Study on long noncoding RNA in enterovirus 71-induced severe hand-foot-mouth disease

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Research article

Keywords: Long noncoding RNA, Enterovirus 71, Severe hand-foot-mouth disease, Target gene

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Abstract

Background: To identify the critical type of severe hand-foot-mouth disease (HFMD) in early stage, long noncoding ribonucleic acid (LncRNA) microarray was used to screen specific LncRNA and to predictpossible pathogenesis and target genes of HFMD.

Methods: Twenty cases of EV71-induced severe HFMD were collected in Children's Hospital of Chongqing Medical University from September 2015 to September 2016. LncRNA microarray was used to analyze the expression of LncRNA and mRNA in peripheral blood of children between severe type and critical type, and then verify the validity by real-time polymerase chain reaction. Bioinformatic methods were used to investigate differentially expressed mRNA by gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

Results: We obtained differentially expressed profiles of LncRNA and mRNA from peripheral blood of children with EV71-induced severe HFMD, and the number of LncRNA was 1949, the total mRNA profiles were 1422. GO analysis found that differentially expressed mRNAs were involved in the processes of immune response, immune defense, nucleic acid metabolism, and protein and molecule binding. KEGG analysis revealed that the differentially expressed mRNAs were involved in the p53 signaling pathway, NOD-like receptor signaling pathway, osteoblast differentiation, and so on. The target gene SLPI was closely related to the critical type of severe HFMD by cis/trans target gene prediction, and the pathogenic processes was regulated by LncRNA NR_038337.

Conclusion: Since differentially expressed LncRNAs and mRNAs are present in severe HFMD, our findings provide a new method to explore the pathogenesis of the critical type of severe HFMD, andfurther study on specific LncRNA and target genes in critical type has great significance for diagnosis and treatment of severe HFMD.

Keywords: Long noncoding RNA; Enterovirus 71; Severe hand-foot-mouth disease; Target gene

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Background

Hand-foot-mouth disease (HFMD), an infectious disease named after clinical features, was first reported in New Zealand in 1957. It is mainly prevalent in the Asian-Pacific region and has become an urgent situation to global public health security. HFMD is more likely to occur in children younger than 5 years, usually causing mild clinical symptoms. However, some children may suffer from convulsions, cerebral hernia, brainstem encephalitis, pulmonary hemorrhage, neurogenic pulmonary edema, myocarditis, circulatory disturbance, and other complications, which are known as severe HFMD. According to the degree of organ injuries, severe HFMD can be divided into severe type and critical type[1]. Severe type can be cured in about 1 week, but critical type has a very high mortality rate. An epidemiological survey has

shown that enterovirus 71 (EV71) is the major cause of severe HFMD, and the positive rate of EV71 is 93% in the fatal cases[2]. Therefore, it is particularly important to identify EV71-induced severe HFMD, especially in its early stage.

The pathophysiological process of severe HFMD is extremely complex. The levels of viral receptors, inflammatory factors, and immune cells are varied, with abnormal expression of genes in vivo[3,4].With the deepening of genomics studies, researchers are paying more attention to those genes that cannot encode proteins. Over the last decades, the importance of long noncoding RNA (LncRNA) has been realized to participate in chromatin structure, regulation of gene expression, subcellular tissue structure stabilization, cytoplasmic transportation and other processes. They may bewidly related to coronary disease, diabetes, depression, breast cancer, and so on. In this article, LncRNA microarray was used to analyze gene expression in peripheral blood of children with EV71-induced severe HFMD to screenspecific LncRNA related to the critical type and to predict possible pathogenesis and target genes.

Materials And Methods

1. Objects: Twenty cases of EV71-induced severe HFMD were collected in Children's Hospital of Chongqing Medical University during September 2015 to September 2016. Of these 20 cases, 10 cases of critical type were regarded as the test group, and 10 cases of severe type were regarded the control group. There was no significant difference in age and sex between the two groups (P□0.05).

2. Selected standards: The cases were selected in compliance with severe HFMD diagnostic criteria of Diagnosis and Treatment Guideline on Hand-foot-mouth Disease promulgated by Ministry of Health of the People's Republic of China in 2010. Etiology of EV71-IgM in peripheral blood or EV71-PCR in stool must be positive, and CA16-IgM and EVU-PCR should be negative. Other infectious diseases, chronic diseases, and complications were excluded.

3. Materials: Human lymphocyte separation medium, red blood cell lysis buffer, TRIzol, Quick Amp Labeling Kit, RNeasy Mini Kit, ArrayStar human LncRNA microarray, Agilent Gene Expression Hybridization Kit and Gene Expression Wash Buffer, Agilent Microarray Scanner, Primer 5.0, GeneAmp PCR System 9700, and so on.

4. Methods: Peripheral blood mononuclear cells were obtained by Ficoll density gradient centrifugation, and cRNA was obtained from total RNA extracted by TRIzol after being purified, qualified, and labeled. LncRNA microarray hybridization was then performed, followed by image scanning and data extraction. Finally, we obtained the LncRNA and mRNA gene expression profiles. RT-PCR assays were performed after we selected the LncRNA of interest. Finally, we conducted in-depth analysis of microarray data through gene ontology analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, and cis/trans target gene prediction.

