Research Article

MicroRNA Expression Profile of Whole Blood Is Altered in Adenovirus-Infected Pneumonia Children

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Human adenovirus (Adv) infection is responsible for most community-acquired pneumonia in infants and children, which results in significant morbidity and mortality in children every year. MicroRNAs (miRNAs) are associated with viral replication and host immune response. Knowing the miRNA expression profile will help understand the role of miRNAs in modulating the host response to adenovirus infection and possibly improve the diagnosis of adenovirus-infected pneumonia. In our study, total RNA extracted from whole blood of adenovirus-infected pneumonia children and healthy controls were analyzed by small RNA deep sequencing. Expression profiles of whole blood microRNAs were altered and distinctly different in adenovirus-infected children. The top 3 upregulated miRNA (hsa-miR-127-3p, hsa-miR-493-5p, and hsa-miR-409-3p) were identified in adenovirus-infected children and provided a clear distinction between infected and healthy individuals. Potential host target genes were predicted and validated by qRT-PCR to study the impact of microRNAs on the host genes. Most of the target genes were involved in the MAPK signaling pathway and innate immune response. These highly upregulated microRNAs may have crucial roles in Adv pathogenesis and are potential biomarkers for adenovirus-infected pneumonia.

1. Introduction

Human adenovirus (Adv) infection is responsible for most community-acquired pneumonia in infants and children [1, 2]. Adv causes infections for 5–10% of upper and lower respiratory tract infections in children, which results in pneumonia and nearly 1.3 million deaths of children every year [3, 4]. The fatality rates for untreated severe pneumonia or disseminated disease caused by Adv may even exceed to 50% [5, 6]. There are no efficacious antiviral drugs for Adv treatment until now. Also, the traditional diagnosis of Adv infection is limited. Therefore, to discover the interaction between the virus and its host will help us to find novel treatment and diagnosis for Adv infection.

Human Ads are nonenveloped double-stranded DNA viruses and belong to the Adenoviridae family [7, 8]. Human Ads are divided into seven subgroups including 53 serotypes based on immunologic and biological characteristics. Adv replicates efficiently in human cells and triggers an innate immune response such as inflammatory response in the host cells. Also, viral infection has proved to have a great impact on cellular small RNA expression and gene expression [9–11]. Adv infects the host cells through binding to different cellular receptors such as coxsackievirus and adenovirus receptor (CAR) [12]. During Adv DNA replication, host cellular proteins such as nuclear factor I and POU2F1 are used by Adv [13, 14]. In turn, the host will trigger an innate immune response against Adv infection. However,
the details of Adv and host interaction still remains poorly defined.

Small RNAs are important regulators that modulate development, proliferation, differentiation, and apoptosis of organisms [15, 16]. Small RNAs include microRNA (miRNA), siRNA, tRFs, piRNA, and rasiRNAs, which regulate gene expression in a wide range of processes such as viral replication and host immune response. miRNAs are the most well-studied small RNAs during the latest decades. miRNAs are very important regulators that modulate transcriptome changes [17]. miRNAs regulate gene expression in a wide range of physiological and pathological processes such as in immune response and viral replication [18]. Although miRNAs have been examined in Adv type 3-infected human laryngeal epithelial cells and Adv type 2-infected human lung fibroblast cells [19, 20], there is no research about small RNA profiling in whole blood of Adv-infected pneumonia children. In our study, we sought to present the different miRNA profiles between Adv-infected pneumonia children and healthy controls, identify candidate diagnostic biomarkers for pneumonia with Adv infection in children, and examine the role of miRNAs in host defense response in Adv-infected children.

2. Materials and Methods

2.1. Patients. The whole blood samples used in the study were obtained from Guangzhou Women and Children’s Medical Center. Children diagnosed with human Adv pneumonia were included in the study. The diagnosis of human Adv pneumonia was considered certain when it was associated with the following criteria: (1) lower respiratory and/or systemic symptoms, (2) lung infiltration on chest radiography or computed tomography (CT) scan, and (3) positive results for human Adv IgM antibody in sera and/or human Adv DNA by PCR in throat swabs and/or bronchoalveolar lavage (BAL) fluid. A total of 33 samples from patients and 33 samples from healthy volunteers were used in the study. The ages of all patients (male or female) and healthy volunteers (male or female) range from one year to three years. The study was approved by the Ethics Committee at Guangzhou Women and Children’s Medical Center (number 2014121815), and written informed consent was obtained from all guardians.

