Genome-wide profiling of mRNA and IncRNA expression in dengue fever and dengue hemorrhagic fever

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Dengue fever (DF) and dengue hemorrhagic fever (DHF) are recurrent diseases that are widespread in the tropics. Here, we identified candidate genes associated with these diseases by performing integrated analyses of DF (GSE51808) and DHF (GSE18090) microarray datasets in the Gene Expression Omnibus (GEO). In all, we identified 7,635 differentially expressed genes (DEGs) in DF and 8,147 DEGs in DHF as compared to healthy controls (P < 0.05). In addition, we discovered 215 differentially expressed long non-coding RNAs (DElncRNAs) in DF and 225 DElncRNAs in DHF. There were 1256 common DEGs and eight common DElncRNAs in DHF vs DF, DHF vs normal control, and DF vs normal control groups. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that signal transduction (false discovery rate = 8.33E-10), ‘toxoplasmosis’, and ‘protein processing in endoplasmic reticulum’ were significantly enriched pathways for common DEGs. We conclude that the MAGED1, STAT1, and IL12A genes may play crucial roles in DF and DHF, and suggest that our findings may facilitate the identification of biomarkers and the development of new drug design strategies for DF and DHF treatment.

Abbreviations
AUC, area under the ROC curve; BP, biological process; DC, dendritic cell; DEG, differentially expressed gene; DElncRNA, differentially expressed long non-coding RNA; DEmRNA, differentially expressed mRNA; DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; ER, endoplasmic reticulum; FDR, false discovery rate; GEO, the Gene Expression Omnibus database; GO, Gene Ontology; IFN, interferon; IncRNA, long non-coding RNA; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAGED1, melanoma-associated antigen D1; MF, molecular functions; NC, normal control; PPI, protein–protein interaction; ROC, receiver operating characteristic; STAT1, signal transducer and activator of transcription 1.
Dengue fever (DF) is the second most severe infectious disease worldwide by mortality and morbidity after malaria [1]. Clinically, DF is a mosquito-borne illness that is caused by infection with dengue virus (DENV), especially affecting children in endemic, mostly tropical areas [2]. In total, 33% of the world’s population is at risk of infection with the DENV. The majority of DENV infections are symptomless or produce a slight illness with flu-like symptoms, such as headache, fever, myalgia and decreased platelet counts and leucopenia. These symptoms are known as DF, which is an acute, self-limited, febrile illness. However, some DF patients develop a severe syndrome known as dengue hemorrhagic fever (DHF), in which patients may display hematomas with marked thrombocytopenia or extremely low platelet counts [3]. The clinical hallmark of DHF is plasma leakage, which usually lasts for approximately 48 h and leads to reduced circulatory volume [4].

During DENV infection, overproduction of cytokines and chemokines is considered to contribute to the increased vascular permeability, disruption of the coagulation system and shock associated with DHF [5]. Despite its high burden on global health, no accessible antivirals or vaccines have been approved for clinical use [6]. At present, there is no specific therapy available for DF and DHF. Appropriate fluid management to correct hypovolemia has been successful in reducing the mortality of DF and DHF [7,8], but access to medical services remains problematic in many developing countries. Mosquito control, which is costly and often ineffective, still remains the only method of preventing DF and DHF currently available [2].

In this study, through integrated analysis, we aimed to obtain more accurate results with a large sample size than those through individual studies [9]. In order to obtain the key long non-coding RNAs (lncRNAs), mRNAs associated with DF and DHF, the study analyzed the blood transcriptome of DF patients, DHF patients and normal controls, seeking to identify early detection biomarkers of DF and DHF.

**Methods**

**Eligible gene expression profiles of DF and DHF**

We selected gene expression datasets of DF and DHF from the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo) which is the largest database of high-throughput gene expression data [10]. Search keywords were (‘dengue’ [MeSH Terms] OR ‘dengue fever’ [All Fields]) AND ‘Homo sapiens’ [porgn] AND ‘gse’ [Filter]. The datasets that met the following criteria were included in our study: (a) the selected dataset was genome-wide mRNA transcriptome profiling by array; (b) the data were derived from DF or DHF patients; and (c) the datasets were normalized or raw datasets.

**Identification of common differentially expressed mRNAs and IncRNAs in the comparisons of DF vs normal control, DHF vs normal control, and DF vs DHF**

Background correction was performed for the downloaded raw data. Using the limma package and the metama package, the inverse normal method was used for P-value consolidation. The adopted standard of differential analysis was \( P < 0.05 \). Finally, the differentially expressed mRNAs (DEmRNAs) and differentially expressed long non-coding RNAs (DElncRNAs) of DF vs normal control (NC), DHF vs NC and DF vs DHF were obtained [11]. The Wilcoxon rank-sum test was used to identify significant differences in the expression of mRNAs and lncRNAs between the different groups.

