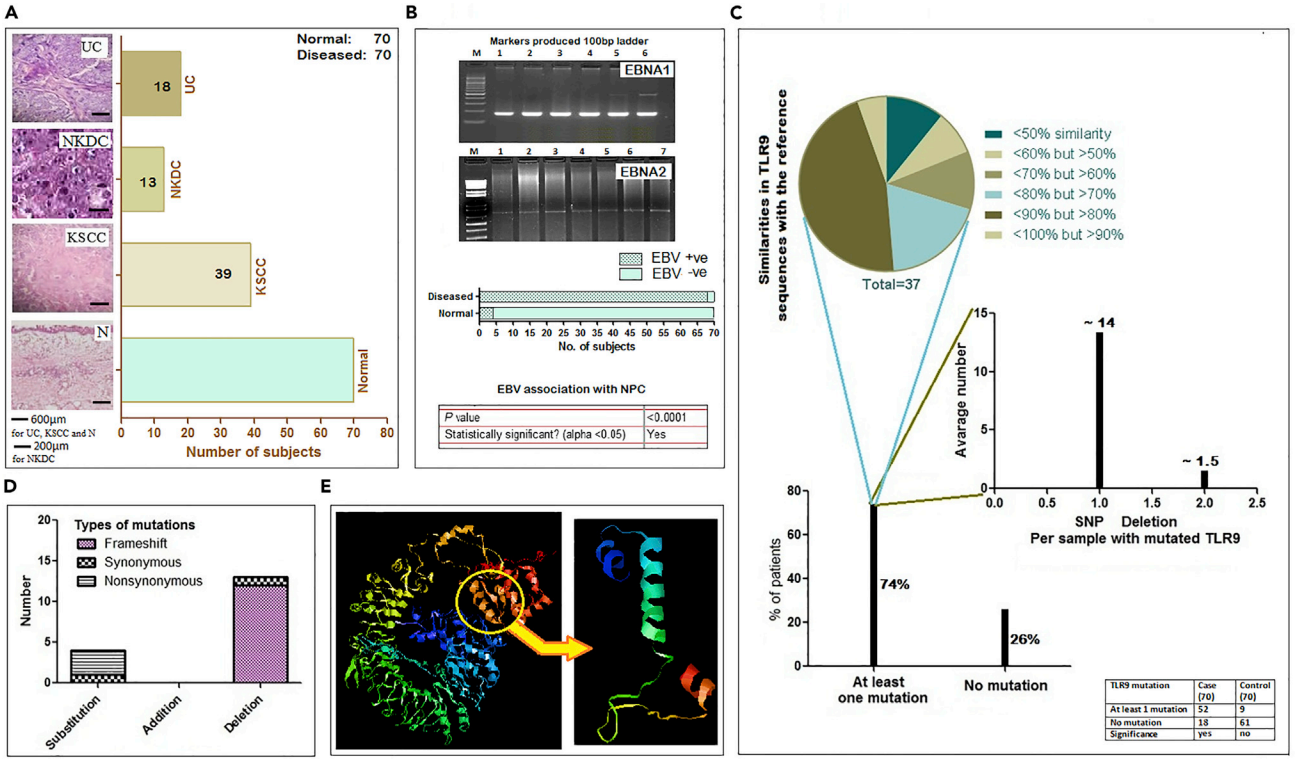

Figure 1. EBV Association with NPC and *TLR9* Mutations

(A–E) NPC has three types: keratinizing squamous cell carcinoma (KSCC), non-keratinizing carcinoma (NKC), and basaloid squamous cell carcinoma. Non-keratinizing carcinoma of NPC is subdivided into two varieties: the differentiated type (NKDC) and the undifferentiated type (UC).We have performed histopathological examinations of the biopsy samples taken from the patients and found KSCC, NKDC, and UC, but no basaloid type (A). After initial infection, the EBV nuclear antigens (EBNAs, EBNA-1 and EBNA-2) are expressed, both of which were detected in the blood samples of patients with NPC and in a few controls. After PCR amplification, all those samples showed the presence of EBNA-1 or EBNA-2, and a significant association of EBV with NPC is found (B, only a few representatives are shown). Mutation screening in *TLR9* sequences from patients and controls reveal some non-synonymous and synonymous substitutions, addition, and deletion in the exon 2 of *TLR9* gene of some samples, of which occurrence of mutations in patients has been found to be significant. At least one mutation is seen in 74% patients, and about 50% of them showed less than 80% similarity with the reference sequence. p value was calculated using GraphPad Prism software (https://www.graphpad.com/scientific-software/prism/) considering the p value of <0.05 as statistically significant. Considering the average number of mutations, more than 13 SNPs and more than 1 deletion are found per sample of mutant *TLR9* (C and D). The three-dimensional structure of full TLR9 sequence and the domain where the mutations are found, as predicted by Phyre[2] software (E). See also Figure S1 and Table S1.

of the collected NPC samples; of those some mutations are non-synonymous and some are synonymous. It was found that all patients with UC carry at least one mutation, five patients with NKDC had at least one mutation, and 29 patients with KSCC were detected with mutated TLR9. Along with base substitutions, we have also found addition and deletion in the exon 2 of *TLR9* gene of some samples (Figures 1C and 1D, Table 1). A 3-dimentional (3D) model of the wild-type reference TLR9 and the domain where the mutations are found is shown in Figure 1E (generated by Phyre[2] and Raswin, as detailed later).

As stated earlier, TLRs recognize conserved molecular patterns on specific classes of pathogens and initiate a series of signaling events that leads to the expression of pro-inflammatory genes. Recent studies show genetic polymorphisms in TLRs in various pathogenic diseases improving our understanding of the relationship between TLRs and pathogenesis. These in future may provide a rational basis for developing novel therapies to treat these important diseases. A role for *TLR9* on viral, fungal, mycobacterial, and *Helicobacter pylori* infections each has been described by some workers earlier (Berrington and Hawn, 2007; Carvalho et al., 2008, 2009). In humans, the *TLR9* gene is located on chromosomes 3 and is polymorphic (Georgel et al., 2009). Among the TLRs that bind nucleic acids, *TLR9* recognizes unmethylated CpG DNA motifs (present at a much higher frequency in the genomes of prokaryotes and virus than that of eukaryotes) as a "danger signal" that activates the innate immune system (Carvalho et al., 2012). In humans, this receptor is expressed in plasmacytoid dendritic cells and B lymphocytes, cells that are known to have a

Sl. No.	SNP	Triplet Code Change	Amino acid Change	Mutation	Addition	Deletion
1	A>52195766T	TGG > AGG	Trp > Lys	+	–	–
2	G > 52195702Del	AGT > A-T	Ser>_	–	–	+
3	52188102A > Del	GAA > GA-	Glu>_	–	–	+
4	52188114CA > Del	CAG>__R	Gln>__	–	–	+
5	52188159C > G	CCC > GCC	Pro > Ala	+	–	–
6	52195757G > C	CCC > GCC	Pro > Ala	+	–	–
7	A>52188093Del	GAA > GA _	Glu > –	–	–	+
8	52188160A > Del	AAA > AA-	Lys > Lys	–	–	+
9	52188093A > Del	AGA>_GA	A rg > –	–	–	+
10	52188152C > A	GCC > GCA	Ala > Ala	+	–	–
11	52188170A > Del	AAG > Del	Lys>_	–	–	+
12	52188186 A > -Del	AGT>_GT	Ser > –	–	–	+
13	52188093A > Del	AAG > A_G	Lys>__	–	–	+
14	52188151C > Del	CCA > -AA	Gln > –	–	–	+
15	521880107C > Del	TTC > TT_	Phe > –	–	–	+
16	52188151C > Del	CCA > -AA	Pro > –	–	–	+
17	52188151C > Del	CCA > -CA	Pro > –	–	–	+

Table 1. Toll-like Receptor 9 (Third Chromosomal Position) Mutations, Deletions, and Additions in Northeast Indian NPC Samples

diverse TLR repertoire with the TLR9 being predominant. EBV lives latently in B lymphocytes and can eventually enter the lytic cycle. During an infection, *TLR9* is stimulated by unmethylated CpG sites of EBV and triggers the activation of macrophages, B lymphocytes, and dendritic cells, leading to the production of various cytokines, chemokines, and immunoglobulins. Later on, some of these molecules, like IL-2, help the T cells to differentiate into activated T helper 1 and cytotoxic T cells (Krieg, 2007). Interestingly, these initial immune responses toward EBV start to subside as LMP genes start to be expressed. It has been reported recently that EBV persistence may be favored by downregulation of *TLR9* via LMP1-mediated NF-κB activation (Fathallah et al., 2010). LMP1 activates NF-κB, which prevents *TLR9* promoter activity, and thus prevents it from its antimicrobial activity. Another protein, which is an EBV lytic phase protein called BGLF5, triggers the degradation of *TLR9* mRNA (van Gent et al., 2011). Persistence of EBV in humans and generation of various cancers including NPC can be justified by such inhibitory methods adopted by the virus to subside the action of *TLR9* (Jordi et al., 2017). The most important finding of these literatures is that loss of function of *TLR9* might result in the initiation and progression of EBV-mediated cancers including NPC. In our study, we found various mutations in the coding region of the gene (in exon 2, actually), of which, most result in the loss of function of *TLR9*. So, such mutations in the NE Indian patients indicate a genetic bias that might be attributed to the higher incidence of NPC in that ethnic group.

Highly Conserved *TLR9* Depicts Mutations in a Specific Domain

The presence of TLRs is restricted to higher eukaryotes only except in most birds and reptiles. The lowest organism where any TLR channel is documented so far is the zebrafish (Yajuan et al., 2017). Comparative genomic analyses of *TLR9* depicted that the *TLR9* gene is conserved on the same locus from fishes to human over a period of 450 million years with sets of conserved flanking genes (Figure 2A). Syntenic analyses also demonstrated that some fishes have multiple copies of *TLR9* like in Atlantic fish; there are five paralogs of *TLR9* known as *TLR9* a–e (Figure 2A).

In most of these organisms including zebrafish, *TLR9*, if present, recognizes CpG-oligodeoxynucleotides and includes signaling pathways related to those of humans (Mutwiri, 2012; Kumagai et al., 2008). *TLR9*