2.2. RNA Extraction and Small RNA Sequencing. The blood samples of the patients and volunteers were collected in anticoagulant tubes. The total RNA was isolated using RiboPure™ Blood RNA Isolation Kit (Ambion, USA) according to the manufacturer’s protocol. The extracted small RNAs were treated with DNase before deep sequencing. RNA concentration was determined using a NanoDrop ND1000 system (Thermo Fisher Scientific, South San Francisco, CA), and their integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Small RNA deep sequencing was performed as previously described [21]. A total of 3 samples from patients and 3 samples from healthy volunteers were used for small RNA deep sequencing in the study. The clinical characteristics of the 3 patients and 3 healthy volunteers are summarized in Table 1.

2.3. Cell and Virus Culture. Human primary lung fibroblasts (IMR-90) and human 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (Invitrogen), streptomycin, and penicillin (Invitrogen). All virus infections were carried out in serum-free medium for 1 h, followed by addition of saved complete medium.

2.4. Transfection. miRNA inhibitors (GenePharma Co., China) were transfected into IMR-90 cells with Lipofectamine RNAiMAX (Invitrogen) at final concentrations of 100 nM according to the manufacturer’s protocol.

2.5. Virus Growth Assay. IMR-90 cells transfected with miRNA inhibitors were infected with HAdV5 (isolated from patients) at an MOI of 10 in serum-free medium. Virus titers were determined 72 h after infection by plaque assays performed on 293T cells [22, 23].

2.6. Quantitative Real-Time PCR (qRT-PCR) Analysis. A qRT-PCR experiment was performed using the Power SYBR Green PCR Master Mix. Each reaction was performed in a 10 μL volume system containing 0.5 μL of cDNA, 0.5 μL of each primer, 5 μL of Power SYBR Green PCR Master Mix, and 3.5 μL of ddH2O. The reactions were incubated in a 96-well plate at 95°C for 10 minutes, followed by 39 cycles of 95°C for 15 seconds and 62°C for 1 minute. For miRNA quantitation, 10 ng of total RNA was reverse-transcribed using specific stem-loop primers. U6 was used as an endogenous control.

2.7. Data Analysis. miRNA cluster and family information from miRBase (miRBase 20, http://www.mirbase.org/) was used to annotate the cluster of miRNAs. miRNA target genes were predicted based on two software: miRDB software (http://mirdb.org/) and TargetScan software (http://www.targetscan.org/).

2.8. Statistical Analysis. Data were analyzed using GraphPad Prism 6.0 software (La Jolla, CA, USA). The two-tailed Student’s t-test was used to determine the significance of statistical data between two experimental groups. Data were considered significant at *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

3.1. Different Expressions of MicroRNAs in Adv-Infected Children vs. Healthy Children. To study the impact of Adv infection on cellular small RNA expression in pneumonia children, deep sequencing of small RNAs was performed in our study. Accordingly, we found an apparent small RNA peak at 21-24 nt for miRNAs (Figure 1(a)). When we analyzed the differently expressed small RNAs, 118 miRNAs were found differently expressed in Adv-infected children vs. healthy children in a volcano plot (Figure 1(b)). We mapped the clean reads from each group to the known miRNA sequences and identified 908 miRNAs in Adv-infected children versus healthy controls (Figure 1(c)).
<table>
<thead>
<tr>
<th>Volunteer or patient</th>
<th>Age</th>
<th>Gender</th>
<th>Fever duration(^a) (days)</th>
<th>WBC ((\times 10^9/\text{L})) (5–12)</th>
<th>HsCRP (mg/L) (&lt;5)</th>
<th>Laboratory characteristics(^b)</th>
<th>HAdV DNA(^d) (throat swabs/BALF)</th>
<th>LDH (U/L) (159–322)</th>
<th>Radiology(^e) Consolidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>1y9m</td>
<td>male</td>
<td>–</td>
<td>8.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V2</td>
<td>2y8m</td>
<td>female</td>
<td>–</td>
<td>9.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V3</td>
<td>2y3m</td>
<td>male</td>
<td>–</td>
<td>5.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A1</td>
<td>2y1m</td>
<td>male</td>
<td>13</td>
<td>10.2</td>
<td>26.5</td>
<td>+</td>
<td>+/+</td>
<td>919</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>1y9m</td>
<td>female</td>
<td>16</td>
<td>8.3</td>
<td>51.14</td>
<td>+</td>
<td>+/+</td>
<td>812</td>
<td>+</td>
</tr>
<tr>
<td>A3</td>
<td>2y8m</td>
<td>female</td>
<td>22</td>
<td>2.8</td>
<td>10.98</td>
<td>+</td>
<td>+/+</td>
<td>948</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\)Fever duration from onset to normothermia. \(^b\)Data extracted from the first test for the children on admission. \(^c\)Specific IgM antibodies against HAdV pneumoniae were detected in 2 mL of acute phase (on admission) patient serum using a commercial ELISA kit. \(^d\)HAdV pneumonia DNA was detected by real-time PCR in throat swabs or bronchoalveolar lavage fluid (BALF). \(^e\)Judged by chest radiograph or CT scan in whole course of the patients.
Furthermore, hierarchical cluster analysis of differentially expressed miRNA is shown in Figure 2. Specially, 77 differentially expressed miRNAs in the 6 samples passed our fold-change filter (log2 fold change > 1.0), among which 20 miRNAs that have high expression (reads up to 1000 in Adv samples) and showed significant different expressions were selected for further analysis (Table 2).

