Original Article

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Identification of CircRNA Complexes Acting on miRNA Sponges within the Exosome in Patients with Alzheimer's Disease: An *In Silico* Approach

Alzheimer Hastalarında Eksozom İçinde Süngerimsi miRNA'ya Etki Eden CircRNA Komplekslerinin Tanımlanması: Bir *In Silico* Yaklaşımı

Rabia KALKAN CAKMAK[1,2], Nail BESLI[1], Bahar SARIKAMIS JOHNSON[1], Nilufer ERCIN[1], Hayati Sencer POLAT[1], Ulkan KILIC[1,2]

[1]University of Health Sciences Türkiye, Institute of Health Sciences, Department of Medical Biology, İstanbul, Türkiye
[2]University of Health Sciences Türkiye, Hamidiye Faculty of Medicine, Department of Medical Biology, İstanbul, Türkiye

ABSTRACT

Objective: Alzheimer's disease (AD) is a neurodegenerative ailment that launches insidiously and progresses slowly, and its prevalence gradually increases with age. In a former study, we explored the plasma exosomal circular RNA (circRNA)-micro RNAs (miRNA) expression profiles in AD patients derived from *next-generation sequencing* data by scanning experimentally validated miRNA-target interaction databases. The current paper focused on computing the integration of circRNA-miRNA and RNA binding proteins (RBP) into the plasma exosomes using the output of our earlier study.

Methods: We identified 24 upregulated circRNAs and 12 downregulated miRNAs from our previous paper. These were then subjected to prediction of circRNA-miRNA via the circMine web server and MiRNet. Subsequently, the circRNA Interactome web server was used to integrate the 24 circRNAs with RBPs. Finally, the obtained numeric scale results were visualized using R studio, ensuring the validity and reliability of our findings.

Results: As an overall outcome of calculations, we found CNTN4, SH3BGRL, *THOC2*, CGGBP1, FLNA, ATP6V1A, and UBN1 from queried 24 circRNAs linked to AD pathology, proposing that circRNAs complexes composed of RBPs and miRNAs might be considered empirically under oncoming studies.

ÖZ

Amaç: Alzheimer hastalığı (AH), sinsi başlayan ve yavaş ilerleyen, prevalansı yaşla birlikte giderek artan nörodejeneratif bir hastalıktır. Önceki bir çalışmada, deneysel olarak doğrulanmış miRNA-hedef etkileşim veritabanlarını tarayarak *next-generation sequencing* verilerinden elde edilen AH'li hastalarda plazma eksozomal dairesel RNA (circRNA)-mikro RNA'lar (miRNA) ekspresyon profillerini araştırdık. Mevcut makale, önceki çalışmamızın çıktısını kullanarak circRNA-miRNA ve RNA bağlayıcı proteinin (RBP) plazma eksozomlarına entegrasyonunu hesaplamaya odaklandı.

Yöntemler: Önceki makalemizden 24 yukarı regüle edilmiş circRNA ve 12 aşağı regüle edilmiş miRNA tanımladık. Bunlar daha sonra circMine web sunucusu ve MiRNet aracılığıyla circRNA-miRNA'nın tahminine tabi tutuldu. Daha sonra, 24 circRNA'yı RBP'lerle entegre etmek için circRNA Interactome web sunucusu kullanıldı. Son olarak elde edilen sayısal ölçek sonuçları R studio kullanılarak görselleştirilerek bulgularımızın geçerliliği ve güvenirliği sağlanmıştır.

Bulgular: Hesaplamaların genel bir sonucu olarak, AH patolojisine bağlı sorgulanmış 24 circRNA'dan CNTN4, SH3BGRL, *THOC2*, CGGBP1, FLNA, ATP6V1A ve UBN1 bulundu.

Address for Correspondence: Ulkan Kilic, University of Health Sciences Türkiye, Institute of Health Sciences, Department of Medical Biology, İstanbul, Türkiye
E-mail: uckilic@yahoo.com **ORCID ID:** orcid.org/0000-0002-6895-8560

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ABSTRACT

Conclusion: Basically, our calculations claim that it may be examined whether these complexes behave as sponge miRNA in AD.

Keywords: Alzheimer's disease, exosomes, miRNA sponges, circRNA

ÖZ

Sonuç: Temel olarak hesaplamalarımız, bu komplekslerin AH'de sünger miRNA gibi davranıp davranmadığının incelenebileceğini iddia etmektedir.