Results

1. Total RNA quality control: Optical density (OD) 260/280 of total RNA in each sample ranged from 1.8 to 2.1 and OD 260/230 > 1.8. There were clear 18s and 28s bands in denatured agarose gel electrophoresis. In addition, the fluorescent value of the 28s band was about twice that of the 18s band (see Fig. 1).

2. LncRNA microarray quality control: The normalized data on microarray are described by box plots (Fig. 2 and Fig. 3). The horizontal axis represents the samples and groups, and the vertical axis shows the normalized signal value. The expression profiles of LncRNA and mRNA are categorized by scatter diagrams (Fig. 4 and Fig. 5). The black spots indicate that there was no significant difference in two groups. The red spots imply that these data are twofold higher in the test group and the green spots shows twofold lower.

3. The differential expression profiles of LncRNA and mRNA: For this analysis, we set a standard, that is fold change ≥ 2 and $P < 0.05$. We obtained differentially expressed profiles of LncRNA and mRNA in peripheral blood mononuclear cells between EV71-induced severe type and critical type. In these profiles, the number of LncRNA was 1949, with 1067 upregulated and 882 downregulated. The total mRNA profiles were 1422, with 881 upregulated and 541 downregulated (Table 1 and Table 2).

4. RT-PCR assay: We use RT-PCR to verify the reliability of microarray. Results show that the expression levels of ENST00000419973 and ENST00000545293 are increased, and the expression levels of ENST00000520944, NR_038337, and ENST00000564152 are decreased in RT-PCR, which are consistent with the results of LncRNA microarray. Therefore, we think that data from LncRNA chips are reliable (Table 3).

5. Bioinformatics analysis

5.1 GO analysis: The highest enrichment scores for upregulated mRNAs were for cytosol, unfolded protein binding, and immune response. The top three enrichment scores for downregulated mRNAs were for nucleus, transcription corepressor activity, and nucleic acid metabolism process.

5.2 KEGG analysis: Through KEGG analysis, in upregulated mRNA profiles, the top three signal pathways were legionellosis, influenza A, complement and coagulation cascades. While in the downregulated database, the highest enrichment pathways were systemic lupus erythematosus, alcoholism, and transcriptional misregulation in cancer (Fig. 6 and Fig. 7).

5.3 Target gene prediction: We selected several LncRNAs of interest and use cis/trans modes to predict their potential target genes in databases (Table 4). Compared with differentially expressed profiles of mRNA, secretory leukocyte protease inhibitor (SLPI) was considered to be the target gene of LncRNA NR_038337.

Discussion

Currently, microarray technology has become more mature and stable. It is based on the principle of complementary base pairing, followed by nucleic acid probes hybridizing to the unknown sequences. After obtaining the total RNA from samples, we should first label them with fluorescent tags and then hybridize the RNA with nucleic acid in microarrays. After elution, the information of matched RNA is successfully saved on the chip. To perform microarray quality control，image processing software is used to convert the hybridization signals to relative gene expression intensity. By researching databases and related articles, detailed information about genes in different groups and samples can be compared.

Currently, LncRNA microarray is usually used for the study of breast cancer, Parkinson disease, depression, systemic lupus erythematosus, and diabetes. However, research on infectious diseases, especially on HFMD, is still rare. A number of studies have found that[5,6] LncRNA in a host can change its expression level to respond to viral infection, thus playing a role in immune defense. Similarly, the virus LncRNA can bind to the host protein and regulate gene expression, thereby promoting reproduction of the virus itself. Yin and other researchers revealed that there were abnormal changes in LncRNA and mRNA in the rhabdomyosarcoma cells that were EV71-infected and mock-infected[7]. Differentially expressed LncRNA may regulate these processes, including selective splicing, splicing diversity, phosphoprotein, and acetylation. This study suggests that LncRNA is involved in the host immune response after EV71 infection and is expected to be an indicator of EV71 infection, but it is still necessary to verify these results in vitro.

Based on this finding, 20 cases of severe HFMD peripheral blood samples were analyzed to obtain different expression profiles of LncRNA and mRNA in severe and critical type EV71 infection. The number of LncRNA was 1949 and the total number of mRNA was 1422. The expression levels of Toll-like receptor (TLR)2, TLR4, TLR8, IL27, and interleukin (IL)17A were significantly upregulated in the test group, whereas the levels of IL23A, IL8, tumor necrosis factor receptor superfamily (TNFRSF)25, and TNFRSF10B were downregulated. Because macrophages in vitro can activate TLRs during the pathogenesis of severe HFMD, we deem that this abnormal phenomenon is associated with immunoreaction after EV71 infection. When patients' condition progresses to critical and TLRs and other related pathways are abnormally activated, a large amount of IL and TNF would be produced, leading to inflammation, tissue injury and apoptosis. A worst case scenario is that cell destruction can also activate TLRs, resulting in gradual increase in the inflammatory effect. Therefore, the differentially expressed mRNAs may play roles in immune response, inflammatory response, apoptosis, and other aspects of critical type. Because most mRNAs are regulated by LncRNAs, the differentially expressed LncRNAs in this experiment are likely to regulate the above three aspects and thus affect the pathogenesis of critical type of HFMD.

