Upregulation of GLE1 and LCP2 Genes in H5N1 Influenza Virus Infected Patients

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Abstract

Previous study showed that the Gle1 RNA export mediator-like (Gle1l) gene and the lymphocyte cytosolic protein 2 (Lcp2) gene were upregulated in response to influenza virus A/Puerto Rico/8/1934 (H1N1) in a mouse mode. To determine whether these two genes were upregulated in humans after influenza A virus infection, nasopharyngeal swabs were collected from eleven patients with flu-like symptoms for viral RNA extraction and PCR amplification. Sequencing analysis revealed that nucleoprotein (NP) gene fragments amplified from nasopharyngeal swabs of four patients shared the highest similarity with the NP gene from avian influenza A (H5N1) virus (A/goose/Shantou/753/2002). Peripheral blood samples were then collected from four patients for quantitative analysis of GLE1 and LCP2 gene expression. Our results demonstrated that both GLE1 and LCP2 genes were upregulated in H5N1 influenza A virus infected patients, suggesting that upregulation of GLE1 and LCP2 genes may be important for the host defense against influenza A viruses.

Keywords

Influenza, H5N1, GLE1, LCP2, Upregulation, Host Defense

1. Introduction

Disease outbreaks and human infections of highly pathogenic H5N1 avian influenza virus in Hong Kong in 1997 [1], H7N9 avian influenza virus in China in 2013 [2] [3], and H5N6 avian influenza virus in China in 2014

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were mainly caused by close contact of humans with infected birds or influenza virus-contaminated environments. These events demonstrate that a future pandemic from avian influenza viruses is potentially one of the biggest global health threats. The ability of influenza viruses from different origins to infect humans and cause diseases depends largely on the distribution and expression status of influenza virus receptors on host cells [5] [6], internalization of bound viruses through multiple endocytic pathways [7], and host-pathogen interactions [8].

Results from studies in mice infected with influenza A viruses have documented that many differentially expressed host genes are related to host-pathogen interactions such as the inflammatory response and intracellular signaling pathways [9]-[11]. Our previous study showed that the Gle1 RNA export mediator-like (Gle1l) gene and the lymphocyte cytosolic protein 2 (Lcp2) gene were upregulated in response to influenza virus A/PR/8/34 in a mouse mode [10].

The human GLE1 gene (Gene ID: 2733), also known as GLE1l and LCCS1 (lethal congenital contracture syndrome-1) gene, encodes a predicted 75-kDa protein with high sequence and structure homology to the yeast Gle1p protein. The Gle1 protein can stimulate the DExD/H-box RNA-dependent ATPase Dbp5’s activity and is one of the principal components of the mRNA nuclear export machinery required for poly(A)+RNA export [12]-[14]. The multifunctional Gle1 protein plays an important role in nuclear mRNA export and translation, and mutations in the human GLE1 gene are responsible for the autosomal recessive LCCS1 [15]. Structure prediction and functional analysis suggest that the LCCS1 and lethal arthrogryposis with anterior horn cell disease (LAAHD) mutations disrupt the function of Gle1, indicating the potential impact of altered mRNA transport and gene expression in human diseases [16]. The human LCP2 gene (Gene ID: 3937), also known as SLP-76 (SH2 domain-containing leukocyte cytosite protein of 76 kilodaltons) gene, encodes a 76-kDa protein, which was originally identified as a substrate of the ZAP-70 (70-kDa zeta-associated protein kinase) protein tyrosine kinase (PTK) following T cell receptor (TCR) ligation in the leukemic T cell line Jurkat [17]-[19].

Identification and characterization of genes that are upregulated after influenza A virus infection can provide insights into the mechanism by which the host interacts with the virus, and potential biomarkers for diagnosis, prevention, and treatment of influenza virus infections. Because of their important functions in host-pathogen interactions, human GLE1 and LCP2 genes were selected for this study to determine whether they were upregulated in influenza A virus infected patients. In the present study, we collected nasopharyngeal swabs from eleven patients with flu-like symptoms and two healthy controls for viral RNA extraction and PCR (polymerase chain reaction) amplification of influenza A virus nucleoprotein (NP) gene. We then sequenced NP gene fragments amplified from four patients to determine the sequence similarity with known NP genes in the GenBank. We further collected peripheral blood samples from four patients at different time points after the disease onset for quantitative analysis of GLE1 and LCP2 gene expression. Our results demonstrated that both GLE1 and LCP2 genes were upregulated at different time points after the disease onset in influenza A virus infected patients.