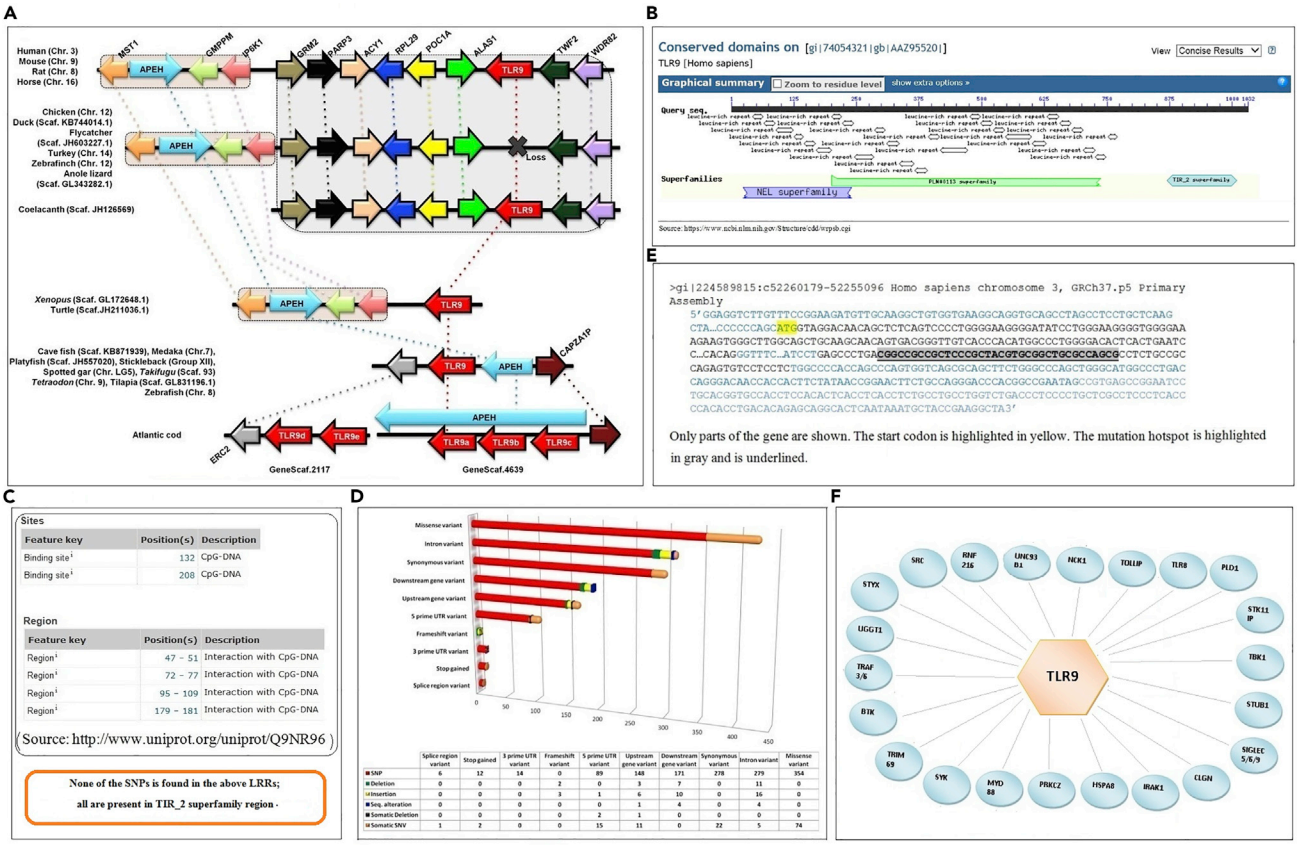

Figure 2. Genetic Locus of *TLR9*, Conserved Domain, and Mutation Hotspot

(A–F) Synteny analyses of *TLR9* gene reveals that *TLR9* gene is conserved from fishes to human for about 450 million years (A). *TLR9* gene codes for a 1,132-aa-long protein with some conserved domains, most of which are leucine-rich repeats (LRRs). (B) Both the regions (aa 132 and aa 208) that can bind pathogenic unmethylated CpG are located in LRRs. These two amino acids interact with four other peptide stretches along the *TLR9* protein, three of which are located in LRRs (C, upper panel). None of the SNPs in our study has been found in these regions, suggesting that either the IL-mediated signaling is affected or any structural alteration of *TLR9* makes it nonfunctional (C, middle and lower panels). None of the SNPs in our study is found to be located in LRRs; all are present in an N-terminal region belonging to TIR_2 superfamily (Toll/interleukin-2 receptor). Each sequence of exon 2 of *TLR9* from each patient with NPV who was EBV positive was aligned with the standard *TLR9* sequence, and we have found various sequence alterations in the full *TLR9* gene (D). A total of 17 different types of mutations are found to be present in the TIR-2 domain of exon 2 of the *TLR9* gene of mutant samples. These mutations are found in a small region of the gene at its 3′ end, a region that can be considered as the mutation hotspot (E). Calculations include 5% or fewer errors. In humans, *TLR9* protein can interact with more than 20 other proteins and initiate downstream signaling thereof (F). See also Table S2.

gene codes for an 1132-amino acid (aa)-long protein, which contains some conserved domains, most of which are the leucine-rich repeats (LRRs) (Databse: https://www.ncbi.nlm.nih.gov/cdd) (Figure 2B). LRRs are generally 20- to 30-aa-long and contain a significantly higher amount of the hydrophobic amino acid leucine; contributing to the β-sheet structures in the corresponding protein. These regions function as the major areas of molecular interactions. In *TLR9*, LRRs can interact with microbial lipopolysaccharides and/or other pathogenic molecules, thereby facilitating the recognition of, and innate immunity to, foreign invaders. Both the regions (aa 132 and aa 208) that can bind pathogenic unmethylated CpG are located in LRRs (http://www.uniprot.org/uniprot/Q9NR96). Of note, no post-translational modification is seen in these two amino acid positions, and in our study, no SNP is found in these two positions. For the CpG-binding function, these two amino acids interact with four other peptide stretches along the *TLR9* protein, three of which are located in LRRs (Figure 2C). Interestingly, none of the SNPs in our study is found to be located in LRRs; all are present in a region belonging to the TIR_2 superfamily (Toll/interleukin-2 receptor) (Table 1). This indicates two possible effects of the SNPs. First, as none of those are present in LRRs, the interaction of *TLR9* with EBV is not altered at all. These SNPs make the specific NE Indian population susceptible toward NPC by altering the IL-mediated signaling. The second possibility is that the SNPs alter the folding pattern of *TLR9* in such a way that its function becomes compromised (Figure 2C).

Each sequence of exon 2 of *TLR9* from each patient with NPC who is EBV-positive was aligned with the standard *TLR9* sequence as mentioned earlier. We have found various sequence alterations in the full *TLR9* gene (Figure 2D), of which at least 1 of 17 types of mutations was found to be present in the TIR-2 domain of exon 2 of the *TLR9* gene of 74% patient samples (Table S1). This value is quite high, and we found it statistically significant considering the p value of < 0.05 (Figure 1C, right lower part). Depending on these data, it has also been found that these mutations are found in a small region of the gene at its 3′ end, a region that can be considered as the mutation hotspot (Figure 2E and Table S2). About half of the patients with mutated *TLR9* show less than 80% sequence similarity with the reference *TLR9* sequence, and most of those are due to SNPs (Figure 2E). It is of note that the N-terminal region of the final *TLR9* protein plays a vital role in the interaction of the protein with the unmethylated CpG of the pathogens, although the exact molecular mechanism is not known (Ohto et al., 2015). The C-terminal domain is responsible for downstream signaling and interactions with various cellular proteins (Figure 2F).Therefore it may be inferred that mutation(s) in the 3′ end of the gene results in lack of function of TLR9 for immunity against EBV, leading to higher susceptibility toward NPC (Figure 2C, lower right panel). However, elucidation of the role of any individual SNP needs a detailed investigation of *TLR9* structure-function relationship.

As living habitats and environments encountered by different species are tightly linked with detection of immunity and further recruitment of downstream signaling, *TLR9* provides an example of a unique molecule that can be studied in the context of molecular evolution. In humans, this protein can interact with more than 20 other proteins and can initiate downstream signaling thereof (Figure 2F). Most of these proteins are found in other higher eukaryotes having *TLR9* signaling cascade. Therefore the subtle changes and retention of domains and motifs that are essential for the function can also be explored by comparing the multiple sequences from different organisms. We have retrieved the *TLR9* sequences of different species available in public databases and have analyzed the molecular evolution of *TLR9* by fragmenting the *TLR9* amino acid sequence in different domains and motifs (Figure 3A). We specifically analyzed the regions that are conserved across those species and also analyzed the respective selection pressure imposed on these regions from their degree of conservation. We used MUSCLE alignment program to align the amino acid sequences of *TLR9* for the purpose of phylogenetic analysis. We implemented a Bayesian phylogenetic tree constructed by the Bayesian approach (5 runs, 7,500,000 generations, 25% burn-in period, WAG matrix-based model in the MrBayes 3.2 program; Figure 3B). The aligned data were subsequently imported into R statistical tool for statistical analysis. As the complete *TLR9* sequences from certain species are not available (mostly due to sequencing errors at certain regions), the analysis was conducted with the available sequences only. As expected, domains and motifs show conservation among varied species in due course of evolution, reflecting the important role of *TLR9* in survival. Polymorphisms are present, but they do not affect the structure-function relationship along the evolutionary lineage.

Absence of TLR9 Protein Is Not Attributed by These Mutations

Previously we have shown that none of the mutations are found in the CpG-interacting region of TLR9; therefore the ability of this protein to interact with and recognize foreign DNA is not compromised. This finding led us to hypothesize two phenomena. First, recognition of EBV as the causative agent of NPC does not play the major role; instead, any loss of function actually contributes to the EBV-mediated carcinogenic process. Like other TLRs, TLR9 can recognize a variety of microbes bearing similar PAMPs, e.g., unmethylated CpG (Martínez-Campos et al., 2017). This may initiate similar kinds of downstream signaling, but the success of pathogen clearance depends on activation of specific second messengers and other immune molecules. As mentioned earlier, the proper execution of TLR9 function is dependent on cellular proteins like MyD88 and IRAK4, as well as pathogenic proteins like LMP1 and BGLF5 (Du et al., 2000; Takeshita et al., 2001; Doyle et al., 2007; van Gent et al.). The balance between the direct activation of NF-κB pathway by TLR9 and suppression of TLR9 by LMP1-mediated activation of NF-κB pathway might play a vital role in this process, as the latter mechanism is related to brown adipose tissue (BAT)-generating lipid metabolism seen in the Mongoloids (Fathallah et al., 2010; Jimenez-Preitner et al., 2011, 2011; Talukder et al., 2012; Kostjuk et al., 2012; Nagajyothi et al., 2012; Köberlin et al., 2016).

The second thing we hypothesized is that the presence or absence of TLR9 protein is not critically affected, at least due to these mutations. The functional regulation of TLR9 is outlined in the previous paragraph and is also detailed in Introduction. Therefore we tried to test whether these mutations are responsible for any loss of TLR9 protein or not. We performed immunohistochemical staining of TLR9 in deparaffinized NPC tissue sections by streptavidin-peroxidase method and diaminobenzidine. Hematoxylin was used to stain

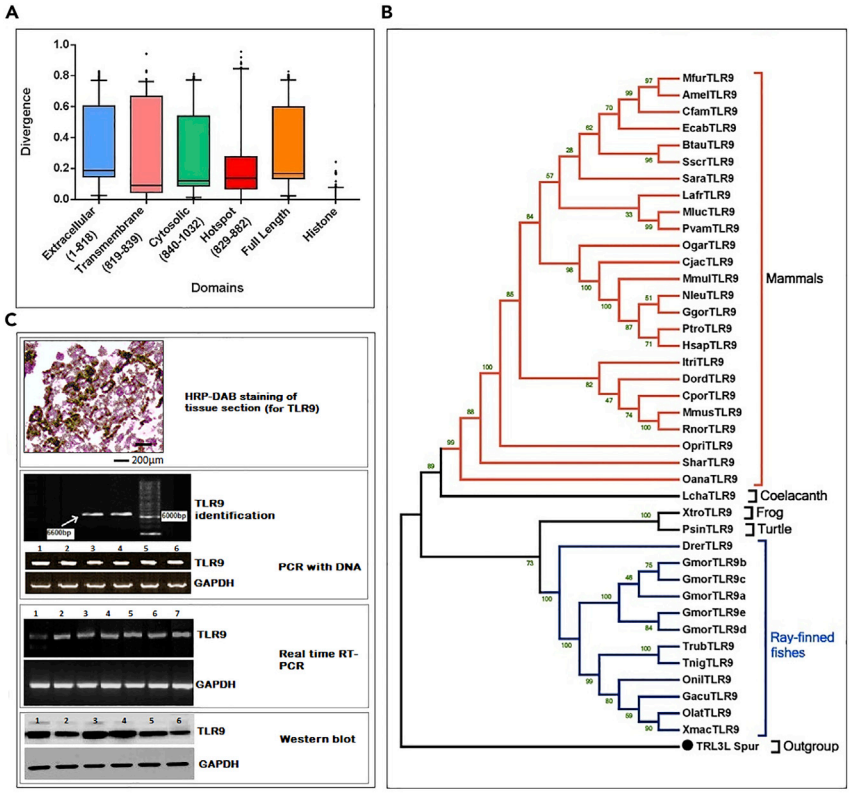

Figure 3. Functional and Evolutionary Importance of *TLR9*

Divergence analysis shows that the mutation hotspot is more conserved than the full-length *TLR9* throughout the vertebrate evolution, suggesting high selection pressure on this region. However, this region contains conserved sequence patterns within different ethnic groups in humans. Although our study needs further experiments to infer about the structure-function relationship affected by the mutations, presence of polymorphisms in this region suggests a strong such role for the protein (A). Phylogenetic analysis of domains and motifs shows conservation among varied species in due course of evolution, reflecting the important role of *TLR9* in survival. Polymorphisms are present in other regions also, but they do not affect the structure-function relationship along the evolutionary lineage. Phylogenetic analysis of domains and motifs shows conservation among varied species in due course of evolution, reflecting the important role of *TLR9* in survival. Polymorphisms are present in other regions also, but they do not affect the structure-function relationship along the evolutionary lineage (B). Presence of TLR9 has been seen in all samples tested by streptavidin-peroxidase method and diaminobenzidine (DAB) (C, upper panel). We further tested the presence of TLR9 gene, RNA, and protein by performing PCR, real-time RT-PCR, and western blot (C, second, third, and fourth panels, respectively). No significant absence of TLR9 was noted. See also Tables S1–S3.

the base tissue, and the sections were examined microscopically. Presence of TLR9 has been seen in all samples tested (Figure 3C, upper panel). We further tested the presence of TLR9 by performing real-time RT-PCR from RNA extracted from fresh tissues. First we identified the specific band of TLR9 gene by performing PCR with DNA extracted form NPC tissue samples (Figure 3C, second panel). For testing RNA, we have taken random samples and run all the PCR products simultaneously. No significant absence of TLR9 RNA was noted (Figure 3C, third panel). To complement our findings, we performed western blot analyses from protein samples extracted from the same tissues as used in the above-mentioned PCR experiment. We found no significant absence of TLR9 protein in random samples (Figure 3C, lower panel). Therefore, we inferred that the detected mutations or SNPs affect the function of TLR9 and eventually the downstream signaling is altered.

