MAJOR ARTICLE

Single Nucleotide Polymorphisms in TLR9 Are Highly Associated with Susceptibility to Bacterial Meningitis in Children

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Background. Bacterial meningitis (BM) is a severe infection mainly caused by *Streptococcus pneumoniae* and *Neisseria meningitidis* (NM). However, genetically determined susceptibility to develop severe infections by these microorganisms is variable between individuals. Toll-like receptor 9 (TLR9) recognizes bacterial DNA leading to intracellular inflammatory signaling. Single nucleotide polymorphisms (SNPs) within the *TLR9* gene are associated with susceptibility to several diseases, no such association with meningitis has been described.

Methods. We studied the role of *TLR9* SNPs in host defense against BM. Two *TLR9* SNPs and 4 *TLR9* haplotypes were determined in 472 survivors of BM and compared to 392 healthy controls.

Results. Carriage of the *TLR9*+2848-A mutant was significantly decreased in meningococcal meningitis (MM) patients compared with controls (*p*: .0098, odds ratio [OR]: .6, 95% confidence interval [CI]: .4–.9). *TLR9* haplotype I was associated with an increased susceptibility to MM (*p*: .0237, OR 1.3, 95% CI: 1.0–1.5). In silico analysis shows a very strong immunoinhibitory potential for DNA of NM upon recognition by TLR9 (CpG index of -106.8).

Conclusions. We report an association of TLR9 SNPs with susceptibility to BM, specifically MM indicating a protective effect for the *TLR9*+2848-A allele. We hypothesize that the *TLR9*+2848-A mutant results in an up-regulation of TLR9 induced immune response compensating the strong inhibitory potential of NM CpG DNA.

BACKGROUND

Bacterial meningitis (BM) is a serious, life-threatening infectious disease of the central nervous system (CNS) that often occurs in young children. Despite adequate antibiotic treatment and the use of adjunctive therapy such as corticosteroids, the rates of mortality and morbidity remain high [1]. The 2 most common pathogens causing BM in children in the Western world are *Streptococcus pneumoniae* (SP) and *Neisseria meningitidis* (NM). The incidence of BM, defined as bacteria isolated from the cerebrospinal fluid (CSF), was 1.2 per 100.000 inhabitants for pneumococcal meningitis (PM) and .4 per 100.000 for meningococcal meningitis (MM) in 2008 in the Netherlands [2] but is much higher in developing countries [3] where immunization rates are low.

A crucial step in the first-line defense against BM is the recognition of bacteria by innate immune mechanisms, using pathogen recognition receptors (PRRs) expressed on antigen presenting and phagocytic cells present in neural structures and non-neural structures (including macrophages and dendritic cells) in direct contact with the CSF [4]. Toll like receptors (TLRs) are a key example of those PRRs. Within the CNS, TLRs are expressed on antigen presenting astrocytes and

Received 16 May 2010; accepted 18 November 2010.

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Clinical Infectious Diseases 2011;52(4):475–480

1058-4838/2011/524-0001$37.00
DOI: 10.1093/cid/ciq155

monocyte derived microglia [5]. TLR9 is an intracellular PRR that recognizes unmethylated cytosine-fosfate-guanine (CpG) motives in bacterial and viral DNA. Binding of TLR9 to non-self DNA triggers a cascade of intracellular receptor signaling, finally resulting in transcription of nuclear factors and the production of pro- and anti-inflammatory cytokines [6].