3.2. Validation of Differentially Expressed miRNAs. To confirm the differential expression of miRNAs in Adv patients vs. healthy controls, we performed qRT-PCR assays in our study. The result showed that hsa-miR-127-3p, hsa-miR-379-5p, hsa-miR-493-5p, hsa-miR-409-3p, hsa-miR-99b-5p, hsa-miR-370-3p, and hsa-miR-381-3p were upregulated in whole blood samples from 5 Adv-infected children vs. 5 healthy controls (Figure 3(a)), while hsa-miR-101-3p, hsa-miR-150-5p, hsa-miR-29a-3p, and hsa-miR-342-3p were downregulated in whole blood samples from 5 Adv-infected children vs. 5 healthy controls (Figure 3(a)), which was comparable with our sequencing data. To identify candidate diagnostic miRNA biomarkers, we focused on the upregulated miRNAs. More samples (15 Adv-infected children vs. 15 healthy controls) were collected to verify the expression of the upregulated miRNAs (Figure 3(b)). From the result, we found that hsa-miR-127-3p, hsa-miR-493-5p, and hsa-miR-409-3p were significantly increased. Also, after depleting these miRNAs with miRNA inhibitors, the viral replications were significantly decreased (Figure 3(c)). These findings

**Figure 1:** miRNA expression profile of whole blood from adenovirus-infected pneumonia children. (a) Deep sequencing shows the distribution of small RNAs of whole blood from adenovirus-infected pneumonia children. The miRNA peaks appear around 22 nt. (b) Volcano plot of differently expressed miRNAs between Adv-infected children and healthy controls. (c) Venn diagram of differently expressed miRNAs between Adv-infected children and healthy controls.
Cluster analysis of differentially expressed miRNA

Figure 2: Hierarchical cluster analysis of differentially expressed miRNAs.
imply that our selected miRNAs may reflect the infection of Adv, and such miRNAs can likely serve as biomarker candidates for Adv-infected patients.

3.3. Predict Target Genes of Differentially Expressed miRNAs.

To study the biological significance of miRNAs, we then predicted the computational target genes of miRNAs. We focused on the targets of verified miRNAs with distinct expression profiles. GO enrichment of the predicted target genes of the miRNAs showed that the target genes were mainly involved in cellular process and molecular function (Figure 4(a)). In particular, most of the target genes participated in the MAPK signaling pathway and Ras signaling pathway (Figure 4(b)). The top five predicted target genes of hsa-miR-127-3p, hsa-miR-493-5p, or hsa-miR-409-3p are listed in Table 3 by the highest miRNA target score from two miRNA predictive software.

The target genes were selected for further validation with qRT-PCR, and we found that the mRNA expression of 8 genes (PSMB5, ITGA6, MYCBP2, TCF7L2,UBE2V2, HIPK1, UBE2D2, and KANSL1) were downregulated in Adv-infected patients compared to healthy controls (Figure 5). Most of them are transcription factors or factors involved in the ubiquitin pathway. The downregulated miRNAs may indicate the mechanism of Adv infection and Adv-induced pneumonia.