The most significant second messenger of TLR9 is MyD88. It interacts directly with TLR9 protein to start immune activation. Therefore we tried to check the effect of presence or absence of TLR9 on MyD88 and some other downstream proteins like TRAF6, IRAK1, and IRAK4. Although these proteins are important in TLR9 signaling, it should be noted that these proteins are expressed from unrelated genes (Du et al., 2000;

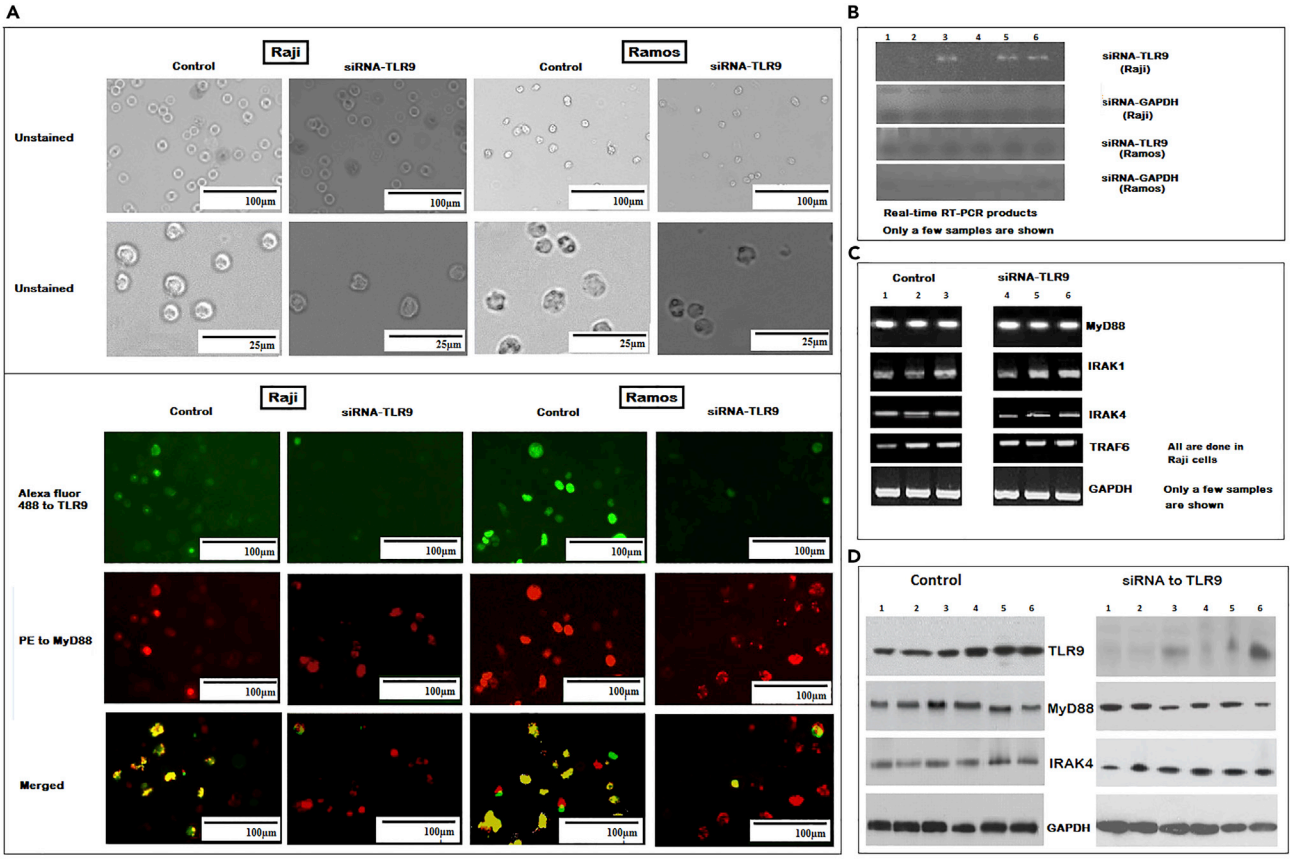

Figure 4. Alteration of Downstream Signaling Is Not Attributed to Total Absence of TLR9 Protein

(A–D) Cultures of Raji (EBV-positive B cell line of head and neck cancer origin) and Ramos (EBV-negative B cell line of head and neck cancer origin) cells were transfected with siRNA for TLR9. Treated and control sets were examined, but no significant morphological change was observed (A, two upper rows). Each of these cells was then treated with fluorescently tagged antibodies to visualize the presence and interaction of TLR9 and MyD88 (A, third and fourth rows). As seen under the microscope, absence of TLR9 in siRNA-treated cells does not affect the presence of MyD88. However, MyD88 seems to colocalize and interact with TLR9 when both are present (A, lower row). Lack of or reduced TLR9 RNA is seen in RT-PCR in siRNA-treated cells, as expected (B). Presence of MyD88, TRAF6, IRAK1, and IRAK4 were also tested in RT-PCR, and no significant reduction is noted (C). Western blot analyses of TLR9, MyD88, and IRAK4 were done, but no significant alterations were found (D). For experimental control, siRNA to GAPDH was tested. See also Table S3.

Takeshita et al., 2001; Doyle et al., 2007). Therefore, lack of TLR9 should not imply any compulsion on the presence or absence of these proteins. We have established the cultures of Raji (EBV-positive B cell line of head and neck cancer origin) and Ramos (EBV-negative B cell line of head and neck cancer origin) and transfected those with small interfering RNA (siRNA) for TLR9. For experimental control, siRNA to GAPDH was tested. We have performed real-time RT-PCRs and western blot experiments for TLR9 and a few proteins as stated above. Four sets were established for these; Raji control, Raji with TLR9-siRNA, Ramos control, and Ramos with TLR9-siRNA. Each set was run in triplicate. Cells from each set were examined microscopically, but no significant morphological change was observed (Figure 4A, two upper rows). Transfection efficiency was not measured for any as transfection in both Raji and Ramos are quiet difficult. Only a few cells survived and run for around a week before examination. Each of these cells were then treated with fluorescently tagged antibodies to visualize the presence and interaction of TLR9 and MyD88 (Figure 4A, third and fourth rows). As seen under the microscope, absence of TLR9 in siRNA-treated cells does not affect the presence of MyD88. However, MyD88 seems to colocalize and interact with TLR9 when both are present (Figure 4A, lower row).

Total RNA and total protein were extracted from each of these sets of cells for further experiments. Lack of or reduced TLR9 RNA is seen in RT-PCR in siRNA-treated cells, as expected (Figure 4B). Presence of MyD88, TRAF6, IRAK1, and IRAK4 was also tested in RT-PCR, and no significant reduction is noted (Figure 4C). Western blot analyses of TLR9, MyD88, and IRAK4 were done to further complement the PCR experiments

(Figure 4D). Analyzing these results, we inferred that the mutations do not affect the presence or absence of the protein, but alter the function of TLR9 by altering its structure-function relationship.

Radical Mutations Might Affect the Function of *TLR9*

The variations in amino acid sequences of *TLR9*, as revealed in 1,000 human genome sequences collected from public databases (Database: www.ncbi.nlm.nih.gov), have been used in our study for Grantham distance calculation. Align-GVGD is an extension of the original Grantham difference to multiple sequence alignment. It combines the biophysical characteristics of amino acids and protein multiple sequence alignments to predict where missense substitutions in genes of interest might have deleterious or neutral effects. The probability of amino acid substitutions are negatively correlated with the physicochemical distances, which are based on amino acid properties. As higher Grantham score reflects a greater evolutionary distance, higher scores are considered more deleterious and their effects are more damaging. In our study, we have found amino acid substitutions having significant physicochemical distances among them, thus a possible alteration of structure-function relationship leading to inactivation of *TLR9*. We have also found some deletions in the DNA resulting in frameshift during protein production. This again indicates a loss of function of *TLR9* leading to disease susceptibility. A region of the transmembrane (TM) part and two regions in the cytoplasmic (C) part contain the possible mutation hotspot (Figure 5). Hence, these amino acid substitutions or frameshift mutations might affect the signaling cascade initiated by *TLR9*. The signal peptide shows no mutation; therefore the subcellular localization of *TLR9* is not affected in the patients with NPC, at least in our study. Similarly, no mutation is found in the CRAC (cholesterol recognition amino acid consensus) and CARC (the reverse version of CRAC) domains, indicating no alteration for association with cholesterol. Interestingly, none of these domains is present in the TM part of *TLR9*, but two CRAC domains are present close to the mutation hotspot present in the cytosolic region. Therefore any mutation, which can affect the structure of *TLR9*, might affect the CRAC-related downstream signaling cascades.

According to the Align-GVGD analysis, it can be noted that amino acid substitutions with high risks of deleterious effects are seen in NE Indian patients. Polymorphisms in various amino acid positions have low through moderate to high risks for NPC (Figure 6A, left panel). In our study, we have found a few polymorphisms that can be considered with a very high risk for disease susceptibility (Figure 6A, middle and right panels, red dots). We have also analyzed the amino acids by predicting their SIFT (scale-invariant feature transform) scores and PolyPhen-2 scores. These two are the two most commonly used algorithms for predicting if an SNP has any effect (generally negative effect) on a protein's structure. Both scores use the same range, 0.0–1.0, but with opposite meanings; a PolyPhen score of 0.0 is predicted to be benign, whereas a SIFT score of 1.0 is predicted to be benign. After analyzing all the sequences we got a few mutations that can be considered deleterious in accordance with all three scores, i.e., GD score, SIFT score, and PolyPhen-2 score (Figure 6B). Therefore, loss of function of *TLR9* is expected in mutants and susceptibility toward NPC may be inferred (Figure 6C). As stated earlier, genetic polymorphisms in TLRs, like other genes, are conserved among ethnic groups. Therefore the findings of our study strongly indicate a role of *TLR9* polymorphisms for susceptibility toward NPC in NE Indian populations with higher incidence.

To further investigate the effects of these mutations on the structure-function relationship of TLR9, we have performed 3D modeling study with the help of two different software named Phyre^2 (http://www.sbg.bio.ic.ac.uk/phyre2) and Raswin (http://rasmol.org/) (Figure 7A). Only the reference part of wild-type TLR9 and three other mutated samples are shown for simplicity. The uppermost part of the figure shows the sequences aligned with ClustalW, as mentioned earlier. Base substitutions in the mutated sequences are marked in red. Just below this alignment, 3D-folding prediction of each of the above-mentioned four sequences is shown; against each, its PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) analysis is shown. With the help of this software, each amino acid of a sequence has been studied for its character (aatypes), its possibility to be present in a particular type of protein folding (psipred), and its possibility to interact with the membrane (memsat). The Phyre^2 results show that the 3D structures are distinctly different from that of the wild-type sequence, indicating the loss of function due to aberrant structure caused by the mutations. The PSIPRED analysis classified as the "psipred" format shows that none of the mutated sequences carry amino acids required for helical structure formation like the wild-type. This again justifies the possibility of loss of function due to the structure-function relationship. The characters of the substituted amino acids, as shown in the "aatypes" format, complement this. The "memsat" format of the sequence analysis indicates a role of the mutation having an even greater importance. The substituted amino acids mostly are unable to interact with the membrane; thereby indicating that mutated TLR9 protein may be produced inside the cell,

