Identifying the functional pathways and potential genes associated with interferon signaling during human adenovirus type 7 (HAdV-7) by weighted gene co-expression network analysis

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Research

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Abstract

Background

Human adenovirus type 7 (HAdV-7) can cause severe pneumonia in children and severe complications. At present, however, the pathogenesis and genes involved remain largely unknown.

Methods

We collected HAdV-7 infected and mock-infected A549 cells at 24, 48, and 72 hours post-infection (hpi) for RNA sequencing (RNA-seq). And, we identified potential genes and functional pathways associated with HAdV-7 infection using weighted gene co-expression network analysis (WGCNA).

Results

Based on bioinformatics analysis, 12 co-expression modules were constructed by WGCNA, with the blue, tan, and brown modules significantly positively correlated with Ad-24 hpi, Ad-48 hpi, and Ad-72 hpi, respectively. Functional enrichment analysis indicated that the blue module was mainly enriched in DNA replication, cell cycle, and viral process; the tan module was largely enriched in metabolic pathways and regulation of superoxide radical removal; and the brown module was predominantly enriched in mitochondrial respiratory chains and regulation of cell death. Quantitative polymerase chain reaction (qPCR) was used to determine transcript abundance of some identified hub genes, and results were consistent with those from RNA-seq. Based on comprehensive analysis of hub genes and differentially expressed genes (DEGs) in the GSE68004 dataset, we identified *SOCS3*, *OASL*, *ISG15*, and *IFIT1* as potential candidate genes for biomarkers or drug targeting in HAdV-7 infection. Further, we propose a multi-target inhibition of interferon signaling mechanism to explain the HAdV-7 associated with higher severity of clinical consequences.

Conclusions

We constructed a framework of co-expression gene modules from A549 cells infected with HAdV-7, thus providing a basis for identifying potential genes and pathways involved in adenovirus infection and for studying the pathogenesis of adenoviruses.

Background

Human adenoviruses (HAdVs) belong to the family Adenoviridae within the genus *Mastadenovirus* and are classified into seven species (A-G). Since the first adenovirus was characterized [1], over 100 types have been recognized according to genomic sequences (<https://hadvwg.gmu.edu/>). Among these, HAdV species B (HAdV -3, 7, 11, 14, 16, 21, 34, 35, 50, and 55), C (HAdV -1, 2, 5, 6, and 57), and E (HAdV-4) are

known to cause a variety of acute respiratory diseases [2–4]. However, HAdV-3 and HAdV-7 are the major types responsible for lower respiratory diseases [5–8], and HAdV-7 is more likely to cause severe pneumonia and significant mortality, especially in young children [7–10]. Nevertheless, the molecular mechanism underlying adenovirus infection remains largely unknown, especially the potential genes and pathways involved in this process.

Several studies have revealed multiple alterations in gene expression between pathogen and host cells via different -omics analysis, such as RNA and protein expression profiles [11]. For example, quantitative proteomic analysis of HAdV-2-infected A549 cells and transcriptomic analysis of HAdV-2-infected human primary lung fibroblasts at 24 and 48 hours post-infection (hpi) have shown that viral infection affects the expression of genes and proteins involved in different cellular pathways [12, 13]. However, other HAdVs in different host cells using different methodologies also need to be explored to improve our understanding of adenovirus infection. RNA sequencing (RNA-seq) for transcriptome profiling uses deep sequencing technology, with the resulting reads used to produce genome-scale transcription maps composed of both the transcription structure and expression level of each gene for a specific developmental stage or physiological condition, thus generating a tremendous amount of data [14].

Many genes are involved in the pathogenesis of adenovirus infection, thus constituting a complicated network. The exploration of gene-network signatures associated with complicated diseases and biological processes can be achieved by weighted gene co-expression network analysis (WGCNA) [15]. Through this algorithm, gene expression data are transformed into co-expression modules and trait data are effectively integrated to identify functional pathways and key genes implicated in the pathogenesis process [16].

Therefore, in this study, we constructed co-expression modules using expression data obtained from HAdV-7-infected A549 cells and identified modules containing highly co-expressed genes. Functional enrichment analysis was performed on the modules of interest and hub genes were identified in the corresponding modules. Candidate genes with biomarker and therapeutic target potential were also identified. This study provides a foundation for further investigations on the pathogenesis of adenovirus infection and potential therapies.

