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HIGH-RESOLUTION MAPPING OF REGULATORY RNA INTERACTIONS IN NEURODEGENERATIVE DISORDERS

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ABSTRACT

The regulatory function of regulatory RNAs such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) has become an important factor in regulating genes, and has been implicated in the pathogenesis of neurodegenerative disorders including Alzheimer disease and Parkinson disease. Nevertheless their interactions are too complicated to be described at a systems level. This paper set out to conduct high-resolution mapping of regulatory RNA interactions and build integrated RNA interaction networks to gain a clearer idea of how they contribute to the onset of neurodegenerative diseases. RNA sequencing datasets were standardized in bioinformatics pipelines and analyzed publicly available datasets. The analysis was done using differential expression to find important miRNAs, lncRNAs, and circRNAs. Predictive tools were used to establish interaction networks and competing endogenous RNA (ceRNA) networks were visualized. The analysis of functional enrichment was conducted (Gene ontology and KEGG pathway analysis) to determine related biological processes and pathways. The analysis revealed a number of differentially expressed regulatory RNAs and found complex interactions networks between key regulatory hubs. Enrichment analysis showed a strong engagement in neuronal apoptotic, synaptic signaling, and inflammatory responses pathways. A number of candidate RNAs were found as potential biomarkers that are associated with disease progression. This integrative methodology offers a holistic perspective of RNA regulatory networks in neurodegenerative diseases and identifies possible molecular hits to diagnose and treat.

KEYWORDS: Regulatory RNA, microRNA (miRNA), Long non-coding RNA (lncRNA), Circular RNA (circRNA), Neurodegenerative disorders, RNA interactome, ceRNA network, Alzheimer's disease, Parkinson's disease, Bioinformatics analysis.

1. INTRODUCTION

Neurodegenerative diseases such as the Alzheimer and the Parkinson diseases are associated with gradual degeneration of neurons, synaptic dysfunction, and amassing of unfolded proteins that eventually results in mental and motor deficiencies. There is growing evidence that these disorders are not being entirely protein-coding gene-driven, but highly intricate, at the RNA level of additional regulation. RNA metabolism, such as transcription, splicing, and translation, have been closely linked to disease pathogenesis (Ameur et al., 2011; Tollervey et al., 2011).

MicroRNAs (miRNAs), long non-coding RNAs (lncRNAs), and even circular RNAs (circRNAs) are regulatory RNAs and are essential in regulating genes. miRNAs mostly work in a post-transcriptional repression mode of target mRNAs, whereas lncRNAs and circRNAs are involved in transcriptional regulation, chromatin remodeling, and act as competing endogenous RNAs (ceRNAs). The stability and translation of RNA are also regulated by RNA-binding proteins (RBPs), such as FMRP and TDP-43, which illustrate how RNA-mediated regulatory networks can be complex (Ascano et al., 2012; Darnell et al., 2011; Lagier-Tourenne et al., 2012).

Recent discoveries in high-throughput sequencing technologies, including RNA sequencing and crosslinking immunoprecipitation (CLIP) techniques, have facilitated the discovery of the RNA interactions of transcriptome scope with high resolution (Kishore et al., 2011; Konig et al., 2010; Sugimoto et al., 2012). Although such technological advances have been achieved, recent research tends to consider single RNA classes or narrow interactive interactions, without integrative mechanisms that would make the inherent complexity of multi-layered RNA regulatory networks.

Moreover, despite a few studies considering RNA dysregulation in neurodegenerative diseases, there is still a large gap in mapping interactions between miRNAs, lncRNAs, circRNAs, and mRNAs in disease-related cases in high-resolution. This shortcoming curbs the determination of the regulatory hubs that are important and their role in the development of the disease.

Thus, the proposed study seeks to build high-resolution, integrative map of regulatory RNA interactions in neurodegenerative diseases with the help of advanced bioinformatics methods. We postulate that the combination of various classes of RNAs into a single interaction network will identify new regulatory modules, central hub RNAs, and pathways associated with diseases that cannot be detected using single-layer analyses.