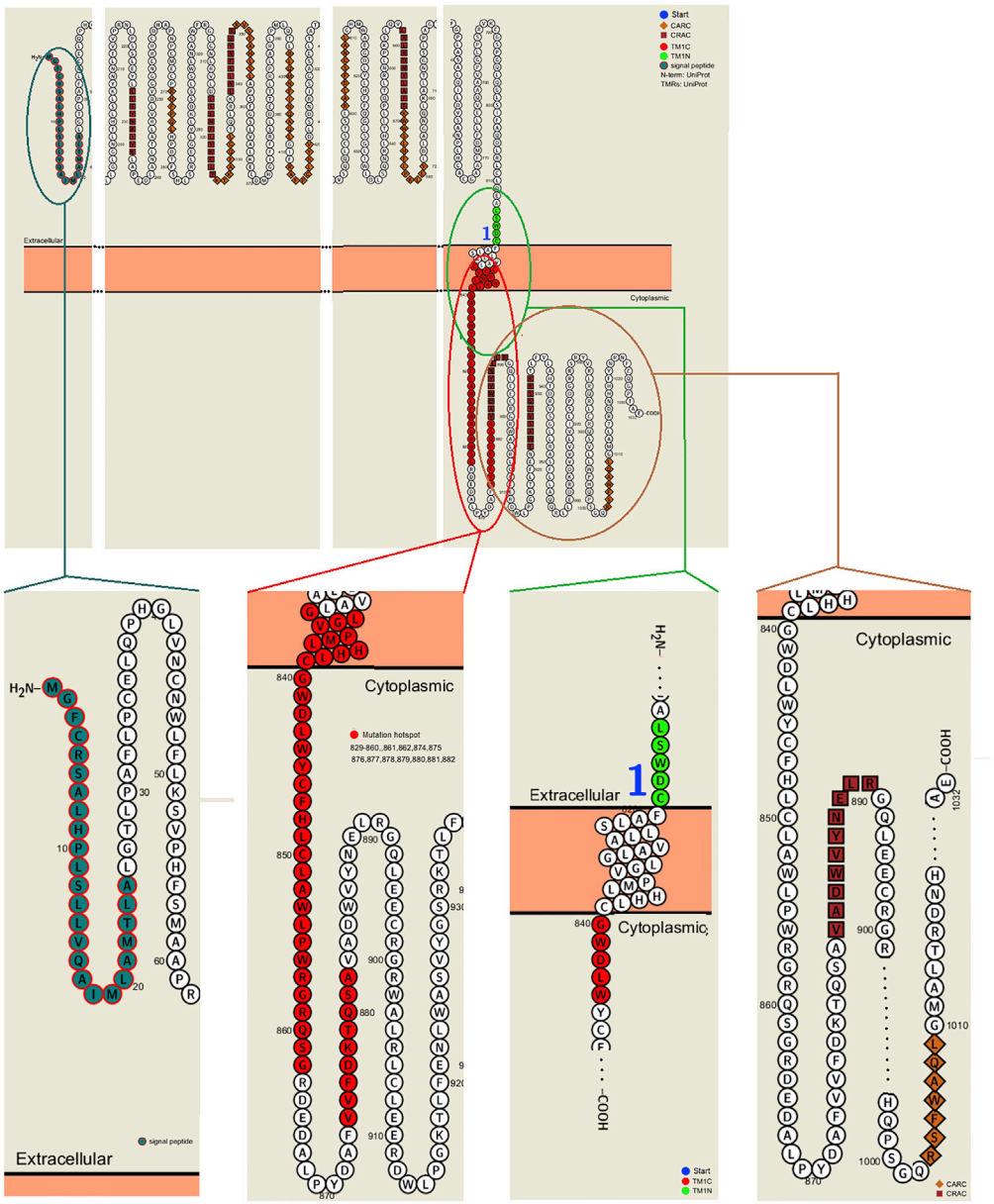

Figure 5. Important Regions of *TLR9* and Possible Regulation by Membrane Cholesterol

The signal peptide shows no mutation, therefore the subcellular localization of TLR9 is most likely not affected in patients with NPC. Similarly, no mutation is found in the CRAC (cholesterol recognition amino acid consensus) and CARC (the reverse version of CRAC) domains, possibly indicating no alteration for association with cholesterol. A region of the transmembrane (TM) part and two regions in the cytoplasmic (C) part contain the possible mutation hotspot, and two cytosolic CRAC domains are present close to this hotspot. Hence mutation in this region is expected to affect the structure of *TLR9*, most likely to be influenced by membrane cholesterol, and thus may affect the CRAC/CARC motif-related downstream signaling cascades. See also Tables S1 and S2.

but that it is unable to be integrated into the cellular membranes. This lack of integration can destroy the proper functioning of TLR9 and is reported elsewhere (Cornélie et al., 2004; Mouchess et al., 2011; Majer et al., 2018).

Interestingly, the amino acids found in the transmembrane region and a part of the following carboxy-terminal region in wild-type TLR9 protein matches exactly with the predicted roles of amino acids in

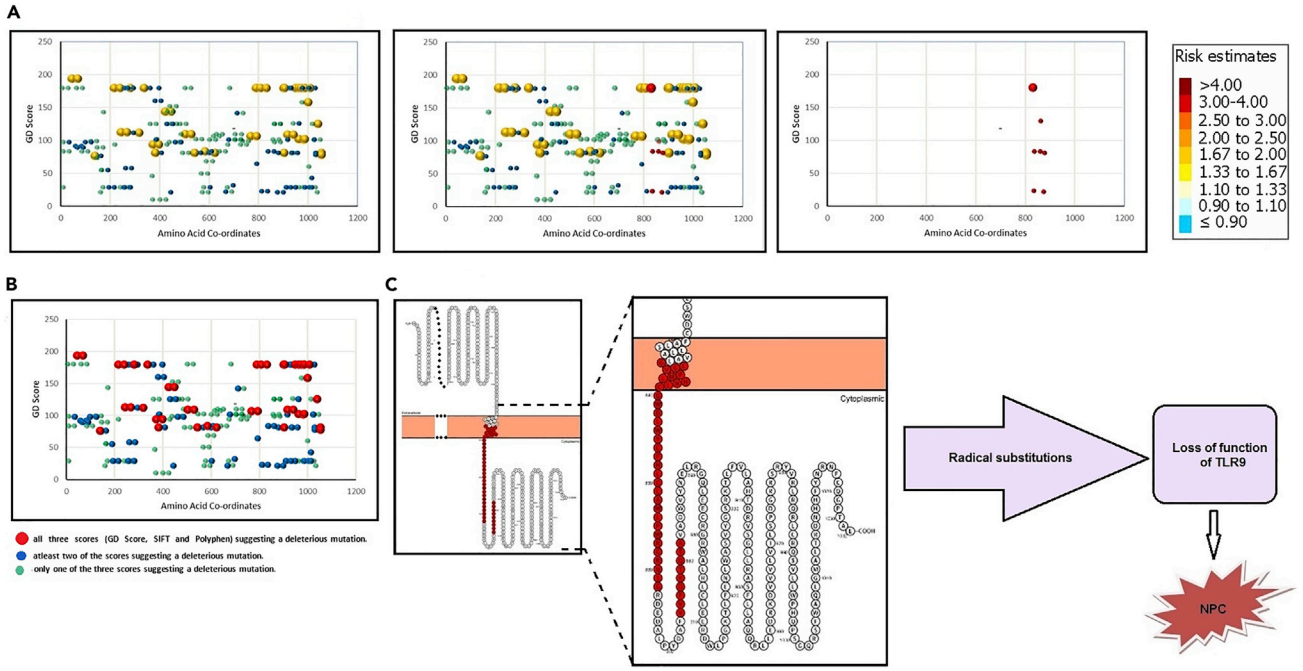

Figure 6. Radical Mutations Might Affect the Function of *TLR9*

(A–C) Align-GVGD is an extension of the original Grantham difference (GD) score to analyze the impact of amino acid substitutions. A higher Grantham score reflects a greater evolutionary distance and is considered more deleterious; effects of such mutations with higher scores are more damaging. Polymorphisms in various amino acid positions have low through moderate to high risks for NPC (A, left panel). We have found seven polymorphisms that can be predicted to be associated with a very high risk for NPC susceptibility (A, middle and right panels, red dots). We have also analyzed the amino acids by predicting their SIFT (scale-invariant feature transform) scores and PolyPhen-2 scores. We got a few mutations that can be considered deleterious in accordance with all the aforementioned three scores, i.e., GD score, SIFT score, and PolyPhen-2 score (B). The cytoplasmic part of *TLR9* contains the possible "mutation hotspot," therefore the amino acid substitutions or frameshift mutations are most likely to affect the signaling cascade initiated by *TLR9* due to loss of function, and susceptibility toward NPC may be inferred (C). See also Tables S1 and S2.

membrane interaction by the "memsat" format. Thereby we may assume that the prediction is close to real in case of mutated sequences also (Figure 7, these regions are marked by red rectangular boxes). All these findings clearly point toward the loss of function of mutated TLR9 proteins due to aberrant structure-function relationship dependent on protein folding, which in turn is dependent on the amino acid composition. To supplement our data, we have also performed western blots for TLR9, MyD88, and IRAK4 with protein extracted from real NPC tissue samples (Figure 7B). Although some bands for mutated TLR9 are not much clear, and thus may indicate the absence of the protein, it should be noted that the poor condition of the tissue might be responsible for that. Overall, both tissue samples and cultured treated/untreated cells show similar results for the presence of TLR9 and other related proteins.

DISCUSSION

NPC is well reported for its ethnicity-specific higher incidences. Although all higher eukaryotes possess a complex immune system to fight for various diseases, higher incidence of any disease linked with any specific ethnic group is of interest. All these populations, sometimes called "Mongoloids," share similar characteristics like broad and small nose with smaller nasal passages, broad face, abundant fat tissues in facial region especially surrounding the nose, and mongoloid folds over the eyes. These characteristics have resulted from cold adaptation during the migration of human populations from middle Africa and are related to lipid metabolism. Cold adaptation provides a particular mechanism for thermal homeostasis in humans, called *nonshivering thermogenesis* (NST), the main site of which is the regions high in BAT (Guthrie, 1996; Montagu, 1951; Cannon and Nedergaard, 2004). In recent literature, it has been reported that biogenesis of BAT requires the transcription factor C/EBPβ and the zinc finger protein Prdm16. Both these genes are activated by an evolutionarily conserved protein Plac8, which can be considered as a critical upstream regulator of brown fat differentiation and function. Plac8 expression and function may be

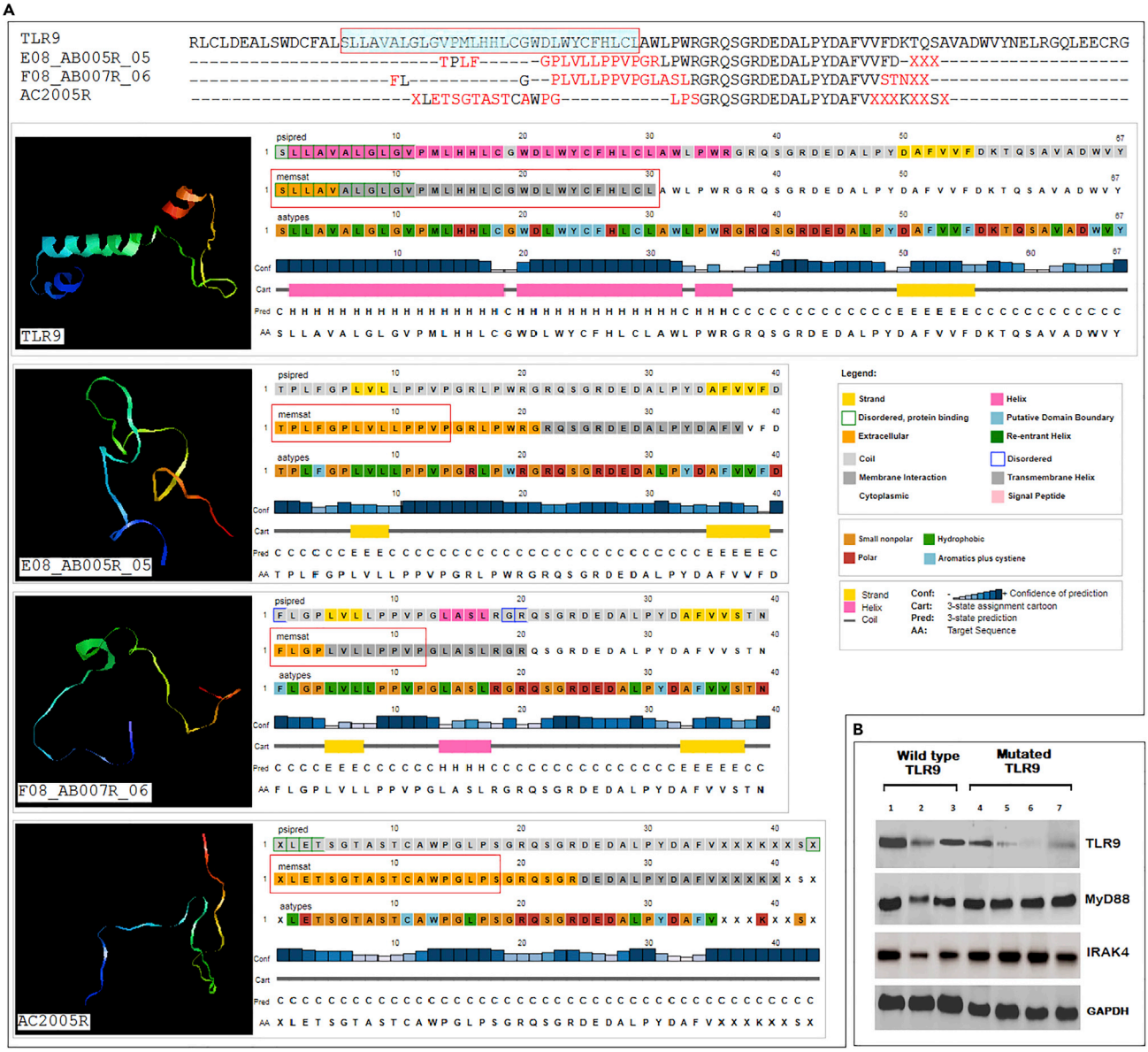

Figure 7. The Molecular Mechanism of Loss of Function of *TLR9* Might Be Related to Structural Aberration Caused by Radical Mutations

Figure360▷ For a Figure360 author presentation of this figure, see https://doi.org/10.1016/j.isci.2020.100937.