Single nucleotide polymorphisms (SNPs) have been identified in different *TLR* genes, and they affect the susceptibility to and severity of several inflammatory diseases [7, 8]. For example, the *TLR4*+896 A > G (Asp299Gly) SNP causes hyporesponsiveness to lipopolysacharide (LPS), an important content in the membrane of NM and has been shown to enhance susceptibility to NM infections [9, 10]. Smirnova et al. examined a large group ($n = 230$) of patients with meningococcal sepsis and compared the frequency of *TLR4* coding changes to those in an ethnically matched control group ($n = 421$). They observed that rare heterozygous missense mutations of *TLR4* significantly contribute to the risk of developing meningococcal sepsis in a white population (p: 2×10^{-6}; odds ratio [OR]: 27.0) [10]. Yuan et al. compared SNPs in *TLR2* and *TLR4* between children with invasive pneumococcal disease and healthy blood donors and concluded that the prevalence of the *TLR4*+896 A > G/ *TLR4*+1196 C > T SNP was significantly lower in patients than in controls ($p < .05$, OR: .3; 95% CI: .1 – 1) [11]. TLR mediated activation by interleukin-1 receptor-associated kinase 4 (IRAK4) is an important enzyme in the functioning of TLRs [12]. Eight different *IRAK4* SNPs are associated with recurrent pneumococcal- and some with meningococcal disease, characterized by the absence of fever and unresponsiveness to LPS in in-vitro whole blood tests [7]. One Gambian study on meningitis exclusively did not show an association between *TLR4* *TLR4*+896 A > G SNPs and susceptibility to MM during epidemics in children ($n = 50$) [13].

Genetically determined defects in innate immunity have been described in both meningococcal and pneumococcal infections within families. A large sibling study in UK whites showed that host genetic factors contribute up to one-third to the susceptibility to meningococcal disease [14]. Although many studies on genetic variation in TLRs and infections with SP and NM have been described, studies focusing on meningitis exclusively are rare.

The aim of this study is to investigate the role of *TLR9* SNPs in the susceptibility to PM and MM. *TLR9* SNPs have been associated with the susceptibility to pouchitis, systemic lupus erythematosus, atherosclerosis, and asthma [8, 15], but no association of *TLR9* SNPs with BM or meningococcal and pneumococcal infections has been described. We hypothesized that SNPs in *TLR9* genes might change the recognition of CpG motifs in bacteria causing BM, leading to a decreased or increased susceptibility to PM and MM. To investigate the role of *TLR9* SNPs in susceptibility to BM, we compared the frequencies of *TLR9* SNPs, representing *TLR9*+2848 and *TLR9*-1237 and their haplotypes, in survivors of BM ($n = 472$) with those in healthy controls ($n = 392$) without a known history of BM. To determine the importance of the CpG in SP and NM and its immunostimulatory ability, we also performed an in silico analysis, a computer-based scan of bacterial genomes for stimulatory and inhibitory CpG motives.

METHODS

Patients

The study population consists of 472 Dutch white school-aged children and adolescents who survived PM or MM. These former patients were selected from data on bacterial CSF isolates of the Netherlands Reference Laboratory for Bacterial Meningitis. The mean age of infection was 2 years. Of the 472 participating children, 83 (17.6 %) had PM and 389 (82.4 %) had MM. In total, 397 children had meningitis between January 1990 and December 1995, and this cohort was described in detail by Koomen et al. [16–18]. A similar cohort of 75 children had BM between 1997 and 2001. Children with "complex onset' of meningitis (defined as: meningitis secondary to immune deficiency states, CNS surgery, cranial trauma or CSF shunt infections or relapsing meningitis) were excluded (14 patients [3%]).

Controls

The control group consists of 392 healthy adult Dutch whites without a known history of BM. Serum samples were taken from healthy employees of the VU University Medical Center in Amsterdam and the Erasmus University in Rotterdam, both in The Netherlands. Since the SNPs studied are stable over ages, no age matched control were needed.

Patients were mailed and asked to return a sterile swab after collecting their buccal DNA. Of these patients, 472 (71.9% of all patients who were asked to participate) returned the swab and an informed consent form. The Medical Ethical Committee of the VU University Medical Center approved this study.

DNA Isolation

DNA was isolated from the buccal swabs using the following procedure: after addition of 250 μL 10 mmol/L Tris-HCl (pH 7.4) the sample was heated at 96 degrees Celsius for 10 minutes. After mixing for 10 seconds the swabs were removed and the sample was centrifuged (14.000 rpm). In controls, venous blood (5–10 mL) was drawn and genomic DNA was isolated using standard protocols; 5–100 ng of genomic DNA was used each cycle of genotyping.