Although LncRNA cannot directly play a biological role in encoding the protein, it can participate in epigenetic, transcriptional, and posttranscriptional levels to regulate the functional and conformational changes in mRNA, thus affecting the process of critical type HFMD. Classifying the differentially expressed mRNA, we can see that cytosol and cytoplasm have higher enrichment scores in the aspect of cellular component. Meanwhile, unfolded protein binding, heat shock protein binding, and peptidoglycan

binding occur many times in the molecular function, which indicate that the different mRNA expression of critical HFMD is predominantly in the cytoplasmand the major biological behavior is binding various proteins, molecules, and the like. During the biological process, upregulated mRNAs are involved in immune response, defense response, and innate immune response, and downregulated mRNAs are enriched in the nucleic acid metabolism process and regulation of biosynthetic process and gene expression, suggesting that mRNA is related to immune response in critical HFMD, and the expression of mRNA in nucleic acid metabolism and biosynthesis regulation is limited, which indicate the mechanism differences between severe and critical type of EV71-induced HFMD and the abnormally activated immune system in patients.

KEGG analysis is used to describe changes between the two groups. Both in upregulated and downregulated pathways, mRNAs are enriched in the p53 signal pathway, transcriptional misregulation in cancer, and osteoclast differentiation. Because of the different pathogenesis and clinical manifestations in severe and critical HFMD, it can be considered that these pathways are significantly different between the two groups, which remains to be confirmed by further studies. At the same time, we found that discrepant mRNA also appears in pathways of conditions including legionella, influenza A, and measles. It is speculated that pathways between severe HFMD and other infectious diseases share similarities, constituting a complex genetic network to regulate the process of critical type in EV71-induced HFMD.

Subsequently, we selected five LncRNAs of interest for cis/trans target gene prediction and found that SLPI is the target gene of LncRNA NR_038337.NR_038337 and is a long noncoding RNA located on chromosome 20, which is also known as OSER1 antisense RNA1 (OSER1-AS1). Although some researchers want to study the molecular epidemiology of OSER1-AS1 in nonsmall-cell lung cancer, currently, no studies have reported on EV71-induced HFMD. NR_038337 was downregulated 5.1 times in the test group and SLPI was downregulated 3.3 folds. SLPI, is an endogenous immune-related protein that can fight against infection, promote cell proliferation, prevent apoptosis, and play an important role in a variety of lung diseases[8,9]. SLPI levels decrease in the acute stage of many infectious diseases. When a patient's condition improves, the level of SLPI gradually increases[10,11]. Previous studies have shown that the anti-inflammatory effect of SLPI is to inhibit the activation of nuclear factor-κB in nonmacrophages, protecting tissues from excessive inflammatory responses[12]. Because the pathogenesis of severe HFMD is closely related to the inflammatory response and immune response, we hypothesize that NR_038337 can decrease the expression level of its target gene *SLPI,* which inhibits the anti-inflammatory effect after EV71 infection and causes significant damages to tissues and organs, ultimately resulting in the difference in clinical manifestations and prognosis between severe and critical type of EV71-induced HFMD; however, these results should be verified again in vivo.

In conclusion, LncRNA and mRNA expression profiles in peripheral blood from children with EV71-induced severe HFMD were obtained for the first time by LncRNA microarray. We conducted a preliminary analysis of the differentially expressed LncRNAs and mRNAs, providing a reference for the in-depth follow-up analysis of pathogenesis of critical type of HFMD. However, because these two groups had EV71 infection in this study, we could distinguish them only through clinical manifestations, so analyzing

differentially expressed LncRNA is based on EV71 infection, and we could not classify samples to infected group and control group of mRNA expression, which is one of the difficulties and also innovation of this study. If samples at every stage of EV71-induced HFMD could be collected, the trends of gene expression in the entire pathogenesis process would be more clear, and the different stages of disease could be analyzed from the genetic viewpoint.

Abbreviations

HFMD Hand-foot-mouth disease

EV71 Enterovirus 71

LncRNA long noncoding RNA

KEGG Kyoto Encyclopedia of Genes and Genomes

OD Optical density

Declarations

Ethics approval and consent to participate:

This study was approved by the Institutional Review Broad,Children's Hospital of Chongqing Medical University,and already registered in Chinese Clinical Trail Registry(ChiCTR-DCD–15007192). Written informed consent was obtained from all the participants.

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:

We have read and understood BMJ policy on declaration of interests and declare that we have no competing interests.

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Authors' contributions:

HY and FF contributed equally to this work and should be considered co-first authors. XF was the corresponding author.XF designed the study.FF and HY collected and analyzed the data,drafted the manuscript.All authors have read and approved the final manuscript.