Anahtar Sözcükler: Alzheimer hastalığı, eksozomlar, miRNA süngerleri, sirkülerRNA

Introduction

Alzheimer's disease (AD) is an onward neurological illness, which nowadays influences more than 5.5 million people (1,2). Progressively recognized as a serious, global public health concern, the widespread consensus in the pathological diagnosis of AD is the accumulation of extracellular amyloid beta plaques and neurofibrillary tangles (3). As AD commenced insidiously and progressed unsteadily rise over the years, investigations in the discovery of candidate biomarkers for the early diagnosis of AD urgently need to be exerted seeing that lack of novel biomarkers has existed for many years.

Exosomes are tiny vesicles and different membrane structures of endosomal origin, based on biomarker scanning, are a trend and rapidly developing area in the diagnoses of neurodegenerative disease since they generally cross the blood-brain barrier and include many types of non-coding RNA (ncRNA)s (4).

Circular RNAs (circRNAs) that belong to the class of ncRNA molecules are covalently closed and endogenous biomolecules with neither 3' poly(A) tails nor 5' end caps (5). Given the advances in the next-generation sequencing (NGS) and bioinformatics techniques, researchers have identified a great number of circRNAs and found these RNAs in tissue and cell-specific expression patterns in eukaryotes (6). Since the milestone finding of ciRS-7/CDR1as (circRNA sponge for miR-7) in 2013, circRNAs have turned out a valuable issue in RNA research (7,8). CircRNAs are believed to be structurally more stable than other RNAs, owing to being covalently closed circular biomolecules. This stability makes them have longer half-life than linear RNAs and will probably display a model feature of circRNAs during their forthcoming evolution as biomarkers (9,10). This suggests that circRNAs may be considered as biomarkers in the early diagnosis of diseases. Additionally, circRNAs are established to be proper biomolecules as therapeutic targets for several disorders such as cancer, cardiovascular diseases, chronic inflammatory diseases, and neurological disorders (11-13).

The presence of circRNA accumulation in various species during aging suggests that circRNA may be a factor associated with aging and the pathogenicity of age-related disease such as AD (14). Moreover, a great number of differentially expressed circRNAs in the brains of AD patients have been reported (9). Some differentially expressed circRNAs have been monitored to ease AD-like pathological conditions in cellular and animal models of AD, suggesting that circRNAs are presumably related to regulating the neuropathophysiology of AD (15).

Even though little has been recognized about the functions of circRNAs as yet, two main functions have been identified as playing a role including as miRNA sponges and circRNA-protein interactions. The most notable proteins interacting with RNA molecules are the RBPs. RBPs are a category involved in the metabolic functioning of RNAs by mediating their translation, transport, localization, and maturation; in fact, these proteins take part in the shape of ribonucleoprotein complexes (10).

What we know about sponge miRNAs and RBPs is primarily based on empirical studies that investigate how they are. Besides, bioinformatics methods can predict the binding sites of RBPs and miRNAs on circRNAs, so providing the opportunity to perform preliminary analysis prior to proceeding to experimental studies. Thus, the main purpose of this paper is to compute the interaction with RBP proteins and miRNAs in the exosomal circRNA complex, and to describe with bioinformatics approaches that possible circRNAs may be the first target molecules for further experimental biomarker research.

Methods

Data Collection

As a work-schema of the present study is summarized in Figure 1, we extended our previous data to demonstrate the possible interaction of miRNA and RBP with circRNA. The list of differential expression of miRNAs and circRNAs was obtained from analysis of dataset (ID: MTAB-11222) in the repository of ArrayExpress (16) (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-11222).

Prediction of Interaction of miRNA and RBPs with circRNAs

The list of differential expression of miRNAs and circRNAs was submitted to MiRNet 2.0 (17) as multiple query types to observe possible integrations in the network with selecting no specific tissue. To identify the potential interactions between miRNA and circRNA based on their sequence matching feature, the circRNA-miRNA prediction tool was employed by utilizing the miRanda, miRBase, and circBase databases through circMine web server (18). The parameters of score cutoff and energy cutoff were set at 140 and -7 to assign the significant interacting miRNA. To determine the possible binding sites of RBPs to the circRNAs, circRNA Interactome (19) that serves for RBPs binding to the circRNA junctions using Targetscan prediction tool was utilized. Furthermore, the flanking regions of circRNAs were evaluated and considered the highest number of binding sites for each RBP.