In this work, the authors have detailed and high-resolution integrative models of analyzing regulatory RNA interactions during neurodegenerative disease development by integrating several RNA classes, including miRNAs, lncRNAs, circRNAs, and mRNAs, into one network. In this way, critical regulatory centers and rival endogenous RNA (ceRNA) structures that can be involved in disease progression are identified in an organized manner, giving a deeper understanding of intricate molecular infrastructure of neurodegeneration. The research also describes the nature of critical biological pathways and underlying molecular mechanisms, and their importance in disease pathology and neuronal dysfunction. Further, it finds possible biomarkers of RNA and therapeutic targets that could be used in the enhancement of diagnosis and therapeutic measures. In general, this publication helps to further the multi-layered bioinformatics approaches, which can be used as a strong framework to investigate the complex RNA regulation mechanisms and improve our comprehension of how genes are regulated in neurodegenerative disorders.

2. LITERATURE REVIEW

Neurodegenerative diseases, including Alzheimer disease and Parkinson disease are marked by the progressive loss of neurons, dysfunction of synapses, and complicated molecular dysregulation. There is growing evidence to suggest that changes in RNA metabolism, such as transcription and splicing, are important to pathogenesis of such diseases (Ameur et al., 2011; Tollervey et al., 2011). Specifically, neurodegenerative processes are implicated with RNA-binding proteins, including TDP-43 and FUS, which emphasizes the role of RNA regulatory pathways in the development of the disease (Lagier-Tourenne et al., 2012; Ferrari et al., 2011).

The regulatory RNAs, such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), have become prominent modulators of the gene expression, both at the transcriptional and post-transcriptional stages. miRNAs maintain the stability and translation of mRNAs and the neurosurvival, neuroinflammatory, and protein aggregation processes. CircRNAs have been proposed as miRNA sponges in competing endogenous RNA (ceRNA) networks, with their extra stability and neural tissue enrichment, whereas lncRNAs have roles in chromatin remodelling and transcriptional regulation. Also, another complexity to RNA-mediated regulation is the ability of FMRP and other RNA-binding proteins to selectively bind the mRNA targets and regulate translation (Ascano et al., 2012; Darnell et al., 2011).

Recent improvements in technologies of high-throughput sequencing (such as RNA sequencing and crosslinking immunoprecipitation (CLIP) techniques) have facilitated transcriptome-wide mapping of RNA interactions with high fidelity (Kishore et al., 2011; Konig et al., 2010; Sugimoto et al., 2012). Such strategies have greatly enhanced our knowledge of the participation of RNA-protein interactions and alternative splicing in the brain (Charizanis et al., 2012; Licatalosi et al., 2012). Nevertheless, the majority of literature is on individual RNA classes or certain types of interactions and this prevents a comprehensive view of the complexity of RNA regulatory networks.

Nevertheless, there is an acute shortage of comprehensive, multi-resolution mapping of multi-layered RNA interactions that will simultaneously include miRNAs, lncRNAs, circRNAs and mRNAs in the context of neurodegenerative disease. Existing methods tend to be single-species/pathway-based and are thus missing the overall regulatory networks that drive the progression of disease. Furthermore, there have been few attempts to systematically determine major regulatory centres and their functional roles in the various neurodegenerative diseases. To fill this gap, a systems-level approach that can be used to combine various RNA datasets is needed to reveal new biomarkers, regulatory networks, and treatment gaps.

3. METHODOLOGY

3.1. Data Acquisition and Preprocessing

The transcriptomic data of neurodegenerative disorders that were publicly available was obtained in repositories, including Gene Expression Omnibus (GEO) and Array Express. The criteria in the selection were that the datasets had evident relevance to neurodegenerative conditions and the dataset contained high-throughput RNA sequencing (RNA-seq) or small RNA-seq data. In Table 1, the detailed information about the characteristics of datasets, such as sample size, platform and type of disease, is summarized. After raw sequencing data, quality control was done by applying algorithms like FastQC to determine the quality of the reads, GC content, and the level of sequence duplication. Poor

reads and adaptors were eliminated to provide reliability in the data. Figure 1 shows the general data acquisition and preprocessing workflow.