**Functional annotation of common differentially expressed genes**

To identify the function and the potential pathways of common differentially expressed genes (DEGs), Gene Ontology (GO) classification (molecular functions, biological processes and cellular component) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were performed by using the online software GENECODIS3 (http://genecodis.cnb.csic.es/analysis) [12]. False discovery rate (FDR) < 0.05 was defined as the criterion of statistical significance.

**Protein–protein interaction network construction of common DEGs**

To further research the biological functions of common DEGs, Cytoscape was used to search protein interaction of the top 100 up-regulated common DEGs and top 100 down-regulated common DEGs in the comparison of DHF vs DF in the BioGRID database. After removing non-common genes, a protein–protein interaction (PPI) network was constructed [13–15]. The network consisted of nodes and edges in which the nodes represent the proteins and the lines represent the interactions between them [16].

**Validation in the Gene Expression Omnibus dataset and receiver operating characteristic analysis**

The GSE38246 dataset was obtained from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/), and the amount of samples of normal:DF:DHF is 8:53:32. The expression pattern of selected DEmRNAs was verified using the GSE38246 dataset. In order to access
the diagnostic value of DEmRNAs for DF, the ‘pROC’ package was used to calculate the receiver operating characteristic (ROC), and the area under the ROC curve (AUC) was further calculated. When the AUC value was > 0.6, the DEmRNAs were considered to be capable of distinguishing patients with DF from NC with excellent specificity and sensitivity.

Results

Differential expression analysis of genes in DF and DHF

The probes corresponding to multiple genes were removed, and for multiple probes corresponding to only one gene, the one with the largest average expression was retained. Finally, 18 756 genes were obtained for the differential analysis, of which 968 were lncRNAs and 17 788 were mRNAs according to the GRCh38.p7 reference genome.

Compared with NC, 7635 DEGs in DF were obtained with \( P < 0.05 \), among which 4190 genes were up-regulated and 3445 genes were down-regulated. Likewise, 8147 DEGs in DHF were obtained, with 4239 up-regulated and 3908 down-regulated genes. The hierarchical clustering of the top 100 most significantly up- or down-regulated genes was performed, and listed in the heatmap [Fig. 1A (DF) and Fig. 1B (DHF)]. Compared with the DF group, 2677 DEGs were obtained, among which, 1390 genes

![Fig. 1. Heatmap image displaying genes that were significantly up-regulated or down-regulated (\( P \)-value < 0.05) in DF and DHF compared to NC. (A) DF vs NC. (B) DHF vs NC. (C) DHF vs DF.]

![Fig. 2. Heatmap image displaying lncRNAs that were significantly up-regulated or down-regulated (\( P \)-value < 0.05) in DF and DHF compared to NC. (A) DF vs NC. (B) DHF vs NC. (C) DHF vs DF.]

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were up-regulated and 1287 genes were down-regulated in the DHF group. The top 100 most significantly up- or down-regulated genes are listed in Fig. 1C.

**Differential expression analysis of IncRNAs in DF and DHF**

Compared with NC, 215 DEIncRNAs in DF were obtained with $P < 0.05$, among which, 57 IncRNAs were up-regulated and 158 IncRNAs were down-regulated. Likewise, of 225 DEIncRNAs in DHF, 74 up-regulated and 151 down-regulated IncRNAs were obtained [Fig. 2A (DF) and Fig. 2B (DHF)]. Compared with the DF group, there were 81 DEIncRNAs, among which, 42 DEIncRNAs were up-regulated and 39 DEIncRNAs were down-regulated in DHF group (Fig. 2C).

**Common DEGs and IncRNAs of DHF vs DF, DHF vs NC and DF vs NC**

In Fig. 3, there were 1256 common DEGs in DHF vs DF, DHF vs NC and DF vs NC, of which 834 DEGs were up-regulated, and 422 DEGs were down-regulated in DF or DHF compared to NC. A hierarchical clustering heatmap for the top 100 (DHF vs DF) common genes is shown in Fig. 4A. A total of 18 common DEIncRNAs were obtained in DHF vs DF, DHF vs NC and DF vs NC, of which 16 DEIncRNAs were down-regulated and two DEIncRNAs were up-regulated in DF or DHF compared to NC. The hierarchical clustering heatmap of all common IncRNAs is shown in Fig. 4B.

