Cord Blood Gene Expression in Infants Hospitalized with Respiratory Syncytial Virus Bronchiolitis

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Background. Only a few infants develop acute bronchiolitis when exposed to respiratory syncytial virus (RSV), and host, environmental, and viral properties are probably all of importance in determining the severity of infection.

Methods. Microarray analysis was used to identify differentially expressed single genes and gene sets in cord blood from 5 infants hospitalized with RSV bronchiolitis versus cord blood from 5 control infants exposed to RSV without bronchiolitis during infancy. Quantitative real-time polymerase chain reaction (QRT-PCR) was performed on single genes in both the 5 infants selected for microarray analysis and 13 more infants hospitalized with the same disease. Gene set enrichment analysis (GSEA) was performed to identify differentially expressed gene sets within the microarray experiments.

Results. Microarray analysis identified 15 single genes to be significantly differentially expressed between case and control infants. Eleven of these genes were evaluated with QRT-PCR, and the genes FAM102A, TNFRSF25, and STMN3 were down-regulated in all but 1 of the 18 infants. A pathway involved in regulation of the actin cytoskeleton was found to be clearly down-regulated when analyzed with GSEA.

Conclusions. FAM102A, TNFRSF25, and STMN3 and a pathway involved in regulation of the actin cytoskeleton are down-regulated in cord blood from infants hospitalized with RSV bronchiolitis.

Respiratory syncytial virus (RSV) is an important cause of childhood respiratory tract infection worldwide, and most children are infected by age 2 years. The clinical course varies from mild upper respiratory tract symptoms (“common cold”) to bronchiolitis or pneumonia. Reinfections are common, but the first infection is usually the most severe and leads to hospitalization in 2%–3% of cases, with the majority occurring in otherwise healthy infants [1, 2]. Why some infants develop bronchiolitis when exposed to RSV is poorly understood, and several aspects of environmental and host immunity have been extensively studied [3]. Genetic studies have shown an association between variations in the interleukin-8 and -10 genes and susceptibility to severe RSV bronchiolitis [4, 5]. Other studies have proposed a possible protective role of antenatal RSV sensitization to bronchiolitis during early life [6].

The aim of the present study was to identify genes expressed in cord blood from infants hospitalized with RSV bronchiolitis during infancy and to discuss the impact of these genes on severity of infection.

Microarray analysis can detect the simultaneous expression and interactions of thousands of genes [7]. This technology was used as a hypothesis-generating tool to identify the most differentially expressed genes and gene sets in cord blood samples from infants hospitalized with RSV bronchiolitis. To evaluate our microarray gene-expression results, quantitative real-time polymerase chain reaction (QRT-PCR) was performed [8]. To do this, cord blood samples from 13 infants hospitalized with the same disease, as well as cord blood samples from 5 infants selected for microarray analysis, were analyzed.

MATERIALS AND METHODS

Patients and Control Infants

From a total of 3500 deliveries in Akershus University Hospital during the period 20 January 2002 to 15 Feb-
ruary 2003, a basic birth cohort of 2108 healthy infants was
established. For each delivery, a venous blood sample for iso-
lolation of total RNA was collected from the umbilical cord, and
informed consent for further studies was obtained from the
parents. If a child was hospitalized with acute bronchiolitis
during infancy, another venous blood sample for RNA isolation
and a sample of mucous from nasopharyngeal aspirate (NPA)
for microbiological diagnosis were collected as part of the ad-
mission routine. When the follow-up period ended on 15 Feb-
uary 2004, a total of 57 infants from the basic birth cohort
had been hospitalized with acute bronchiolitis. Of these, 27 had
RSV subtype B bronchiolitis and were selected for further eval-
uation. Eighteen of the 27 cord blood samples had acceptable
RNA quality and were selected for final analyses. Cord blood
samples from 5 boys in this group were randomly selected for
microarray analysis, whereas the remaining 13 infants took part
in the QRT-PCR studies (tables 1 and 2).