Methods

1. Cell culture and adenovirus infection

The HAdV-7 strain (CQ45_2019, MT113943) used in this study was originally isolated from nasal aspirates of a child infected with HAdV-7 and was stored at -80°C . The strain was cultured in A549 cells obtained from the American Type Culture Collection (ATCC) and subsequently maintained in our lab. The HAdV-7 particles were purified by standard caesium chloride gradient centrifugation and suspended in Hank's Balanced Salt Solution (HBSS) with 0.5% fetal bovine serum (FBS, Gibco), as described previously [17]. The median tissue culture infectious dose (TCID₅₀) of the virus was calculated according to our previous methods [18]. A549 cells were infected with HAdV-7 (multiplicity of infection (MOI) = 1),

and three biological replicates of infected and mock-infected cells were collected at 24, 48, and 72 hpi for RNA-seq. All virus experiments were performed in biosafety level 2 facilities following governmental and institutional guidelines.

2. RNA library construction and sequencing

Total RNA was isolated and purified from six samples of HAdV-7-infected and mock-infected A549 cells (three each) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedures. Poly(A) RNA was purified from 1 µg of total RNA using Dynabeads Oligo (dT)25-61005 (Thermo Fisher, CA, USA) with two rounds of purification. The poly(A) RNA was then fragmented into small pieces using the Magnesium RNA Fragmentation Module (NEB, cat.e6150, USA) under 94 °C for 5–7 min. The cleaved RNA fragments were then reverse transcribed to create cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen, cat. 1896649, USA) for the synthesis of U-labeled second-stranded DNA with *E. coli* DNA polymerase I (NEB, cat.m0209, USA), RNase H (NEB, cat.m0297, USA), and dUTP solution (Thermo Fisher, cat. R0133, USA). An A-base was then added to the blunt ends of each strand for ligation to the indexed adapters. Each adapter contained a T-base overhang to ligate the adapter to the A-tailed fragmented DNA, and single- or dual-index adapters were ligated to the fragments. After heat-labile UDG enzyme (NEB, cat.m0280, USA) treatment of the U-labeled second-stranded DNA, the ligated products were amplified by polymerase chain reaction (PCR). The average insert size for the final cDNA library was 300 ± 50 bp. Lastly, 2 × 150-bp paired-end sequencing (PE150) was performed on an Illumina NovaSeq™ 6000 (LC-Bio-Technology Co., Ltd., Hangzhou, China) following the vendor's recommended protocols.

3 Bioinformatics analysis

3.1 Correlation analysis of replicas and principal component analysis (PCA)

According to gene expression in the samples, we performed R correlation analysis of two parallel experiments to evaluate repeatability. PCA was performed using the R package model (<http://www.r-project.org/>) to detect possible clusters or outliers among samples.

3.2 Differentially expressed gene (DEG) screening

RNA differential expression analysis was performed using the “DESeq2” R package [19] between non-infected and adenoviral-infected groups. Genes with fold-change > 2 or < 0.5 and *P*-value < 0.05 were considered as DEGs.

3.3 Construction of co-expression network

A co-expression network based on HAdV-7-infected and mock-infected control samples was constructed using the R package “WGCNA” [15]. The gradient method was applied to test the independence and average connectivity degree of different modules with different power values (ranging from 1 to 30). We selected a soft-thresholding power of 4 when the degree of independence was 0.9. The minimum number

of genes was set to 40 for the high reliability of results. To merge possible similar modules, we defined 0.1 as the threshold for cut height. Co-expression modules were identified using the WGCNA algorithm, and similar modules (module eigengenes) were merged into a single module, and then used to further interpretation.

3.4 Functional enrichment analysis

The constructed modules were arranged by number of genes. To gain insight into the function of the genes in the modules most related to HAdV-7 infection, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID)[20] (<https://david.ncifcrf.gov/home.jsp/>) to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. A *P*-value of < 0.05 was set as the cut-off threshold, and the top 10 records were extracted (if there were more than 10 records). The R package “ggplot” was used to show the results graphically.

3.5 Hub gene identification and validation

Genes in each module of interest with module membership (MM) > 0.8 and gene significance (GS) > 0.2 were identified as important intramodular genes, indicating a significant correlation with HAdV-7 infection. Subsequently, the DEGs obtained by RNA-seq were overlapped with the corresponding important intramodular genes to identify hub genes, the results of which are presented as a table and Venn diagrams[21] (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). To further validate the confidence of the high-throughput transcriptome sequencing, five hub genes were randomly selected for analysis via quantitative PCR (qPCR).