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Tables

Due to technical limitations, Tables 1-4 are provided in the Supplementary Files section.

Figures

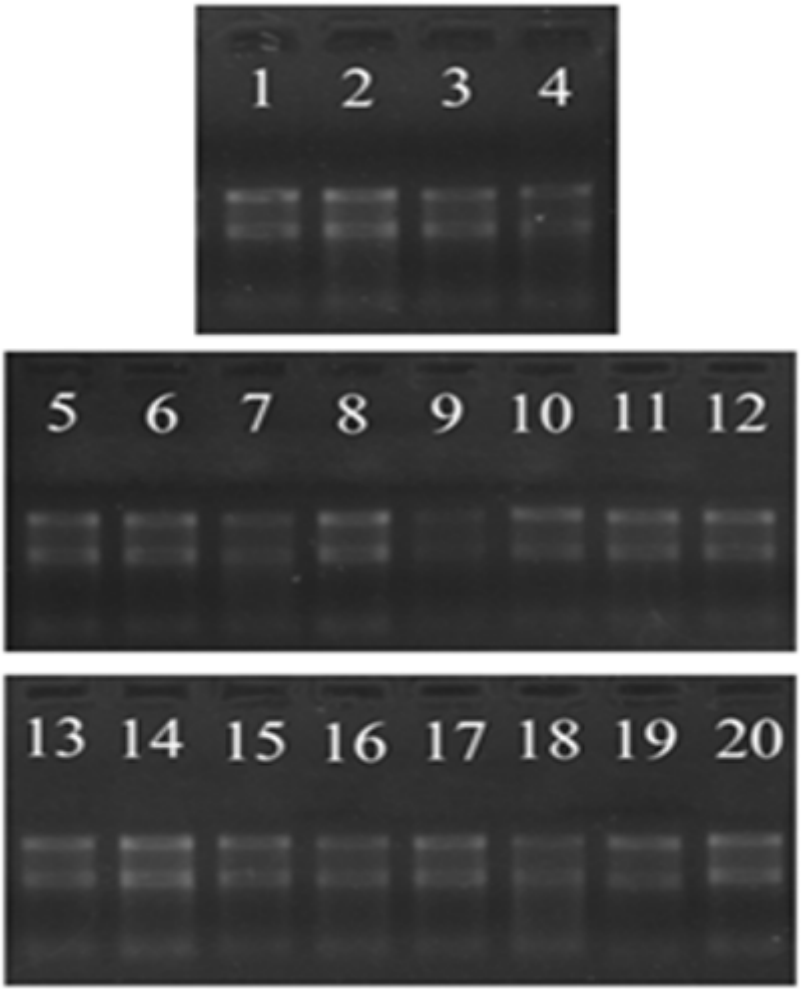

Figure 1

Denaturing agarose gel electrophoresis

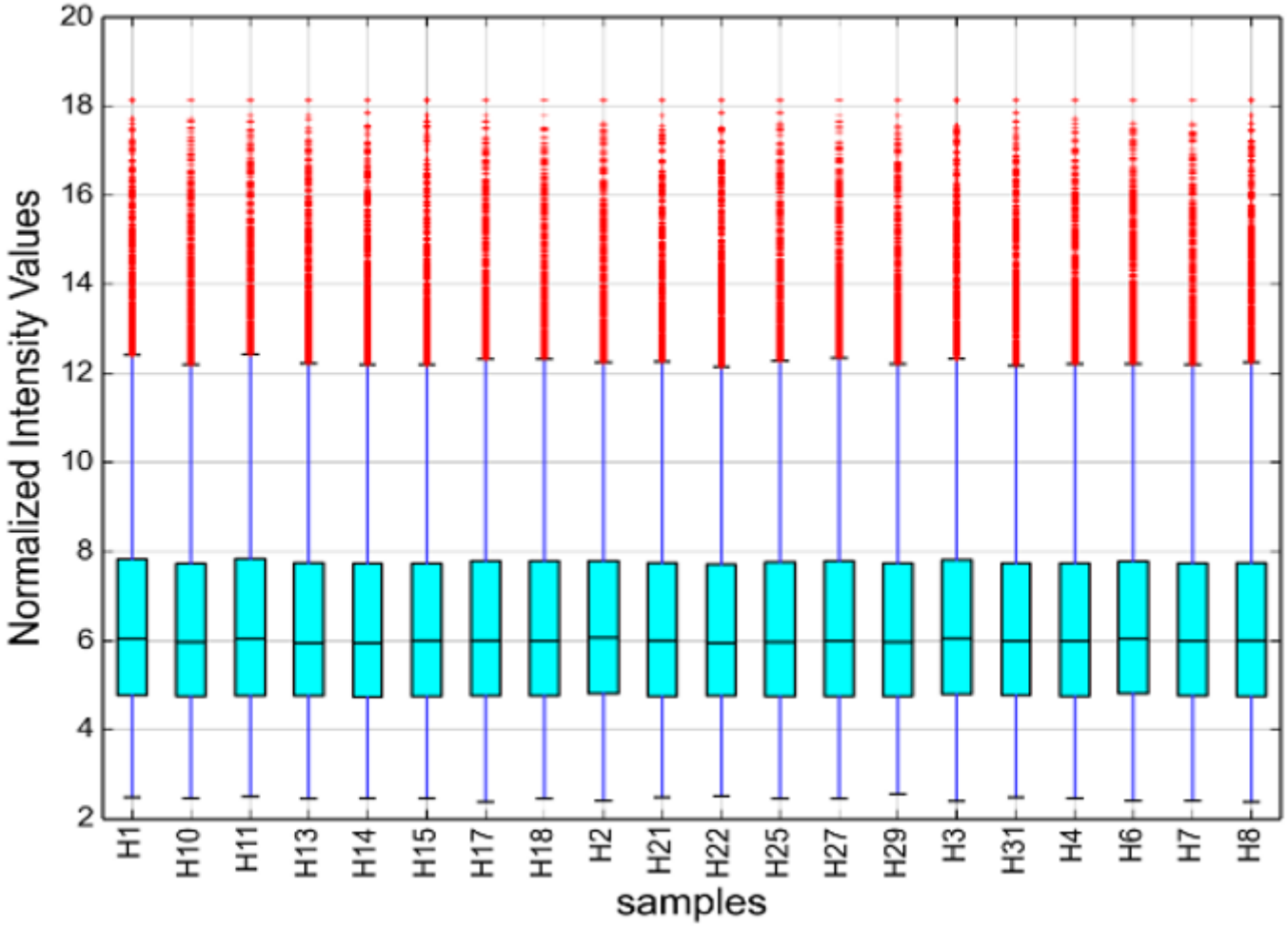

Figure 2

Box plot of LncRNA