Data Visualization

The interaction network of the miRNAs and circRNAs was constructed using the Cytoscape 3.9.1 (20), which is one of the most well-known network visualizations. Heatmap plots representing the numerical scale of the interaction of circRNAs with both miRNAs and RBPs were conducted with R studio software (version 4.1.2, https://rstudio.com/).

Results

In the present computational study, 24 upregulated circRNAs and 12 downregulated miRNAs derived from our previous paper (21) were used for the possible integration of circRNA and miRNAs, and calculating the potential miRNA sponges through circMine with binding energy scores. As shown in Figure 2, 14 miRNAs and 18 circRNAs were integrated via miRnet web server, whilst the heatmap in Figure 3 that a total of 23 micro RNAs and 24 circRNAs were computed according to their energy score. However, there is no integration of HDAC1 from circRNAs and 9 miRNAs in the network in Figure 2. There is also a main network indicating potential circRNA to miRNAs and miRNAs to their target genes in a Supplementary Figure 1.

It is apparent from Figure 4 that Heatmap provides an account of potential 23 RBP binding proteins with interacting 129 circRNAs with distinct IDs. In the supplementary file, overall, 22 circRNAs are indicated by 129 circBase IDs.

Discussion

The circRNAs have been reported to incline to augment in the aging-brain and to be abundant in mammalian brain, however their function and expression in the nervous system are poorly understood (22). To conduce to the content of further studies related to AD pathology, as regarding two main tasks of circRNAs, the present study explores the plasma exosomal upregulated circRNAs and downregulated miRNAs in AD patients from our former study by calculating potential sponge miRNAs and RNA Binding proteins (RBPs) through computational tools. The main reason for analyzing the upregulated circRNAs is that miRNAs may be downregulated when circRNAs act as sponges to miRNA.

In the present study, in Figure 3, there is a hierarchically top binding energy score in some circRNAs including CNTN4, SH3BGRL, *THOC2*, CGGBP1, FLNA, ATP6V1A and UBN1 in the heatmap. In CNTN4 and CGGBP1 from these circRNAs, there is no any integration with miRNAs in Figure 2. Despite no correlation with miRNAs in the network analysis in Figure 2, interestingly, CNTN4 is predominantly expressed in brain, particularly in cerebellum (23). Also, CGGBP1 is extently expressed in cerebellum and cerebral cortex (24). In accordance with the calculation in Figure 3, CNTN4 interacts with hsa-miR-485-5p and hsa-miR-185-5p at peak value, as well as it is interacting with all miRNAs significantly, but CGGBP1 is interacting with hsa-miR-374a-3p at the highest matching score. In Figure 4, fused in sarcoma (FUS), Methyltransferase like-3 (METTL3) and TAR DNA *binding* protein 43 (TDP43) have much more binding sites on CNTN4 but not CGGBP1. CNTN4 is a member of the Contactin family, and functionally serves as one of the axon guidance molecules critical for the accurate construction of the optic system. Despite there are presently very limited studies that display a correlation between CNTN4 and AD pathology, a correlation has been reported in a few papers between CNTN4 and APP by dysregulation of expression of Contactin protein in the autopsy brain tissue of AD patients (25,26).