By random telephone interview of parents, we invited a lim-
ited number of children from the same basic birth cohort to
a follow-up visit at age 1 year. These children had not been
hospitalized and/or treated for acute bronchiolitis during in-
fancy. Venous blood, including a sample for isolation of total
RNA and a sample for serological testing, was collected, and
the parents were once again asked whether the child had been
hospitalized and/or treated for acute bronchiolitis during in-
fancy. All clinical examinations and interviews with the parents
of these children were performed by the principal investigator,
and answers given by the parents were checked against hospital
discharge records. Five boys with cord blood of acceptable RNA
quality were used as matched control infants in the cord blood
microarray experiments, and a pooled cord blood RNA sample
from the same 5 boys was used as an exogenous control in the
QRT-PCR studies (tables 1 and 2). All 5 boys tested positive
at age 1 year for previous RSV exposure when their samples
were analyzed for RSV antibodies by use of a standard com-
plement-fixation test [9].

Clinical and Virological Diagnosis
The infants received a diagnosis of bronchiolitis if they had
symptoms in the lower airways characterized by wheezing,
dyspnea, respiratory distress, poor feeding, tachypnea, and fine
crepitations on auscultation when examined by the doctor on
call in the emergency room [10]. The doctor conducted a struc-
tured clinical interview with the parents, and NPAs were ob-
tained for viral examination by multiplex RT-PCR [11].

Microarray Experiments
RNA extraction, amplification, labeling, and cRNA synthesis.
Cord blood samples were collected in PaxGene RNA collection
tubes (PreAnalytiX) in accordance with the manufacturer’s rec-
ommendations and were stored at −80°C until RNA extraction
[12]. Total RNA was extracted from the PaxGene RNA collection
tubes using the PaxGene blood RNA isolation kit (Pre-
AnalytiX). RNA quality was assessed using the RNA integrity
number of each sample as determined using the Agilent 2100
Bioanalyzer [13]. Fluorescent cRNA was generated by RNA
amplification using the Agilent Low Input linear amplification
kit (Agilent Technologies) in accordance with the manufac-
turer’s instructions, and each sample was labeled with either
Cy3 or Cy5 (PerkinElmer). The final cRNA concentration
was determined using the ND-1000 spectrophotometer (NanoDrop
Technologies).

Hybridization, scanning, and storage of raw data. One
microgram of Cy3-labeled cRNA from each of the 5 infants
was mixed with the same amount of reverse-color, Cy5-labeled
cRNA from the corresponding control infant. All experiments
were also dye-swapped with reverse coloring. The mixed, la-
beled cRNA was hybridized onto Agilent 60-mer human 1A
(V2) oligomicroarrays (G4110B). The hybridization of the
cRNA-labeled targets to the glass slide was done using the
Agilent 60-mer oligomicroarray processing protocol (version
4.1; Agilent Technologies), and the slides were scanned using
the Agilent Microarray Scanner (version G2505B; Agilent Tech-
nologies). The raw data were loaded into the public domain
MIAME (minimum information about a microarray experi-
ment)–compliant database BASE (version 1.2.10) [14].

Experimental design and statistical analysis. A dye-swap
case-control experimental design was chosen in which each of
the 5 dye swaps consisted of cord blood samples from 1 patient
and 1 control infant. Each patient and each control infant were
included in exactly 1 dye swap. Feature extraction was done
using Agilent G2567AA Feature Extraction software (version
v.7.5; Agilent Technologies) with linear and Lowess normali-
ization using default parameter settings. All control spots and
all spots with intensities <300 in either channel were flagged.

Testing the genes one by one. For finding the differentially
expressed genes, statistical analyses using Bayesian analysis of
variance for microarrays (BAMarray; http://www.bamarray.
com) were done on the normalized data [15]. To use the
BAMarray software, an expression matrix was made. To get rid
of the dye effect, the mean of the 2 values obtained from each of
the 2 experiments in the corresponding dye swap was com-
puted for each case-control pair and gene. If 1 of the 2 values
was missing, the mean value was set as equal to the nonmissing
value in the pair. If both values were missing, the mean value
was also missing. After the removal of genes with data from
only 1 or 0 case-control pairs, missing values were imputed
using the K−nearest neighbor imputation method in Signifi-
cance Analysis of Microarrays statistical software (http://www
-stat.stanford.edu/~tibs/SAM). The differentially expressed genes
were then determined from the resulting expression matrix
using BAMarray software.
Table 1. Medical history of 18 infants hospitalized with respiratory syncytial virus bronchiolitis and of five 1-year-old control infants without prior hospitalization or treatment for bronchiolitis.

