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Research Paper

Variation of 46 Innate Immune Genes Evaluated for their Contribution in Pneumococcal Meningitis Susceptibility and Outcome

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ABSTRACT

Pneumococcal meningitis is the most common and severe form of bacterial meningitis. Early recognition of the pathogen and subsequent innate immune response play a vital role in disease susceptibility and outcome. Genetic variations in innate immune genes can alter the immune response and influence susceptibility and outcome of meningitis disease.

Here we conducted a sequencing study of coding regions from 46 innate immune genes in 435 pneumococcal meningitis patients and 416 controls, to determine the role of genetic variation on pneumococcal meningitis susceptibility and disease outcome.

Strongest signals for susceptibility were rs56078309 *CXCL1* ($p = 4.8e-04$) and rs2008521 in *CARD8* ($p = 6.1e-04$). For meningitis outcome the rs2067085 in *NOD2* ($p = 5.1e-04$) and rs4251552 of *IRAK4* were the strongest associations with unfavorable outcome ($p = 6.7e-04$). Haplotype analysis showed a haplotype block, determined by *IRAK4* rs4251552, significantly associated with unfavorable outcome ($p = 0.004$). Cytokine measurements from cerebrospinal fluid showed that with the *IRAK4* rs4251552 G risk allele had higher levels of IL-6 compared to individuals with A/A genotype ($p = 0.04$).

We show that genetic variation within exons and flanking regions of 46 innate immunity genes does not yield significant association with pneumococcal meningitis. The strongest identified signal IRAK4 does imply a potential role of genetic variation in pneumococcal meningitis.

1. Introduction

Community-acquired meningitis is a life-threatening infection of the membranes surrounding the brain and spinal cord. Pneumococcal meningitis is the most common and severe form of bacterial meningitis. Fatality rates are substantial, and long-term sequelae develop in about half of survivors (Brouwer et al., 2010; Schut et al., 2012; van de Beek et al., 2012; van de Beek et al., 2004; Zoons et al., 2008). Vaccination has decreased the incidence of invasive pneumococcal disease in infants and recently also in the adult population (Bijlsma et al., 2016; McIntyre et al., 2012; Tsai et al., 2008).

Streptococcus pneumoniae is a human commensal strain adapted to colonize the nasopharynx (Brown et al., 2015). However, after asymptomatic colonization translocation of the pneumococcus to the respiratory tract, sinuses and nasal cavity, *S. pneumoniae* can cause pneumonia, acute sinusitis, otitis media, bacteremia, sepsis and meningitis (Brown et al., 2015; Mook-Kanamori et al., 2011; van de Beek et al., 2006). One of the first host determinants of developing an infection is the recognition and clearance of the pneumococcal strains with initiation of an inflammatory response by the innate immune response. The innate immune response depends on specific pathogen-associated molecular pattern molecules (PAMPS) of the pneumococcus. For example, peptidoglycan and lipoteichoic acid (LTA), are recognized by membrane

Abbreviations: CSF, cerebrospinal fluid; FDR, False Discovery Rate; GATK, genome analysis tool kit; GOS, Glasgow Outcome Scale; HWE, Hardy–Weinberg equilibrium; IPD, invasive pneumococcal disease; LTA, lipoteichoic acid; MAF, minor allele frequency; MDS, multidimensional scaling; NLRs, NOD-like receptors; NRLBM, Netherlands Reference Laboratories For Bacterial Meningitis; OR, odds ratio; PAMPS, pathogen-associated molecular pattern molecules; PCR, polymerase chain reaction; RVIS, residual variation intolerance score; SKAT, SNP-set (Sequence) Kernel Association Test; TLR, Toll-like receptors.

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surface and intracellular Toll-like receptors (TLRs) found on leukocyte cells (Santos-Sierra et al., 2006). After PAMP recognition, intracellular signaling initiates the activation of transcription factors. This leads to the induction of small cell signaling proteins, called cytokines, which are responsible for the inflammatory response and attracts immune cells to the site of infection (Akira et al., 2006). TLRs, like TLR2, TLR4, TLR9 and NOD-like receptors (NLRs) as NOD2 are known to be important in the recognition of invasive pneumococcal strains (Koppe et al., 2012). Differences within these or underlying signaling proteins, caused by genetic variations, can contribute to differences in the immune response affecting the susceptibility to disease and its severity.

Genetic variations *TLR4, TIRAP, NFKBIA and NFKBIB, TNF, IL10, IL-6, IRAK4* and *IKBKG* have been previously described to influence susceptibility to invasive pneumococcal disease (Brouwer et al., 2009; Chapman et al., 2007). The role of genetic variation in innate immune genes in pneumococcal meningitis and its effect on disease outcome is less well known. In this study, we sequenced the exons and flanking intron borders of all known genes that are important in innate immune signaling pathway in a pneumococcal meningitis patient population for which detailed clinical data has been collected. This gives us the opportunity to study association of innate immune gene variation with disease susceptibility and outcome, and to gain further insight in the role of genetic variation in the innate immune system during pneumococcal meningitis.