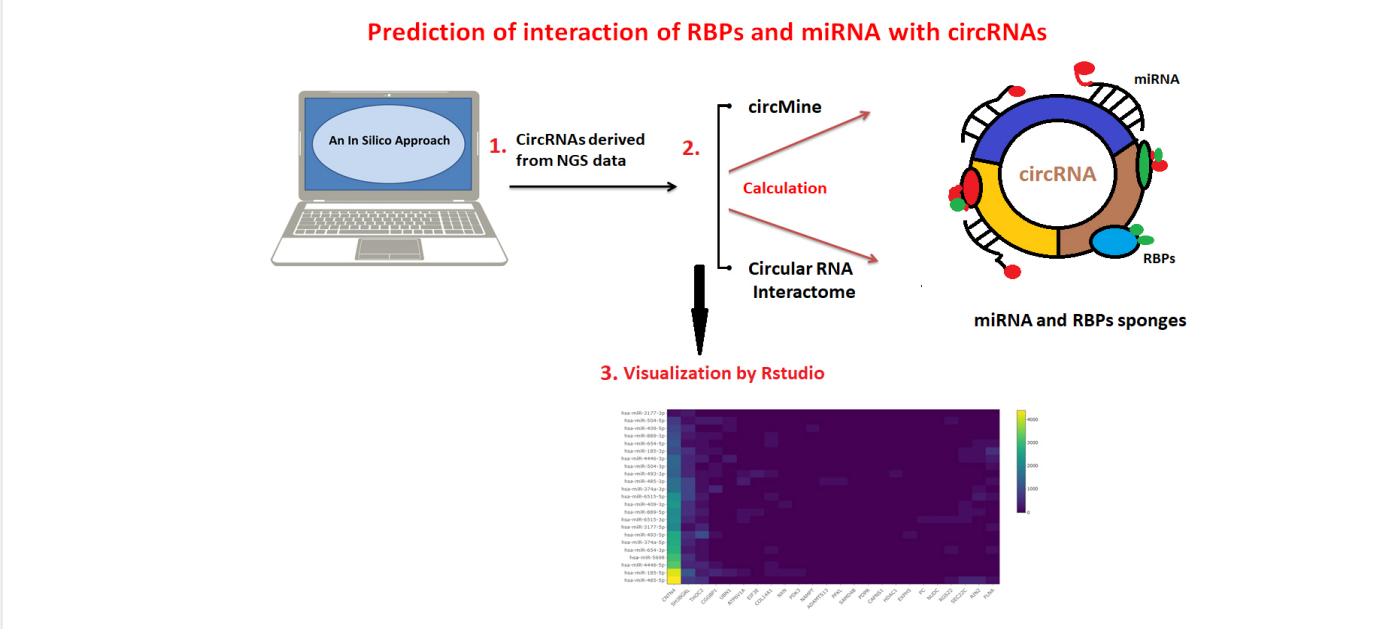

Figure 1. The work flow of the current study is composed of three main steps

NGS: Next-generation sequencing, RBPs: RNA binding proteins

THOC2 is also a highly expressed gene in the brain, and calculations have shown that it potentially interacts with a dozen of miRNAs as circRNAs. Importantly, *THOC2* is a functionally mRNA transport protein and has been uncovered to be associated with the pathogenesis of ALS (27). *THOC2* is integrating with both has-mir-374a-3p and hsa-miR-485-3p in Figure 3, but it is potentially interacting with hsa-miR-493-5p and hsa-miR-485-5p as the highest matching score by far than other miRNAs in Figure 3. In Figure 4, it can be clearly observed that the number of binding sites for Argonaute 2 (AGO2) as well as Eukaryotic initiation factor 4A-3 (EIF4A3) is by far greater for *THOC2*.

ATP6V1A, which is in many sites in the subcellular location as well as mostly located in secretory vehicles, has been reported to be down-regulated in AD and related to Synaptic Vesicle Cycle, Phagosome, and Oxidative Phosphorylation (28). Surprisingly, Wang et al. (29) have also claimed that the deficiency of ATP6V1A might be associated with neuronal disorder and neurodegeneration. In Figure 2, ATP6V1A is interacting with