| Characteristic                      | 810 | 880 | 1501 | 1826 | 1859 | 82 | 135 | 333 | 829 | 1374 | 1558 | 1643 | 1651 | 1713 | 1915 | 1926 | 577 | 589 | 668 | 775 | 832 |
|------------------------------------|-----|-----|------|------|------|----|-----|-----|-----|------|------|------|------|------|------|-----|-----|-----|-----|-----|
| Sex                                 | M   | M   | M    | M    | M    | F  | F   | F   | F   | F    | F    | F    | F    | M    | M    | M    | M   | M   | M   | M   | M   |
| Gestational age, weeks              | 40  | 39  | 40   | 40   | 40   | 40 | 40  | 41  | 36  | 38   | 39   | 39   | 40   | 40   | 40   | 40   | 40  | 40  | 40  | 40  | 40  |
| Delivery mode                       | V   | V   | V    | V    | V    | V  | V   | V   | V   | V    | V    | C    | V    | V    | V    | V    | V   | V   | V   | V   | V   |
| Obstetrical complications           | N   | N   | Y    | N    | N    | N  | N   | N   | N   | N    | N    | N    | N    | N    | N    | N    | N   | N   | N   | N   | N   |
| Normal postpartum pediatric examination | Y  | Y   | Y    | Y    | Y    | Y  | Y   | Y   | Y   | Y    | Y    | Y    | Y    | Y    | Y    | Y    | Y   | Y   | Y   | Y   | Y   |
| Preexisting atopic disease          | N   | N   | N    | N    | N    | N  | N   | N   | N   | N    | N    | N    | N    | N    | N    | N    | N   | N   | N   | N   | N   |
| Heredity for atopic disease         | Y   | Y   | Y    | Y    | Y    | Y  | Y   | Y   | Y   | Y    | Y    | Y    | Y    | Y    | Y    | Y    | Y   | Y   | Y   | Y   | Y   |

**NOTE.** Infants 810, 880, 1501, 1826, and 1859 took part in a cord blood mRNA case-control microarray experiment, and samples were analyzed vs. cord blood from control infants 577, 589, 668, 775, and 832. Samples from all 18 infants and a pooled sample from the 5 control infants were used in a quantitative real-time polymerase chain reaction study of cord blood mRNA samples. C, elective cesarean section; F, female; M, male; N, no; V, vaginal; Y, yes.

* a Breech position.
* b Vacuum extraction.
* c Sister with asthma.
* d Brother with asthma.
* e Mother with asthma.
* f Mother with pollen allergy.
* g Mother with eczema.
Table 2. Clinical observations, routine laboratory evaluations, and treatment in 18 infants hospitalized with respiratory syncytial virus bronchiolitis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Identification no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>810</td>
</tr>
<tr>
<td>Actual symptoms before hospitalization, days</td>
<td>4</td>
</tr>
<tr>
<td>Age at admission, months</td>
<td>8</td>
</tr>
<tr>
<td>Weight at admission, kg</td>
<td>9.0</td>
</tr>
<tr>
<td>Rectal temperature at admission, °C</td>
<td>39.1</td>
</tr>
<tr>
<td>Respiration at admission, breaths/min</td>
<td>50</td>
</tr>
<tr>
<td>O₂ saturation at admission, %</td>
<td>100</td>
</tr>
<tr>
<td>C-reactive protein at admission, mg/L</td>
<td>&lt;7</td>
</tr>
<tr>
<td>Venous pCO₂ analysis at admission, kPa</td>
<td>5.17</td>
</tr>
<tr>
<td>Supplementary oxygen given during hospitalizationᵃ</td>
<td>No</td>
</tr>
<tr>
<td>Treatment for bronchial obstructionᵃ</td>
<td>Yes</td>
</tr>
<tr>
<td>Visible retractions at admission as reported by doctor on call</td>
<td>Yes</td>
</tr>
<tr>
<td>Expiratory stridor at admission as reported by doctor on call</td>
<td>Yes</td>
</tr>
<tr>
<td>Coughing at admission as reported by doctor on call</td>
<td>Yes</td>
</tr>
<tr>
<td>Other medical examination as reported by doctor on call</td>
<td>N</td>
</tr>
<tr>
<td>Complications, including need for assisted ventilation and treatment for bacterial infection</td>
<td>No</td>
</tr>
<tr>
<td>Duration of hospitalization including admission day, days</td>
<td>4</td>
</tr>
</tbody>
</table>

**NOTE.** 

N, normal.  