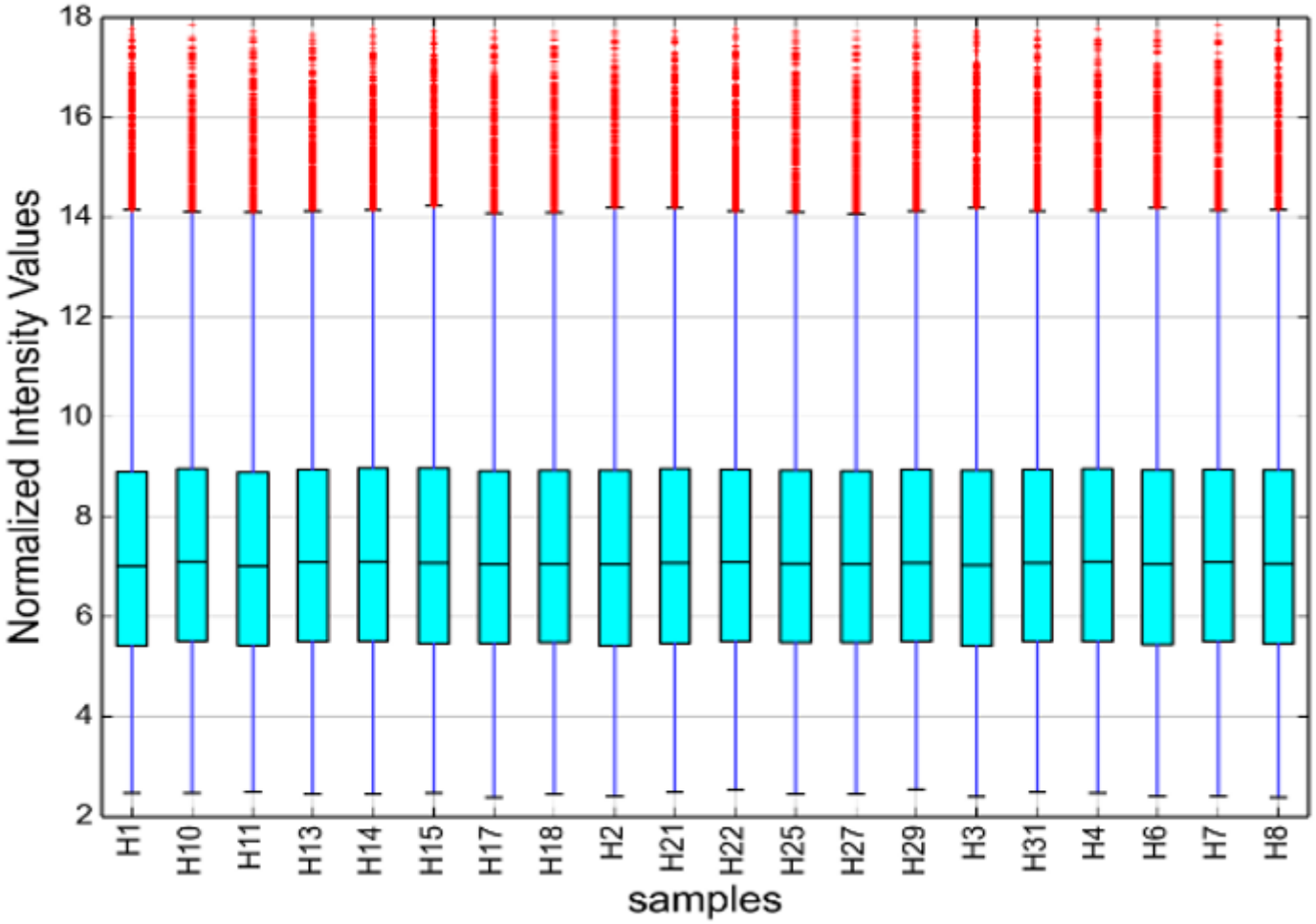

Figure 3

Box plot of mRNA

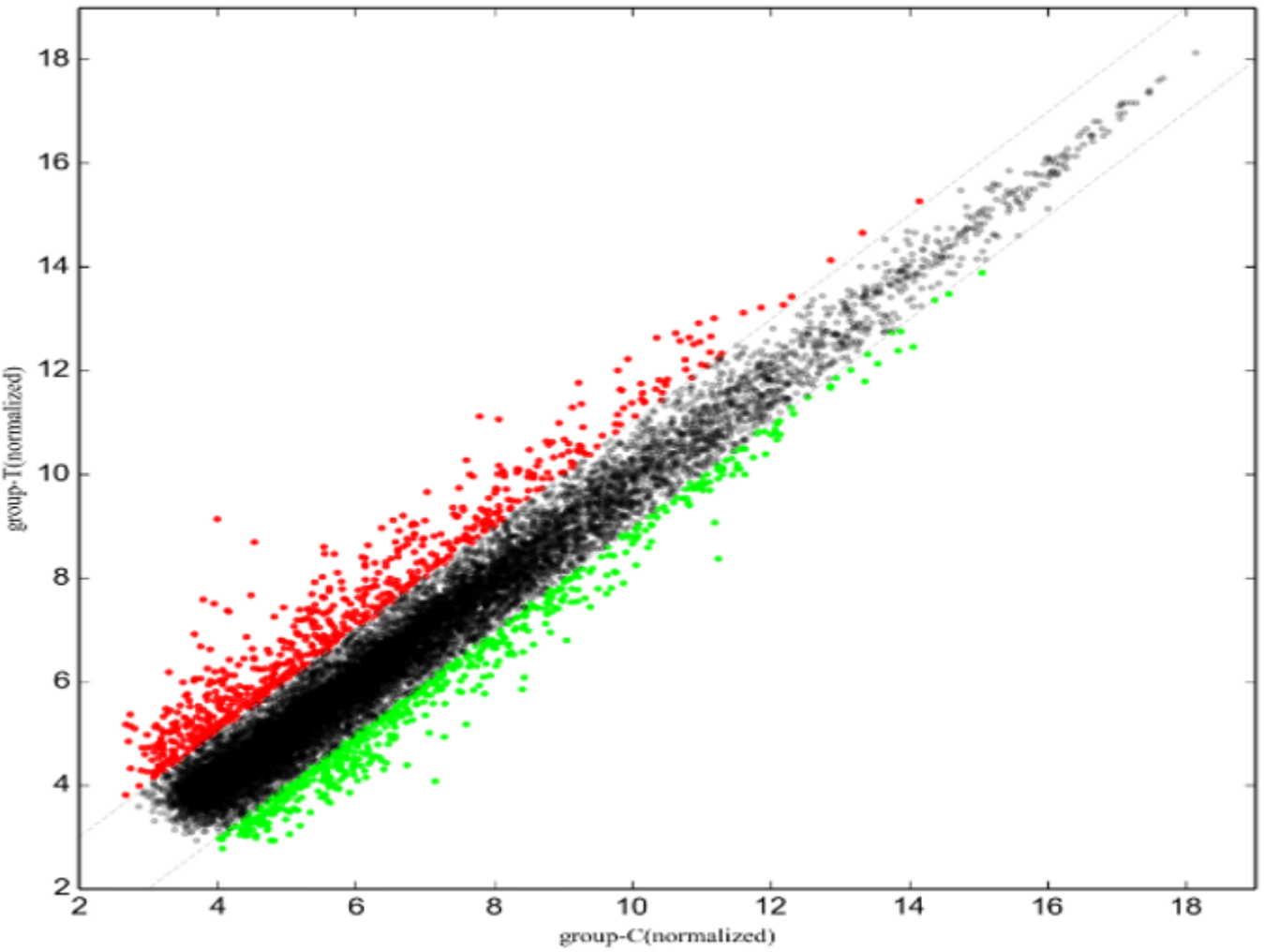

Figure 4

Scatter diagram of LncRNA