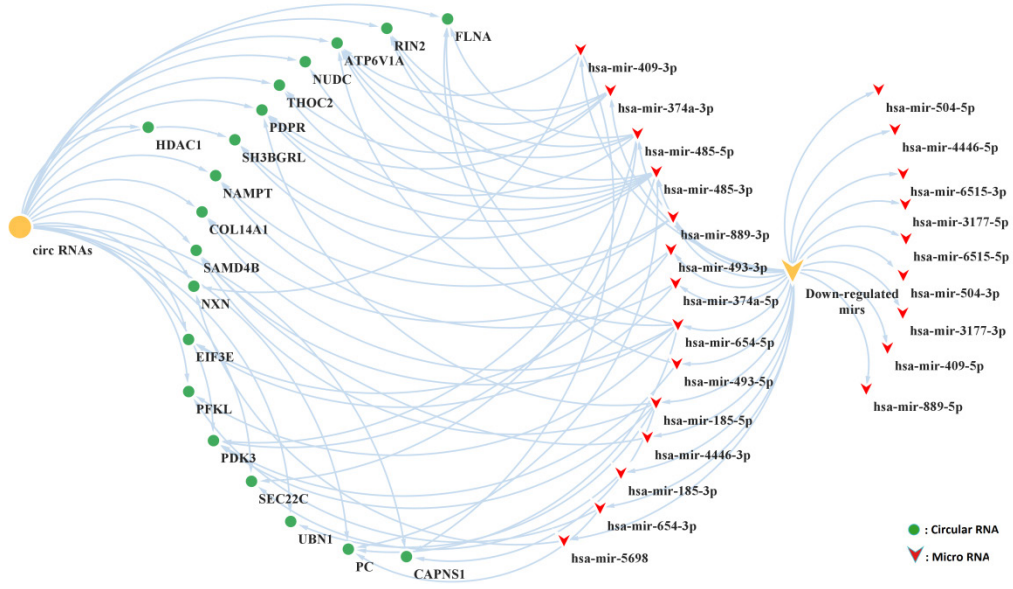

Figure 2. It represents the results obtained from multiple queries of exosomal circRNAs and miRNAs, the prediction of interaction network in the MiRNet 2.0 web server. Upregulated circRNAs are colored as circular nodes in green whereas downregulated miRNAs are colored by red nodes. The type of network is a source >> target that is indicated by the blue arrow edges. The network was designed as two subgroups circRNAs and Down-regulated miRs

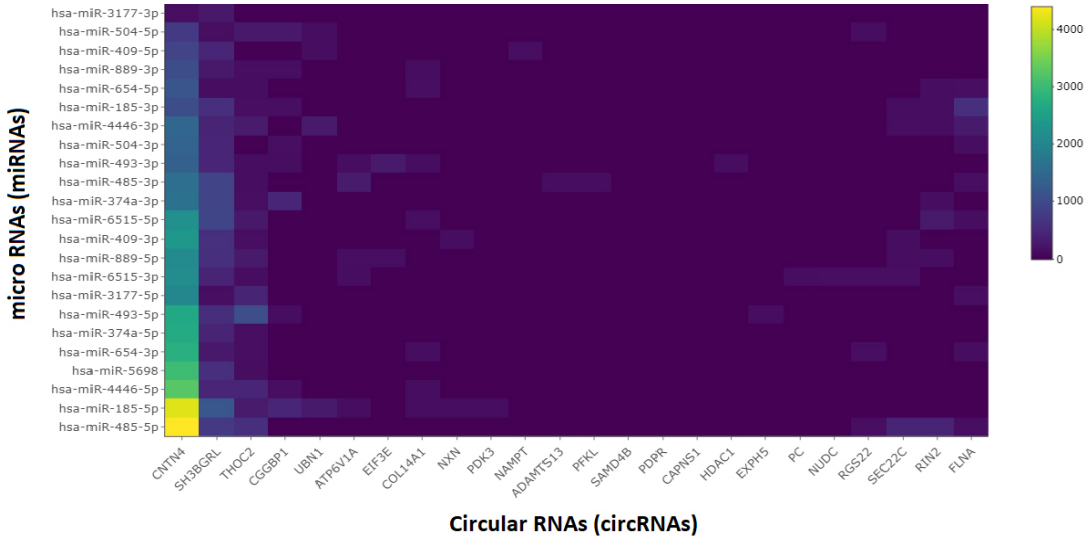

Figure 3. It gives an account of that Heatmap analysis of exosomal circRNAs and miRNAs that outcomes acquired from circMine server with respect to total energy score scale indicating in the range of 0 to 4407. The coloring varies from navy blue to yellow regarding the total score energy

has-mir-409-3p, has-mir-374a-3p, has-mir-485-5p, and has-mir-654-5p, whereas it is possibly interacting with hsa-miR-185-5p, hsa-miR-889-5p in Figure 3. However, the results of the correlational analysis for ATP6V1A set out a commonly interaction with hsa-miR-485-3p in Figures 2, 3. Taken together, just as AGO2 and EIF4A3 are commonly having highest binding sites on ATP6V1A, so DiGeorge syndrome critical region 8 gene, Fragile X mental retardation protein (FMRP), FUS and Hu