(A and B) To investigate the effects of these mutations on the structure-function relationship of TLR9, 3D modeling and secondary structure prediction were done and analyzed by Phyre[2], Raswin, and PSIPRED (A). Only the reference part of wild-type TLR9 and three other mutated samples are shown for simplicity. The uppermost part of the figure shows the sequences aligned with ClustalW. Base substitutions in the mutated sequences are marked in red. Against each 3D structure, the PSIPRED analysis of that sequence has been shown. With this software, each amino acid of a sequence has been studied for its character (aatypes), its possibility to be present in a particular type of protein folding (psipred), and its possibility to interact with the membrane (memsat). These results indicate that the loss of function of TLR9 occurs due to aberrant structure caused by the mutations. To supplement our data, we have also performed western blots for TLR9, MyD88, and IRAK4 with protein extracted from real NPC tissue samples (B). See also Tables S1 and S2.

regulated by its interaction with phospholipid scramblase 1 (PLSCR1), which is a calcium-binding protein associated with lipid rafts (Jimenez-Preitner et al., 2011).

Interestingly, this *PLSCR1* has been reported to regulate *TLR9*-mediated type I IFN production. *PLSCR1* also contributes to cell proliferation and differentiation and helps avoid apoptosis, indicating that cancer cells might get benefited for their survival (Talukder et al., 2012). Of note, *TLR9* itself and its unmethylated

CpG-binding property both are reported to be related to lipid metabolism (Köberlin et al., 2016; Kostjuk et al., 2012; Nagajyothi et al., 2012). The main gene for NST from BAT is the uncoupling protein 1 (UCP1) gene, which uncouples the proton gradient in the inner mitochondrial membrane from the formation of ATP. In a very recent study it has been shown that this gene is also closely related to the expression of *TLR9*. In colder climate, presence of UCP1 decreases *TLR9* function, thereby make the animals susceptible to pathogenic infections (Kazak et al., 2017). After initial infection and EBNA-2 expression, LMP1 starts to be expressed and then activates the NF-κβ signaling pathway. This is a broad pathway affecting various cellular processes including lipid metabolism. NF-κβ signaling pathway itself can suppress the promoter of *TLR9* and make it nonfunctional, as mentioned earlier. Therefore the genes involved in lipid metabolism and *TLR9* regulation mentioned earlier, and the NF-κβ-mediated signals, might play the contributing role in NPC occurrence. With regard to cell survival, *TLR9* helps p53 expression; therefore lack of *TLR9* function might result in lack of p53 and thus lack of apoptosis in cancers (Holm et al., 2017). These findings indicate a possible role of *TLR9* in controlling susceptibility toward EBV for the mongoloid populations, who show higher occurrence of NPC.

It is very clear that lack of function of *TLR9* renders a cell very much susceptible to pathogenic infections like infection with EBV. Therefore the main path of EBV-mediated NPC generation might involve the loss of function of *TLR9* (by any pathway as discussed earlier) by deleterious SNPs found in our study (Figure 8). Single-nucleotide variations (SNVs) in *TLR9* are documented in various cancers including the head and neck cancers, of which NPC is a part (https://hive.biochemistry.gwu.edu/cgi-bin/prd/biomuta/servlet.cgi?gpageid=11&searchfield1=gene_name&searchvalue1=TLR9). Compared with other cancers, SNVs are less common in head and neck cancers and are much less reported in NPC. Some polymorphisms are also documented in the exon 2 of *TLR9* (http://bioinf.umbc.edu/dmdm/gene_prot_page.php?search_type=protein&id=20140872), but no relationship of those with NPC is shown. In the present study, we report for the first time a set of polymorphisms in exon 2 of *TLR9* gene, i.e., amino acid modifications in a specific region of the *TLR9* protein. We have noted an interesting thing from *in silico* analyses; all the mutated sequences show modifications in the amino acids responsible for the membrane interaction of TLR9 protein. Membrane-interacting proteins always show functional aberration if their membrane interaction is compromised, and we have already mentioned that with supporting references in a previous part of this article. Although the present study shows no conclusive data for the association of specific *TLR9* polymorphisms with NPC, we cannot entirely exclude the role of *TLR9* as a candidate gene for this rare but fatal disease. Other *TLR9* gene SNPs that were not analyzed in our study may also be associated with NPC, and further studies are needed to elucidate the role of *TLR9* in the pathogenesis of NPC in NE Indian populations and other similar ethnic groups. Detailed investigation on the mechanism of UCP1-mediated and PLSCR1-mediated repression of TLR9 will also add to the knowledge for explaining the ethnicity bias of EBV-associated NPC occurrence. Non-mongoloid persons with EBV infection and TLR9 mutation should also be studied in detail to find out the molecular mechanism for such bias. Nevertheless, the importance of our findings lies in the possibility of early detection of the disease and/or susceptibility toward the disease, which can help prevention and cure of this fatal cancer. Most of the patients experienced unexplained chronic headache, and it indeed is an initial symptom of NPC generation (Figure S1B). Complaint of such headache even without any other symptom can be taken care of by screening the polymorphic status of the *TLR9* gene/protein of the subject. Presence of any deleterious mutation(s) can then suggest other extensive tests for diagnosis of an early NPC or susceptibility toward it. The treatment plan and/or preventive measures, for example, a change in diet and lifestyle, might therefore save a life. Sequencing of full-length TLR9 or even the ''mutation hotspot'' at early age can be used as a possible biomarker to access the risk factors associated with fatal disease.

Limitations of the Study

The present report documents a novel possible mechanism for NPC generation and poor prognosis, thereby paving the way for newer preventive and/or curative techniques for this fatal cancer. However, the study has a few limitations that should be considered before planning any diagnostic and/or curative method(s) for NPC in such populations. First, NPC itself is rare; therefore our accessible sample size was small enough to conclude for the model pathway (Figure 8). Second, a complete list of genetic polymorphisms responsible for ethnicity-specific characters is not available and was not created by us due to lack of required data for specific genetic makeup of different human races. Third and the last limitation we are deeply concerned of is that neither the exact molecular mechanism for cholesterol signaling, diet, and BAT in these ethnic populations nor the details of *TLR9* signaling cascade related to BAT is known.

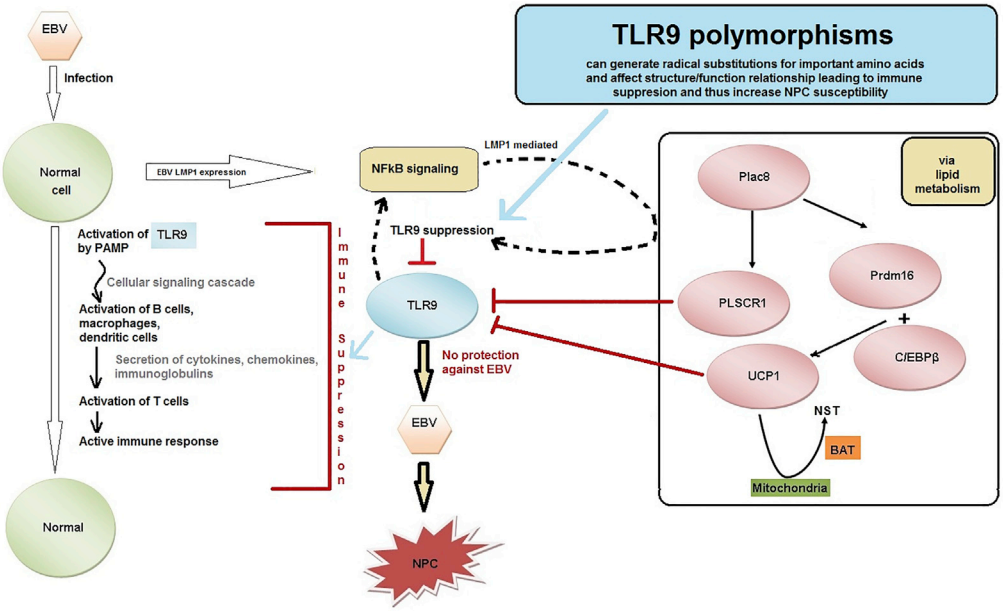

Figure 8. The Molecular Mechanism of Loss of Function of *TLR9* Might Play a Major Role for NPC Susceptibility in Specific Ethnic Groups of NE India

Some populations, called "mongoloids," show a significantly higher incidence for NPC. Their similar characteristics are related to lipid metabolism via NF-κβ signaling pathway. Biogenesis of BAT in these populations requires an evolutionarily conserved protein Plac8, the transcription factor C/EBPβ, and the zinc finger protein Prdm16. Plac8 expression and function may be regulated by its interaction with phospholipid scramblase 1 (PLSCR1), which can regulate *TLR9*-mediated type I IFN production. Another gene called the uncoupling protein 1 (UCP1) decreases *TLR9* expression in mongoloids during colder climates. NF-κβ signaling pathway itself can suppress the promoter of *TLR9* and make it nonfunctional. Therefore the genes involved in lipid metabolism and *TLR9* regulation, and the NF-κβ-mediated signals, all might contribute to NPC susceptibility. In the present study, we report a set of polymorphisms in the 3′ end of exon 2 of *TLR9* gene, i.e., amino acid modifications in the N-terminal region of the *TLR9* protein. Polymorphisms in this region might affect the structure-function relationship of *TLR9* and downstream signaling pathways related to EBV-infected NPC occurrence in NE Indian populations.

Therefore our model needs a more detailed study for the *TLR9* downstream signaling pathway and the structure-function alterations resulting from polymorphisms reported here. Nevertheless, the major importance of our findings is that it provides new insights for studying the ethnicity bias of NPC.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY

In the Supplemental Information, we have provided all the required data needed for reproducibility of this work. Other data are available in https://data.mendeley.com/datasets/bd6xyp274j/draft?a=d7e11c13-04d5-4b65-bd51-1b318c70e245.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100937.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.C. and C.G., Methodology, T.C., C.G., and N.R.C.; Sample collections and histology, S.D.R., A.K.D., S.T., K.R., Z.P., E.Z., and Y.I.S.; Investigations, N.R.C. and N.T.; Supportive actions in performing the experiments: K.C., S.C., S.K.S, A.G., R.R.R., P.D.,B.B.K., S.M., A.R.S., A.K., and D.G.; Formal Analysis, N.R.C., N.T., C.G., and T.C.; Writing – Original Draft, N.R.C.; Writing – Review & Editing, N.R.C. and T.C.; Resources, C.G., A.K., D.G., and T.C.; Supervision, T.C.

DECLARATION OF INTERESTS

The authors have declared that no competing interests exist.

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Supplemental Information

TLR9 Polymorphisms Might Contribute to the Ethnicity Bias for EBV-Infected Nasopharyngeal Carcinoma

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Supplementary Information

Table S1: Alignment of reference TLR9 gene done with TLR9 sequences retrieved from EBV-infected NPC patients. Related to Figures 1c, 3a, 5, 6, and 7.

