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ORIGINAL RESEARCH

Identification of miRNA-TF Regulatory Pathways Related to Diseases from a Neuroendocrine-Immune Perspective

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Abstract

The neuroendocrine-immune (NEI) network is fundamental for maintaining body's homeostasis and health. While the roles of microRNAs (miRNAs) and transcription factors (TFs) in disease processes are well-established, their synergistic regulation within the NEI network has yet to be elucidated. In this study, we constructed a background NEI-related miRNA-TF regulatory network (NEI-miRTF-N) by integrating NEI signaling molecules (including miRNAs, genes, and TFs) and identifying miRNA-TF feed-forward loops. Our analysis reveals that the number of immune signaling molecules is the highest and suggests potential directions for signal transduction, primarily from the nervous system to both the endocrine and immune systems, as well as from the endocrine system to the immune system. Furthermore, disease-specific NEI-miRTF-Ns for depression, Alzheimer's disease (AD) and dilated cardiomyopathy (DCM) were constructed based on the known disease molecules and significantly differentially expressed (SDE) molecules. Additionally, we proposed a novel method using depth-first-search algorithm for identifying significantly dysregulated NEI-related miRNA-TF regulatory pathways (NEI-miRTF-Ps) and verified their reliability from multiple perspectives. Our study provides an effective approach for identifying disease-specific NEI-miRTF-Ps and offers new insights into the synergistic regulation of miRNAs and TFs within the NEI network. Our findings provide information for new therapeutic strategies targeting these regulatory pathways.

Keywords Nervous · Endocrine · Immune · miRNA-TF feed-forward loops · Neuroendocrine-immune network · Regulatory pathway

Introduction

The nervous, endocrine, and immune systems are the three important systems in human body. Neuroendocrine-immune (NEI) network, first proposed by Besedovsky and Sorkin in 1977, proved that the nervous, endocrine, and immune systems can regulate each other, forming a regulatory network (Besedovsky and Sorkin 1977). NEI network plays a pivotal role in maintaining homeostasis and health (Vela-Patiño et al. 2022). Therefore, the dysregulation of NEI network can lead to the occurrence and development of diseases, including cancers, mental illnesses, and cardiovascular diseases (Fioranelli et al. 2018; Huifang et al. 2022; Jara et al. 2021; Jiang et al. 2020a; Klein 2021; Landgraaf et al. 2023; Sekaninova et al. 2020; Zefferino et al. 2021).

Previous studies have explored the interactions between diseases and NEI systems. Cancer is a systemic disease which manifested by dysfunction of NEI network, and it was found that NEI factors regulated cancer occurrence and metastasis at multiple levels, including central-, organ-, and microenvironment-level manipulation (Jiang et al. 2020a). Additionally, obesity is a multifactorial and multiorgan disease, whose pathophysiology can be explained by a complex crosstalk of NEI systems, and acupuncture,

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as a multi-targeted treatment method, has been found to be able to treat obesity (Landgraaf et al. 2023). It has been reported that the interactions between COVID-19 and NEI system were associated with the susceptibility of old people and patients with autoimmune rheumatic diseases, and explained that other comorbidities could develop into severe complications (Jara et al. 2021). Recently, a study reviewed the mechanism of poor dependence on aromatase inhibitors in breast cancer patients from NEI perspective, and demonstrated that NEI mechanisms play a crucial role in poor adherence to endocrine therapy in breast cancer patients (Huifang et al. 2022). In addition, long-term dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis associated with increased glucocorticoid secretion has been found to be linked to adverse cardiovascular function (Sekaninova et al. 2020). Simultaneously, immune and endocrine properties of the heart as well as the central and autonomic regulation of cardiac functions have also been reviewed (Fioranelli et al. 2018). In summary, the interactions between the nervous, endocrine, and immune systems form a complex biological network to maintain homeostasis.

MiRNAs and transcription factors (TFs) are two types of key regulators in biological networks, both of which are involved in many essential cellular processes, including cell differentiation, proliferation, and apoptosis (Hobert 2008). MiRNAs primarily regulate gene expression at the post-transcriptional level, while TFs regulate gene transcription at the transcriptional level. Studies have demonstrated that miRNAs and TFs could cooperatively regulate the same target genes, and mutually influence each other, thereby forming feed-forward loops (FFLs). The FFLs could form recurrent network motifs and play important roles in mammalian gene regulatory network (Tsang et al. 2007).

Existing researches have investigated the molecular regulatory mechanisms in diseases and biological processes based on collaborative regulation of miRNAs and TFs. For example, transcription factor Yin-Yang1 (YY1) was found to ameliorated liver ischemia/reperfusion injury (I/RI) in mice by repressing miR-181a-5p expression and stimulating ESR1-mediated activation of ERBB2 (Wu et al. 2023). Based on the constructed TF-mRNA-miRNA network associated with medullary thyroid carcinoma, 15 important genes were identified, and a high hub-gene score or a low miRNA score indicated good prognoses of neuroendocrine tumors (Weng et al. 2022). Recently, a modeling framework to reveal co-regulation of transcription factors and noncoding RNAs on cardiac developmental dynamics was constructed, and conserved regulatory network between transcription factors and ncRNA existed in early cells, while significant differentiation occurred in late staged cells (Li et al. 2023). However, exploring the synergistic regulation of miRNAs and TFs on diseases from a neuroendocrine-immune perspective has not been studied.

In the present study, we constructed the background NEI-miRTF-N based on the NEI signaling molecules and NEI-related miRNA-TF FFLs, and disease-specific NEI-miRTF-N was then built using known disease molecules and significantly differentially expressed (SDE) molecules. Furthermore, we proposed a novel method for identifying significantly dysregulated NEI-related miRNA-TF regulatory pathways (NEI-miRTF-Ps) in diseases. The workflow is shown in Fig. 1.