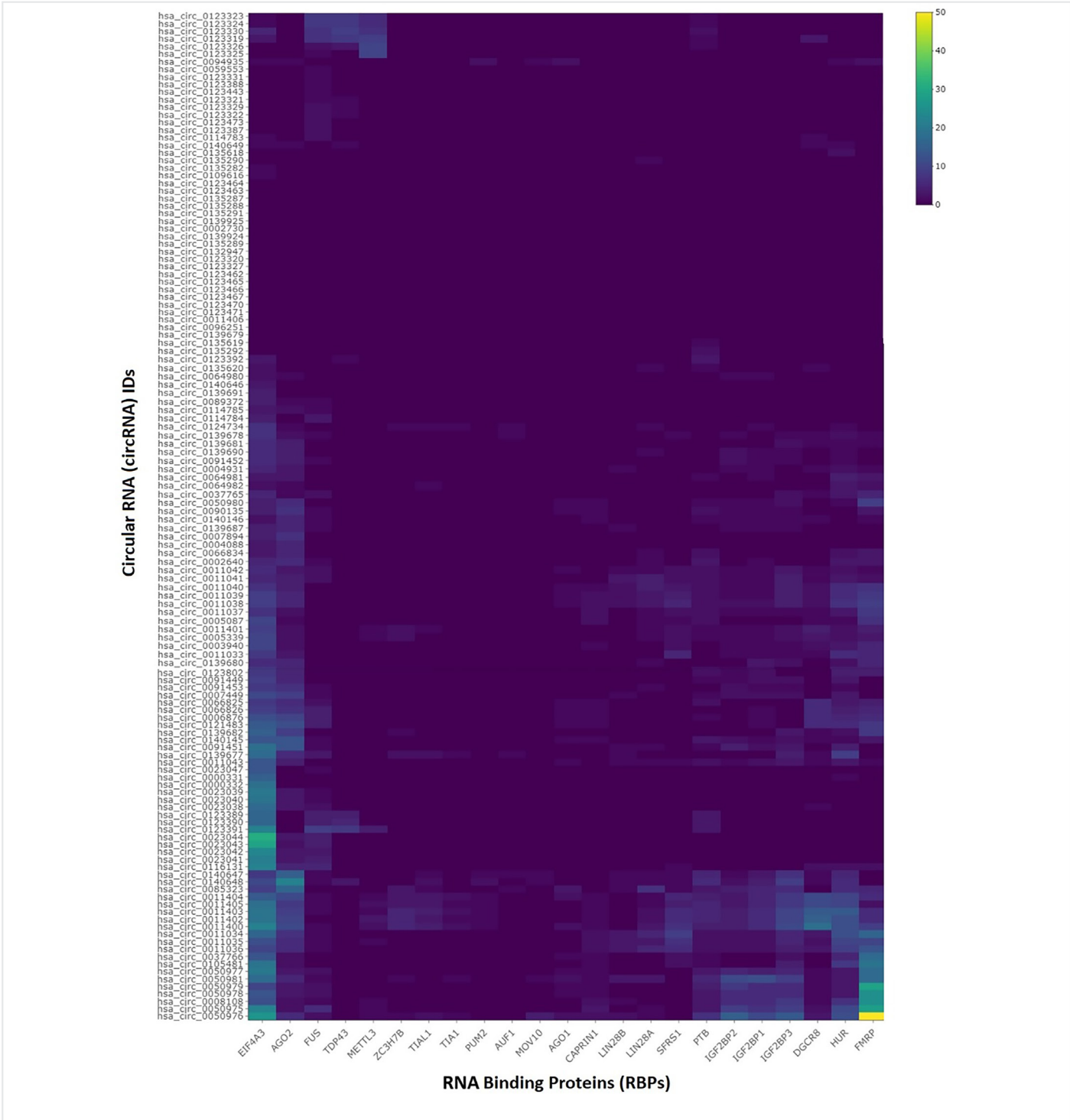

Figure 4. It presents that the possible correlations in the Heatmap analysis among the circRNAs and miRNAs that outcomes retrieved from Circular RNA Interactome server concerning the count of RBPs to the circRNA on a numeric scale indicating in the range of 0 to 50. The coloring varies from navy blue to yellow between the numerical scale of circRNAs and RBPs

RBP: RNA binding proteins

antigen R (HUR) in RBPs in Figure 4 have significant binding sites as well.