Sl. No.	Sequence
1	ACGACCAMATAGAGGCGGCTTGGAGMCKCCTCCGCCGGGGGGAGAGYTAAAGCMCCCACCCCACGMC
2	CSGTMSCTTCCTGKGMGGGGGCGGCAAAKTGGGCGAGATGAKGATGCCCTGCCCTACGATGCCTTCGTGGTCWTARACAAAAMGCAGAGCGCAKTGGCAGACTGGGTGTACAACGASCTTTTAARG
3	TCSKKGCTYMMTGGGCGGGGGGCGGCAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATGCCTTCGTGGTCTTCGAMAAAACGCAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTYAGGGGT
4	GMSSKGCTTYMTKGGGCGGGGGCGGCAAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATGCCTTCGTGGTCTTCGAMAAARSGCAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTTWTG
5	CMYKKRSGTTYYWTGTCGAGACMCGAAGGCATCGTAGGGCAGGGCATCCTCATCTCGCCCACTTTGCCGCCCCCGCARGGAAAGSCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCCAAAAAGT
6	GCTGYTAWCCTCCCKKRRWRRGGGGCGGCAAGTGGGCGAGATGAGGATGYCCTGCCCTACGATGCCTTCGTGGTCTTARACAAAACGCAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTTAAAGGG
7	GWTTCGKYTTMGTTTTKWMMCAKAMCACGAAGGCATCGTAGGGCAGGGCATCCTCATCTCGCCCACTTTGCCGCCCCGAAGGGAGGCCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTTTCCAAARRGG
8	TCCCKGGCCTMCTGGCGGGGGCGGCAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATGCCTTCGTGGTCTTRAMAAAACGCAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTYMAAAGGA
9	GMMYTRSSTTWATKGTYGAGACCACGAAGGCATCGTAGGGCAGGGCATCCTYWTCTCGCCCACTTTGCCGCCCCCGCAGRGAAGCSRGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCCCTTTTRGG
10	GTWAAGGGGGCARGGGCATCCTCWTCTCGCCCACTTTGCCGCCCCCGCTTGGRAARMCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCCYARTGCCTKGCCTGGCTTCCCTGGCWTGGGCGGCAAATGGGCGAGATGAGGATGCCCTGCCTAGATACTTCTGCTTTGAACAGCGAAGAAGGCGACTGGTGTACACGGCCTTAA
11	TCGKCTCTGGCGGGGGCGGCAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATGCCTTCGTGGTCTTCGACAAAACGCAGAGCGCAGTGGCAGACTGGGTGTACAACGASCCTYAAAAGTC
12	ASGTATAGCGCTTATWCGAGTACGTKATRKKGAKKKAKAGKYTTCCCCGCCGGTGCTSGAGTGTGAAWATMWGCAAAGCSWSGTKYAACACGAGKCATCGTRGGCRGGCRTCCTCATCTCGCCCACTTTGCCGCCCCCGCAGGGAAGMSAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGYCCTYTWAGA
13	TASSAAATTCGGKAATACARAAAAGTGCAGCCCTGCCCTAATATGCCTTCGWGGTCCTCTTTAAAMCGCAGAGTASCCTGGCAGACTGGGTGTACAACGCGCTTSARCTTCTTTCMTCGCTTTACAGATGTCTTCKTMAGAAGCACCGTTMACTAGGTTAAYATTTAGGTCAACTACATAAACGTTAGCAGTTGGT
14	TTTCATTTCTTCGCCMACTTTGCCGCCCCCSCTWTKKRAARCCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTTWYYWWTGG

15	TKTCTCCTGGCGGGGGCGGCAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATGCCTTCGTGG TCYYCSACAAAACGCAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTYA
16	CTKSGATTTGTCGAGACCACGAAGGCATCGTAGGGCAGGGCATCCTCATCTCGCCCACTTTGCCG CCCCCGCCMGGGAAGCCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCA
17	TGGCCCRGCCCCCCCCGCYWWRARRAARCCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGT CCCMA
18	TSCCTGCCTTTCTTGGGCGGGGGGSCGGCAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATG CCTTCGTGGTCTARACAAGACGCAGAGCGCAGTGGCAGACTGGGTGTACAACGAGAAAAAAAA RGTC
19	TGAGCTCTAAGCAATGGTCTSAMKAWMAGGSAGGTRGACWSCGTSTTGYAGACAGAAGCATCG TAGCAGCATCCTCWCTCGCCACTTGCGCCCCGCCAACGAATRAYRGCAGTCTGTCAGCAGTACAG ATTCTCTAGGAGA
20	GAAAWAAGGCTCTTTCGWAGGTCTMMCTYMYWAAAMGCAGAGCGCAGTGGCAGACTGGGT GTACAACGAGCTTTAAG
21	CTCCAATTCTTCGGCCCACTTTGCCGCCCCCGCCWKYSKTWARMMAGGCCAGGCACAGGTGGA AGCAGTACCAGAGGTCCYTWAAG
22	TGCTGTGTTCCCCTTTGYGGGGGCGGCAAKTGGGCGAGATGAKGATGCCCTGCCCTACGATGCCT TCGTGGTCTTCGAAAAACGCKRAGCGCAGTGGCAGACTGGGTGTACAACGAGCTCAATAAAAA WT
23	TCSTKSSKTTTKKTCGAAGACCACGAAGGCATCGTAGGGCAGGGCATCCTCATCTCGCCCACTTTG CCGCCCCCGCCAGGGAAGCCGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCAAAAAGAGG GGT
24	CTTTCATTCTCGCCCACTTTGCCGCCCCCSCTWYRGWAARMCRGGCCAGGCACAGGTGGAAGCA GTACCAGAGSYTWYWAAG
25	TCGGTCTCTGGCGGGGGCGGCAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATGCCTTCGTG GTCTTGACAAAASRMAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCCAAAAGGARGG
26	TYSKSSGGTTTGTCGAGACCACGAAGGCATCGTAGGGCAGGGCATCCTCATCTCGCCCACTTTGCC GCCCCCGCAGGGAAGCCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCCAAAAAAG
27	ATCSGCCCCCCCGGCCCMYKWKKWWAAAMCAGGCCAGGCACAGGTGGAAGSAGTACCAGAG GTCWYYAATKC
28	CGGGGGGAACCGCCGCCCYTATGGGGTCGGGGGAGGGSGGGGGAAGGAAAAAGGG
29	GKCYWGCCMCCTKGRMRGGGGCGGCAAAGTGGGCGAGATGAGGATGCCYTGCCCTACGATGC CTTCGTGGTCTTGAMAAAACGMRGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTTTAARG AGG
30	TKSMMKMGKTCTTKRWCRAAACMCGAAGGCATCGTAGGGCAGGGCATCCTYATCTCGCCCACT TTGCCGCCCCCGTAGGGAGGCCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCTAAAAA AARRGG
31	GRYSKKGSCYTCCYKGGCRGGGGGCGGCAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATGC CTTCGTGGTCTTARAMAAAACGCAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTATGGG GGG
32	GKKWKSGKSSKYYTTKKYCRAGACMACGAAGGCATCGTAGGGCAGGGCATCCTCATCTCGCCCAC TTTGCCGCCCCCGAAAGGGAAGCCAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCTCAAA RAAARG
33	TMMKKKRSGGTYYTGTCGAGACCACGAAGGCATCGTAGGGCAGGGCATCCTCATCTCGCCCACT TTGCCGCCCCCGCCARRRAAGSSAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCYWWAA G
34	CTTKTRSSTYGTCTTTKTYRAAGACCACGAAGGCWTCGTAGGGCAGGGCATCYTCATCTCGCCCAC

	TTTGCCGCCCCCGAARGGAAGMSRGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCCTAAK RGGGC
35	GYCKKGGCCTTMMCTGGCGGGGGCGGCAAAGTGGGCGAGATGAGGATGCCCTGCCCTACGAT GCCTTCGTGGTCTTGAMAAAAMGSAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTYTAG GGGGT
36	TMMWYKKSSGTYMYTKGTYRAAGACCACGAAGGCATCGTAGGGCAGGGCATCCTYATCTCGCCC ACTTTGCCGCCCCCGAAGGGAAGCMAGGCCAGGCACAGGTGGAAGCAGTACCAGAGGTCCCCT TAWAR
37	TWSSRWMGGTCYTKRYYGAAGACCACGAAGGCATCGTAGGGCAGGGCATCCTCATCTCGCCCA CTTTGCCGCCCCCGCAGGGAAGCCRGGMCAGGCACAGGTGGAAGCAGTACCAGAGGTCCYATA AAGGG

Table S2: The probable mutation hotspot. Related to Figures 2c, 2e, 3a, 5, 6, and 7.

TLR9 reference

5.084KB

>gi|224589815:c52260179-52255096 Homo sapiens chromosome 3, GRCh37.p5 Primary Assembly

1. GGAGGTCTTGTTTCCGGAAGATGTTGCAAGGCTGTGGTGAAGGCAGGTGCAGCCTAGCCTCCTGCTCAAG
2. CTACACCCTGGCCCTCCACGCATGAGGCCCTGCAGAACTCTGGAGATGGTGCCTACAAGGGCAGAAAAGG
3. ACAAGTCGGCAGCCGCTGTCCTGAGGGCACCAGCTGTGGTGCAGGAGCCAAGACCTGAGGGTGGAAGTGT
4. CCTCTTAGAATGGGGAGTGCCCAGCAAGGTGTACCCGCTACTGGTGCTATCCAGAATTCCCATCTCTCCC
5. TGCTCTCTGCCTGAGCTCTGGGCCTTAGCTCCTCCCTGGGCTTGGTAGAGGACAGGTGTGAGGCCCTCAT
6. GGGATGTAGGCTGTCTGAGAGGGGAGTGGAAAGAGGAAGGGGTGAAGGAGCTGTCTGCCATTTGACTATG
7. CAAATGGCCTTTGACTCATGGGACCCTGTCCTCCTCACTGGGGGCAGGGTGGAGTGGAGGGGGAGCTACT
8. AGGCTGGTATAAAAATCTTACTTCCTCTATTCTCTGAGCCGCTGCTGCCCCTGTGGGAAGGGACCTCGAG
9. TGTGAAGCATCCTTCCCTGTAGCTGCTGTCCAGTCTGCCCGCCAGACCCTCTGGAGAAGCCCCTGCCCCC
10. CAGCATGGTAGGACAACAGCTCTCAGTCCCCTGGGGAAGGGGATATCCTGGGAAGGGGTGGGGAAAGAAG
11. TGGGCTTGGCAGCTGCAAGCAACAGTGACGGGTTGTCACCCACATGGCCCTGGGGACACTCACTGAATCC
12. TGAAGACTTCAGAGCCGAAGCCCTCTTCTTTTTTCTTTTTTTTTTTTTTTGAGACGGAGTTTCGCTCTTG
13. TTGCCCAGGCTGGAGTGCAGTGGCTTGATCTCAGCTCACTGCAATCTCCACCTCCCGGGTTCAAGTGATT
14. CTCCTGCCTCAGCCTCCCAAGTAGCTGGGATTATGGCTTGCGCCACCATGCCTGGCTAATTTTGTATTTT
15. TAGTAGAGCCAGGGTTTCTCCATGTTGGTCAGGCTGGTCTTGAACTCCAGACCTCAGGTGATCTGCCCAC
16. TTCGGCTTCCCAAACTGCTGGGATTACAGGTGTGAGCCACCGTGCCCAGCCACTGAAGCCCTCTTCTGAG
17. CAATGAGACCCAAACCCCCAGAGGGGACGAGGAGACTGAGGCACTGATGGAGCTGGGGCTGGAGCCTGGG
18. TTTGGGTCTCACTGGGCCTGTAGCTTGGGGCTGGCTCTGTCTGGCACTTTGCAGGCCACCCCTCTCTCCA
19. TCTGTTCTGACCCATAAGGCAAAGGCTCTCGGGCTAGGTGAGGCAGGCAGAGAGTGATGGAGCTGTGCAG
20. TGCCCTCTGGCCAGGGTCCCTGGGAGGCAGCCGGCCTGGGCCATAGTGGGAGACTCTCACACCTGCCTGA
21. ATCTTGGCCGAAGGAAAAGGAAGGGGTTGTGGGGTGGCTGGTGGAGGGCAGAGAGCGGATTTCAGGGCAG
22. CTGGAGGGGAGGGAGCCAGCTGCCACCTTGCTCAGGAGCAGGAAGGGAAAGTCGGGGAGAGACCAGGGGG
23. GGCCGTGGGCCACCTTGCCTGGCCTGGTATAGCCAGGGATTGGTTAAGTGAAAGCTGTAGATCATAAGTC
24. TGGATTCTAGGTCTCAGTCCTGGTTCTGAAGCCTAATTCTGAGTCCAAGACTGGGTCTGAGACTGAGTTC
25. AGGTTCCCCACAAGCCATGGGGGATGGGAATGGGGCTTGGCAGCCAGGAAGAACTTCTGCAGGTAGGGCT
26. TGGAGAGAGGGGTTGGAAGATGCTAGAAGATGCCCATGAAGTGGAGTGGGTGGAGGTAGAGCTGGGGGCC
27. GGCCACTCACACAGCCCTCCCACCCCCACAGGGTTTCTGCCGCAGCGCCCTGCACCCGCTGTCTCTCCTG
28. GTGCAGGCCATCATGCTGGCCATGACCCTGGCCCTGGGTACCTTGCCTGCCTTCCTACCCTGTGAGCTCC
29. AGCCCCACGGCCTGGTGAACTGCAACTGGCTGTTCCTGAAGTCTGTGCCCCACTTCTCCATGGCAGCACC
30. CCGTGGCAATGTCACCAGCCTTTCCTTGTCCTCCAACCGCATCCACCACCTCCATGATTCTGACTTTGCC
31. CACCTGCCCAGCCTGCGGCATCTCAACCTCAAGTGGAACTGCCCGCCGGTTGGCCTCAGCCCCATGCACT
32. TCCCCTGCCACATGACCATCGAGCCCAGCACCTTCTTGGCTGTGCCCACCCTGGAAGAGCTAAACCTGAG
33. CTACAACAACATCATGACTGTGCCTGCGCTGCCCAAATCCCTCATATCCCTGTCCCTCAGCCATACCAAC
34. ATCCTGATGCTAGACTCTGCCAGCCTCGCCGGCCTGCATGCCCTGCGCTTCCTATTCATGGACGGCAACT
35. GTTATTACAAGAACCCCTGCAGGCAGGCACTGGAGGTGGCCCCGGGTGCCCTCCTTGGCCTGGGCAACCT
36. CACCCACCTGTCACTCAAGTACAACAACCTCACTGTGGTGCCCCGCAACCTGCCTTCCAGCCTGGAGTAT
37. CTGCTGTTGTCCTACAACCGCATCGTCAAACTGGCGCCTGAGGACCTGGCCAATCTGACCGCCCTGCGTG
38. TGCTCGATGTGGGCGGAAATTGCCGCCGCTGCGACCACGCTCCCAACCCCTGCATGGAGTGCCCTCGTCA
39. CTTCCCCCAGCTACATCCCGATACCTTCAGCCACCTGAGCCGTCTTGAAGGCCTGGTGTTGAAGGACAGT
40. TCTCTCTCCTGGCTGAATGCCAGTTGGTTCCGTGGGCTGGGAAACCTCCGAGTGCTGGACCTGAGTGAGA
41. ACTTCCTCTACAAATGCATCACTAAAACCAAGGCCTTCCAGGGCCTAACACAGCTGCGCAAGCTTAACCT
42. GTCCTTCAATTACCAAAAGAGGGTGTCCTTTGCCCACCTGTCTCTGGCCCCTTCCTTCGGGAGCCTGGTC
43. GCCCTGAAGGAGCTGGACATGCACGGCATCTTCTTCCGCTCACTCGATGAGACCACGCTCCGGCCACTGG
44. CCCGCCTGCCCATGCTCCAGACTCTGCGTCTGCAGATGAACTTCATCAACCAGGCCCAGCTCGGCATCTT
45. CAGGGCCTTCCCTGGCCTGCGCTACGTGGACCTGTCGGACAACCGCATCAGCGGAGCTTCGGAGCTGACA
46. GCCACCATGGGGGAGGCAGATGGAGGGGAGAAGGTCTGGCTGCAGCCTGGGGACCTTGCTCCGGCCCCAG
47. TGGACACTCCCAGCTCTGAAGACTTCAGGCCCAACTGCAGCACCCTCAACTTCACCTTGGATCTGTCACG