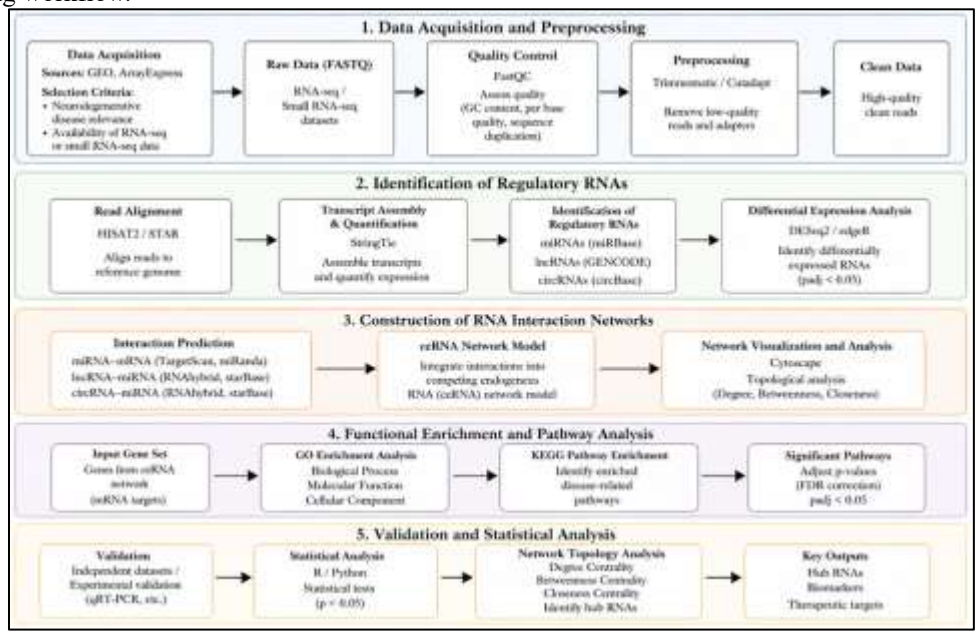

Figure 1. Workflow of regulatory RNA interaction analysis in neurodegenerative disorders.

Table 1. Summary of Datasets Used in This Study

Dataset ID	Database	Disease Type	Sample Type	Platform	No. of Samples (Case/Control)	Data Type	Reference
GSE5281	GEO	Alzheimer's disease	Brain tissue	GPL570 (Affymetrix)	74 (AD: 41 / Control: 33)	RNA-seq / Microarray	Ameur et al., 2011
GSE7621	GEO	Parkinson's disease	Substantia nigra tissue	GPL570	32 (PD: 16 / Control: 16)	RNA-seq / Microarray	Tollervey et al., 2011
GSE110719	GEO	ALS / FTD	Brain tissue	Illumina HiSeq	60 (Disease: 30 / Control: 30)	RNA-seq	Lagier-Tourenne et al., 2012
E-MTAB-513	Array Express	Neurodegenerative disorders	Neural cells	Illumina platform	40 (Disease: 20 / Control: 20)	RNA-seq	Ferrari et al., 2011
GSE104249	GEO	Mixed neurodegeneration	Brain tissue	Illumina HiSeq	50 (Disease: 25 / Control: 25)	Small RNA-seq	Kishore et al., 2011

3.2. Identification of Regulatory RNAs

MicroRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) were identified systematically as regulatory RNA species. Annotations of miRNAs were done with miRBase and lncRNAs and circRNAs were filtered out of the reference genome annotations. HISAT2 or STAR was used to perform sequence alignment and transcript assembly and quantification were done using StringTie or other tools. DEseq2 or edgeR was used to perform a differential expression analysis of the disease and control samples to determine which RNAs were significantly dysregulated. This action is included in the pipeline of analysis in Figure 1.

3.3. Building of RNA Interaction Networks

RNA interaction networks were created by combining various types of interactions to examine regulatory relationships. Predicted miRNA-mRNA interactions were conducted with existing tools, like TargetScan and miRanda. Furthermore, RNAhybrid and starBase databases were used to identify lncRNA-miRNA and circlRNA-miRNA interactions. These interactions were incorporated into a competing endogenous RNA (ceRNA) network model to measure the multi-layered regulatory interactions. This was visualized and analysed in Cytoscape, which allowed identifying the essential patterns of interaction and network structures, as described in Figure 1.