More importantly, FLNA, the most interacting one with miRNAs in circRNAS in Figure 3, has been recently elicited by Tsujikawa et al. (30) enriching tau pathological lesions in post-mortem *Progressive supranuclear palsy*-PSP brains as well as tau aggregation through their interactions with F-actin. Given that obtained data from Figure 2 and 3, hsa-miR-654-5p, hsa-miR-485-5p are commonly interacting with FLNA, however, the calculation output of FLNA with hsa-miR-185-3p is higher than the other miRNAs (Supplementary Figure 1). In spite of flanking region on FLNA, FMRP has 35 binding sites on FLNA by far compared to other RBPs in Figure 4.

As far as concern the computational Heatmap in Figure 4, there is an unambiguous relationship between CNTN4 and EIF4A3, FUS, METTL3, TDP43 with a higher number of binding sites than other RBPs. Considerably, reliable evidence display that TDP43 and FUS proteins related to the AD pathology, emphasizing the function of RBPs in neurodegeneration (31,32). Besides, TDP43 protein acts as a neuropathological marker in Alzheimer's disease (33). On the other hand, EIF4A3 possesses the highest number of binding sites in RBPs. Particularly, HDAC1, PC, *THOC2*, and PFKL from circRNAs have far more binding sites of EIF4A3 than other circRNAs. This output points out a need to understand the various perceptions of EIF4A3 that exist among the first genes to be evaluated in experimental analysis steps such as *in vitro* or *in vivo*.

SH3BGRL is a protein that is regularly localized in vesicles and has been reported to be highly expressed in Parkinson's disease with the method of MALDI-ToF-MS (34). Correspondingly, we also found the SH3BGRL in the exosome by the differential gene expression analysis via NGS in AD patients. From both data in Figures 2 and 3, potential interactions between hsa-miR-485-5p, hsa-miR-889-3p and SH3BGRL are obvious. In Figure 2, however, hsa-miR-185-5p is the best matching score with SH3BGRL, suggesting as acting sponge by its much more value. Considering potential circRNA complexes, as shown in Figure 4, AGO2, EIF4A3, HUR, and Insulin-like growth factor 2 mRNA-binding protein 1-2-3 (IGF2BP1-2-3) have much more binding sites on SH3BGRL (Supplementary Figure 1).

In Figure 4, FMRP with the largest number of binding sites has 50 binding sites, specifically on SAMD4B. It is an mRNA binding protein associated with translational inhibition of target transcripts of the amyloid precursor protein mRNA. Regarding the data, it has been suggested by Renoux et al. (35) that FMRP expression is not directly to be a primary contributor AD pathogenesis, despite decreased expression of FMRP in the brain resulting in Fragile X Syndrome. In Figure 2, SAMD4B has interactions with hsa-miR-185-5p and hsa-miR-4446-3p, but no matching score related to SAMD4B in Figure 3, proposing that variable outputs might be depended on different algorithms in web servers. Additionally, FUS, HUR, PTP and IGF2BP1-2-3 from RBPs have more binding sites on SAMD4B by far than other RBPs in Figure 4.

On the other hand, UBN1 is the most statistically significant gene expression in our former study (21) and has the potential to interact alternatively with hsa-miR-185-5p, hsa-miR-409-5p, hsa-miR-504-5p and hsa-miR-4446-3 in Figure 3 (Supplementary Figure 1), whereas it solely interacts with hsa-miR-185-5p in the network of Figure 2 that is also the highest matching score with the other miRNAs in Figure 3. Hence, UBN1 might be strongly described as a correlation with hsa-mir-185-5p concerning both calculation outcomes in Figure 2 and 3. Moreover, AGO2, EIF4A3, FMRP, FUS, HUR and IGF2BP3 are RPBs that have much more binding sites on UBN1 than other RBPs, suggesting that these RPBs should be given primarily consideration in further experimental analysis.

In this study, we attempt to explore the probable complexes that exosomal circRNAs related to AD pathology can form with RBPs and miRNAs by the computational biology approach of preliminary analysis, which will assist in the detailed analysis in oncoming experimental studies. However, there has been limited research on RBPs and miRNAs composed of circRNAs complexes, and more documents are needed. To raise the accuracy and reliability of our preliminary analysis, we were based on the inspiration of the findings in our earlier study (21) and employed more than one bioinformatics tool including circMine (18), circRNA Interactome (19) and MiRNet (17) to integrate all related data, therefore our calculations resulted meaningfully.