Materials and Methods

Collection of NEI Signaling Molecules and the Regulatory Relationships Between Them

NEI signaling molecules including miRNAs, genes, and TFs were collected. According to previous studies (Jiang et al. 2020b; Loscalzo 2011), NEI signaling genes were derived from Gene Ontology (GO) (Carbon et al. 2019) using keywords "neurotransmitters", "neuropeptides", "hormones", and "cytokines". To improve the reliability of the results, molecular functions (MF) and biological processes (BP) branches were searched respectively, and the intersection of them was considered as NEI signaling molecules. Six TF-related databases including Transfac (Matys et al. 2003), Tred (Jiang et al. 2007), TransmiR (version 2.0) (Tong et al. 2019), AnimalTFDB (version 4.0) (Shen et al. 2023), TF-marker (version 1.0) (Xu et al. 2022), and hTF target (Zhang et al. 2020) and a previous report (Vaquerizas et al. 2009) were used to extract human TFs. Thus, NEI signaling genes and TFs were obtained (Supplementary Table S1).

NEI signaling miRNAs were retrieved by conducting a comprehensive literature review. We searched PubMed using the same keywords mentioned above and the keywords "microRNA", "miRNA", and "miR". After unifying names according to miRbase (version 22) database and removing redundancy, NEI signaling miRNAs were collected (Supplementary Table S1).

The acquisition of regulatory relationships between signaling molecules was as follows. Firstly, experimentally verified miRNA-gene relationships were extracted from miRTarbase (Release 9.0) (Huang et al. 2022) and Tarbase (version 8) (Karagkouni et al. 2018) databases. Secondly, by using the human TF list obtained above, miRNA-TF interactions were extracted from the miRNA-gene relationships. Thirdly, TF-gene relationships were obtained from TransFAC (Matys et al. 2003), TRED (Jiang et al. 2007), and htfTarget databases (Zhang et al. 2020), and experimentally confirmed interactions between TFs and miRNAs were derived from TransmiR (version 2.0) (Tong et al. 2019) (Supplementary Tables S2 and S3). It is worth noting that precursor miRNAs

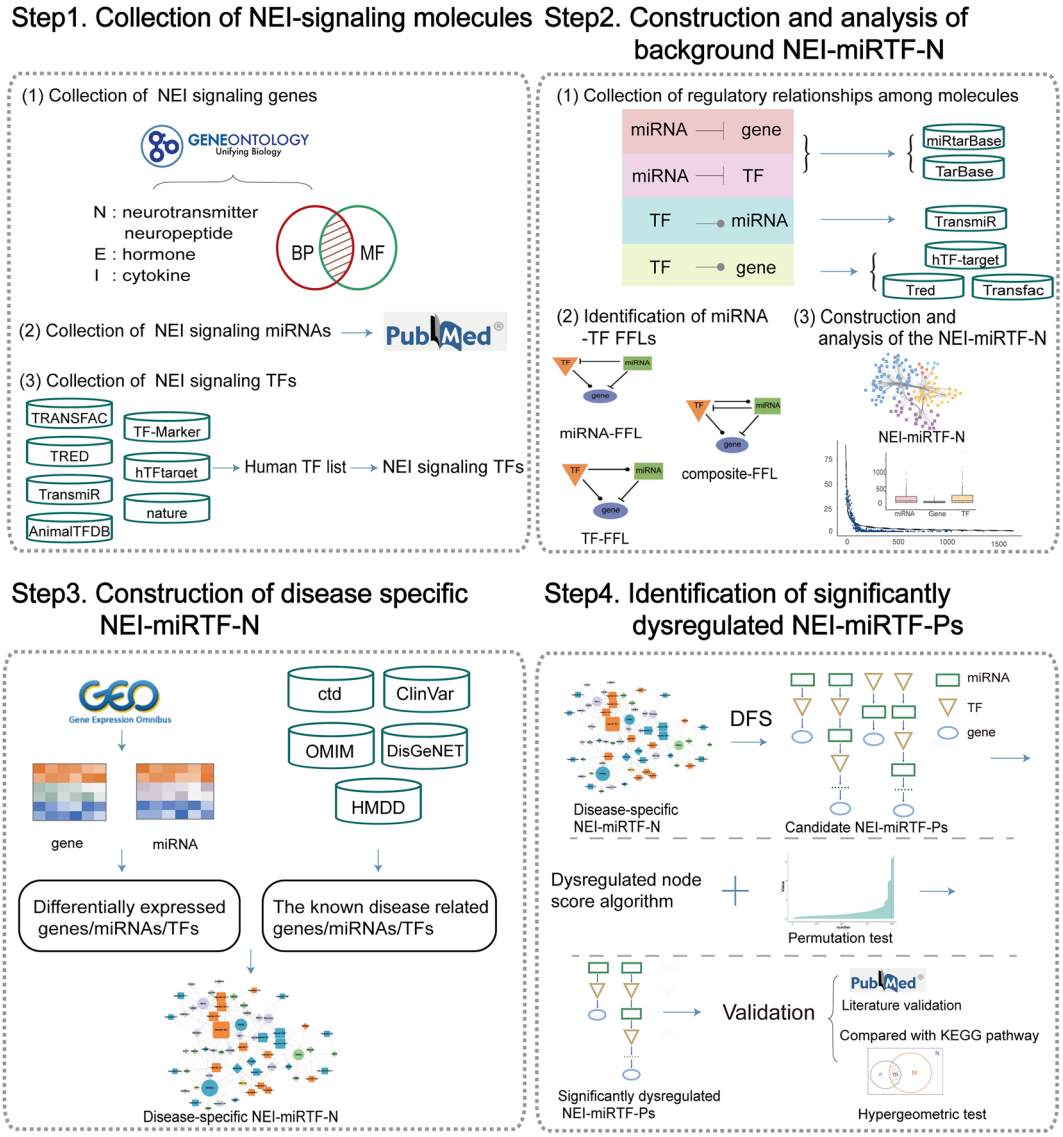

Fig. 1 Workflow of the present study. Step 1. Collection of NEI signaling molecules, including collection of NEI signaling genes, miRNAs, and TFs. Step 2. Construction and analysis of the background NEI-miRTF-N, including collection of regulatory relationships among molecules, identification of miRNA-TF FFLs, construction of the background NEI-miRTF-N and analysis of basic characteristics of the background NEI-miRTF-N. Step 3. Construction of disease-specific NEI-miRTF-N. We mapped the known disease molecules and significantly differentially expressed (SDE) molecules into the background NEI-miRTF-N, ensuring all nodes within the FFLs are composed of these two types of molecules, ultimately deriving a disease-specific NEI-miRTF-N. Step 4. Identification of significantly dysregulated NEI-miRTF-Ps. Dysregulated NEI-miRTF-Ps were identified based on the depth-first-search (DFS) approach and the algorithm we developed. The "ggplot2" R package was used to generate all figures, with Adobe Illustrator employed for final layout adjustments

were used, we mapped mature miRNAs to their corresponding precursors based on the miRBase database.