ᵃ Standard treatment for bronchial obstruction in acute bronchiolitis include aerosolized racemic epinephrine mixed with 2–3 mL of 0.9% NaCl given with 100% oxygen in a nebulizer as needed.
Testing gene sets. The approach described above tests for each gene whether it is differentially expressed or not. A difficulty with this approach is that the power of the tests often is low, which implies that too few of the truly differentially expressed genes are identified. A solution to this problem is to test sets of genes instead of checking each gene separately. The gene sets might be, for example, the genes in an already known pathway, or it might be a set of coexpressing genes found in previous studies. The method, called "gene set enrichment analysis" (GSEA), is one method for checking whether an a priori–defined gene set S is differentially expressed between 2 groups (e.g., 2 different phenotypes) [16]. GSEA is performed by ranking the genes occurring in the expression matrix on the basis of their correlation with the phenotype. The goal is then to determine whether members of the gene set S tend to occur toward the top or bottom of the ranked list of genes. If this is the case, the gene set S is correlated with the phenotypic group distinction, and we conclude that the gene set S is up-regulated or down-regulated in group 1, compared with group 2. To decide whether S is correlated with the phenotypic class distinction, a statistic called an “enrichment score” (ES) is calculated, and the leading-edge subset can be interpreted as the core of a gene set that accounts for the enrichment signal. Examination of the leading-edge subset can reveal a biologically important subset within a gene set. The absolute value of the ES becomes high if the gene set S tends to occur toward the top or bottom of the ranked list of genes and becomes small otherwise. Whether the observed ES is large enough to conclude that S is differentially expressed between 2 groups is decided using a permutation-based test. Because we might want to test several different gene sets, we need to account for multiple testing. In the GSEA software, this is done by controlling the false discovery rate (FDR). The GSEA program was run, mainly with default parameter settings, using the same expression matrix as that used for the BAMarray analysis. The GSEA package includes an initial catalog of human gene sets. From this catalog, we chose the functional sets and included gene sets with at least 15 and at most 500 genes also occurring in the expression matrix. These functional sets contain genes whose products are involved in specific metabolic and signaling pathways, as well as genes coregulated in response to genetic and chemical perturbations. Because the GSEA software assumes that the expression matrix consists of some columns with measurements for group 1 and other columns with measurements for group 2, we had to modify the software such that the expression matrix could consist of columns where the measurements are log ratios of the measurements from group 1 and group 2.