4. Discussion

Human Advs are common causative pathogens of acute respiratory infections in children. The treatment of human Adv infections is limited because prospective, randomized therapeutic trials have not been done. Therefore, it is very important to discover the mechanism of Adv-infected pneumonia and search the biomarkers for Adv-infected pneumonia in children. The regulatory potential of miRNA is well defined, and the different profiles of miRNA expression are the result of diverse diseases including viral infections. In our study, we found that the miRNA profile in whole blood of Adv-infected children was different from that of healthy children. Blood samples of Adv-infected children reflect the associated pathology of Adv infection and thus provide a better understanding of the disease.

Profiling of miRNA expression from Adv-infected blood samples identified a cluster of 118 miRNAs significantly altered. The altered blood miRNA profile was similar to cells infected with Adv reported previously [19, 20], indicating that the different expressed miRNAs identified in our study could be taken as the candidate diagnostic biomarkers for pneumonia with Adv infection in children. Among these different expressed miRNAs, hsa-miR-127-3p was reported to involve in the replication of influenza infection [24]. hsa-miR-127-3p was reported to affect the Epstein-Barr virus-associated lymphoma through targeting the PTEN-AKT-mTOR pathway [25]. Altogether, these miRNAs play important roles in innate immune response or viral replication, thus affecting the outcome of the disease.

Through miRNA target gene GO analysis, we found that most target genes of different expressions of miRNAs were involved in the MAPK signaling pathway. The MAPK signaling pathway is activated by Toll-like receptors [26], which play important roles in innate immune response against viral infection. Also, the activation of the MAPK pathway will result in the activation of the NF-κB signal pathway, which

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**Table 2: Top 20 different expressed miRNAs.**

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Reads in ADV</th>
<th>Reads in control</th>
<th>Log2 fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>has-miR-381-3p</td>
<td>9358</td>
<td>3507</td>
<td>1.17</td>
<td>0.00511</td>
</tr>
<tr>
<td>has-miR-486-5p</td>
<td>5480</td>
<td>2240</td>
<td>1.12</td>
<td>0.00434</td>
</tr>
<tr>
<td>has-miR-409-3p</td>
<td>4916</td>
<td>1223</td>
<td>1.61</td>
<td>0.00023</td>
</tr>
<tr>
<td>has-miR-486-3p</td>
<td>4745</td>
<td>1922</td>
<td>1.12</td>
<td>0.00402</td>
</tr>
<tr>
<td>has-miR-127-3p</td>
<td>3950</td>
<td>755</td>
<td>2.10</td>
<td>6.35E − 09</td>
</tr>
<tr>
<td>has-miR-182-5p</td>
<td>2396</td>
<td>870</td>
<td>1.17</td>
<td>0.00762</td>
</tr>
<tr>
<td>has-miR-99b-5p</td>
<td>2241</td>
<td>650</td>
<td>1.46</td>
<td>0.00066</td>
</tr>
<tr>
<td>has-miR-379-5p</td>
<td>1687</td>
<td>378</td>
<td>1.88</td>
<td>3.31E − 07</td>
</tr>
<tr>
<td>has-miR-370-3p</td>
<td>1682</td>
<td>617</td>
<td>1.18</td>
<td>0.00621</td>
</tr>
<tr>
<td>has-let-7e-5p</td>
<td>1239</td>
<td>429</td>
<td>1.33</td>
<td>0.00030</td>
</tr>
<tr>
<td>has-miR-493-5p</td>
<td>1089</td>
<td>237</td>
<td>1.87</td>
<td>1.63E − 06</td>
</tr>
<tr>
<td>has-miR-494-3p</td>
<td>1070</td>
<td>342</td>
<td>1.54</td>
<td>3.94E − 09</td>
</tr>
<tr>
<td>has-miR-101-3p</td>
<td>57,893</td>
<td>150,004</td>
<td>− 1.12</td>
<td>0.00825</td>
</tr>
<tr>
<td>has-miR-142-3p</td>
<td>24,616</td>
<td>77,702</td>
<td>− 1.25</td>
<td>0.00841</td>
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<tr>
<td>has-miR-150-5p</td>
<td>15,854</td>
<td>41,850</td>
<td>− 1.25</td>
<td>0.00025</td>
</tr>
<tr>
<td>has-miR-29a-3p</td>
<td>6679</td>
<td>18,944</td>
<td>− 1.40</td>
<td>9.01E − 07</td>
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<tr>
<td>has-miR-186-5p</td>
<td>6093</td>
<td>13,066</td>
<td>− 1.07</td>
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<tr>
<td>has-miR-27a-3p</td>
<td>5558</td>
<td>11,953</td>
<td>− 1.00</td>
<td>0.00170</td>
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<tr>
<td>has-miR-342-3p</td>
<td>3373</td>
<td>13,980</td>
<td>− 1.87</td>
<td>1.37E − 09</td>
</tr>
<tr>
<td>has-miR-29b-3p</td>
<td>1339</td>
<td>3404</td>
<td>− 1.25</td>
<td>1.60E − 05</td>
</tr>
</tbody>
</table>
will stimulate the production of inflammatory cytokines and MMPs [27] and finally lead to pneumonia. Thus, the altered miRNA expression profile of whole blood from Adv-infected children partly reflected the mechanism of Adv-infected pneumonia.