2. Material and Methods

2.1. Dutch Bacterial Meningitis Cohort

In a nationwide prospective cohort study (MeninGene) we included adult patients, age of 16 years and older, with community-acquired bacterial meningitis with positive CSF cultures who were identified by the Netherlands Reference Laboratories for Bacterial Meningitis (NRLBM). The cohort and inclusion procedure is described elsewhere (Bijlsma et al., 2016).

A total of 1300 patients and partners or non-related proxies, living in the same residence, used as controls included during 2006 and 2011, were included in this study. Patient data were collected in an online case record form and included presenting characteristics, treatment, complications and outcome. Patient outcome was graded at discharge according the Glasgow Outcome Scale (GOS) (Jennett et al., 1976). A score of one on this five point scale indicates death, score of two vegetative state, score three severe disability, score four moderate disability, and a score of five mild or no disability. We considered a score of 5 a favorable outcome and scores 1 to 4 were defined as unfavorable outcome.

Blood for DNA extraction was withdrawn from the patients and collected in sodium/EDTA tubes. Isolation of the DNA was performed with the Gentra Puregene isolation kit (Qiagen) according to manufacturer's protocol, thereafter the yield and quality of the extractions were determined to ensure appropriate conditions for genotyping.

2.2. Ethical Approval

This study was approved by the ethics committee of the Academic Medical Center, Amsterdam, the Netherlands. Informed consent was obtained from all participating individuals or legally authorized representatives. The study was conducted according to the principles of the Declaration of Helsinki (version of 2013. Fortaleza, Brazil) and in accordance with the Medical Research Involving Human Subjects Act (WMO) and other guidelines, regulations and acts.

2.3. Marker Selection

Forty-six innate immune genes were selected for exome sequencing (Supplementary Table 1). Inclusion criteria were: (1) genes with known involvement of pneumococcal recognition, (2) genes with variations that have previously been associated with invasive pneumococcal disease and meningitis and (3) genes that encode for related downstream signaling and transcription proteins in the TLR, NOD and inflammasome signaling pathways. This led to the inclusion of 46 innate immune genes (Supplement Table 1).

2.4. Solid Sequencing

DNA concentration was determined by means of fluorometric measurement (Qubit, Thermo) and quality was checked by means of determining the absence of degradation and presence of High Molecular weight DNA. Circa 1,5 μg DNA was sheared by sonication followed by barcoded adaptor ligated library construction, using the Biomek FX automated liquid handler (Beckman Coulter). Solid fragment Library preparation kit and solid barcoded adaptors were used according manufacturers protocol (Life Technologies, 5500 SOLID™ Fragment Library Core Kit, Catalog number 4464412). Each sample was generated using a separate 10 bp barcode incorporated in the adaptor sequence. After 8 cycles of amplification using the library prep kit supplied PCR primers, PCR mix and PCR protocol and purified twice-using ampure. Two rounds of hybridization capture was performed using a Custom Complement Capture (Nimblegen, pn 130204_HG19_CompCapV2_MJ_EZ_HX3). Equimolar pooling of the captured libraries was based on concentration and average sample size. Emulsion PCR was performed using the Solid EZ Bead Emulsifier and Amplifier (Applied Biosystems). Sequencing was performed on the Solid 5500xl sequencer (Life technologies) generating paired-end reads (50 bp forward and 35 bp reverse).

2.5. Alignment and Variation Calling Pipeline

Paired-end reads for each individual were merged using Picard (version 1.92) and aligned to the GRch37/HG19 reference genome using the Lifescope aligner (version 2.5.1, Applied Biosystems). To minimize mismatched bases between reads, realignment was performed using the RealignerTargetCreator function in GATK (version 2.7-4) using the intervals from Mills_and_1000G_gold_standard.indels.hg19.vcf. Mate information can be changed during realignment and changes were fixed using Picard. Samples were recalibrated with GATK Recalibrate and variants were called using GATKs HaplotypeCaller (version 3.3-0) with default settings and only adjusting the minimal mapping quality score to 30. Variants were then filtered on a minimal read depth of 20, allele balance between 0.2 and 0.8 for heterozygotes, >0.9 for homozygotes and genotype quality of 99. After filtering, all samples were combined and genotyped by using GATK CombineGVCFs and GenotypeGVCFs (version 3.3-0) and converted to PLINK (Chang et al., 2015). Finally, all chromosomal locations for found variants were annotated using SnpEff and the UCSC variant annotation integrator tool (https://genome.ucsc.edu/) (Cingolani et al., 2012).

2.6. Variants and Sample QC

After alignment and variation calling, we removed all individuals of which we found that there was a third, or higher, degree of relatedness. Reported ancestry was used to exclude non-European ancestry to account for population stratification. Because the ancestry was reported we also evaluated the effect of ancestry for all common variants (MAF ≥ 0.01) by calculating the multidimensional scaling (MDS) analysis with PLINK and including them as covariates.

All individuals with missingness of 5% or heterozygosity above or below 3 standard deviations from the mean were removed. Finally, variations were removed from the analysis when having a missingness > 5% and HWE p-value < 1.0e − 05, QC steps were conducted with PLINK (Chang et al., 2015). Variations in short insertions and deletions (indels) are difficult to correctly detect with the short read