Classification of genes. The gene list with individual Agi-

Figure 1. Genes highly differentially expressed by microarray analysis in cord blood of 5 infants hospitalized with respiratory syncytial virus bronchiolitis vs. control infants.
Table 3. Genes highly differentially expressed by microarray analysis in cord blood from 5 infants hospitalized with respiratory syncytial virus bronchiolitis vs. control infants.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene namea</th>
<th>IDb</th>
<th>Major biological processb</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM102A</td>
<td>Family with sequence similarity 102, member A</td>
<td>399665</td>
<td>Unknown</td>
</tr>
<tr>
<td>POLC1</td>
<td>PQ loop repeat containing 1</td>
<td>80148</td>
<td>Electron transporter activity</td>
</tr>
<tr>
<td>TNFRSF26</td>
<td>Tumor necrosis factor receptor superfamily, member 25</td>
<td>8718</td>
<td>Immune response</td>
</tr>
<tr>
<td>GZMA</td>
<td>Granzyme A (granzyme 1, cytotoxic T lymphocyte–associated serine esterase 3)</td>
<td>3001</td>
<td>Immune response</td>
</tr>
<tr>
<td>C3orf54</td>
<td>Chromosome 3 open reading frame 54</td>
<td>389119</td>
<td>Unknown</td>
</tr>
<tr>
<td>STMN3</td>
<td>Stathmin-like 3</td>
<td>50861</td>
<td>Nervous system development</td>
</tr>
<tr>
<td>CA1</td>
<td>Carbonic anhydrase 1</td>
<td>759</td>
<td>One-carbon compound metabolism</td>
</tr>
<tr>
<td>RPL32</td>
<td>Ribosomal protein L32</td>
<td>6161</td>
<td>Protein biosynthesis</td>
</tr>
<tr>
<td>RPL27A</td>
<td>Ribosomal protein L27A</td>
<td>6157</td>
<td>Protein biosynthesis</td>
</tr>
<tr>
<td>TRA@</td>
<td>T cell receptor (TCR) alpha locus</td>
<td>6955</td>
<td>Cellular defense response</td>
</tr>
<tr>
<td>GLTSCR2</td>
<td>Glioma tumor suppressor candidate region gene 2</td>
<td>29997</td>
<td>Unknown</td>
</tr>
<tr>
<td>ZAP70</td>
<td>Zeta-chain (TCR)–associated protein kinase 70 kDa</td>
<td>7535</td>
<td>Immune response</td>
</tr>
<tr>
<td>IL7R</td>
<td>Interleukin 7 receptor</td>
<td>3575</td>
<td>Immune response</td>
</tr>
<tr>
<td>RPL11</td>
<td>Ribosomal protein L11</td>
<td>6135</td>
<td>Protein biosynthesis</td>
</tr>
<tr>
<td>SLC22A8</td>
<td>Solute carrier family 22 (organic anion transporter), member 8</td>
<td>9376</td>
<td>Organic anion transport</td>
</tr>
</tbody>
</table>

a Obtained from Entrez Gene, a database run by the National Center for Biotechnology Information.
b Obtained from Gene Ontology Annotation, a database run by the European Bioinformatics Institute.

lent probe identification numbers (IDs) was curated with tools from the gene database Genetools (http://www.genetools.no), developed by the Norwegian Microarray Consortium (http://www.mikromatrise.no), and annotated with gene ID, symbol, and name according to the National Center for Biotechnology Information database Entrez Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = gene). Further classification of gene involvement in biological processes was done using the Gene Ontology Annotation database, developed by the European Bioinformatics Institute (http://www.ebi.ac.uk/GOA/).

QRT-PCR Studies

Patients and control infants. To evaluate the gene-expression results obtained by microarray analysis, QRT-PCR was performed on genes in cord blood samples from the 13 other

Table 4. Gene sets down-regulated in cord blood of 5 infants hospitalized with respiratory syncytial virus (RSV) bronchiolitis vs. control infants, using Gene Set Enrichment Analysis (GSEA) software.

<table>
<thead>
<tr>
<th>Gene set systematic name</th>
<th>Standard name</th>
<th>Brief description</th>
<th>Genes in set, no.</th>
<th>Source of gene set</th>
<th>Genes detected in assays, no.</th>
<th>FDR q value, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2:PATH:0248</td>
<td>SIG regulation of the actin cytoskeleton by rho</td>
<td>Genes related to regulation of the actin cytoskeleton</td>
<td>35</td>
<td>Signalling Alliance</td>
<td>25</td>
<td>0.7</td>
</tr>
<tr>
<td>C2:PATH:0141</td>
<td>Intrinsic pathway</td>
<td>The intrinsic prothrombin activation pathway is activated by traumatized blood vessels and induces clot formation</td>
<td>23</td>
<td>BioCarta</td>
<td>16</td>
<td>5.7</td>
</tr>
<tr>
<td>C2:PATH:0211</td>
<td>Rac1 pathway</td>
<td>Rac-1 is a Rac family G protein that stimulates formation of actin-dependent structures such as filopodia and lamellipodia</td>
<td>22</td>
<td>BioCarta</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>C2:PERT:0005</td>
<td>NFKB up. Hinata</td>
<td>Genes up-regulated by NF-κB</td>
<td>111</td>
<td>Broad Institute</td>
<td>78</td>
<td>8.1</td>
</tr>
</tbody>
</table>