Total RNA was extracted from HAdV-7-infected and mock-infected A549 cells using Quick-RNA Miniprep Plus kit (R1057, ZYMO RESEARCH, Inc.) according to the manufacturer's instructions. Purified RNA was reverse transcribed into cDNA using a Transcriptor cDNA Synthesis Kit 2 (Roche). Newly synthesized cDNA was analyzed by qPCR using SYBR green PCR master mix (Vazyme) on a CFX96 Touch Real-Time PCR Detection System in 20-μl reactions. Relative mRNA expression was quantified using the comparative threshold ($2^{-\Delta\Delta CT}$) method, with GAPDH as a calibrator. Details on primer sequences are shown in Table 1.

3.6 Identification of potential candidate genes

To verify the hub genes that play important roles in adenovirus infection in public database, we selected an appropriate dataset, GSE68004, from the NCBI Gene Expression Omnibus (GEO[20]; <https://www.ncbi.nlm.nih.gov/geo/>) by searching the keyword ‘HAdV’. This dataset contains blood RNA data from 76 pediatric patients with complete Kawasaki disease (KD), 13 with incomplete KD, 19 patients with HAdV, 17 patients with Group A streptococcal disease (GAS) disease, and 37 healthy controls (HC). The GEO2R tool in the GEO database was employed to analyze samples from patients with HAdV and HC. Criteria for identifying DEGs via GEO2R analysis were set to *P* < 0.05, logFC > 1, or logFC < -1. The DEGs and hub genes in the key modules were assessed through reciprocal overlap

(<http://bioinformatics.psb.ugent.be/webtools/Venn/>) and overlapping genes were regarded as candidate genes. And their interaction network was constructed by using GeneMANIA (<http://genemania.org/>, accessed on 5 January 2021)[22, 23].

4. Statistical analysis

Statistical analyses of candidate gene expression were performed using GraphPad Prism 8.0 (GraphPad Software Inc.). Results are expressed as mean $\pm$ standard error of the mean (SEM). Data were analyzed using two-way analysis of variance (ANOVA) for multiple comparisons. Differences with $P < 0.05$ were considered statistically significant.

Results

1. Transcriptomic analysis of HAdV-7-infected and mock-infected A549 cells

To better understand the underlying mechanisms of HAdV-7 infection, we conducted comparative transcriptomic analysis of HAdV-7-infected and mock-infected A549 cells. Pearson correlation analysis based on sample expression was used to evaluate differences between groups. As shown in Fig. 1a, samples were closely correlated, with a minimum coefficient of 0.971 between groups, indicating good parallelism between biological replicates and reliable sequencing results. As a powerful tool for reducing dimensionality of complex datasets, PCA shows high performance in excluding outliers and artifacts [24]. Figure 1b presents a PCA plot of the 18 specimens in the two-dimensional plane. The first and second principal components (PC1 and PC2) explained 93.86% and 5.42% of sample variance, respectively. At each time point, samples from the infected and non-infected (control) groups were separated, while the closeness of samples within groups showed good repeatability. Based on differential expression analysis, 834 DEGs (598 up- and 236 down-regulated) at 24 hpi, 945 DEGs (638 up- and 307 down-regulated) at 48 hpi, 1 399 DEGs (1 066 up- and 273 down-regulated) at 72 hpi were identified between the mock-infected and HAdV-7-infected groups (Fig. 1c). Distribution of the obtained DEGs is shown in the volcano plot in Fig. 1d-f.

2. Construction of co-expression networks

To characterize alterations in gene expression triggered by HAdV-7 infection, a total of 56 873 genes, only 14 446 gene left after filtering, in 18 samples from HAdV-7-infected and mock-infected A549 cells at three time points were used to construct the co-expression network. Hierarchical clustering analysis of samples was performed, with results shown in Figure S1. Network topology analysis was conducted to determine the soft-threshold power, which affected scale independence and mean connectivity of the network. Following screening, 4 was the lowest power value for which scale independence was below 0.9 (Fig. S2a). To specify the groups of co-regulated genes identified as modules, the dynamic cut-tree algorithm was applied (Fig. S2b), yielding the dendrogram and dynamic cut tree before and after merging the

modules (indicated in different colors) (Fig. S2c). As a result, 12 modules ranging in size from 54 genes (midnight-blue) to 8 034 genes (turquoise) were identified, each containing genes with similar co-expression traits (Fig. 2). The eigengene dendrogram and eigengene adjacency heatmap indicated that these modules could be divided into several smaller groups, suggesting that co-expression clusters with varying functions were present in the genetic networks of HAdV-7 infection.