Genotyping

The polymorphisms were analyzed by TaqMan analysis using the standard TaqMan protocol. The AbiPrism 7000 Sequence Detection System (Applied Biosystems) was used to obtain data.

Primer and probes used for *TLR9*-1237 were: forward primer 5′-GGCCTTGGGATGTGCTGTT-3′ and reverse primer 5′-GGTGACATGGGAGCAGAGACA-3′ and dual-labeled fluorogenic hybridization MGB-probes CTGCCTGAAAACT-5′ Fluor Label (FAM, 6-carboxyfuorescein) and CTGGAAACTCCCC-5′ Fluor Label (VIC). The primers and probes used for *TLR9*+2848 were: forward primer 5'-CCGCTGTGCAGGTGCTAGAC-3' and reverse primer 5′-CCAAAGGGCTGGCTGTTGTA-3′ and dual labeled fluorgenic hybridization MGB probes AGCTACCGCGACTGG-5′ Fluor Label (FAM) and AGCTACCACGACTGG A-5′ Fluor Label (VIC).

Haplotypes

The 2 *TLR9* SNPs we analyzed, were chosen on the basis of a study by Lazarus et al. [19] in which a set of 4 frequent *TLR9* SNPs (*TLR9*-1486, *TLR9*-1237, *TLR9*+1174, and *TLR9*+2848) were described. Genotyping of both *TLR9*-1237 T > C (NCBI SNP CLUSTER ID: rs5743836) and *TLR9*+2848 G > A (NCBI SNP CLUSTER ID: rs352140) allows all 4 locus haplotypes to be distinguished. (Haplotype I: -1486T/-1237T/+1174A/+2848G, Haplotype II: -1486C/-1237T/+1174G/+2848A, Haplotype III: -1486T/-1237C/+1174G/+2848A, Haplotype IV: -1486T/-1237C/+1174G/+2848G or -1486C/-1237T/+1174G/+2848G or 1486C/-1237C/+1174G/+2848G or -1486T/-1237T/+1174G/+2848A. *TLR9* haplotypes were inferred using PHASE v2.1.1 [20, 21] and SNPHAP [22].)

Statistics

Genotypes were compared between cases and controls for the PM and MM group separately and for all cases of BM. Hardy Weinberg tests were used to test for Mendelian inheritance. Fisher exact test and χ^2 test were employed where appropriate. *P* values < .05 were considered statistically significant.

In Silico Analysis

To determine the immunostimulatory ability of the CpG-DNA in SP and NM we performed an in silico analysis as described by Lundberg et al. [23]. To determine the relative prevalence of canonical stimulatory and inhibitory CpG motifs in the genomic sequences of the various bacteria, we employed a two-step approach. Genbank-retrieved FASTA formatted text files were uploaded to '**Error! Hyperlink reference not valid.**; and the frequency of all 4096 possible hexamers were determined as described by Bikandi et al. [24]. This information and the general composition of each genome was then entered into Microsoft Excel, and common-place formulas were used to count the occurrence of specific CpG motifs. As a measure of SP and NM genome's potential for TLR9 stimulation, the CpG index is used to facilitate comparison of immunostimulatory potentials regardless of genome size, G + C content and overall CpG suppression and was calculated by comparing the frequency of stimulatory and inhibitory CpG motifs [23].

Table 1*A*. *TLR9*+2848 Genotype Distribution in Cases and Controls

TLR9+2848	N	GG Wild type (%)	GA Heterozygous (%)	AA Mutant (%)
Total BM[a]	460	110 (23.9)	214 (46.5)	136 (29.6)
S. pneumoniae	80	17 (21.3)	36 (45.0)	27 (33.8)
N. meningitidis[b]	380	93 (24.5)	178 (46.8)	109 (28.7)
Controls*	392	66 (16.8)	192 (49.0)	134 (34.2)

[a] *TLR9*+2848 mutant allele (genotypes GA or AA) was significantly decreased in BM patients compared to controls (p: .0110, OR .6, 95% CI .5 – .9).