Construction of Background NEI-Related miRNA-TF Regulatory Network (NEI-miRTF-N)

Background NEI-miRTF-N was constructed by combining miRNA-TF feed-forward loops (FFLs) we identified. Based on the main regulator, miRNA-TF FFLs can be categorized into three distinct types: miRNA-FFL, TF-FFL, and composite FFL (Zhang et al. 2015). In a miRNA-FFL, miRNA is the main regulator that regulates a TF and their common target genes. Conversely, in a TF-FFL, TF is the main regulator. In a composite FFL, miRNAs and TFs can regulate each other and jointly regulate the expression of their target genes. Using NEI signaling molecules and the interactions between them, miRNA-TF FFLs were identified, and the background NEI-miRTF-N was constructed. It is noteworthy

that NEI-miRTF-N is a network of NEI signaling miRNAs and TFs co-regulation, and it is a part of NEI network.

Obtaining of Disease-Related Molecules

In this work, three diseases depression, Alzheimer's disease (AD), and dilated cardiomyopathy (DCM) were used as case studies. We compiled disease-related molecules by merging the known disease molecules and SDE molecules.

The known disease genes were retrieved from four widely used disease gene databases, CTD (Aug 2022) (Davis et al. 2021), ClinVar (Jan 2020) (Landrum et al. 2018), OMIM (Jun 2015) (Amberger et al. 2015), and DisGeNET (Jan 2017) (Pinero et al. 2017). The known disease miRNAs were obtained from the HMDD (version 3.2) (Huang et al. 2019) database (Supplementary Table S4).

We extensively collected disease-related expression data in the Gene Expression Omnibus (GEO) database in September 2022, and required that the datasets included case and control samples, and they were based on tissue samples. As a result, 17, 48, and 19 mRNA expression datasets and 2, 2, and 2 miRNA expression datasets for depression, AD, and DCM were obtained, respectively. To increase the reliability of the results, for mRNA datasets of depression and AD, the dataset with a sample size greater than 10 for each of the case and control groups was retained, while the threshold was selected as 5 for DCM due to its limited samples. Because miRNA expression dataset is small, it was not filtered. Finally, 12, 5, and 4 mRNA expression datasets and 2, 2, and 2 miRNA expression datasets for depression, AD, and DCM were derived, respectively (Supplementary Table S5). To increase the credibility of the results, we performed preprocessing and differential expression analysis on each dataset separately, and finally retained the SDE molecules that appeared in multiple datasets, with consistent up- and down-regulation.

For microarray data with raw data, RMA standardization (Rohr et al. 2021) was performed. When the raw data was not available, the standardized data it provided was used. When one probe corresponded to multiple genes, we removed it, and when multiple probes corresponded to one gene, the median value was retained. We then reserved protein-coding genes. The R 'limma' package was used to perform the differential analysis, and the genes with p-value < 0.05 were selected as SDE genes. For RNA-seq data, the genes with zero count in all samples were excluded, and the R 'DESeq2' package (Love et al. 2014) was employed to identify SDE genes. If the count data is not available, we applied R 'limma' package to implement differential analysis. For miRNA data, miRNA names were unified using miRbase (version 22) database, and the procedure was the same as mRNA data. Thus, SDE molecules were obtained (Supplementary Table S4).

Construction of Disease-Specific NEI-miRTF-N

Disease-specific NEI-miRTF-N was constructed based on disease-related molecules collected above. It was required that NEI-related miRNA-TF FFLs with all the nodes in it were disease-related molecules, and the disease-specific NEI-miRTF-N was acquired.

Identification of Significantly Dysregulated NEI-Related miRNA-TF Regulatory Pathways (NEI-miRTF-Ps) in Diseases

Since disease-specific NEI-miRTF-N has a complex structure, in this study, we focused on NEI-related miRNA-TF regulatory pathways (NEI-miRTF-Ps), which are paths linked to NEI signaling molecules in disease-specific NEI-miRTF-N. By applying depth-first-search (DFS) approach, the pathways were identified from nodes with zero in-degree to nodes with zero out-degree. It is required that there were at least three nodes in a directed acyclic path, and that the path contained at least one signaling molecule for N, E, and I systems, respectively. We developed an algorithm to identify significantly dysregulated NEI-miRTF-Ps in diseases.

Firstly, we defined dysregulated score of a node considering two aspects: One is the node expression changes in case and control samples (Z. Bai et al. 2021a, b), and the other is the type of the node, that is known disease molecules (KDM) or SDE molecules (SDEM). S_{node} is dysregulated score of a node, which is defined as formula (1).

$$S_{node} = \omega \cdot Diff_{node} \tag{1}$$

where $Diff_{node}$ represents the differential expression extent of the node,ω is a coefficient, representing the weight of the node. $Diff_{node}$ can be computed using the Eq. (2):

$$Diff_{node} = (-\log_{10}(p - value)) \cdot |\log_2 FC| \tag{2}$$

where p-value indicates the significance of differential expression of a node. FC is the fold change of node gene expression. Since the expression of a molecule is detected in multiple datasets, we chose the dataset with the most samples to calculate the p-value and the FC.