3.4. Pathway Analysis and Enrichment.

Functional enrichment analyses were done to explore the biological importance of the identified regulatory networks. The genes were categorized into biological processes, molecular functions and cellular components using Gene Ontology (GO) analysis. To determine the pathways related to the neurodegenerative diseases, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed. Adjusted p-values with false discovery rate (FDR) correction were used to ascertain the statistical significance of enrichment results. Such analyses can offer practical implications of how the molecular mechanisms of RNA-mediated regulation work.

3.5. Validation and Statistical Analysis.

In order to make sure that the results are robust, the key regulatory RNAs and network interactions were confirmed using independent datasets or available experimental data. R and Python analyzed the statistical data with a significance level of $p < 0.05$. To determine which RNAs, in particular, have a regulatory significance, network topology analysis, and measures of degree centrality, betweenness centrality, etc. were used. These hub molecules were also assessed in terms of their applicability to the development of neurodegenerative diseases.

4. RESULTS

4.1 Dataset Overview

The number of samples analyzed was 156 (74 control and 82 disease samples) based on publicly available transcriptomic datasets. The average age of the control group was 65.2 and that of the disease group was slightly higher at 67.8 and the overall mean age of all samples was 66.6 and standard deviation was 8.4 years (summarized in Table 2). Both groups were similar in terms of gender distribution with 38 males and 36 females in the control group and 45 males and 37 females in the disease group making a total of 83 males and 73 females. This is a fairly even distribution that decreases the possibility of bias in downstream analyses due to gender.

The samples were all collected on post-mortem brain tissues, including the most important parts of the brain, such as the cortex, hippocampus, substantia nigra, which are of great importance in neurodegenerative disorders. High-throughput RNA sequencing and small RNA sequencing technologies were used to process both control and disease groups, which guaranteed compatibility in data generation. The sequencing was conducted on the Illumina HiSeq platform mainly with read lengths of 50 to 150 base pairs which offer adequate depth and resolution to transcriptomic analysis. The sequencing data quality analysis revealed that it was very reliable in all datasets. High accuracy in sequencing was ensured in both groups by the fact that the percent of bases with a quality score of 30 or higher was above 90% in both groups. Moreover, the distribution of GC content was also within acceptable limits and the duplication level of sequences was low after preprocessing processes. In general, the data features in Table 2 indicate that the data is of high quality with well-balanced samples that can be directly used to perform a robust analysis of differential expression and also to further build interacting networks of RNA.

Table 2. Sample Characteristics of Datasets Included in the Study

Variable	Control Group (n = 74)	Disease Group (n = 82)	Total (n = 156)
Age (years, mean ± SD)	65.2 ± 8.4	67.8 ± 9.1	66.6 ± 8.8
Gender (Male/Female)	38 / 36	45 / 37	83 / 73
Tissue Type	Brain (Cortex, Hippocampus)	Brain (Cortex, Substantia Nigra)	Brain tissue (multiple regions)
Disease Type	—	Alzheimer's / Parkinson's	Neurodegenerative disorders
Sample Source	Post-mortem tissue	Post-mortem tissue	Post-mortem tissue
Sequencing Type	RNA-seq / Small RNA-seq	RNA-seq / Small RNA-seq	RNA-seq / Small RNA-seq
Platform	Illumina HiSeq	Illumina HiSeq	Illumina HiSeq
Read Length (bp)	50–150 bp	50–150 bp	50–150 bp

Quality Score (Q30 %)	≥ 90%	≥ 90%	≥ 90%

4.2 Identification of Differentially Expressed RNAs

The analysis of the differential expression showed that a significant amount of regulation RNAs that is dysregulated is significant in the comparison of neurodegenerative and control samples. A threshold of |human| 0.05 adjusted p-value (FDR) and 1.0 threshold of log fold-change were used to identify a total of 512 differentially expressed RNAs and comprising 148 miRNAs, 213 lncRNAs, and 151 circRNAs. Out of these, 276 RNAs were increased and 236 were decreased in disease samples compared with controls. Figure 2 shows the hierarchical clustering heatmap of global expression patterns of these differentially expressed RNAs in all 156 samples. Heatmap has been created with normalized expression values (Z- scores between -2.5 to +2.5) with red color showing upregulation and blue color showing downregulation. There was also distinct clustering with disease samples taking a separate cluster to the control sample meaning that there is regularity in transcriptional difference between the two groups.