2. Materials and Methods

2.1. Study Participants

This study was approved by the Institutional Review Board of Affiliated Hospital of Zunyi Medical College and all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Eleven patients (16 - 55 years old) with flu-like symptoms (such as fever, runny or stuffy nose, cough, sore throat, headaches and/or body aches), and two healthy volunteers were enrolled from October 2010 to February 2011 for this study. Patients were treated and recovered in about two weeks. Nasopharyngeal swabs and peripheral blood samples were collected from patients and healthy volunteers for viral RNA extraction and PCR amplification of the NP gene of influenza A viruses.

2.2. Detection of Influenza Viral RNA

Collected nasopharyngeal swabs from 11 patients with flu-like symptoms were kept in the sample storage buffer and used immediately for viral RNA extraction with the RNA Kit manufactured by Tiangen Biotech (Beijing, China). The concentration of viral RNA was determined with NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, USA). Extracted viral RNA was reverse transcribed into complementary DNA (cDNA) by
using the Quantscript RT Kit manufactured by Tiangen Biotech. Primers specific for the NP gene (Table 1) were designed for PCR amplification of a 1156-bp NP gene fragment (nucleotide positions 236 to 1392) from different subtypes of influenza A viruses including H1N1, H3N2, H5N1, H7N7, and H9N2. The reaction mixture (25 μl) containing both primers and complementary DNA was used for PCR amplification with the following cycling conditions: 95°C for 2 min for denaturation, and 42 cycles of 20 s at 95°C, 20 s at 60°C, and 1 min 30 s at 68°C followed by a final extension at 68°C for 7 min. PCR products were analyzed by 1% agarose gel electrophoresis and sent to Invitrogen (Shanghai, China) for DNA sequencing. Sequencing results were analyzed and compared to viral sequences at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST program [20].

2.3. Extraction of Total RNA from Lymphocytes

Peripheral blood samples (3 ml) were collected from four clinically confirmed influenza patients (P1 to P4) at days 3, 7 and 14 after disease onset. Lymphocytes were harvested from collected peripheral blood samples and used for extraction of total RNA by using the TRIzol reagent (Invitrogen). The concentration of total RNA was determined using the NanoDrop 1000 Spectrophotometer. Extracted total RNA was reverse transcribed into cDNA by using the Quantscript RT Kit.

2.4. Quantitative Real-Time PCR

Primer sets specific for human GLE1 and LCP2 genes and the endogenous control beta-globin (HBB) gene (Gene ID: 3043) were purchased from Invitrogen and used for the analysis of gene expression in peripheral blood samples of patients and healthy controls. The gene names, primer sequences, and sizes of predicted PCR products are listed in Table 1. Complementary DNA (2 μl, 50 ng/μl) was used in the quantitative real-time PCR by using the FQ-PCR Reaction System (Takara, Dalian, China) containing 15 μl SYBR II, 0.5 μl forward primer, 0.5 μl reverse primer and 7 μl distilled water. The iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, USA) was used for the quantitative RT-PCR (reverse transcription polymerase chain reaction) with the following parameters: 3 min at 95°C for the initial denaturation followed by 45 cycles of 10 s at 95°C, 30 s at 59°C, and 1 min 10 s at 72°C. The PCR quantification data were collected and analyzed, and quantitative Ct (threshold cycle) results were calculated by using the iCycler iQ5 software with the normalized expression analysis method. Differences between samples from patients and healthy controls were generated by the iCycler iQ5 software. Each sample in the quantitative real-time PCR assay was analyzed in duplicate and normalized to the β-globin mean value. The fold changes between patient samples (P1 to P4) and control samples (C1 and C2) were calculated with the relative standard curve method.