NOTE. GSEA is one method for checking whether an a priori–defined gene set is differentially expressed between 2 groups. In the present study, cord blood from 5 infants hospitalized with RSV bronchiolitis was compared with cord blood from 5 children exposed to RSV during infancy who did not have bronchiolitis. By testing several different gene sets, we needed to account for multiple testing. In the GSEA software, this is done by controlling the false discovery rate (FDR); only gene sets with FDRs <10% are shown.
hospitalized infants as well as on genes in cord blood samples from the 5 infants selected for microarray analysis. A pooled sample of cord blood RNA from the 5 male control infants in the microarray experiments was used as exogenous control in the QRT-PCR studies.

cDNA synthesis. Extracted total RNA was quantified with the ND-1000 spectrophotometer (NanoDrop Technologies) and qualified with the Bioanalyzer platform (Agilent Technologies).

Two micrograms of total RNA was cDNA synthesized by use of the High Capacity cDNA Archive kit (Applied Biosystems). The protocol was performed in accordance with the manufacturer’s instructions for the manual method, and the GeneAmp PCR System 9700 thermalcycler was used for performing the reverse transcription.

QRT-PCR with TaqMan low-density array (TLDA) cards. Quantitative real-time PCR with predesigned gene-expression assays was used in the study. TLDA (Applied Biosystems) is based on a microfluidic technology and allows gene-expression analysis for a number of selected genes [17]. The 7900HT real-time PCR software (SDS 2.2.2; Applied Biosystems) supports real-time relative quantification of nucleic acids using the comparative cycle threshold ($C_T$) method and relative quantification (RQ). The arithmetic formula for RQ is $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is the normalized signal level in a sample relative to the normalized signal level in the corresponding calibrator sample [18].

TLDA technology allows the simultaneous measurement of expression of up to 384 genes in a single sample. Each array has 8 channels. Each channel services 48 wells. In this study, we loaded 4 samples per card. All cards were assayed for the different candidate genes in triplicate and for 3 housekeeping genes (the 18S rRNA gene, GUSB, and RPLOP) per channel.

cDNA was diluted, and an appropriate amount was mixed with nuclease-free water (Ambion) and TaqMan 2× Universal Mastermix (Applied Biosystems) in accordance with protocols. The cDNA concentration corresponds to 700 ng of RNA per channel. The cards were then centrifuged at 331 g for 2 × 1 min,
Table 5. Genes involved in regulation of the actin cytoskeleton and down-regulated in cord blood.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Other aliases</th>
<th>Entrez Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTR2</td>
<td>Actin-related protein (ARP) 2 homolog (yeast)</td>
<td>ARP2</td>
<td>10097</td>
</tr>
<tr>
<td>ACTR3</td>
<td>ARP3 homolog (yeast)</td>
<td>ARP3</td>
<td>10096</td>
</tr>
<tr>
<td>CFL1</td>
<td>Cofilin 1 (nonmuscle)</td>
<td>CFL</td>
<td>1072</td>
</tr>
<tr>
<td>MYH2</td>
<td>Myosin, heavy polypeptide 2, skeletal muscle, adult</td>
<td>MYH2A, MYHSA2, MYHas8, MyHC-2A, MyHC-IIA</td>
<td>4620</td>
</tr>
<tr>
<td>PAK3</td>
<td>P21 (CDKN1A)–activated kinase 3</td>
<td>RP5-914P14.3, CDKN1A, MRX30, MRX47, OPHN3, PAK3beta, bPAK, hPAK3</td>
<td>5063</td>
</tr>
<tr>
<td>PAK6</td>
<td>P21 (CDKN1A)–activated kinase 6</td>
<td>PAK5</td>
<td>56924</td>
</tr>
<tr>
<td>PFN1</td>
<td>Profilin 1</td>
<td>Profilin-1</td>
<td>5216</td>
</tr>
<tr>
<td>ROCK1</td>
<td>Rho-associated, coiled-coil containing protein kinase 1</td>
<td>MGC131603, MGC43611, P160ROCK</td>
<td>6093</td>
</tr>
<tr>
<td>ROCK2</td>
<td>RHO-associated, coiled-coil containing protein kinase 2</td>
<td>KIAA0619</td>
<td>9475</td>
</tr>
<tr>
<td>WASF1</td>
<td>WAS protein family, member 1</td>
<td>FLJ31482, KIAA0269, SCAR1, WAVE, WAVE1</td>
<td>8936</td>
</tr>
<tr>
<td>WASL</td>
<td>Wiscott-Aldrich syndrome–like</td>
<td>DKFZp779G0847, MGC48327, N-WASP</td>
<td>8976</td>
</tr>
</tbody>
</table>