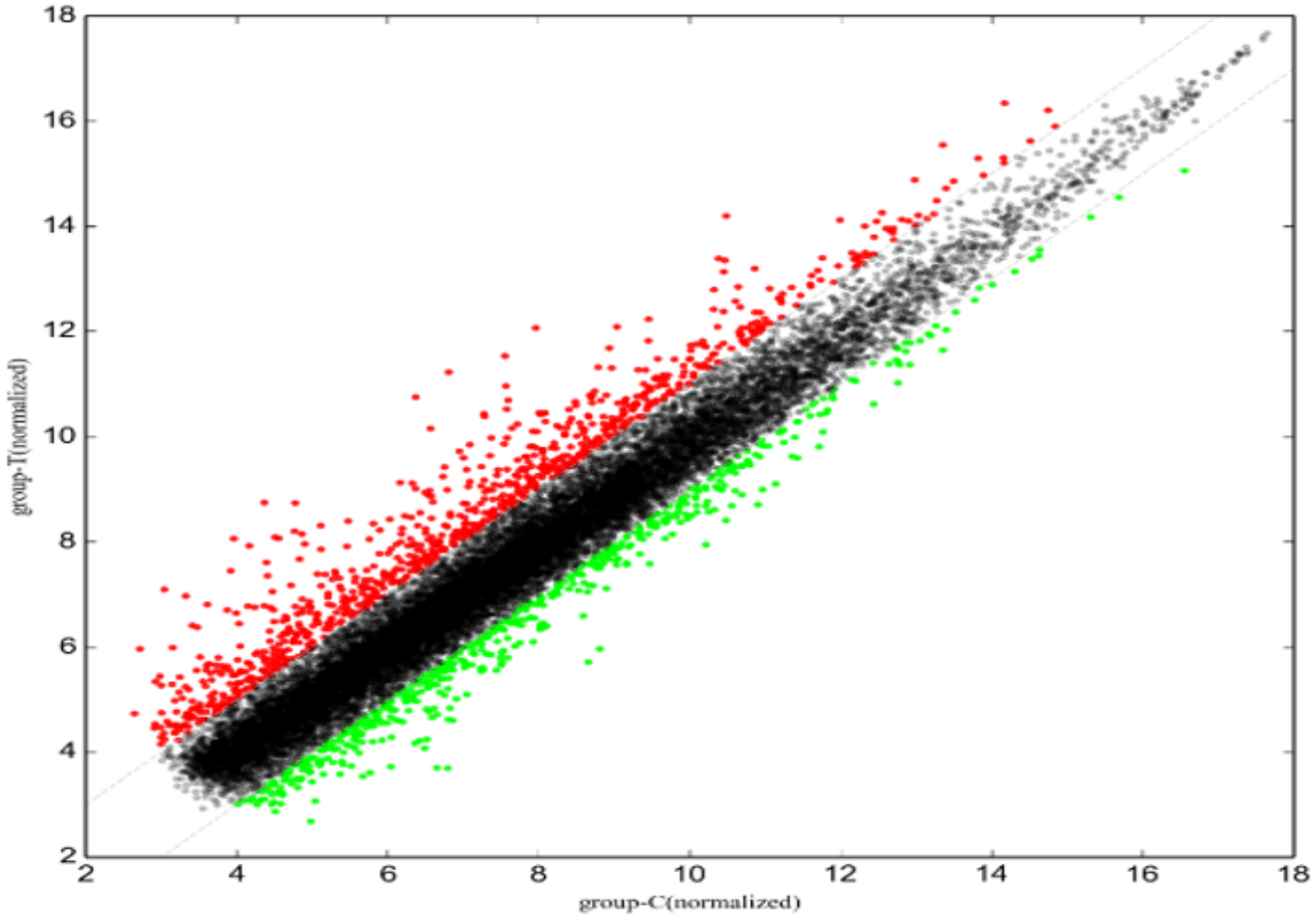

Figure 5

Scatter diagram of mRNA

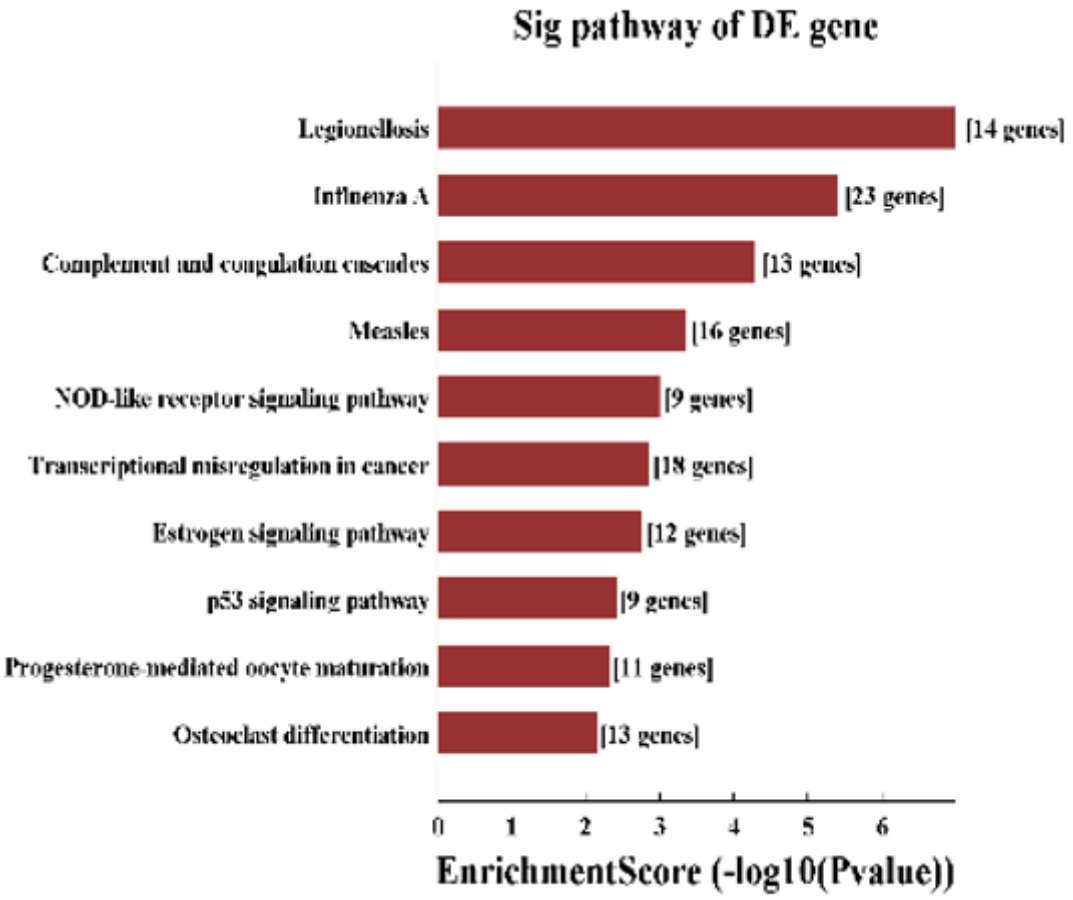

Figure 6

KEGG analysis of upregulated mRNA