3. Gene co-expression modules correspond to HAdV-7 infection

Module-trait associations were analyzed by correlating module eigengenes with clinical traits (i.e., HAdV-7 infection). As shown in Fig. 4a, multiple modules were related to Ad-24 hpi, Ad-48 hpi, and Ad-72 hpi. Among them, the blue module (containing 2 934 genes) was significantly associated with Ad-24 hpi ($r = 0.73$; $P < 0.001$), the tan module (containing 167 genes) was significantly associated with Ad-48 hpi ($r = 0.63$; $P = 0.005$), and the brown module (containing 1 259 genes) was highly positively correlated with Ad-72 hpi ($r = 0.95$; $P < 0.001$). Therefore, these three modules were identified as key modules for HAdV-7 infection. We constructed a scatterplot of GS vs. MM in the key modules (Fig. 2b-d). Genes in the blue, tan, and brown modules most significantly associated with HAdV-7 infection characteristics (GS) were also the most important elements of the modules (MM), as demonstrated by the upper right-hand genes in the plot.

4. Functional enrichment analysis of genes in modules of interest

To better understand the function of genes in key modules, GO and KEGG pathway enrichment analyses were performed, with the top terms of each category presented in Fig. 3-5. KEGG analysis demonstrated that genes in the blue module were primarily enriched in pathways related to cell cycle regulation and cell proliferation, including DNA replication, mismatch repair, RNA transport, and spliceosome, consistent with biological process (BP) and molecular function (MF) enrichment analysis (Fig. 3a-b). Genes in the blue module associated with cell component (CC) terms were primarily enriched in cell nucleus, including nucleoplasm and nucleolus. Genes in the tan module were enriched in mucin type O-glycan biosynthesis, circadian entrainment, retrograde endocannabinoid signaling, and metabolic pathways. GO enrichment showed that tan module genes were significantly associated with cell metabolism, such as glycolipid metabolic process, polypeptide N-acetylgalactosaminyltransferase activity, and glucosyltransferase activity (Fig. 4a-b). Genes in the brown module were enriched in oxidative phosphorylation, ErbB signaling pathway, and metabolic pathways. Furthermore, GO analysis indicated that genes in the brown module were primarily associated with mitochondrial respiratory chain functions, protein translation, and apoptosis, as well as cellular structure of mitochondrial respiratory chain and protein synthesis (Fig. 5a-b).

5. Screening and validation of hub genes

Important intramodular genes in the three main modules are shown in the upper right section of Fig. 4b-c, suggesting that they are not only functionally important elements of HAdV-7 infection but also significantly associated with its characteristic traits. Furthermore, genes in the blue, tan, and brown modules were overlapped with DEGs based on the RNA-seq results, with the intersecting genes defined as hub genes (Fig. 3-5c). These genes were with high intra-module connectivity, meanwhile, with significant differences in the expression (shown in Table SI). Of the genes in the blue module, two are involved in the positive regulation of *ERK1* and *ERK2* cascade, including *TNFAIP8L3*, which belongs to the tumor necrosis factor-alpha (TNF- α)-induced protein 8 (TNFAIP8/TIPE) family and participates in the regulation of inflammatory responses, immune homeostasis, and proliferation/apoptosis axis by regulating *PI3K* and downstream mediators, including nuclear factor- κ B (NF- κ B), mitogen-activated protein kinases (MAPKs; e.g., *ERK-1* and *ERK-2*, *JNK*, and *p38*), and interferon-regulatory factors (IRF; e.g., *IRF3* and *IRF7*) [25–27]. Among the 26 hub genes in the tan module, *PIK3AP1* and *ARID5A* are related to the inflammatory response and innate immune system, and *KIF5C* and *PID1* are associated with energy and metabolism, indicating that energy production and metabolism may play an important role in HAdV-7 infection. Moreover, the presence of *SFRP5* and *TCF7* in the tan module suggest that the WNT signaling pathway may be involved in adenovirus infection. Of the 103 hub genes in the brown module, many participate in the regulation of transcription and cell death. Importantly, *TP73*, *JUND*, *JUNB*, *FOSB*, and *BATF3* are involved in crosstalk among multiple pathways. Five hub genes were selected for qPCR to validate differential expression levels identified by RNA-seq. Comparing transcriptome data with qPCR results, we found that the patterns of gene transcript abundance were consistent (Fig. 6a-e), not only confirming the reliability of our transcriptome data but also the network centrality and potential roles of key genes in HAdV-7 infection.