**Functional annotation of common DEGs**

A total of 1256 common DEmRNAs were subjected to GO enrichment and KEGG enrichment analysis using the R language (GSEABASE package). GO enrichment analysis and KEGG pathway analysis indicated that these common DEGs were significantly involved in the biological processes of signal transduction ($FDR = 8.33E-10$), apoptotic process ($FDR = 1.46E-08$), cell cycle ($FDR = 3.04E-10$) and protein transport ($FDR = 5.19E-10$) (Fig. 5A). In addition, cytoplasm ($FDR = 3.19E-60$), membrane ($FDR = 7.16E-58$) and nucleus ($FDR = 3.37E-32$) were remarkably enriched cytology components (Fig. 5B), and protein binding ($FDR = 3.37E-32$), nucleotide binding ($FDR = 3.58E-25$) and metal ion binding ($FDR = 7.45E-08$) were significantly involved molecular functions (Fig. 5C). Protein processing in...
endoplasmic reticulum (FDR = 6.36E-41), N-glycan biosynthesis (FDR = 1.44E-17) and toxoplasmosis (FDR = 2.14E-05) were significant enriched KEGG pathways (Fig. 5D).

PPI network and module analysis of common DEGs

To identify potential interactions among common DEGs, a PPI network was constructed. The PPI results identified 330 nodes (genes) and 419 edges (Fig. 6: all points are proteins encoded by common DEGs, a green oval indicates proteins encoded by a down-regulated DEG (DHF vs DF) and a red oval indicates proteins encoded by an up-regulated DEG (DHF vs DF). Among them, those of higher degree are ESR1 (degree = 57), AKT1 (degree = 29), TUBA1A (degree = 23), CAV1 (degree = 23), RAB7A (degree = 17), FBXO6 (degree = 14), DERL2 (degree = 1), TMEM216 (degree = 11), MAGED1 (degree = 10) and DNAJB11 (degree = 10).
Fig. 6. Protein–protein interaction network of common genes. All points are differentially expressed genes; green represents down-regulated and red represents up-regulated.

Table 1. Differential expression of common lncRNAs and adjacent differentially expressed common genes.

| chr | LncRNA        | Start – 100 kb | End + 100 kb | Nearby mRNA | Symbol | Start | End   |
|-----|---------------|----------------|--------------|-------------|--------|-------|-------|
| 9   | LOC100129034  | 124253473      | 124459186    | NEK6        | 124257606 | 124353307 |
| 17  | TNRC6C-AS1    | 78007398       | 78211799     | AFMID       | 78187317 | 78207701 |
| 17  | TNRC6C-AS1    | 78007398       | 78211799     | TK1         | 78174075 | 78187233 |

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Proximity analysis of DElncRNA-DEmRNA

Three pairs of DElncRNA-adjacent DEG (including two lncRNAs and three DEGs) were obtained by searching 100 kb upstream and downstream of common DElncRNAs. The expression of common DElncRNAs and adjacent DEGs is listed in Table 1.

Validation of the expression of DEmRNAs by GSE38246

Based on GSE38246, expression of four DEmRNAs (DNAJB11, IL12A, MAGED1, and STAT1) was validated (Fig. 7). Expression of three DEmRNAs (DNAJB11, IL12A, and MAGED1) was up-regulated in DF and DHF compared to NC. These results were generally consistent with the results of our integrated analysis.

ROC curve analysis

ROC curve analyses and the AUC were used to assess the discriminatory ability of four DEmRNAs (DNAJB11, IL12A, MAGED1, and STAT1). The AUCs of all these four DEmRNAs, including DNAJB11 (0.739), IL12A (0.605), MAGED1 (0.722) and STAT1 (0.658), were more than 0.6 (Fig. 8), which had great diagnostic value for DF.

Discussion

In severe cases, DENV, which is an alarming emerging disease, can be fatal. The activation of multiple inflammatory pathways is involved in the pathogenesis of severe critical disease following DENV infection. The response to DENV infection is complicated and characterized by the production of numerous cytokines [9]. In our study, we aimed to identify the gene and lncRNA expression profiles of DF or DHF. We found 1256 common DEGs in the comparisons of DHF vs DF, DHF vs NC and DF vs NC groups, and interestingly the expression trend of these common genes was essentially identical for both DF and DHF patients compared to the NC samples. A total of 18 common DElncRNAs were obtained, of which 16 DElncRNAs were down-regulated, two DElncRNAs were up-regulated (DF vs NC and DHF vs NC). These indicated that DF and DHF may have similar features. For the common DEGs and DElncRNAs, we also constructed the PPI network, functional annotation and adjacent analysis of mRNAs and lncRNAs. Together with retrieved literature, we obtained three genes that may be involved in DF and DHF, namely MAGED1, STAT1, and IL12A. These three genes were among 1256 common differentially expressed genes in DHF vs DF, DHF vs NC and DF vs NC.