We assumed that the weight of the KDM node is one, which is higher than that of SDEM node. If a node is both the KDM and SDEM, the weight is their sum.ω is the weight of the node, which defined as the following formulas:

$$\omega = \begin{cases} 1 \text{ node} \in \text{KDM} \\ \omega' \text{ node} \in \text{SDEM} \\ 1 + \omega' \text{ node} \in \text{KDM} \cap \text{SDEM} \end{cases} \tag{3}$$

$$\omega' = \frac{Diff_{node} - \left(\min_{node_i \in network}\{Diff_{node_i}\} - \sigma\right)}{\left(\max_{node_i \in network}\{Diff_{node_i}\} + \sigma\right) - \left(\min_{node_i \in network}\{Diff_{node_i}\} - \sigma\right)} \quad (4)$$

where σ is the standard deviation of $Diff_{node}$ for all nodes in the network.

Secondly, we calculated dysregulated score of a NEI-miRTF-P by taking the average of all the node scores in the pathway.

$$S_{pathway} = \frac{\sum_{node \in pathway} s_{node}}{N} \quad (5)$$

where N is the number of nodes in the pathway.

Finally, p-values were calculated to screen significantly dysregulated NEI-miRTF-Ps. For a NEI-miRTF-P, we constructed a random NEI-miRTF-P by randomly selecting the same number of miRNAs, genes and TFs as the pathway, and then computed the score of the pathway based on the above procedure. This process is repeated 1000 times. The p-value was defined as the proportion of the randomly obtained path scores larger than the real path score as below:

$$p\text{-}value = (\text{Number}of\ S^{random}_{pathway} > S_{pathway})/1000$$

In this work, the NEI-miRTF-Ps with p-value < 0.01 was selected as significantly dysregulated NEI-miRTF-Ps in depression and DCM, while the threshold 0.05 was chosen for AD due to its relatively small number of pathways.

Results

NEI signaling molecules

Signaling molecules in NEI systems were collected using key words "neurotransmitters", "neuropeptides", "hormones", and "cytokines" (detailed in "Materials and methods"). As a result, a total of 3144 protein-coding genes (2715 genes and 429 TFs) and 581 miRNA signaling molecules in NEI systems were obtained. Among them, there were 147 miRNAs, 578 genes, and 18 TFs in nervous system, 414 miRNAs, 1092 genes, and 259 TFs in endocrine system, and 435 miRNAs, 1473 genes, and 262 TFs in immune system (Supplementary Table S1). The Venn diagrams of the collected NEI miRNA, gene and TF signaling molecules were shown in Supplementary Fig. S1a, b, and c, most of the signaling molecules in nervous system were also the signaling molecules of the other two systems.

Background NEI-miRTF-N

Background NEI-miRTF-N was constructed by integrating NEI-related miRNA-TF FFLs, which were identified through NEI signaling molecules and the interactions between them. As a result, 370,885 FFLs were obtained, comprising 230,214 miRNA-type FFLs (62.07%), 92,248 TF-type FFLs (24.87%), and 48,423 composite FFLs (13.06%) (Fig. 2a, Supplementary Table S6). The number of nodes and links in these FFLs was represented in Table 1. Merging these FFLs resulted in the background NEI-miRTF-N, which consists of 2,658 nodes (494 miRNAs, 1991 genes, and 173 TFs) and 98,629 edges. For the background NEI-miRTF-N, there were 123 miRNAs, 160 genes and 4 TFs in nervous system; 379 miRNAs, 925 genes and 117 TFs signaling molecules in endocrine system; 394 miRNAs,1,263genes and 102 TFs signaling molecules in immune system (Fig. 2b, c, and d).

The node degree and its distribution in NEI-miRTF-N were investigated. As shown in Fig. 2e, we found that only a small portion of nodes highly connected with other nodes, while most nodes had relatively small degrees. This was consistent with the characteristics of biological networks. The average node degree of miRNAs, genes, and TFs was 150.84 (range 2–1005), 44.34 (range 2–256), and 199.25 (range 2–1663), respectively. Additionally, the node betweenness was examined (Supplementary Fig. S2), miRNAs and TFs have higher degrees and betweenness, indicating their greater importance in the network.

We further examined the distribution of neural, endocrine, and immune signaling molecules and their interactions in the network. As demonstrated in Fig. 2f and g (Supplementary Tables S7 and S8), the number of immune signaling molecules is the highest, while the number of neural signaling molecules is the lowest. At the same time, the mutual regulation between immune signaling molecules and endocrine signaling molecules is also the most. Additionally, we investigated the significance of regulation relationships between signaling molecules compared with that of theoretical expectation using hypergeometric test. As shown in Fig. 2h and Supplementary Table S8, we found that signaling molecules in neural system significantly regulate those in endocrine and immune system, and signaling molecules in endocrine system significantly regulate those in immune systems (p-value < 0.0001). This implied that the directions of signal transduction might mainly be from nervous system to the endocrine and immune systems, as well as from the endocrine to immune system.

Disease-Specific NEI-miRTF-N

Disease-specific NEI-miRTF-N was constructed by mapping the known disease molecules and SDE molecules to the background NEI-miRTF-N. The method is applicable to all