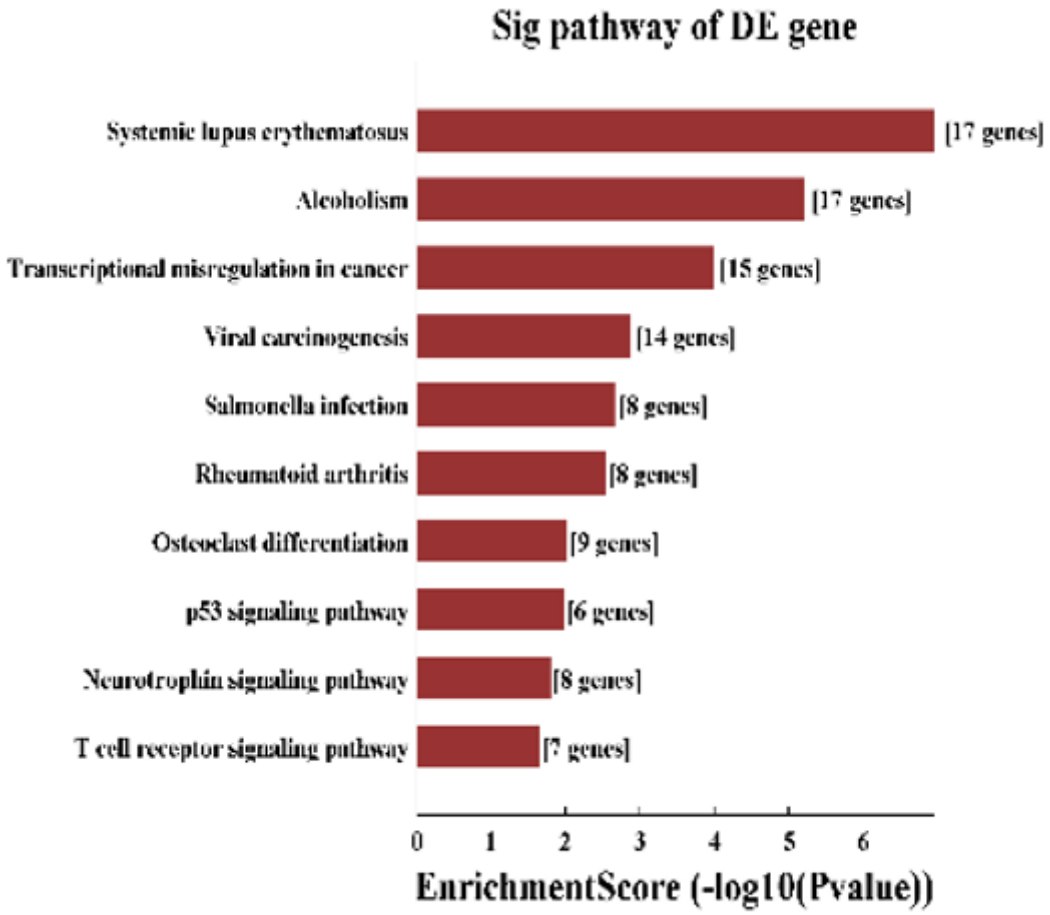

Figure 7

KEGG analysis of downregulated mRNA

Supplementary Files

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- Tables.docx