[b] *TLR9+2848* mutant allele was most significantly decreased in MM patients compared to healthy controls (p: .0098, OR: .6, 95% CI: .4 – .9)

RESULTS

Susceptibility Analysis

Single SNP analysis. For susceptibility analysis, BM patients were compared to the control group. The PM and MM groups were also separately compared to the controls. PM and MM patients were also compared to each other to discover associations between SNPs and susceptibility to a specific pathogen. The results are summarized in Table 1*A* and 1*B*. Because concentrations and quality of DNA varied among samples, some samples that could not be genotyped after 3 or more TaqMan analyses were excluded. This explains the difference in numbers of included patients and numbers of genotyped patients in the tables.

Carriage of the *TLR9*+2848 mutant allele (genotypes GA or AA) was significantly decreased in BM patients compared with controls (*p*: .0110, OR .6, 95% CI .5–.9). Carriage of the *TLR9+2848* mutant allele was also significantly decreased in MM patients compared with healthy controls (*p*: .0098, OR: .6, 95% CI: .4—.9), but the genotype distribution in PM patients did not differ significantly from healthy controls. There were no significant differences between PM and MM patients. The *TLR9*-1237 SNP was not significantly associated with the susceptibility to BM.

Haplotype analysis. Genotyping of both *TLR9*-1237 and *TLR9*+2848 allows all 4 locus haplotypes to be distinguished. The results are summarized in Table 2. *TLR9* haplotype I was

Table 1*B*. *TLR9*-1237 Genotype Distribution in Cases and Controls

TLR9-1237	N	TT Wildtype (%)	TC Heterozyous (%)	CC Mutant (%)
Total BM	464	341 (73.5)	113 (24.4)	10 (2.2)
S. pneumoniae	82	61 (74.4)	20 (24.4)	1 (1.2)
N. meningitidis	382	280 (73.3)	93 (24.4)	9 (2.4)
Controls	392	274 (69.9)	112 (28.6)	6 (1.5)

Table 2. Frequencies of *TLR9* Haplotypes in Cases and Controls

TLR9 haplotypes (2n)		I[a] (%)	II[b] (%)	III[c] (%)	IV[d] (%)
Total BM*	914	419 (45.8)	363 (39.7)	124 (13.6)	8 (0.9)
S. pneumoniae	160	69 (43.1)	69 (43.1)	21 (13.1)	1 (0.6)
*N. meningitidis**	754	350 (46.4)	294 (39.0)	103 (13.7)	7 (0.9)
Controls*	784	319 (40.7)	341 (43.5)	118 (15.1)	6 (0.8)

[a-d]:Haplotypes as defined by Lazarus *et al.* [19]

* *TLR9* haplotype I was significantly increased in BM patients compared to the control group; (p: .0348, OR 1.2, 95% CI 1.0 – 1.5). *TLR9* haplotype I was significantly increased in the MM patients compared to healthy controls; (p: .0237, OR 1.3, 95% CI: 1.0 – 1.5).

significantly increased in BM patients compared with the control group; (*p*: .0348, OR 1.2, 95% CI 1.0—1.5). TLR9 haplotype I was significantly increased in the MM patients compared to healthy controls; (*p*: .0237, OR 1.3, 95% CI: 1.0—1.5). There were no significant differences in haplotype I distribution between the PM group and controls, neither between the PM and the MM group. For the distribution of other haplotypes no significant differences were found between the control group and the total group of BM patients, nor between the PM and MM patients separately.

In Silico Analysis

In Table 3 we summarized CpG indices for causative agents of BM. Positive CpG indices indicate immunostimuatory properties, whereas negative values indicate immunoinhibitory properties. Increasing values for the CpG indices indicate stronger effects. SP showed a mildly immunostimulatory potential with a CpG index of 8.6. This was comparable to another causative agent of meningitis, *H. influenzae*. Interestingly, for NM we found a very strong immunoinhibitory CpG index of -106.8.

DISCUSSION

This study describes a strong association between TLR9 SNPs and bacterial meningitis in a large cohort of BM survivors. Based on our findings we propose that TLR9 genetic variation can compensate for the inhibitory effects of *Neisseria meningitidis* CpG DNA resulting in a reduced susceptibility for NM meningitis.