3. Results and Discussion

To the best of our knowledge, neither GLE1 gene nor LCP2 gene has been linked to host responses to influenza A virus infections in humans. Therefore, we collected nasopharyngeal swabs from 11 patients with flu-like symptoms and peripheral blood samples from four of the patients as well as two healthy controls to conduct the current study. Collected nasopharyngeal swabs were used in RT-PCR for amplification of DNA fragment with primers specific for the NP gene of influenza A viruses (Table 1). Analysis of PCR products showed that the NP gene fragment (1156 bp) was amplified from four of 11 nasopharyngeal swabs collected from patients with flu-like symptoms, but none from 2 healthy controls. These results confirmed that four of the 11 patients were positive for influenza A virus infection.

Table 1. Primer sequences and sizes of PCR products for four genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>AGAGGATGTTGCTTTCTGC</td>
<td>CCATCATCTTTATAACTTCTG</td>
<td>1156 bp</td>
</tr>
<tr>
<td>GLE1</td>
<td>ATGGCCTTGGAGGACTATCA</td>
<td>TCTGTCCCTGAGCTCTGTGATG</td>
<td>145 bp</td>
</tr>
<tr>
<td>LCP2</td>
<td>CCTCTCAGAAGTGAAGGCAC</td>
<td>CAATAATATCTGATACAGAC</td>
<td>177 bp</td>
</tr>
<tr>
<td>β-globin</td>
<td>ACACAACCTGTTGTCACTAGC</td>
<td>CAACTTCATCCACGTTACC</td>
<td>110 bp</td>
</tr>
</tbody>
</table>
infected by influenza A viruses. However, it was unclear whether the rest seven patients were infected by influenza A viruses or not. Therefore, we only collected peripheral blood samples from these four patients (P1 to P4) and two healthy controls (C1 and C2) for further studies. To find out which influenza A virus might have caused the infection of these patients, amplified PCR products were used for DNA sequencing and results were compared to viral sequences at the NCBI website [20]. Sequence analysis revealed that NP gene fragments amplified from four patients shared the highest identity (from 89% to 99%) with the NP gene (GenBank: CY029140.1) from an avian influenza virus A/goose/Shantou/753/2002 (H5N1), indicating that these four patients were infected by an influenza A virus (Table 2).

Total RNAs extracted from peripheral blood samples of two healthy controls (C1 and C2) and four patients (P1 to P4) at days 3, 7 and 14 after disease onset were used for quantitative analysis of GLE1 and LCP2 gene expression. Total RNAs were reverse transcribed into cDNA by using the Quantscript RT Kit. Primer sets specific for human GLE1 and LCP2 genes, and the endogenous control β-globin (HBB) gene (Gene ID: 3043) were used for the analysis of gene expression in peripheral blood samples (Table 1). The PCR quantification data were analyzed and quantitative Ct (threshold cycle) results were calculated by the iCycler iQ5 software using the normalized expression analysis method. Each sample was analyzed in duplicate and normalized to the β-globin mean value. The differences between samples from patients (P1 to P4) and healthy controls (C1 and C2) were calculated with the relative standard curve method.

As shown in Figure 1, GLE1 gene expression was upregulated more than ten-fold in patient 2 at Day 3, and in patients 3 and 4 at Day 14; upregulated more than five-fold in patients 2 and 4 at Day 7; upregulated more than two-fold in patients 1 and 2 at Day 14 and in patient 3 at Day 7; but unchanged in patients 1, 3 and 4 at Day 3 after the disease onset compared to the healthy controls. Results from this study demonstrated that GLE1 gene was upregulated between two and ten-fold in influenza A virus infected patients at different time points after disease onset. In eukaryotic cells, the GLE1 protein is one of the principal components of the nuclear pore complex (NPC) required for poly(A) RNA export through NPC to the cytoplasm, which is an important step for proper gene expression [14] [21]. The fact that human GLE1 gene is upregulated in influenza A virus infected patients suggests that the GLE1 protein may be involved in the virus-host interactions and could potentially become a novel therapeutic target for the treatment of influenza. Recently, GLE1 gene was identified as one of the targets of microRNA miR-376a-3p which induced significant inhibition of cell proliferation, migration, colony formation and spheroid formation when transfected into giant cell tumor of bone (GCTB) derived stromal cells, indicating that miR-376a-3p and its target gene, GLE1, could be valuable therapeutic targets for treating the GCTB and possibly other types of cancers [22].