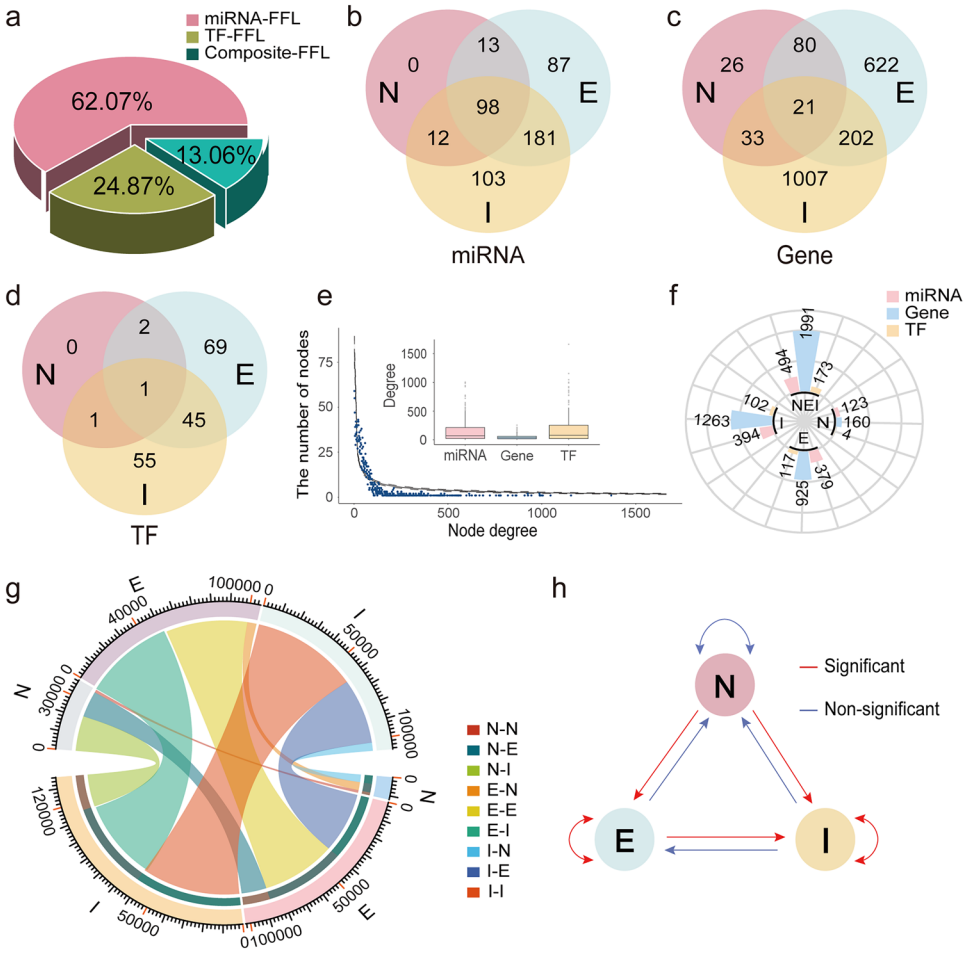

Fig. 2 Background NEI-miRTF-N. **a** The distribution of three types of FFLs in background NEI-miRTF-N. **b** The Venn diagram of NEI signaling miRNAs. **c** The Venn diagram of NEI signaling genes. **d** The Venn diagram of NEI signaling TFs. **e** Degree distribution of all nodes in background NEI-miRTF-N and degree distribution of miRNAs, genes, and TFs. **f** The number of NEI signaling miRNAs, genes, and TFs in background NEI-miRTF-N. **g** The regulatory relationships between NEI signaling molecules in background NEI-miRTF-N. **h** The significance of regulation relationships between signaling molecules compared with theoretical expected cases using hypergeometric test

Table 1 Summary of three types of NEI-related miRNA-TF FFLs

Motif	Number of FFLs	Number of nodes				Number of links				
		Genes	miRNAs	TFs	Total	miRNA-gene	miRNA-TF	TF-gene	TF-miRNA	Total
miRNA-FFL	230,214	1957	438	173	2568	56,414	5981	22,953	–	85,348
TF-FFL	92,248	1862	399	106	2367	39,593	–	16,968	2721	59,282
Composite-FFL	48,423	1751	224	87	2062	27,786	826	11,772	826	41,210
Total	370,885	1991	494	173	2658	64,159	6807	24,116	3547	98,629

the diseases, provided that known disease-related molecules and SDE molecules can be obtained. Depression, AD, and DCM were used as case studies. The acquisition of SDE molecules for the three diseases is as follows.

For depression, AD, and DCM, 12, 5, and 4 mRNA expression datasets and 2, 2, and 2 miRNA expression datasets were retained after filtration (details see Materials and methods). Molecules with p-value < 0.05 were selected as SDE molecules. To ensure the reliability of the data, up- and down-regulation of SDE molecules in multiple datasets were required to be consistent. According to UpSet plots, it is required that SDE genes were differentially expressed in at least 2, 5, and 3 datasets for mRNA expression of depression, AD, and DCM, respectively (Fig. 3a, d, g and Supplementary Table S9). Due to the limited number of miRNA datasets, SDE miRNAs were taken as union sets, and it is required that the miRNAs in the intersection set had consistent up- and down-regulation in datasets. Finally, 62 SDE miRNAs, 720 SDE genes, and 96 SDE TFs in depression; 102 SDE miRNAs, 1085 SDE genes, and 72 SDE TFs in AD; 259 SDE miRNAs, 858 SDE genes, and 97 SDE TFs in DCM were obtained (Supplementary Table S4).

We thus constructed depression, AD and DCM-specific NEI-miRTF-Ns, respectively (Fig. 3b, e, and h,