Carriership of the *TLR9*+2848-A mutant decreases the susceptibility to BM, specifically MM ($P = .0098$). Carriage of *TLR9* haplotype I was associated with an increased risk to develop MM. This can be explained for the greatest part by the fact that this haplotype does not contain the protective *TLR9*+2848-A allele. KO-mice data showed that the presence of the TLR9 is essential to combat meningococcal infection [25]. The in silico analysis showed a very strong immunoinhibitory potential (CpG index of -106.8) for the DNA from NM. Combining the SNP, haplotype, KO-mice data and in silico analyses, one might hypothesize that the *TLR9*+2848-A mutant results in an up-regulation of TLR9 induced immune response compensating the strong inhibitory potential of MM CpG DNA.

Besides activation of microglia and astrocytes [5, 26]. and complement mediated bacterial lysis [27] aso antibacterial responses like antimicrobial peptides and reactive nitrogen and oxygen radicals are relevant in the pathogenesis of BM. A recent study using human embryonic kidney cells reported TLR9 activation by NM [28]. Sjolinder et al. reported that *TLR9* (-/-) mice displayed reduced survival, elevated levels of bacteremia, and reduced bactericidal activity in vivo compared with wild-type mice during meningococcal bacteraemia [25]. They also found that antigen presenting cells relied entirely on TLR9 to induce activation of signal transduction and induction of proinflammatory cytokine gene expression. Together, these studies show the importance of TLR9 upon bacteremia with NM. We propose that the *TLR9* SNPs we described are able to

Table 3. Results of the In Silico Analysis

	CpG motif deviation[a]						
	Genome				Consensus motifs[b]		
Bacterium	Size (Mb)	G + C (%)	CPG/kb[c]	Total CpG[d]	Stimulatory[e]	Inhibitory[f S]	CpG index[g]
Streptococcus pneumoniae	2.22	39.5	78.0	69.5	82.4	66.5	8.6
Neisseria meningitidis	2.27	51.5	132.7	130.6	78.4	140.0	-106.8
Haemophillus influenzae	1.91	38.2	72.8	109.1	105.5	96.4	7.2

[a] Deviations in specified motif occurrences relative to those expected based on genomic G + C content.

[b] Consensus stimulatory and inhibitory CpG hexamer motifs are based on published analysis. They are used in this table as indicators of the general frequencies of stimulatory and inhibitory CpG hexamer motifs in each genome.

[c] Number of CpG hexamer motifs (NNCGNN) occurring in each genome normalized to 100 kb.

[d] Total frequency of CpG hexamer motifs (NNCGNN); expected number based on nucleotide composition of the genome.

[e] Frequency of consensus stimulatory hexamer motifs (RRCGYY).

[f] Frequency of consensus inhibitory hexamer motifs (NCCGNN and NNCGRN).

[g] Calculated from frequencies of stimulatory less inhibitory consensus hexamer motifs as an indicator of stimulation versus inhibition multiplied by total CpG number (normalized to 1 kb) and the overall frequency of CpG (NNCGNN).

enhance TLR9 function and prevent high levels of bacteremia, an important step in the pathophysiology of BM.

Once inside the CNS, bacteria are recognized by antigen presenting astrocytes and microglia. Intracellular recognition of (bacterial) CpG motifs leads to immune activation inside the CNS by the proinflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) and the granulocytic chemoattracktant IL-8 [29]. The effects of continuous intrathecal CpG DNA exposure to the brain of TLR9 (-/-) mice in the CNS were studied by Tauber et al. Microglia from TLR9 -/- mice could not be activated by CpG DNA. Brains of wild-type mice showed more pronounced neuronal damage with microglial activation and reactive astrogliosis. These results suggest that the unfavorable effects of CpG DNA in the brain are dependent on TLR9 and may contribute to neuroinflammation [30]. Very recently, Ribes et al. reported increased phagocytic activity by murine microglia activated with agonists for TLR2, -4, and -9 upon exposure by SP [31]. We speculate that this might also happen in MM and that the *TLR9* SNPs we described result in enhanced phagocytosis by microglia inside the CNS. In contract, by using mice with single- or combined deficiencies of TLR2, TLR4, or TLR9, Klein et al. demonstrated that TLR2 and TLR4 play an essential role in PM, whereas additional TLR9 deficiency did not result in further attenuation of the inflammatory reaction observed TLR2-TLR4-double deficient mice [32]. We did not find this protective TLR9 effect in our SP cohort either.