NOTE. Microarray analysis was performed on cord blood samples from 5 infants with respiratory syncytial virus (RSV) bronchiolitis vs. 5 control infants exposed to RSV during infancy without bronchiolitis. Samples were analyzed using the Gene Set Enrichment Analysis tool (http://www.broad.mit.edu/gsea/).

sealed, and loaded onto the 7900HT real-time PCR system (Applied Biosystems) in accordance with the manufacturer’s recommendations.

Statistical Analysis

QRT-PCR data were quantified using the SDS software package (version 2.2.2; Applied Biosystems), and results from each card were quantified using the RQ method. RQ results were analyzed by manual removal of poor amplifications, automatic determination of baseline and threshold, and automatic outlier removal. The RQ minimum and maximum confidence level was set at 95%.

RESULTS

Microarray Experiments

Feature Extraction software identified 21,073 unique gene sequences. After normalization and filtering of control spots and

Figure 3. Quantitative real-time polymerase chain reaction analysis of genes highly differentially expressed by microarray analysis in cord blood of 18 infants hospitalized with respiratory syncytial virus bronchiolitis vs. control infants.
spots with signal intensities <300 in either channel, 15,239 genes were available for further evaluation.

**Testing the genes one by one.** When we performed statistical analysis of the data using the BAMarray software, we found 15 genes to be significantly differentially expressed between patients and control infants. Five of these genes (GZMA, ZAP70, TNFRSF25, IL7R, and TRA@) were down-regulated in all 5 microarray experiments and are associated with immune responses. Most of the remaining 10 genes were also down-regulated in the microarray experiments but are associated with several different other biological processes, according to the Entrez Gene database (figure 1 and table 3).

**Testing gene sets.** By using the GSEA method, we identified 30 gene sets to be down-regulated and 12 gene sets to be up-regulated, all with FDR q values <30%. Among these pathways, 4 had an FDR q value <10%; the pathway involved in regulation of the actin cytoskeleton was in a class of its own, with an FDR q value of 0.7% (table 4). In the microarray assays, 25 of 35 genes in this gene set were identified, of which 11 genes were identified within the leading-edge subset. Ten of these 11 genes are found in this leading-edge pathway (figure 2 and table 5), as seen in the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/).

**QRT-PCR studies.** Of the 15 genes differentially expressed in the cord blood microarray experiments, 11 were further evaluated by QRT-PCR using TLDA cards both in the 5 infants selected for microarray analysis and in 13 other infants with RSV infection. The 3 genes RPL11, RPL27A, and RPL32 were excluded from analysis with TLDA cards, and the gene SLC22A8 failed amplification. We found the genes FAM102A, TNFRSF25, and STMN3 to be down-regulated in both the microarray experiments and the QRT-PCR study, except for the results from one particular infant who showed results discordant from those of the other 17 infants for all 3 genes (figure 3 and table 6).

**DISCUSSION**

The present study used microarray analysis as a hypothesis-generating tool to identify the most differentially expressed genes and gene sets in cord blood from children hospitalized with RSV bronchiolitis during infancy. To further evaluate these genes, QRT-PCR analysis was performed. We found the genes FAM102A, TNFRSF25, and STMN3 to be the most differentially expressed single genes in our sample. When we analyzed the microarray gene-expression assays for gene sets with GSEA, we found a pathway regulating the actin cytoskeleton to be highly correlated with a predisposition toward subsequent RSV bronchiolitis.