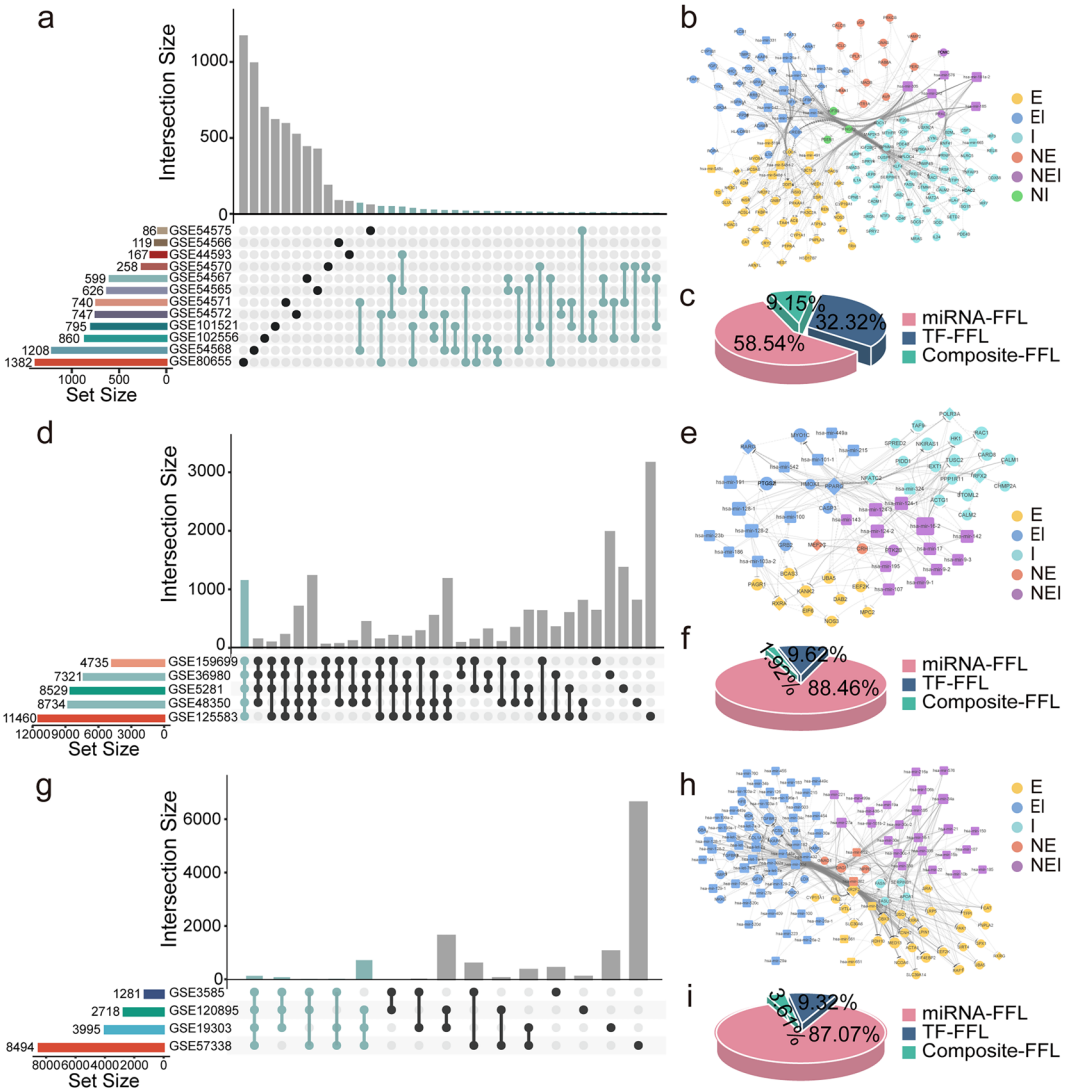

Fig. 3 **a** UpSet plots of SDE genes for depression. **b** Disease-specific NEI-miRTF-N for depression. **c** The distribution of three types of FFLs for depression. **d** UpSet plots of SDE genes for AD. **e** Disease-specific NEI-miRTF-N for AD. **f** The distribution of three types of FFLs for AD. **g** UpSet plots of SDE genes for DCM. **h** Disease-specific NEI-miRTF-N for DCM. **i** The distribution of three types of FFLs for DCM

Supplementary Table S6). There were 162 nodes and 669 edges, 62 nodes and 181 edges, 124 nodes and 652 edges in the three networks, respectively. For depression-specific NEI-miRTF-Ns, 492 FFLs were included, comprising of 288 miRNA-type FFLs (58.54%), 159 TF-type FFLs (32.32%), and 45 composite FFLs (9.15%) (Fig. 3c). For AD-specific NEI-miRTF-Ns, 104 FFLs were included, comprising of 92 miRNA-type FFLs (88.46%), 10 TF-type FFLs (9.62%), and 2 composite FFLs (1.92%) (Fig. 3f). For DCM-specific NEI-miRTF-Ns, 526 FFLs were included, comprising of 458 miRNA-type FFLs (87.07%), 49 TF-type FFLs (9.32%), and 19 composite FFLs (3.61%) (Fig. 3i).

Significantly Dysregulated NEI-Related miRNA-TF Regulatory Pathways (NEI-miRTF-Ps) in Depression, AD, and DCM

In this study, considering dysregulated extent of the known disease molecules and SDE molecules, we developed an algorithm to identify significantly dysregulated NEI-miRTF-Ps in diseases (details see "Materials and Methods"). As a result, 553, 11, and 290 significantly dysregulated NEI-miRTF-Ps in depression, AD, and DCM were identified, respectively (Supplementary Tables S10, 11, and 12). For dysregulated NEI-miRTF-Ps in depression, there are 17 miRNAs, 9 mRNAs, and 16 TFs in them, which included

5, 33, and 27 signaling molecules for N, E and I systems, respectively. The length of these pathways ranges from 4 to 16. For dysregulated NEI-miRTF-Ps in AD, there are 8 miRNAs, 2 mRNAs and 2 TFs in them, which included 8, 11, and 10 signaling molecules for N, E and I systems, respectively. The length of these pathways ranges from 3 to 5. For dysregulated NEI-miRTF-Ps in DCM, there are 25 miRNAs, 42 mRNAs, and 5 TFs in them, which included 13, 69, and 42 signaling molecules for N, E, and I systems, respectively. The length of these pathways ranges from 3 to 11.

The top 5 dysregulated NEI-miRTF-Ps in depression, AD and DCM were investigated, which were shown in Table 2 and Fig. 4a, b and c. Although the molecules in the pathway and the interactions between them were collected from relevant databases, most of them have not been experimentally validated. We examined these dysregulated NEI-miRTF-Ps from four aspects: (1) The associations between the molecules and diseases, as well as NEI systems. (2) The regulatory relationships between the molecules in the pathway. (3) The relations between biological functions of the molecules and diseases, as well as NEI systems. (4) The comparison of these dysregulated NEI-miRTF-Ps with the known disease-related KEGG pathways.

We performed a comprehensive literature search, as represented in Supplementary Tables S13 and S14, the vast majority of molecules are associated with N, E or I and corresponding diseases, and most of the regulatory relationships between molecules have been verified. Additionally, for each pathway in top 5 significantly dysregulated NEI-miRTF-Ps, functional enrichment analysis was implemented using g:Profiler (Kolberg et al. 2023), significantly enriched GO BP terms and KEGG pathways were identified with Benjamini–Hochberg adjusted p-value < 0.05 (Supplementary Tables S15, S16, and S17). As shown in Fig. 4d, e, and f, we found that the large majority of significantly enriched biological functions have been validated to be associated with corresponding diseases as well as the nervous, endocrine, or immune systems.