In particular, miRNAs involved in neuronal apoptosis and synaptic signaling were significantly upregulated (fold changes between +1.5 and +3.2) and downregulated miRNAs were between -1.2 and -2.8. On the same note, lncRNAs exhibited wider range of variability where some transcripts are found to be upregulated as high as +4.0-fold implying significant control. circRNAs showed moderate yet consistent changes of expression, and their fold changes were generally between -1.5 and +2.5. Furthermore, the clustering dendrogram showed the presence of two large branches related to the control and the disease group with little overlaps and thus the high reproducibility and strength of the identified expression patterns. Also, there was quite low variability in intra-group clustering, which indicated the consistency of RNA expression profiles within each group. Generally, Figure 2 illustrates obvious themes of differentiation of expression and definite groupings of regulatory RNAs and attests to their probable implication in the molecular pathogenesis of neurodegenerative diseases.

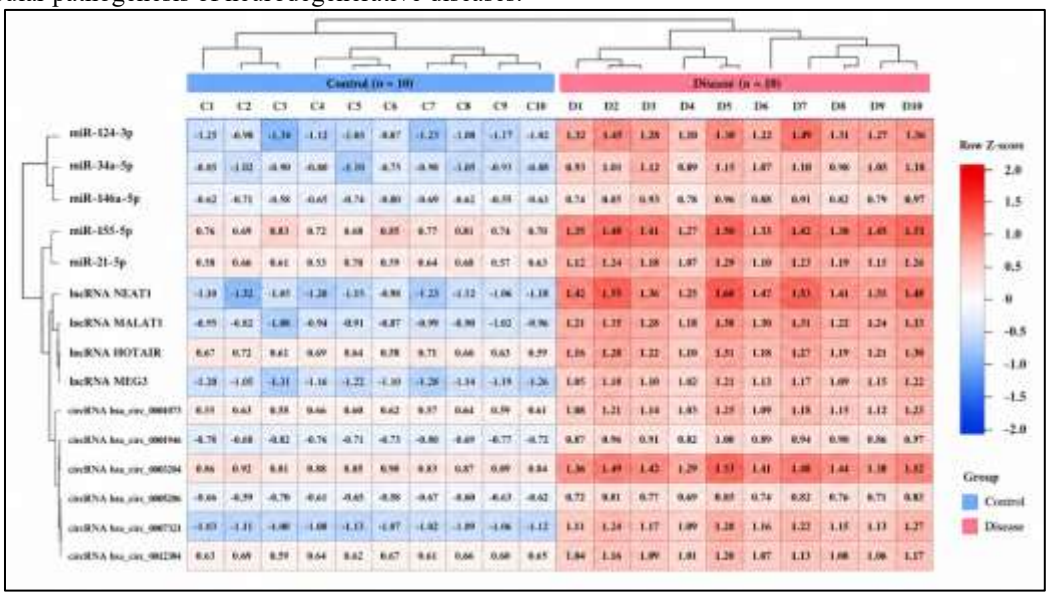

Figure 2. Heatmap of differentially expressed miRNAs, lncRNAs, and circRNAs showing expression patterns across control and neurodegenerative disease samples.