48. GAACAACCTGGTGACCGTGCAGCCGGAGATGTTTGCCCAGCTCTCGCACCTGCAGTGCCTGCGCCTGAGC
49. CACAACTGCATCTCGCAGGCAGTCAATGGCTCCCAGTTCCTGCCGCTGACCGGTCTGCAGGTGCTAGACC
50. TGTCCCACAATAAGCTGGACCTCTACCACGAGCACTCATTCACGGAGCTACCGCGACTGGAGGCCCTGGA
51. CCTCAGCTACAACAGCCAGCCCTTTGGCATGCAGGGCGTGGGCCACAACTTCAGCTTCGTGGCTCACCTG
52. CGCACCCTGCGCCACCTCAGCCTGGCCCACAACAACATCCACAGCCAAGTGTCCCAGCAGCTCTGCAGTA
53. CGTCGCTGCGGGCCCTGGACTTCAGCGGCAATGCACTGGGCCATATGTGGGCCGAGGGAGACCTCTATCT
54. GCACTTCTTCCAAGGCCTGAGCGGTTTGATCTGGCTGGACTTGTCCCAGAACCGCCTGCACACCCTCCTG
55. CCCCAAACCCTGCGCAACCTCCCCAAGAGCCTACAGGTGCTGCGTCTCCGTGACAATTACCTGGCCTTCT
56. TTAAGTGGTGGAGCCTCCACTTCCTGCCCAAACTGGAAGTCCTCGACCTGGCAGGAAACCAGCTGAAGGC
57. CCTGACCAATGGCAGCCTGCCTGCTGGCACCCGGCTCCGGAGGCTGGATGTCAGCTGCAACAGCATCAGC
58. TTCGTGGCCCCCGGCTTCTTTTCCAAGGCCAAGGAGCTGCGAGAGCTCAACCTTAGCGCCAACGCCCTCA
59. AGACAGTGGACCACTCCTGGTTTGGGCCCCTGGCGAGTGCCCTGCAAATACTAGATGTAAGCGCCAACCC
60. TCTGCACTGCGCCTGTGGGGCGGCCTTTATGGACTTCCTGCTGGAGGTGCAGGCTGCCGTGCCCGGTCTG
61. CCCAGCCGGGTGAAGTGTGGCAGTCCGGGCCAGCTCCAGGGCCTCAGCATCTTTGCACAGGACCTGCGCC
62. TCTGCCTGGATGAGGCCCTCTCCTGGGACTGTTTCGCCCTCTCGCTGCTGGCTGTGGCTCTGGGCCTGGG
63. TGTGCCCATGCTGCATCACCTCTGTGGCTGGGACCTCTGGTACTGCTTCCACCTGTGCCTGGCCTGGCTT
64. CCCTGGCGGGGGCGGCAAAGTGGGCGAGATGAGGATGCCCTGCCCTACGATGCCTTCGTGGTCTTCGACA
65. AAACGCAGAGCGCAGTGGCAGACTGGGTGTACAACGAGCTTCGGGGGCAGCTGGAGGAGTGCCGTGGGCG
66. CTGGGCACTCCGCCTGTGCCTGGAGGAACGCGACTGGCTGCCTGGCAAAACCCTCTTTGAGAACCTGTGG
67. GCCTCGGTCTATGGCAGCCGCAAGACGCTGTTTGTGCTGGCCCACACGGACCGGGTCAGTGGTCTCTTGC
68. GCGCCAGCTTCCTGCTGGCCCAGCAGCGCCTGCTGGAGGACCGCAAGGACGTCGTGGTGCTGGTGATCCT
69. GAGCCCTGA**CGGCCGCCGCTCCCGCTACGTGCGGCTGCGCCAGCG**CCTCTGCCGCCAGAGTGTCCTCCTC
70. TGGCCCCACCAGCCCAGTGGTCAGCGCAGCTTCTGGGCCCAGCTGGGCATGGCCCTGACCAGGGACAACC
71. ACCACTTCTATAACCGGAACTTCTGCCAGGGACCCACGGCCGAATAGCCGTGAGCCGGAATCCTGCACGG
72. TGCCACCTCCACACTCACCTCACCTCTGCCTGCCTGGTCTGACCCTCCCCTGCTCGCCTCCCTCACCCCA
73. CACCTGACACAGAGCAGGCACTCAATAAATGCTACCGAAGGCTA

The probable mutation hotspot.

CGGCCGCCGCTCCCGCTACGTGCGGCTGCGCCAGCG

Table S3: List of primers used for PCR. Related to Figures 3c, 4b, 4c, and Table 1.

For Mutation screening	T.C_TLR9_Exon2.1_ LEFT PRIMER T.C_TLR9_Exon2.1_ RIGHT PRIMER	ATGAAGTGGAGTGGGTGGAG GAGTGACAGGTGGGTGAGGT
	T.C_TLR9_Exon2.2_ LEFT PRIMER T.C_TLR9_Exon2.2_ RIGHT PRIMER	TTCATGGACGGCAACTGTTA GGGCCTGGTTGATGAAGTT
	T.C_TLR9_Exon2.3 LEFT PRIMER T.C_TLR9_Exon2.3 RIGHT PRIMER	TCTTCTTCCGCTCACTCGAT CTCAGGCCTTGGAAGAAGTG
	T.C_TLR9_Exon2.4 LEFT PRIMER T.C_TLR9_Exon2.4 RIGHT PRIMER	CGAGGGAGACCTCTATCTGC CACAGGTGGAAGCAGTACCA
	T.C_TLR9_Exon2.5 LEFT PRIMER T.C_TLR9_Exon2.5 RIGHT PRIMER	CCCTCTCCTGGGACTGTTTC ATTCTCCCTTCCCTCCTCCT
For Real-time RT-PCR	MyD88 Forward: Reverse:	 GGTTGGTGTAGTCGCAGACA GTCTCCTCCACATCCTCCCT
	IRAK4 Forward: Reverse:	 CAACATATGTGCGCTGCCTC GACTTGAGGAGTCAGGTGGC
	IRAK1 Forward: Reverse:	 CTCTCCCCAGCTTTTCCAGG ACACCGTGTTCCTCATCACC
	TLR9 Forward: Reverse:	 GTGTACAACGAGCTTCGGGG GAGGCCCACAGGTTCTCAAA

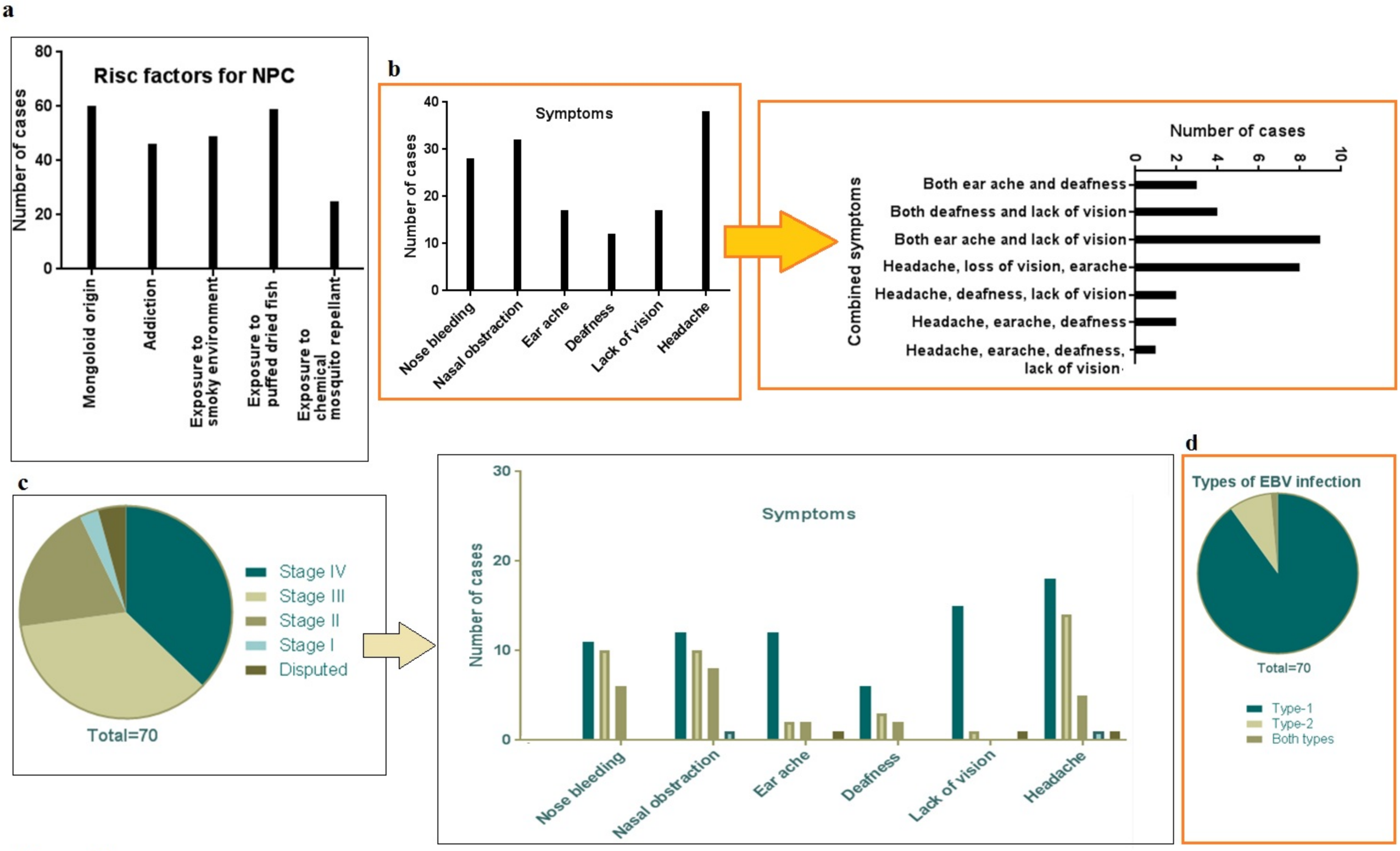
a
Risc factors for NPC
Number of cases
80
60
40
20
0
Mongoloid origin
Addiction
Exposure to smoky environment
Exposure to puffed dried fish
Exposure to chemical mosquito repellant
b
Symptoms
Number of cases
40
30
20
10
0
Nose bleeding
Nasal obstraction
Ear ache
Deafness
Lack of vision
Headache
Number of cases
0
2
4
6
8
10
Combined symptoms
Both ear ache and deafness
Both deafness and lack of vision
Both ear ache and lack of vision
Headache, loss of vision, earache
Headache, deafness, lack of vision
Headache, earache, deafness
Headache, earache, deafness, lack of vision
c
Stage IV
Stage III
Stage II
Stage I
Disputed
Total=70
Symptoms
Number of cases
30
20
10
0
Nose bleeding
Nasal obstraction
Ear ache
Deafness
Lack of vision
Headache
d
Types of EBV infection
Total=70
Type-1
Type-2
Both types