Furthermore, we searched the KEGG pathways of the three diseases. There were 60, 384, and 103 genes in depression, AD, and DCM KEGG pathways, respectively. For each disease, we examined the enrichment of genes of significantly dysregulated NEI-miRTF-Ps in the KEGG pathway by applying hypergeometric test. When only considering genes and TFs in dysregulated NEI-miRTF-Ps, the p-values were not significant for the three diseases. While taking into account of miRNAs, they were all significantly enriched (p-value $= 0.001$ for depression, p-value $= 4.77 \times 10^{-5}$ for AD, and p-value $= 0.001$ for DCM). When we limited it to the top 5 dysregulated NEI-miRTF-Ps, it was not significantly enriched for depression (p-value $= 0.130$), marginally significantly enriched for AD (p-value $= 0.069$), and significantly enriched for DCM (p-value $= 0.019$). The above results showed that the identified dysregulated NEI-miRTF-Ps were credible.

Table 2 The top 5 significantly dysregulated NEI-miRTF-Ps in depression, AD, and DCM

Disease	Rank	Dysregulated NEI-miRTF-P	Pathway length	Pathway p-value	Pathway score
Depression	1	ARNTL -• hsa-mir-576 •⊣ CREB1 -• MRAS	4	0	120.645
	2	ARNTL-•hsa-mir-576 ⊢• HDAC2 -• MRAS	4	0	120.640
	3	ARNTL -• hsa-mir-576 •⊣ CREB1 -• hsa-mir-185 ⊣ MRAS	5	0	96.516
	4	ARNTL -• hsa-mir-576 ⊢• CREB1 -• hsa-mir-212 ⊣ HDAC2 -• MRAS	6	0	80.435
	5	ARNTL -• hsa-mir-576 •⊣ CREB1 -• TIMP2	4	0	74.958
AD	1	hsa-mir-16–2 ⊣ PPARG -• CRH	3	0.019	8.394
	2	hsa-mir-324 ⊣ PPARG -• CRH	3	0.019	8.375
	3	hsa-mir-16–2 ⊣ PPARG -• hsa-mir-142 ⊣ CRH	4	0.023	6.301
	4	hsa-mir-324 ⊣ PPARG -• hsa-mir-142 ⊣ CRH	4	0.026	6.286
	5	hsa-mir-17 ⊣ RARG -• KANK2	3	0.035	5.423
DCM	1	hsa-mir-144 ⊣ NR2F2 -• NPPB	3	0	10.860
	2	hsa-mir-183 ⊣ CBX3 ⊢• hsa-mir-103a-2 ⊣ RXRA ⊢• hsa-mir-21 ⊣ NR2F2 -• NPPB	7	0	6.818
	3	hsa-mir-183 ⊣ CBX3 ⊢• hsa-mir-103a-2 ⊣ RXRA ⊢• hsa-mir-21 ⊣ EIF4EBP2	6	0	6.453
	4	hsa-mir-144 ⊣ NR2F2 -• hsa-mir-30d ⊣ CBX3 ⊢• hsa-mir-103a-2 ⊣ RXRA ⊢• hsa-mir-21 ⊣ TGFBR2	8	0	6.085
	5	hsa-mir-182 ⊣ FOXO3 ⊢• hsa-mir-21 ⊣ TFPI	4	0.001	8.616

A-•B denotes A regulates B; A⊣B represents A inhibits B, A•⊣ B denotes A inhibits B and B regulates A