6. Identification of representative adenovirus infection-related candidate genes

We also explored the relationship between expression profile changes in adenovirus-infected cells and clinical samples. Differential gene analysis was performed on the GSE68004 dataset of HAdV and HC groups, resulting in 374 DEGs. Candidate genes were selected based on the intersection of significant genes and DEGs from the GSE68004 dataset (Fig. 3-5c). Only brown module obtained the intersection of the three different categories of genes, including suppressor of cytokine signaling (*SOCS3*), oligoadenylate synthetases-like (*OASL*), interferon (IFN)-stimulated gene product 15 (*ISG15*), IFN-induced protein with tetratricopeptide repeats 1 (*IFIT1*), hemoglobin subunit alpha 1 (*HBA1*).

7. Hypothesized confrontation between adenovirus and host cells

Transcriptome sequencing showed that the expression levels of *SOCS3*, *OASL*, *ISG15*, and *IFIT1* increased significantly over time, especially at 48 hpi (except *SOCS3*) and 72 hpi (Fig. 7a), indicating that their high expression may play an important role in adenovirus infection. Furthermore, there were expression correlations among the four genes ($p < 0.05$) (excluding *SOCS3* and *ISG15*, *SOCS3* and *IFIT1*) (Fig. 7b),

demonstrating a specific interaction between candidate genes. Additionally, We used the online software GeneMANIA (<http://genemania.org/>, accessed on 5 January 2021) to construct the gene–gene interaction network. Besides the four candidate genes, the network included another 20 potentially frequently interacting genes and 1122 links (interactions), and they were mainly regulation of viral process and response to interferon (Fig. 7c). The innate immune system provides a robust first line of defense against viral infection, and IFN-I is considered a key component of antiviral innate immunity. Upon host invasion, however, a complex and intense battle occurs between host cells and viruses, Virus may also evade the host's immune response by inducing some molecule that in turn inhibit interferon signaling[28], and that SOCS3, OASL, ISG15, and IFIT1 are involved. Based on previous literature and our analysis, we propose the following confrontation signaling between the adenovirus and host cells. Adenovirus binds to its corresponding receptor and enters the target cell. Toll-like receptor 9 (TLR9), the only known endosomal localized DNA sensor that can detect viral un-methylated cytosine-phosphate-guanosine (CpG) motif-containing DNA, recruits myeloid differentiation primary response gene 88 (MyD88), leading to activation of IRF7 and NF- κ B, which induce IFN-I and various chemokines and cytokines [29]. The viral genome can be sensed by RNA polymerase III, IFN gamma-inducible protein 16 (IFI16), DEAD-box helicase 41 (DDX41), cyclic GMP-AMP (cGAMP) synthase (cGAS), and several other DNA sensors, of which RNA pol III transcribes dsDNA into 5'-triphosphate double-stranded RNA (5'-ppp-dsRNA). Once dsRNA binds to retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated gene 5 (MDA5), the antiviral RIG-I-mitochondrial antiviral-signaling protein (MAVS) and MDA5-MAVS signaling pathways are activated [30]. cGAS, IFI16, and other DNA sensors then activate the adaptor protein stimulator of IFN genes (STING), which further recruit and phosphorylate TANK-binding kinase 1 (TBK1) to relay signals to IFN regulatory factor 3 (IRF3) to induce the production of IFN-I [29, 30]. IFN-I interacts with its universally expressed receptors (IFNARs) and successively phosphorylates signal transducer and transcription activator (STAT) family proteins through Janus protein kinase (JAK) family members. The phosphorylated STAT1/STAT2 heterodimer associates with IFN regulatory factor 9 (IRF9) to form the transcriptional factor complex IFN-stimulated gene factor 3 (ISGF3), which translocates to the nucleus and binds the IFN-response elements (IRSE) in ISG promoters to induce the expression of ISG products, including OASL, ISG15, and IFIT1 [31]. The ISGs exert complex functions in adenovirus infection. For instance, IFIT1 can disrupt viral DNA replication [32] and translation [33] and dampen virus-induced innate immune signaling by binding to STING [32]. ISG15 down-regulates RIG-I-mediated signaling to reduce IFN promoter activity, but also inhibits viral replication by sustaining IRF3 activation [34]. IFN induction and signaling induces OASL, which then binds to and inactivates cGAS to negatively regulate IFN production [35]. SOCS3 binds to JAKs to inhibit their activity and ubiquitinates and degrades JAKs via SOCS box, thereby inhibiting JAK/STAT signaling(Fig. 8) [36, 37].