The strength of this study is that we focused exclusively on meningitis patients to find the host genetic determinant of this specific disease. We recruited a large group of MM survivors and found very significant results. Our cohort of PM survivors was relatively small however, and we could not find significant results for this group. In our study we used a healthy adult control group, but we have no specific information on a history of BM. However, since the maximum incidence of BM in the Netherlands was low (incidence per 100.000 inhabitants varied between 4.8 and 1.6 during the period 1999–2008 in the Netherlands) [2], we considered it a representative control group for immunogenetic research on BM. DNA from children with fatal BM who suffered the most severe infections was not available. We expect, however, that including DNA of fulminant cases will only make the association we found for susceptibility to BM stronger, although future studies focusing on the severity of BM specifically would be of interest in this respect but these cases are rare and collecting 400 cases would be near to impossible.

The results we found in this study will guide future research on genetic studies in susceptibility to and severity of BM in our cohort. Genetic studies can reveal relevant SNPs in immune response genes influencing the pathogenesis of BM. This might also identify potential drug targets. For example, TLR activation leads to cytokine production mostly via nuclear factor kappa B (NFκB) and the mitogen-activated proteins kinase (MAPK) family. Clinical trials are prepared to block NFκB and MAPK transcription [33, 34]. However, none of such potential drugs are used for clinical practice yet. Identification of specific SNPs could be used to develop a customized treatment fitting the patient's genetic profile and decreasing side effects. Patients carrying SNPs known to influence the immune response might receive medication to either stimulate or inhibit this response. Better understanding of the role of immunogenetics in the pathogenesis of BM may allow the prediction of individual risk to develop BM, enabling a tailored approach to follow up. Besides clinical factors, host-genetic factors (SNPs) may be valuable markers for the prediction of long term consequences of BM including hearing loss, and neuropsychological complications [17, 18, 35] in an early stage of disease. Multiple associations have already been described between SNPs in innate immunity genes and the outcome of SP and NM infections [36–38].

Three important steps have to be made to translate these finding into patient management: (1) the study has to be confirmed and addition SNPs in other genes have to added in a genetic trait to obtain synergy in the prediction of susceptibility or protection to BM, (2) this genetic trait has to added to potentially strengthen current clinical prediction rules on complication rates after BM which include hearing loss and academic and behavioral limitation [16–18], and (3) implement strategies to promote a faster path for genetic knowledge from bench to bedside. The various stakeholders in public health play a key role in translating the implications of genomics such as deriving from molecular epidemiology and host-pathogen genomics. This knowledge will not only enable clinical interventions but also health promotion messages and disease prevention programs to be targeted at susceptible individuals as well as subgroups of the population based on their genomic profile (personalized healthcare) [39, 40]. The field involved in this translation is called Public Health Genomics which has as major task "*the responsible and effective translation of genome-based knowledge and technologies into public policy and health services for the benefit of population health*" (Bellagio statement, 2005: see www.graphint.org for details).

In summary, our findings provide another step toward the use of SNPs in immune response genes as valuable markers to assess the risk to develop BM. Additional immunogenetic studies with larger sample sizes, validation cohorts, and genotyping including multiple synergistic genes and more complete haplotype information will help to elucidate the pathophysiology of meningitis and to explain the inter-patient variability in BM, largely depending on the ability of the (innate) immune system to clear the infection, with as an ultimate goal to predict the course and outcome of infection.

Acknowledgments

The authors acknowledge Jolein Pleijster at the Laboratory of Immunogenetics of the VU University Medical Center for expert technical assistance

and Rogier de Jonge, neonatologist at the department of Pediatrics of the VU University Medical Center, together with Arisja Mauritz, medical student of the VU University for their excellent support in data inclusion.

Potential conflicts of interest. none reported.

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