Study Limitations

The main limitations of the study need to be validated by further experimental investigations, due to the lack of adequate data in the databases, all miRNAs and circRNAs could not be integrated into those associated with AD, and the sample size of the NGS utilized here was not big sufficiently, solely comprising 9 tissue samples.

Conclusion

Given the functions and stability of circRNAs, they are promised as target molecules to be explored in diseases such as AD, where early diagnosis is crucial. Hence, circRNAs are paving the way for further investigation of new biomarkers in AD. To this end, prior to undertaking further experimental investigations, we sought to predict the miRNAs and RBPs involved in the structure of circRNA complexes in AD patients by sequence-based matching calculation. Our present report points out several potential integrations of circRNA-miRNAs and circRNA-RBPs that contribute to the pathology of AD.

Ethics

Ethics Committee Approval: Ethics committee approval is not required.

Informed Consent: Informed consent is not required.

Footnotes

Authorship Contributions

Concept: R.K.C., N.B., U.K., Design: R.K.C., N.B., Data Collection or Processing: R.K.C., N.B., Analysis or Interpretation: B.S., N.E., H.S.P., U.K., Literature Search: B.S., N.E., H.S.P., Writing: R.K.C., N.B.

Conflict of Interest: No conflict of interest was declared by the authors.

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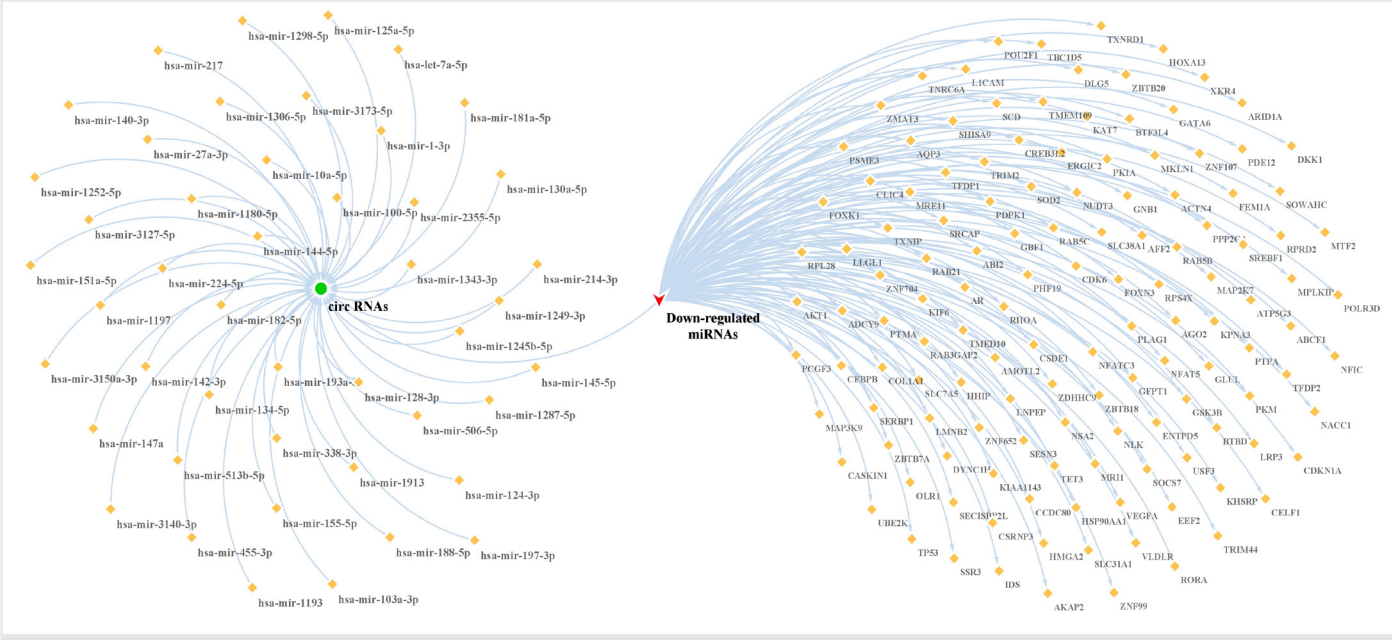

Supplementary Figure 1. The network reveals potential circRNA-miRNA interactions and miRNA-target gene interactions