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Original article

Transcriptome signature in young children with acute otitis media due to *Streptococcus pneumoniae*

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Abstract

Streptococcus pneumoniae (*Spn*) is the predominant causative organism of acute otitis media in children. To better understand the genes that are regulated at the onset of AOM caused by *Spn* infection in the middle ear, the transcriptome profile of peripheral blood mononuclear cells isolated from children prior to and during an AOM event was evaluated by microarray. We found that 1903 (6.2%) of 29,187 genes were differentially regulated greater than 2-fold at the onset of AOM compared to the pre-infection stage of the same children. The ontology of differentially regulated genes was dominated by those involved with the immune response. At onset of infection, genes associated with bacterial defenses were significantly up-regulated, including beta-defensin123, S100 protein A12, Toll-like receptor 5, IL-10, and those involved in the classical and alternative complement pathways. Genes associated with inhibition of bacterial entry through clathrin-dependent endocytosis were also up-regulated. In contrast, genes associated with cell-mediated immune responses were broadly down-regulated. The results provide the first human transcriptome data identifying genes differentially regulated at the onset of AOM in children.

Keywords: *Streptococcus pneumoniae*; Acute otitis media; Transcriptome profile

1. Introduction

Acute otitis media (AOM) is the most frequent reason that children seek medical care for illness and receive antibiotics. AOM is associated with significant middle ear inflammation, mainly caused by bacterial infection. *Streptococcus pneumoniae* (*Spn*) is one of the most frequent pathogens isolated from middle ear fluid (MEF) of children with AOM [1]. The pathogenesis of AOM in humans involves nasopharyngeal (NP) colonization by the bacteria achieved by adherence and invasion of host NP epithelial cells. The bacterial inoculum in the NP then refluxes up the eustachian tube near the adenoid tissues and gains entry to the middle ear. In *in vitro* experiments and an animal model, a robust inflammatory response with significant gene regulation has been described during *Spn* infection [2,3] but data on human infection are needed.

Here we describe, for the first time in human children, an evaluation of the gene expression profile of peripheral blood mononuclear cells (PBMCs) associated with onset of AOM caused by Spn. This analysis was made possible during a prospective study on the immune responses of children to *Spn* during AOM with comparable PBMC samples taken within 4 weeks prior to onset of AOM. Double sampling from the same children reduced the complexities of analysis due to genetic heterogeneity among individuals. Closely timed sampling avoided the influence of changing age on responses in pediatric subjects. Our previous work identified significant changes in blood of a limited number of specific pro-inflammatory molecules during AOM caused by *Spn* [4–6]. However, with transcriptome analysis, we were able to identify a much broader range of significant changes in gene regulation.

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2. Materials and methods

2.1. Subjects

The samples were collected from children (age 10–15 months) as approved by the IRB at Rochester General Hospital. The diagnosis of AOM was based on symptoms of fever, irritability or ear ache, and physical signs of inflammation of the tympanic membrane (bulging) with the presence of middle ear fluid (pus-laden fluid) documented by tympanocentesis. The inclusion criteria for children were: Caucasian children; pre-infection PBMC/serum sample within 4 weeks before the onset of AOM and the collection of an acute stage of illness PBMC/serum sample within 24 h of onset of AOM; age 10–15 months at onset of AOM; AOM caused by *Spn* as proven by tympanocentesis; and moderate to severe AOM as judged by the physician. Exclusion criteria were: a history of immunodeficiency; chronic or recurrent AOM; any chronic disease; any other concurrent infectious disease; or receipt of steroids or other immunomodulatory agents within 1 month of any PBMC/serum sample.

2.2. Identification of pathogens

Identification of *Spn* in MEF was performed based on presence of α-hemolysis and inhibition of optochin and confirmed by a positive slide agglutination test according to established CLSI procedures (BBL™ Pneumoslide™ Kit, BD, Tetax, USA). *Nontypeable Haemophilus influenzae (NTHi)*, *Moraxella catarrhalis* and other otopathogens were identified as described previously [7]. The culture results were further verified by multiplex PCR as described previously [8].

2.3. RNA isolation

4–10 ml of heparinized peripheral venous blood was collected for isolation of PBMCs and sera. PBMCs were isolated by Ficoll gradient and total RNA was extracted from PBMCs using a QIAamp RNA blood Mini Kit (Qiagen, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA was treated with DNase I to remove contaminating DNA (Qiagen). RNA quality and integrity were determined utilizing an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only high quality RNA, having a RIN of >7.0, and an A_{260}/A_{280} absorbance ratio of >1.8, was utilized for further experimentation.

2.4. Microarray analysis

DNA microarray analysis was performed in a blinded manner by Phalanx Biotech using the Human Whole Genome OneArray™ (Phalanx Biotech, Palo Alto, CA), which included 29,187 human genome probes and 1088 experimental control probes. RNA was converted to double-stranded cDNA and amplified using *in vitro* transcription that included amino-allyl UTP, and the aRNA product was subsequently conjugated with Cy5™ NHS ester (GEH Life sciences, Piscataway, NJ). Fragmented aRNA was hybridized at 42 °C overnight using the HybBag mixing system with 1× OneArray Hybridization Buffer (Phalanx Biotech, Belmont, CA), 0.01 mg/ml sheared salmon sperm DNA (Promega, Madison, WI), at a concentration of 0.025 mg/ml labeled target. After hybridization, the arrays were washed according to the OneArray protocol. For the hybridization, the controls included dye intensity controls, positive controls containing a mixture of probes from 58 robustly expressed housekeeping genes and negative controls containing eight 60-mer oligonucleotide probes that do not cross-hybridize with human genomic probes on the Human OneArray DNA microarray. Gene expression was assessed in three replicates of each PBMC sample.

2.5. Quantitative real-time reverse transcriptase PCR analysis

Real-time PCR was performed in our laboratory at the Rochester General Hospital Research Institute using a saved aliquot of the RNA preparation from the microarray experiments as templates. 100 ng of total RNA was reverse transcribed to cDNA using an RT^2 First Strand Kit (QIAGEN). Detection and quantification of gene expression in PBMCs was performed using an RT^2 Profiler Human Custom Kit according to the manufacturer's instructions (QIAGEN). Quantitative real-time reverse transcriptase PCR was performed using a CFX 96 Thermocycler (Bio-Rad, Fairfield, CA). The threshold and baseline were set automatically using the pcr/array analysis method according to the manufacturer's instructions (SABiosciences). C_T data were uploaded into the data analysis template on the manufacturer's website (http://www.sabiosciences.com/pcr/arrayanalysis.php). The relative expression of genes compared with the expression in control samples was calculated on the website using the ΔC_T method with five housekeeping genes as controls.

2.6. Proteome assays

The protein levels of S100A12 in the serum and MEF samples were determined from the same children where microarray was performed using the CircuLex S100A12 ELISA Kit according to standard instructions in the protocol. IL-10 levels were determined in the same samples using a Bio-Plex Pro Assay (Bio-Rad Laboratories, Hercules, California, USA) according to manufacturer's instructions and read with a Luminex instrument.

2.7. Data analysis

Primary data analysis was performed by Phalanx Biotech. Raw intensity signals for each microarray were captured using a Molecular Dynamics™ Axon 4100A scanner, measured using GenePixPro™ Software, and stored in GPR format. The data from all microarrays in each experimental set was then passed to Rosetta Resolver (Rosetta Biosoftware, Cambridge, MA) for analysis. Testing was performed by combining technical replicates (http://www.phalanxbiotech.com) and