Discussion

HAdVs are considered the common viral agent of pediatric severe pneumonia, with HAdV-7 in particular causing more severe clinical consequences[38], which is often associated with the high replication competence of HAdV-7 in human lung[39], and prolonged virus shedding and persistence of HAdV-7 in

targeting cell[40]. Some of which possible mechanisms have been previously reported. for example, adenovirus's own protein (e.g. E1A) can suppress of type I interferon signaling. However, the underlying pathophysiological mechanisms of HAdV-7 infection are complicated and remain unclear.

Viral infection usually results in changes in the expression of host genes following replication. Thus, host cell transcriptomes can reflect the changes in specific genes and pathways that occur during infection [41, 42]. WGCNA may be an effective method of mining valuable data to analyze complicated genetic networks in HAdV-7 infection. One of the advantages of WGCNA is its high reliability and biological significance because the analysis focuses on the association between co-expression modules and infection traits [43].

In this study, using gene expression data obtained by RNA-seq from HAdV-7-infected and mock-infected A549 cells, a total of 12 co-expression modules were constructed using WGCNA. Among them, the blue, tan, and brown modules were positively correlated with HAdV-7-infected traits at 24, 48, and 72 hpi, respectively.

Genes in the same module are considered functionally related to each other [44]. Functional enrichment analysis indicated that genes in the blue module were primarily enriched in cell cycle regulation and DNA replication, consistent with previous studies noting that adenovirus infection has proceeded far into the early phase at 24 h and expression changes favor its DNA replication, with about 50% of those genes with known functions involved in cell cycle control [45]. Genes in the tan module were significantly associated with cell metabolism. At this time, the virus has gained control of the cellular metabolic machinery to create conditions in which the viral genome can replicate efficiently [13]. Genes in the brown module were mainly enriched in mitochondrial respiratory chain functions, protein translation, and apoptosis. During this period, considerable energy and proteins are needed to complete virus assembly. Moreover, cell death is thought to be imminent, facilitating efficient release and spread of the virion progeny.

DEGs with high intramodular connectivity were identified as hub genes for HAdV-7 infection. Furthermore, concordant genes among hub genes from the blue, tan, and brown modules and DEGs in the GSE68004 dataset were identified using a Venn diagram (Fig. 3-5c). Among them, *SOCS3*, *OASL*, *ISG15*, and *IFIT1* were closely involved in the process of adenovirus infection and were thus regarded as candidate genes.

During viral invasion, the host rapidly establishes several defensive mechanisms by eliciting the innate immune response. Conventionally, IFN-I is the major component of the innate immune system against viral infection via induction of various ISGs [34, 46]. *OASL*, *ISG15*, and *IFIT1* are ISGs and are involved in confrontation between adenovirus and host cells (Fig. 8). *OASL* codes for an important ISG and plays different roles in DNA and RNA viruses by inhibiting cGAS-mediated IFN production or enhancing RIG-I-mediated IFN induction, respectively [35, 47]. Several studies have found that *OASL* inhibits the replication of some RNA viruses, such as vesicular stomatitis virus (VSV), Sendai virus (SeV), and respiratory syncytial virus (RSV) [47–49]. Unlike RNA viruses, however, much less is known about the effects of *OASL* in the context of DNA virus infection. Human *OASL* and mouse *Oasl2* are reported to

promote replication of certain DNA viruses, including herpes simplex virus (HSV), mouse cytomegalovirus (MCMV), and adenovirus [50]. Therefore, OASL may serve as a biomarker to improve discrimination between DNA and RNA virus infection. As one of the most highly up-regulated genes during viral infections, *ISG15* acts as both an effector and a signaling molecule in various phases of the innate immune response [51]. ISG15 is involved in many antiviral-signaling pathways, both intracellular and extracellular, and activates various immune cells and promotes the production of many antiviral cytokines to facilitate viral clearance [34, 51]. Several studies have reported on *ISG15*-deficient patients and found that human *ISG15* is redundant for antiviral immunity and negatively regulates IFN-I immunity [33, 52]. Although the multiple biological functions of ISG15, including as a biomarker of antiviral treatment [53], offer promise for intervention in disease progression, several important questions remain to be answered in future research. The expression of IFIT1 is strongly induced by IFN-I, double-stranded RNAs, and viral infection [54, 55], and increasing evidence has demonstrated that IFIT1 has antiviral activity during both DNA and RNA virus infection, mainly by intervening translation by differentially recognizing the 5' terminus of target RNA [56], but it could also inhibit the interferon signal pathway [32]. As one of the most studied members of the SOCS family, SOCS3 can be stimulated by JAK/STAT signals to regulate the proinflammatory response via negatively regulating cytokine receptors [57]. There is considerable evidence that multiple viruses can up-regulate SOCS3 expression and dampen host antiviral responses to promote viral replication through various immune evasion strategies [36]. For example, influenza A virus [58], HSV [59], and RSV [60] can suppresses IFN-I production and response by stimulating SOCS3 expression [61], consistent with our data showing that SOCS3 was up-regulated after adenovirus infection. SOCS3, as viral virulence factor, may also have great therapeutic potential. For example, SOCS3 expression could be manipulated to restore antiviral immune responses. In addition, SOCS3 antagonists have shown antiviral effects on a broad range of viruses in cell and animal models [36, 37, 62].