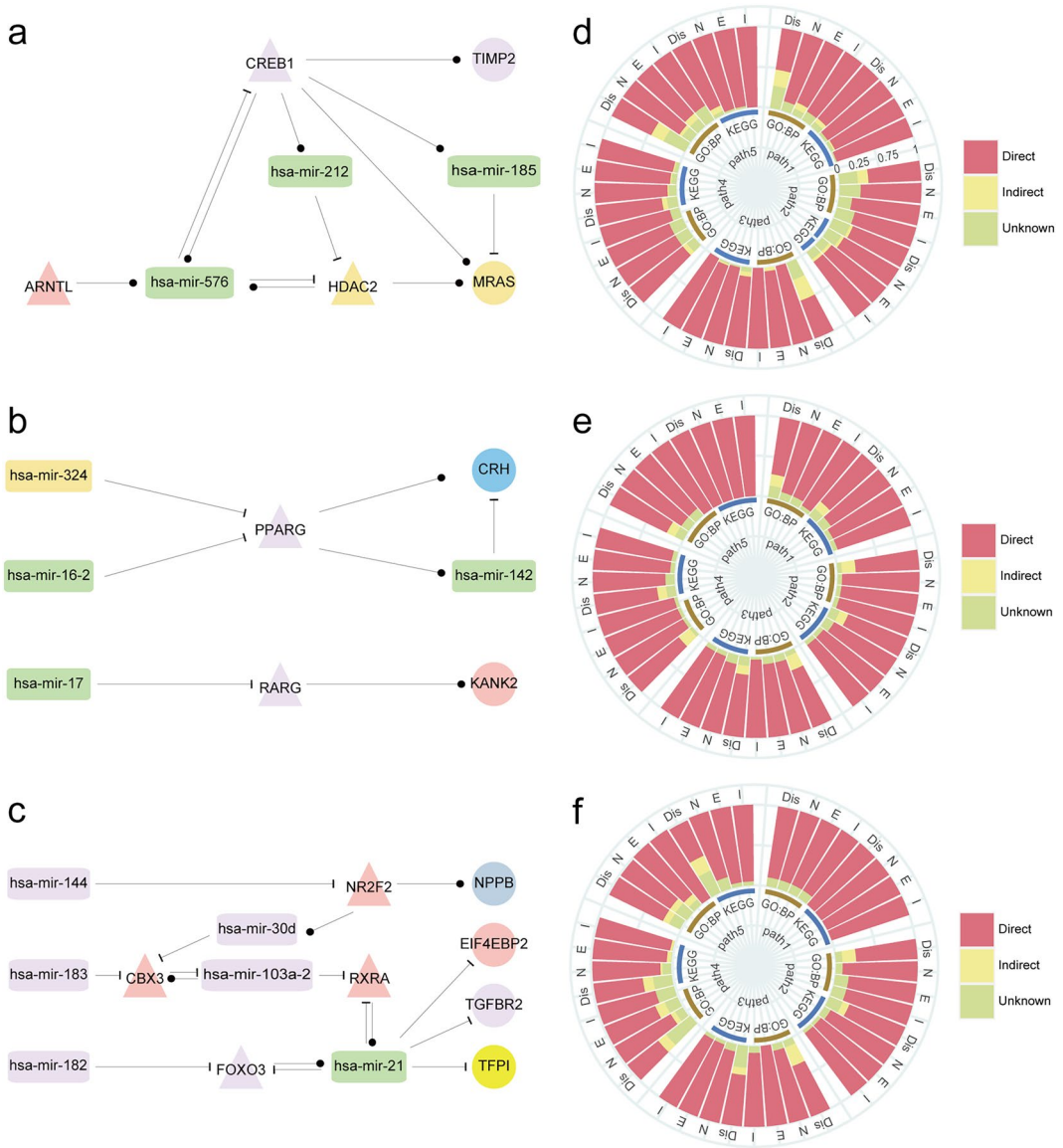

Fig. 4 **a** The top 5 significantly dysregulated NEI-miRTF-Ps in depression. **b** The top 5 significantly dysregulated NEI-miRTF-Ps in AD. **c** The top 5 significantly dysregulated NEI-miRTF-Ps in DCM. **d** Literature validation of significantly enriched biological functions of molecules from the top 5 dysregulated NEI-miRTF-Ps in depression. **e** Literature validation of significantly enriched biological functions of molecules from the top 5 dysregulated NEI-miRTF-Ps in AD. **f** Literature validation of significantly enriched biological functions of molecules from the top 5 dysregulated NEI-miRTF-Ps in DCM

Discussion

To investigate the synergistic regulation of miRNAs and TFs from an NEI perspective, in this study, we constructed the background NEI-miRTF-N by collecting NEI signaling molecules and identifying NEI-related miRNA-TF FFLs. Three disease-specific NEI-miRTF-Ns for depression, AD, and DCM were then constructed based on the known disease molecules and SDE molecules. We further proposed a novel method for identifying significantly dysregulated NEI-miRTF-Ps in disease-specific NEI-miRTF-Ns, and we verified the reliability of the identified pathways from various perspectives.

In order to ensure the reliability of the results, the data used in this study was screened rigorously. For retrieving NEI signaling genes and TFs, we used BP and MF branches in GO database and took the intersection. For collecting NEI signaling miRNAs, we manually inquired 4678 articles from PubMed using the keywords. For regulatory relationships between the signaling molecules, we integrated multiple commonly used databases and the vast majority of them were experimentally verified. For

collecting disease-related expression profile data, it is required that the datasets were based on tissue samples and mRNA dataset for depression and AD with a sample size greater than 10 for each of the case and control groups was retained, while the threshold was selected as 5 for DCM due to its limited samples. For screening SDE molecules, we required SDE molecules to be differentially expressed across multiple datasets, with consistent up- and down-regulation.

For the background NEI-miRTF-N, we analyzed the regulations between the signaling molecules in the three systems. It was found that the signaling molecules in nervous system significantly regulated the signaling molecules in the other two systems, and the signaling molecules in endocrine system also significantly regulated the signaling molecules in immune system, while the regulations in other directions were not significant. This did not mean that there was no regulation in other directions, but might be relatively weak.

We analyzed the top 5 dysregulated NEI-miRTF-Ps in depression, AD, and DCM, discovering that the majority of these molecules are linked to N, E, or I, as well as their respective diseases. Furthermore, most of the regulatory interactions between these molecules have been validated. For example, ARNTL, a clock gene, whose gene expression has been reported to be significantly higher in controls than in depression in peripheral mononuclear blood cells (Bengesser et al. 2022). Simultaneously, ARNTL acts through neurons and hormones, with expression in liver, kidney, lung, heart, suprachiasmatic nucleus of brain, and other various cell types of tissue (Pan et al. 2020). It is reported that co-expression of hsa-miR-182-5p and hsa-miR-154-5p may potentially show diagnostic value for DCM (Zaragoza et al. 2019), and miR-182 has been shown to be related with endocrine and immune (Bai et al. 2021a, b; Zhao et al. 2022). Additionally, transcription factor CREB1 activates expression of a gene locus that produces hsa-miR-212 in newborn hippocampal neurons in young adult mice (Magill et al. 2010).

There are some limitations in the work. For synergistic regulation of miRNAs and TFs, we focused on miRNA-TF FFLs, and the constructed background NEI-miRTF-N was also based on the FFLs, other synergistic regulatory modes between miRNAs and TFs were not considered. When constructing disease-specific NEI-miRTF-Ns, all three nodes in an FFL were required to be either the known disease molecules or SDE molecules, so certain important molecules, but which are neither the known disease molecules nor SDE molecules, were not included. For identifying significantly dysregulated NEI-miRTF-Ps, it is required that the pathways contained at least one signaling molecule for nervous, endocrine, and immune systems respectively, while other situations were not taken into account. However, the identified significantly dysregulated NEI-miRTF-Ps for the three diseases have been validated from multiple perspectives, demonstrating their credibility.

In summary, we constructed the background NEI-miRTF-N, and developed an effective approach for identifying significantly dysregulated NEI-miRTF-Ps in diseases. Our results will shed new light on deciphering the pathogenesis of diseases, and provide a theoretical basis for studying the synergistic regulation of disease-related miRNAs and TFs from an NEI perspective.

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Author Contributions H.S, G.Z and Y.L designed the research. C.W, M.W, Z.W, and X.W performed the research and analyzed the results. C.W, Z.W, H.Y, S.J, G.L, R. L and Q.W analyzed the data. H.S and C.W wrote the paper. All authors read and approved the final paper.

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Data Availability All datasets used in this study can be found in online repositories. The names of the repository and accession numbers can be found in the article and Supplemental Material.

Declarations

Conflict of interest The authors declare no competing interests.

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