With a developmental framework or disease process, miRNAs exhibit dynamic expression patterns. In our study, we characterized the miRNA expression profile of Adv-infected children using deep sequencing analysis and identified that many miRNAs were differentially expressed in

Figure 3: Quantification of miRNA expression levels by qRT-PCR. (a) Quantification of top 20 differently expressed miRNAs, including upregulated and downregulated miRNAs. Five samples from Adv-infected children and 5 from healthy controls were used in the experiment. (b) Quantification of top 5 upregulated miRNAs. 15 samples from Adv-infected children and 15 from healthy controls were used in the experiment. (c) The effect of miRNA inhibitors on the replication of Adv. After transfection with miRNA inhibitors, cells were infected with HAdV5 at an MOI of 10. Virus was harvested, and the titer was determined on 293T cells at the indicated time points. Data are shown as means ± SD from three independent experiments. **P < 0.01 and ***P < 0.001 (Student t test).
Figure 4: Go enrichment of predicted target genes. (a) The GO classification enrichment of target genes. (b) The KEGG pathway scatterplot of target genes.
Adv-infected children when compared with healthy children. Those with the greatest differences were chosen for further verification. In particular, the expressions of hsa-miR-127-3p, hsa-miR-493-5p, and hsa-miR-409-3p from 20 Adv-infected children were significantly higher than those from 20 healthy controls, indicating that these miRNAs could be taken as good diagnostic biomarkers for Adv-infected pneumonia.

To further explore the possible molecular mechanisms of the differently expressed miRNAs in Adv-infected children, we predicted the possible target genes of hsa-miR-127-3p, hsa-miR-493-5p, and hsa-miR-409-3p and found that most of them are transcription factors or factors involved in the ubiquitin pathway. Especially, after verifying the predicted target genes with qRT-PCR, 8 genes (PSMB5, ITGA6, MYCBP2, TCF7L2, UBE2V2, HIPK1, UBE2D2, and KANSL1) were found significantly downregulated in samples from Adv-infected children. In particular, MYCBP2 is an E3 ubiquitin protein ligase [28, 29], which regulated the cAMP and mTOR signaling pathway. The mTOR signaling pathway plays a critical role in effector T cell function, the downregulation of which will result in impaired cell cytolysis and incapability of virus elimination. UBE2V2 is an ubiquitin-conjugating enzyme and is involved in the differentiation of monocytes, which can produce proinflammatory cytokines like MIP-1β [30, 31]. Thus, the downregulated UBE2V2 will result in abnormal differentiation of monocytes and more proinflammatory will be produced, which in turn aggravates pneumonia. UBE2D2 is essential for the activation of MAVS and RIG-I in response to viral infection [32–34]. The downregulation of UBE2D2 will result in abnormal activation of MAVS and RIG-I signal and virus clearance. These findings imply that the miRNA expression profile changed in Adv-infected children resulting in different transcriptome profiles, which reflects the mechanism of Adv replication and the formation Adv-infected pneumonia.

5. Conclusions

In summary, we identified the 3 most markedly differently expressed miRNAs in whole blood from Adv-infected children, which can be taken as biomarkers for Adv-infected pneumonia. Simultaneously, based on target gene prediction and qRT-PCR analysis, we found that genes MYCBP2, UBE2V2, and UBE2D2 may play important roles in viral replication and Adv-induced pneumonia. However, additional studies are necessary to clarify their roles in these processes, which will provide a physiological basis for the treatment of Adv-infected pneumonia.
Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

Authors’ Contributions

Feng Huang and Junsong Zhang contributed equally to this work.

Acknowledgments

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