Altogether, SOCS3, OASL, ISG15 and IFIT1 are not only involved in confrontation between adenovirus and host cells together with other molecules (mainly disturbing type I interferon signaling), but also have good a correlation in the expression levels. We therefore speculate that HAdV-7 could inhibit interferon production through multiple targets after infecting host cells, which partly explain the possible mechanism of more severe disease and significant morbidity.

Although the present study is the first to investigate co-expression gene networks associated with HAdV-7 infection using WGCNA, our research has several limitations. For instance, we only used a single cell line rather than human specimens, thus, we select the data of children whole blood from a public database for validation of hub genes. On the other hand, we did not further study the exact mechanism of the identified key genes in HAdV-7 infection. Further studies are required to evaluate and confirm the involvement of the candidate genes in different races and samples.

Conclusions

Based on WGCNA, we identified three co-expressed gene modules, several candidate genes, and various functional biological pathways related to the pathogenesis of HAdV-7 infection. Moreover, proteins encoded by *SOCS3*, *OASL*, *ISG15* and *IFIT1* were found to be vital in the confrontation between adenovirus and the host, also could be used as potential biomarkers for drug targeting and therapy. And the more severe clinical manifestations of HAdV-7 may be due to multi-target inhibition of the interferon pathway. However, the exact molecular mechanisms of the candidate genes and functional pathways in HAdV-7 infection require further investigation.

Abbreviations

HAdV

Human adenovirus

WGCNA

Weighted gene co-expression network analysis

RNA-seq

RNA sequencing

qPCR

quantitative PCR

DEGs

Differentially expressed genes

PCA

Principal component analysis

DAVID

Database for Annotation, Visualization and Integrated Discovery

GO

Gene Ontology

KEGG

Kyoto Encyclopedia of Genes and Genomes

MM

Module membership;

GS

Gene significance

GEO

Gene Expression Omnibus;

KD

Kawasaki disease

HC

healthy controls

BP

Biological process

MF
Molecular function
CC
Cell component
TNF- α
Tumor necrosis factor-alpha
NF- κ B
Nuclear factor-Kb
MAPKs
Mitogen-activated protein kinases
IRFs
Interferon-regulatory factors
SOCS3
Suppressor of cytokine signalling
OASL
Oligoadenylate synthetases-like
ISG15
Interferon (IFN)-stimulated gene product 15
IFIT1
IFN-induced protein with tetratricopeptide repeats 1
HBA1
Hemoglobin subunit alpha 1
TLR9
Toll-like receptor 9
MyD88
Myeloid differentiation primary response gene 88
IFI16
IFN gamma-inducible protein 16
DDX41
DEAD-box helicase 41
cGAS
cyclic GMP-AMP (cGAMP) synthase, and several other
RIG-1
Retinoic acid-inducible gene I
MDA5
Melanoma differentiation-associated gene 5
MAVS
Mitochondrial antiviral-signaling protein
STING
Stimulator of IFN genes

TBK1
TANK-binding kinase 1
STAT
Signal transducer and transcription activator
JAK
Janus protein kinase
ISGF3
IFN-stimulated gene factor 3
IRSE
IFN-response elements
VSV
Vesicular stomatitis virus (VSV)
SeV
Sendai virus (SeV)
RSV
Respiratory syncytial virus
HSV
Herpes simplex virus
MCMV
Mouse cytomegalovirus

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZY designed the study, analyzed the results, and wrote the manuscript. JW designed the study, prepared the adenovirus, and supervised the manuscript. YH analyzed partial data. LR modified the manuscript language. SC prepared the adenovirus. YD, EL, and NZ conceptualized the study. EL and NZ designed, supervised, and modified the manuscript.