Fig. 7. Validation of the expression levels of selected DEGs in DF and DHF based on GSE38246. The x-axis shows case and normal groups and y-axis shows gene expression level. (A) DNAJB11; (B) IL12A; (C) MAGED1; (D) STAT1.
MAGED1 (melanoma-associated antigen D1, also known as NRAGE or Dlxin-1) is a member of the MAGE homology domain (MHD)-containing protein superfamily, which includes over 30 members in humans [17]. The possibility that they are candidates for disease is raised by the strong expression of the MAGED1 genes in structures involved in higher function, such as the cerebral cortex, and the hippocampus [18]. MAGED1 is highly expressed throughout the brain [18]. Previous study has found that during the acute phase of dengue, the expression level of MAGED1 was greater in DHF patients compared to DF patients [19]. Interestingly, in patients of dengue and the acute DHF patients, the pro-apoptotic PDRX4 and MAGED1 genes were over-expressed. MAGED1 has been associated with the p75 neurotrophin receptor-mediated programmed cell death pathway [20]. Indeed, DENV infection augments apoptosis in patients with severe DHF, so the expression of pro-apoptotic genes, such as MAGED1, was increased [19]. In our study, MAGED1 expression was up-regulated in patients with DF and DHF compared with NC. In the PPI network, MAGED1 was among the top 10 genes of higher degree. All of these results indicated that MAGED1 may play a role in the pathogenesis of DF and DHF.

IL12A/IL-12p35, which is a subunit of a cytokine, acts on T and natural killer cells and has a broad array of biological activities. IL12A is crucial for the T-cell-independent induction of IFN-γ, and is required for the differentiation of both Th1 and Th2 cells. The responses of lymphocytes to this cytokine are mediated by the activator of transcription protein STAT4 [21]. In the study of de Kruijff, the profile showed characteristics of a general antiviral response with up-regulation of IL12A, which is a potent stimulator of IFN-γ production [9].

Signal transducer and activator of transcription 1 (STAT1), which is encoded by the STAT1 gene, is a transcription factor in humans. STAT1 is a member of the STAT protein family. A recent study of a panel of 184 inflammation-related genes showed that the
*STAT1* gene was one of the most differentially expressed [22]. In the study of Cerny, nanostring gene expression data showed significant up-regulation of *STAT1* upon dengue viral exposure in susceptible dendritic cell populations [23]. Yu et al. demonstrated that STAT1-mediated antiviral interferon responses contribute to the action of schisandrin A against DENV replication [24]. In our study, the *STAT1* expression was up-regulated in DF vs NC and DHF vs NC, and was down-regulated in DHF vs DF.

In our functional annotation, we found that DEGs were significantly involved in the biological processes of signal transduction. Recent studies have shown that DENV can induce apoptosis [25], programmed cell death can be observed in endothelial cells, hepatocytes, neuroblastoma cells and hepatoma cells [26], and its signaling and transduction pathways have been studied in great depth. However, it is reasonable to speculate that the capsid protein can participate in the signal transduction of host cells, cause apoptosis of the host cells, and lead to the development of the disease. In the KEGG results, we found that DF-related genes, *STAT1* and *IL12A*, were enriched in the signaling pathway of ‘toxoplasmosis’, and *MAGED1* was enriched in ‘protein processing in endoplasmic reticulum’. This may indicate that there were some similar features in the pathogenic mechanism of DF and DHF compared with toxoplasmosis. As previously described, endoplasmic reticulum (ER) rearrangement and expansion is an early event in the DENV life cycle that is driven by viral but not host protein synthesis [27]. Reid et al. [28] reported that throughout the viral life cycle, DENV plus- and minus-strand RNAs were highly partitioned to the ER, identifying the ER as the primary site of DENV translation. The KEGG enrichment analysis in the present study supported the previous studies and suggested that ‘toxoplasmosis’ and ‘protein processing in endoplasmic reticulum’ pathways are involved with DF and DHF.

**Conclusion**

This study provides further insight into the molecular aspects of DF and DHF, suggesting new molecular signatures and new targets for development of specific biomarkers. In particular, our findings suggest the possibility that signal transduction-related genes may also be factors indicating a poor prognosis for DF and DHF.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

XL and XZ drafted the manuscript; FS and HY participated in clinical data and collection; ZL and WY carried out the data analysis; ZL and JY had significant roles in the study design and manuscript review. All authors read and approved the final manuscript.

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