The infants in the present study were all born in our hospital after uncomplicated deliveries, and all cord blood samples were collected immediately after birth by trained nursing personnel. All 18 hospitalized infants had acute bronchiolitis of moderate severity during infancy, meaning that they were hospitalized because of severe respiratory difficulties but were not in need of assisted ventilation, including treatment with continuous positive airway pressure or mechanical ventilation. Eight of the 18 hospitalized infants had a family history of atopic disease, which might influence the interpretation of the gene-expression results (tables 1 and 2). When we performed statistical analysis to compare these 2 subgroups, we found no significant differences for the genes analyzed. This was also true for the possible impact of mode of delivery on gene expression (data not shown).

The children in the control group had RSV infection during infancy with only mild symptoms and were chosen to exclude...
children from the control group who could have had a possible genomic predisposition for a more abnormal immune response characterizing the infants with overt bronchiolitis. We cannot give full evidence of prior RSV infection in the control infants. However, the complement fixation test has acceptable specificity and was the only routine analysis available for serological testing of respiratory viruses in Norway at the time of the study.

We found the 3 genes \textit{FAM102A, TNFRSF25,} and \textit{STMN3} and a pathway involved in regulation of the actin cytoskeleton to be down-regulated in cord blood from infants with subsequent RSV bronchiolitis. However, to confirm that a down-regulation of these genes is of importance for susceptibility to RSV bronchiolitis in early life, a much larger study is needed. Furthermore, the specificity or lack of specificity of these cord blood gene expression to RSV must also be analyzed by comparison with that of other viruses causing bronchiolitis in early life [19, 20].

\textit{TNFRSF25}, or death domain receptor 3 (DR3), is a tumor necrosis factor superfamily receptor whose expression can be induced on T cells by T cell receptor activation. Engagement of T cell DR3 does not, however, lead to apoptosis but rather to activation of NF-\textit{kB} and to potentiation of interferon (IFN)–\textit{\gamma} production [21]. Several studies have shown an association between an immunological cord blood imbalance that includes a delayed maturation of IFN-\textit{\gamma} production and increased susceptibility to RSV bronchiolitis in early life. Lack of cord blood \textit{TNFRSF25} activation in the ill infants in our study further supports these observations [22–25].

A significant down-regulation of the gene set involved in the regulation of the actin cytoskeleton was also observed in our study. Other studies have shown that the actin cytoskeleton is important for T cell formation and signaling [26, 27]. In relation to the above findings of \textit{TNFRSF25} down-regulation, it is therefore tempting to propose that an immaturity in the formation of the T cell receptor may explain, at least in part, why \textit{TNFRSF25} is down-regulated and why some infants develop RSV bronchiolitis during early life. This is also interesting with respect to previous studies in our group indicating a delayed maturation of T cell subsets in newborns at high risk for allergy, compared with those at low risk [28, 29].

The 2 genes \textit{FAM102A} and \textit{STMN3} were also down-regulated in our study. However, the possible role(s) of these genes has still to be investigated in relation to RSV infection. A significant down-regulation of the gene \textit{GZMA} was also observed. This is another interesting observation, given its known association with cytotoxic T lymphocyte (CTL) function. CTLs kill their cellular targets by mechanisms that require direct contact between the effector and target cells. In one of these pathways, perforin and granzymes are secreted by exocytosis and together induce apoptosis of the target cell. The granule exocytosis pathway of cytotoxic lymphocytes is therefore crucial for immune surveillance and homeostasis [30]. In a mouse study by Rutigliano and Graham [31], a protective immune response to RSV generated by perforin/granzyme-mediated cytolyis and IFN production was postulated. Because of this and other reports, it is tempting to hypothesize that the down-regulation of granzymes in cord blood from infants with subsequent RSV bronchiolitis is associated with more-severe disease.

Microarray analysis can be used in cord blood studies as a hypothesis-generating tool to identify genes and gene sets of importance for susceptibility to and severity of RSV infection during early life; to our knowledge, this method has not been published before. The present study identified the genes \textit{FAM102A}, \textit{TNFRSF25,} and \textit{STMN3} and a pathway involved in regulation of the actin cytoskeleton to be down-regulated in cord blood from infants hospitalized with RSV bronchiolitis. However, more studies are needed to identify the specificity of these genes in relation to this disease.

\section*{References}