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Tables

Table 1 Sequences of primers used in the study

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
<i>KIF5C</i>	ATCCCACGAATTGCCCATGAT	CCCTTTACATACGGGACTCTGT
<i>SFRP5</i>	CTGAGATGCTGCACTGCCACAA	GTCAGCACTGTGCTCCATCTCA
<i>TP73</i>	CACCTCAGCTCTCCATCTTATTG	GCATGGGTCTTAGCCTTTCT
<i>OASL</i>	CCATTGTGCCTGCCTACAGAG	CTTCAGCTTAGTTGGCCGATG
<i>SOCS3</i>	CATCTCTGTCGGAAGACCGTCA	GCATCGTACTGGTCCAGGAACT

Figures

Figure 1

(a) Correlation heat map of A549 cell samples. Numbers in boxes represent Pearson correlation coefficients between two corresponding samples. (b) PCA of tested samples. (c) Numbers of DEGs between control and HAdV-7-infected groups after 24, 48, and 72 hpi. (e-f) Volcano plot of DEGs.

Figure 2

(a) Correlation heatmap between modules and HAdV-7 infection. Each cell contains corresponding correlation and P -value. Table is color-coded by correlation according to color legend. (b-c) Scatterplot of gene significance (GS) for HAdV-7 infection vs module membership (MM) in blue, tan, and brown modules. One dot represents one gene and corresponding correlation coefficient and P -value are listed above scatter plot.

Figure 3

(a-b) GO and KEGG enrichment analysis of genes in blue module. Gene count is number of genes annotated in GO terms in specific module. (c) Venn diagram of DEGs obtained by RNA-seq, genes in blue module, and DEGs from GSE68004 dataset.

Figure 4

(a-b) GO and KEGG enrichment analysis of genes in tan module. (c) Venn diagram of DEGs obtained by RNA-seq, genes in tan module, and DEGs from GSE68004 dataset.

Figure 5

(a-b) GO and KEGG enrichment analysis of genes in brown module. (c) Venn diagram of DEGs obtained by RNA-seq, genes in brown module, and DEGs from GSE68004 dataset.

Figure 6

(a-e) Quantitative real-time PCR verification of hub genes related to HAdV-7 infection. Data are representative of two independent experiments (3–6 samples per group).

Figure 7

(a) Gene expression level by RNA-seq graphs at three time points. Data are representative of two independent experiments performed on three samples per group. &, $P < 0.05$, &&, $P < 0.01$, &&&, $P < 0.001$, &&&&, $P < 0.0001$, HAdV-7-infected group vs control group for SOCS3; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$, HAdV-7-infected group vs control group for ISG15; ^, $P < 0.05$, ^^, $P < 0.01$, ^^^, $P < 0.001$, ^^^^, $P < 0.0001$, HAdV-7-infected group vs control group for OASL; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$, ####, $P < 0.0001$, HAdV-7-infected group vs control group for IFIT1.

< 0.001, ####, $P < 0.0001$, HAdV-7-infected group vs control group for IFIT1. (b) The correlation between the candidate genes according to genes expression. Correlation coefficients (r , Spearman rank correlation) are reported in the lower left, and significance in the upper right (*, $p < 0.05$, **, $p < 0.01$, *** $p < 0.001$). (c) Gene to gene interaction network of the four candidate genes in the GeneMANIA dataset, wherein the size of each node indicates the strength of interaction, color of lines represents the types of interaction between genes (color code in Interactions legend), and colored node circle sections show the functions of respective genes (color code in Functions legend).

Figure 8

Antiviral innate immune signaling pathways in adenovirus infection and confrontation between adenovirus and host cells. (cGAMP, cyclic GMP-AMP; cGAS, cyclic GMP-AMP synthase; DDX41, DEADV-box polypeptide 41; HAAdV, human adenovirus; IFI16, interferon gamma-inducible protein 16; IFIT1, IFN-induced protein with tetratricopeptide repeats 1; IFN-I, type I interferon; IFNARs, type I interferon receptors;

IRF3, interferon regulatory factor 3; IRF7, interferon regulatory factor 7; IRF9, interferon regulatory factor 9; IRSE, IFN-response elements; ISG15, IFN-stimulated gene product 15; ISGF3, interferon-stimulated gene factor 3; ISGs, interferon-stimulated genes; JAK1, Janus kinase-1; MAVS, mitochondrial antiviral-signaling protein; MDA5, melanoma differentiation-associated gene 5; MyD88, myeloid differentiation primary response gene 88; OASL, oligoadenylate synthetases-like; RIG-I, retinoic acid-inducible gene I; STAT1, signal transducer and activator of transcription 1; STAT2, signal transducer and activator of transcription 2; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; TLR9, Toll-like receptor 9; TYK2, tyrosine kinase 2). Created with BioRender.com.

Supplementary Files

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