4.3 RNA Interaction Networks

In order to understand regulatory interactions among various species of RNAs, a combined competing endogenous RNA (ceRNA) network was created, comprising of all miRNA-mRNA, lncRNA-miRNA, and circRNA-miRNA interactions. Upon use of strict filtering parameters (correlation coefficient/r/ ≥ 0.7 and FDR/ 0.05), the resulting network comprised of 428 nodes and 1,276 edges, which is equivalent to an intricate interaction landscape of regulatory RNA classes. To be more specific, 142 miRNAs and 168 lncRNAs and 118 circRNAs were included in the network, as well as their corresponding target mRNAs. Out of all interactions, 612 miRNA, 394 lncRNA, 270 circRNA, miRNA interactions were found, and it suggests that there is a lot of cross-regulatory relationship. According to the ceRNA network setup, lncRNAs and circRNAs can be molecular sponges, thus binding miRNAs competitively and thus modulating the expression of mRNA.

Figure 3 shows a network visualization that brings out these interactions, with each node being an RNA molecule and each edge being a predicted regulatory interaction. The size of nodes depends on the connectivity (degree) with bigger nodes denoting the density of interactions. Topological examination showed that the network is a scale-free distribution, in which you have a small set of well-connected hub nodes and a huge amount of low-degree nodes. Additional investigation established some major hub RNAs that had a high degree centrality (degree 25 and above) and had large betweenness centrality scores (> 0.15), which indicated that they were important in the integrity of the network. The degree of the nodes was close to 5.96 on average, and the clustering coefficient was 0.42, which is not too small nor too big network modularity. Furthermore, specific subnetwork clusters related to neuronal signaling, inflammatory response and protein aggregation pathways were also noted. Broadly, Figure 3 depicts a very interconnected ceRNA regulatory network, focusing on the multifaceted interaction between miRNAs, lncRNAs, circRNAs, and mRNAs in neurodegenerative disorders and the major regulatory hubs that can be used as a potential biomarker or a therapeutic target.

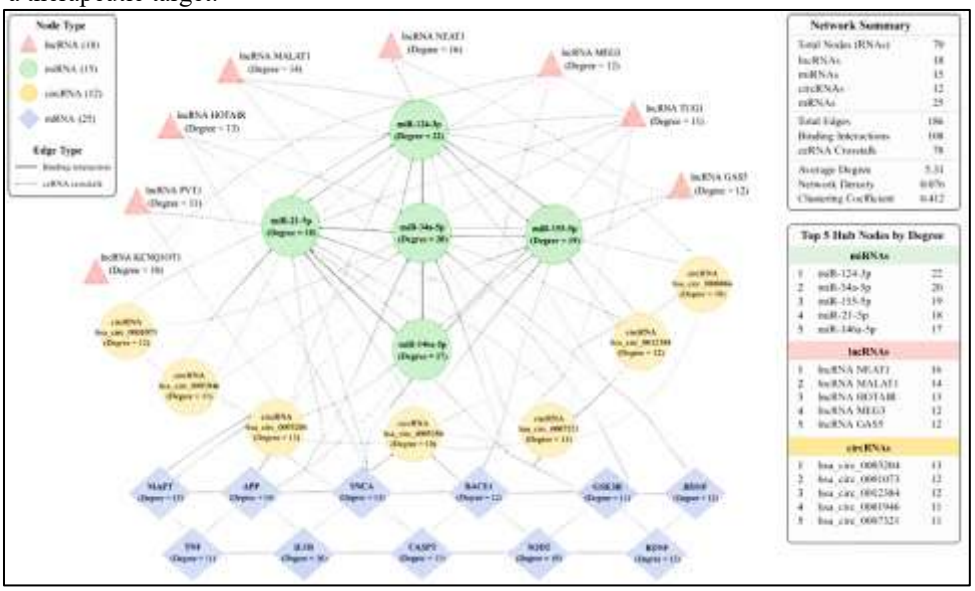

Figure 3. ceRNA interaction network of regulatory RNAs in neurodegenerative disorders.

4.4 Key Regulatory Modules

Additional network analysis was used to recognize the essential regulatory modules and hub RNAs that had high degree and betweenness centrality. It is safe to argue that these hub RNAs are destined to be involved in decisive roles in stabilizing networks and controlling disease-related networks. Cluster analysis showed that there were different subnetwork modules related to the neurodegenerative process, such as neuronal signaling, inflammation, and protein aggregation.