Similar to GLE1 gene, LCP2 gene expression was upregulated more than 40-fold in patient 1 and more than 25-fold in patient 2 at Day 3; more than ten-fold in patients 1, 2 and 4 at Day 7, and in patients 3 and 4 at Day 14; and more than two-fold in patient 1 at Day 14 and in patient 3 at Day 7 after disease onset compared to the healthy controls (Figure 2). Results showed that LCP2 gene was upregulated between two and 40-fold in influenza A virus infected patients at different time points after disease onset, suggesting that upregulation of LCP2 gene may be involved in the activation of intracellular signaling pathways and virus-host interactions. A recent report showed that quantitative reductions of Lcp2 protein in mutant mice produced excessive amounts of proinflammatory cytokines, autoantibodies, and IgE, which revealed a dose-sensitive threshold for Lcp2 protein in the balance of immunity and immune dysregulation [23]. In addition, LCP2 gene was identified as one of the three novel prognostic genes for diffuse large B-cell lymphoma (DLBCL) by using bioinformatic methods [24]. These studies indicate the importance of LCP2 gene in TCR signaling and immune regulations, and suggest that LCP2 gene could be used for subtyping and potential targets for treatment of DLBCL.

Table 2. NP gene fragments amplified from nasopharyngeal swabs of four patients shared the highest identity with the NP gene of influenza virus A/goose/Shantou/753/2002 (H5N1).

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Name and subtype of matched influenza A virus</th>
<th>NP gene identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>A/goose/Shantou/753/2002 (H5N1)</td>
<td>89</td>
</tr>
<tr>
<td>P2</td>
<td>A/goose/Shantou/753/2002 (H5N1)</td>
<td>99</td>
</tr>
<tr>
<td>P3</td>
<td>A/goose/Shantou/753/2002 (H5N1)</td>
<td>91</td>
</tr>
<tr>
<td>P4</td>
<td>A/goose/Shantou/753/2002 (H5N1)</td>
<td>97</td>
</tr>
</tbody>
</table>
Figure 1. Fold change of GLE1 gene expression in peripheral blood samples collected from influenza A virus infected patients at days 3, 7, and 14 after disease onset. P1, Patient 1; P2, Patient 2; P3, Patient 3; P4, Patient 4; C1, healthy control 1; and C2, healthy control 2. Each sample was analyzed in duplicate and normalized to the β-globin mean value. The fold changes between patients (P1 to P4) and healthy controls (C1 and C2) were calculated with the relative standard curve method. Data are presented as the mean and the standard deviation.

Figure 2. Fold change of LCP2 gene expression in peripheral blood samples collected from influenza A virus infected patients at days 3, 7, and 14 after disease onset. P1, Patient 1; P2, Patient 2; P3, Patient 3; P4, Patient 4; C1, healthy control 1; and C2, healthy control 2. Each sample was analyzed in duplicate and normalized to the β-globin mean value. The fold changes between patients (P1 to P4) and healthy controls (C1 and C2) were calculated with the relative standard curve method. Data are presented as the mean and the standard deviation.

4. Conclusion

In summary, results from this study demonstrated that GLE1 and LCP2 genes were upregulated in influenza A virus infected patients, suggesting that upregulation of these genes may be important for the host defense against influenza A virus infections. However, it is unclear what specific roles upregulation of GLE1 and LCP2 genes may play in the host defense against influenza A virus infections. We tried to compare our findings with similar studies but could not find any study which linked either GLE1 gene or LCP2 gene to host responses to influenza A virus infections in humans. One of the limitations of our study is that a relatively low number of participants were enrolled, because some eligible patients did not agree to sign the informed consent forms during the study period. Therefore, to confirm and extend the results from this study, more patients infected by different influenza A viruses will need to be enrolled, and future studies using established cell lines and animal models (such as
which is involved in the infectious disease research.

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Conflicts of Interest

All authors except H.Z. declare no conflicts of interest. H.Z. is employed by and has shares in Z-BioMed, Inc., and Clinical Technology Innovation in Drug-resistant TB [No. (2011) 4011], the Second 2011 Collaborative Innovation Center of Guizhou Province for TB Prevention and Control in Guizhou Province (Incubation Project), and the Forth Talented Individual Base of Guizhou Province for Infectious Disease Prevention and Control.

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