Figure S1

Figure S1. Risk factors, symptoms, and stages of NPC in NE Indian polulations. Related to Figure 1. The risk factors of NPC includes genetic factors*;* diet and other environmental factors*;* and infection with HPV (Human Papilloma virus) or EBV (Epstein-Barr virus). A few dietary habits and lifestyles with poor hygiene are known to be associated with NPC in endemic populations like those in specific NE Indian populations. Most people from these populations, colloquially called 'Mongoloids', consume some kinds of preserved foods, live in poor hygienic conditions like a long time in smoky environments, inhaling various toxic fumes like that of mosquito repellents, habits of smoking etc. (**a**). Symptoms include headache, nasal obstruction, nose bleeding, deafness, loss of vision etc. (**b**); of which, headache is found in most patients and in earlier stages (**c**). Type 1 EBV is present in most of the cases, type 2 is much less common, and only a few have both types of infection (**d**).

Transparent Methods

Patients' history

The study was carried out in collaboration with Civil Hospital Dimapur, Nagaland, RIIMS, Imphal, Manipur and other NE hospitals of India. Seventy freshly diagnosed patients with NPC and seventy age and sex matched controls were taken for this study. Routine histopathological analysis was done to confirm the diagnosis. Detailed questionnaire was completed, in which patients were interviewed particularly for dietary habits, tobacco consumption and type of dwelling unit. Detailed evaluation of clinico-pathological parameters and identifiable clinical variables such as disease severity, age of onset and initial clinical manifestation was obtained from patient's clinical records, operative notes and pathologic reports. Informed consent was obtained from all subjects as per the guidelines of research review committee. Approval was obtained from the Institutional Medical Ethical Committees of the participating centers for the study.

Sample Collection

Peripheral blood sample was taken from each patient prior to treatment for genomic DNA extraction. Tumor tissue sections were obtained from paraffin block and were used for histopathological confirmation of diagnosis. For control group, peripheral blood samples were collected from healthy age and sex matched volunteers from same ethnic background.

Histology

Paraffin sections (5 μm) from samples of nasopharyngeal carcinoma specimens were deparaffinized in 100% xylene and re-hydrated in descending ethanol series(100%, 90%,80%,

70% ethanol) and water according to standard protocols. Then the sections were stained by hematoxylin and eosin. The slides were examined under the microscope.

Processing of Samples

Genomic DNA was extracted and purified from each blood sample using commercially available kits following manufacturer's protocol (The GenElute™ Blood Genomic DNA Kit, Sigma-Aldrich). DNA samples were used immediately after isolation or stored at -80^{0}C for further analysis.

PCR based Detection of EBV

PCR amplifications of EBNA1 and EBNA2 genes were done for each sample. Real-time RT PCR method was applied in both cases and control samples to standardize the optimum PCR conditions for detection of EBV. Primers used in the study are as follows: EBNA-1F: 5'-TGAATACCACCAAGAAGGTG-3' and EBNA-1R:5′-AGTTCCTTCGTCGGTAGTC-3′. EBNA-2F: TGGAAACCCGTCACTCTC, EBNA-2R: TAATGGCATAGGTGGAATG. The primers were synthesized from IDT.

PCR amplification and mutation screening for *TLR9* gene

PCRs were performed in the DNA samples isolated from blood of each subject. For each sample, the PCR amplified DNA was purified and placed for sequencing. Then mutation screening was performed by Finch TV software. The standard genomic sequence of *TLR9* was obtained from the published database of National Center for Biotechnology Information (NCBI) database. Primer pairs were designed for the target region using the web-based software Primer 3.0. Primers used for *TLR9* amplification are 5'TCTGGAGTGACGTGGTGTGT3'(F)

3’CTTCCCAGGATATCCCCTTC5’(R) for exon 1, and for exon 2_1 5’ATGAAGTGGAGTGGGTGGAG3’(F) 5’GAGTGACAGGTGGGTGAGGT3’(R), exon2_2 5’TTCATGGACGGCAACTGTTA3’(F), 5’ GGGCCTGGTTGATGAAGTT3’(R) and exon2_3 5’TCTTCTTCCGCTCACTCGAT3’(F), 5’ CTCAGGCCTTGGAAGAAGTG3’(R). The PCR products were sequenced using Big Dye Terminator Sequencing Kits (ABI) and by loading the samples onto an ABI Automatic Sequencer (Applied Biosystems, Foster City, CA).

Sequence retrieval and alignment

The *TLR9* sequences were retrieved from Ensemble and National Centre for Biotechnology Information (NCBI) database. The *TLR9* sequence from *Zebrafish* was retrieved from NCBI. The sequence alignment was done by using MUSCLE alignment software with its default values. As a highly conserved protein, sequences for histone H4 from different species were downloaded from the Ensembl site (http://www.ensembl.org/index.html).

Phylogenetic tree formation

We used MUSCLE alignment program to align the amino acid sequences of *TLR9* for the purpose of phylogenetic analysis. We implemented a Bayesian phylogenetic tree constructed by the Bayesian approach (5 runs, 7500,000 generations, 25% burnin-period, WAG matrix-based model in the MrBayes 3.2 program. Fragmentation of *TLR9* in different domains and motifs was done. To analyze the conservation of different small regions of the *TLR9* that are important structurally and/or functionally, different domains and motifs were characterized before we analyzed those separately. MUSCLE software was used to align and find out the respective regions present in other species. The aligned data were subsequently imported into R statistical tool for statistical analysis. As the complete *TLR9* sequences from certain species are not

available (mostly due to sequencing errors at certain regions), the analysis aimed to understand the conservation of different domains and motifs of *TLR9* were conducted with the available sequences only (Supplementary material, Table 1).

Box-plot using distance Matrix generation and Statistical tests:

Using the saved alignment files in MEGA6, distance matrices were generated for different aligned data sets. Using this method, pair-wise distances of any two different amino acid sequences within a group can be measured. To estimate the variance, bootstrap method was used. In substitution method, amino acid p-distance was used. In case of data gaps/data missing pair-wise deletion method was used. For each data set there will be one matrix which informs about the pair-wise distances of all sequences in a group. In the matrix window distances between each sequence with another is calculated along with overall mean distance of all sequences. Then the pair-wise distance values (generated in the distance matrix) were imported in ‘‘R’’ software for statistical analysis and graphical representation.

Using R, box-plots were generated to represent the evolutionary relationship of different protein sequences. The Kruskal-Wallis analysis of variance test was done for each set of data to check the reliability and significance of the data points. As we have measured the pair-wise evolutionary distances of protein sequences, the graphical representation reflect values in the Y-axis which is inversely proportional with the conservation. Therefore, the conserved sequences show lower values and divergent sequences show higher values in the Y-axis. Along with this calculation, the median values of each data set were calculated and also represented along with conservation figures. The distances generated were imported to Graphpad Prism and boxplot were generated for different domains.

Immunohistochemistry

Thin paraffin sections (5 μm) from samples of nasopharyngeal carcinoma specimens were deparaffinized in 100% xylene and re-hydrated in descending ethanol series(100%, 90%,80%, 70% ethanol) and water according to standard protocols. For increased specificity and sensitivity, tissues have been warmed in microwave at 95 °C for 15 min to retrieve the antigen. After cooling and rinsing in distilled water, endogenous peroxide activity has been blocked in each with 3% H_2O_2 for 15 min, after which samples were rinsed in 0.01 mol/L phosphate-buffered saline (PBS), pH 7.4 for 10 mins. After blocking of any unwanted proteins, primary antibodies (mouse monoclonal anti-human TLR9) has been applied in proper dilution as per manufacturer's protocol. After secondary anti-mouse biotinylated antibody treatment, antigen-antibody complexes have been detected using the streptavidin-peroxidase method with diaminobenzidine (DAB) as the chromogenic substrate. The sections were stained lightly with hematoxylin, and PBS has been used as negative control. Then the sections were examined under the microscope.

siRNA treatment

Both Raji and Ramos cells were transfected with siRNA against TLR9 as per manufacturer's protocol. siRNA against GAPDH was used as control and both the siRNAs were gifted kindly by Dr. Dipyaman Ganguly, Head, Dendritic Cell Laboratory, IICB, Kolkata and were manufactured by Santa Cruz Biotechnology, Inc.

Immunofluorescence and colocalization

siRNA treated cells and control cells were washed with PBS and pelleted by centrifugation. Cells were then spreaded on clean galss slides and air dried. After acetone-methanol treatment and blocking, each slide was incubated with appropriate antibody. After incubation with appropriate

seconday antibody and addition of antifed, ceels were visualized under microscope. Merging was done by in-built software. Alexa Fluor 488 was used against TLR9 and PE was used against MyD88. All antibodies were procured from Santa Cruz Biotechnology, Inc.

PCR based Detection of various gens

PCR amplifications of TLR9, MyD88, IRAK1, IRAK4, and TRAF3 genes were done for 20 patient samples and 20 controls, chosen randomly. Real-time RT PCR method was applied in both cases and control samples to standardize the optimum PCR conditions for detection of each gene. Primers used in the study are listed in Table 3 of Supplementary material. The primers were synthesized from Eurofins Genomics India Pvt. Ltd.

Western blot analyses

Presence of various proteins checked in the current study is done by standard western blotting techniques. Total protein has been extracted from each cell culture set or tissue sample provided. 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, followed by transfer to PVDF membrane, has been done and the membrane has been incubated with different monoclonal primary antibodies overnight at 4°C. After washing, membranes have been incubated with appropriate polyclonal secondary antibody at room temperature for 4 h. After washing with PBS, color development has been performed with ECL. GAPDH has been used as loading control. Antibodies were purchased from Santa Cruz Biotechnology, Inc.

Grantham Distance calculation

The variations in amino acid sequence of *TLR9* as revealed in 1K humans genome sequence database was used for Grantham Distance calculation. All these changes in a single amino acid

coordinate were analysed and fed to Align GVGD (http://agvgd.hci.utah.edu/index.php), an online tool by IARC, WHO, which combines biophysical characteristics of amino acids and protein multiple sequence alignments to predict where amino acid substitutions in protein of interest fall in a spectrum from enriched deleterious to enriched neutral. Align-GVGD is an extension of the original Grantham differences to multiple sequence alignments and true simultaneous multiple comparisons. Thus, we obtained the GD (Grantham Deviation) score for each amino acid substitution in *TLR9*. The GD scores were plotted against the respective amino acid coordinates and different colour codes were given for different classifiers and were plotted.

Structure-function analysis studies *in silico*

3D-modelling study was done by the help of two different software named Phyre[2] (http://www.sbg.bio.ic.ac.uk/phyre2) and Raswin (http://rasmol.org/). PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) software analysis of each mutated sequence and reference TLR9 sequence has been done to check the effect of mutations on protein structures. By the help of this software, each amino of a sequence has been studied for its character (aatypes), its possibility to be present in a particular type of protein folding (psipred), and its possibility to interact with the membrane (memsat).