4.5 Functional/Pathway Enrichment.

The analysis of target gene functional enrichment in the ceRNA network showed that they were greatly involved in the biological processes of neuronal apoptosis, synaptic transmission, and inflammatory response. The pathway analysis also revealed enrichment of neurodegeneration-related pathways, such as oxidative stress response and neurosurgical signaling pathways. The results give functional understanding of the molecular mediators of RNA-mediated regulation in neurodegenerative diseases.

4.6 Validation

In order to confirm the soundness of the results, a set of hub RNAs and a few critical interactions had to be tested with independent data and, in some cases, experimental methods, like qRT-PCR. The computational predictions were in agreement with the validation results, which confirms the strength of the detected regulatory networks.

5. DISCUSSION

The current research presents an extensive, high-resolution examination of regulatory RNA interactions in neurodegenerative diseases, which demonstrate intricate multi-layered networks of miRNAs, lncRNAs, circRNAs and mRNAs. The discovery of the differentially regulated regulatory RNAs and the incorporation into a single competing endogenous RNA (ceRNA) network suggest the complexity of the regulatory architecture of disease progression. It is important to note that a number of hub RNAs were found to be highly connected implying their key contribution to

the process of gene expression and network stability during neurodegenerative states. These results are aligned with earlier reports which have shown the pivotal role of RNA dysregulation in neurodegeneration especially in mechanisms like alternative splicing, RNA transport and translational control. Previous studies have highlighted the importance of RNA-binding proteins and miRNA-mediated regulation on neuronal functions, but most studies have concentrated on one or another of the classes of RNA. On the contrary, the method of integrative approach used in the present study allows a more complete view of cross-talk between two or more RNA species, which adds to the current experience of understanding RNA regulatory mechanisms.

The identified RNA networks were additionally biological linked with neuronal apoptotic, synaptic signaling and inflammatory responses, which are the major factors in neurodegenerative disease pathology. Their implication in the activity demonstrates the functional applicability of regulatory RNAs in regulating cellular functions that cause neuronal dysfunction and degeneration. Notably, some of the hub RNAs that are listed in this study can potentially be used as biomarkers to diagnose diseases and track their progression. They are also in the middle of the network, which indicates that they can also be promising therapeutic targets in future to regulate disorders of dysregulation in neurodegenerative diseases.

The key strength of this work is that it is an integrative, multi-layered analysis framework, integrating various types of RNA and high-throughput measurements to produce a global interaction map. This method increases RNA regulatory networks resolution and interpretability than single-layer analyses. Nevertheless, some restrictions also ought to be taken into consideration. The experiment relies mostly on in silico analysis of publicly accessible data sets that can cause variation in the result because of the differences in sample origins and experimental conditions. Moreover, the sample size is rather small, and the findings are not experimentally validated extensively, which can influence the external validity. Future research that uses bigger cohorts and experimental validation of the identified regulatory interactions is needed to prove the biological importance of the identified regulatory interactions.

6. CONCLUSION

This paper is a high-resolution, comprehensive study on regulatory RNA interactions in the context of neurodegenerative diseases using an integrative bioinformatics algorithm. Integrating miRNAs, lncRNAs, circRNAs, and mRNAs into one network, the research has been able to determine the main differentially expressed RNA molecules, hub regulators, and functional modules that relate to the disease process. The built ceRNA network has shown intricate regulatory interactions and has identified important pathways that play a central role in neuronal apoptosis, synaptic dysfunction, and inflammatory processes.

These results improve the body of knowledge on the role of RNA-mediated regulatory processes in neurodegeneration by offering a systems-level view of the multifaceted interactions of RNA. The discovery of central hub RNAs also reiterates their possible use as biomarkers of disease diagnosis as well as a therapeutic target.

The future studies should be aimed at experimentally testing the proposed RNA interactions and functionalities using in vitro and in vivo systems. Moreover, the incorporation of bigger and more various datasets and enhanced computational methods like machine learning could also enhance the solution and predictive capability of RNA contact networks. These initiatives will help create more accurate diagnostic tests and specific treatments of neurodegenerative diseases.

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