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A Summary of Circular RNAs in Alzheimer's Disease

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Abstract

Circular RNAs (circRNAs) are recently rediscovered eukaryotic molecules that form a covalently closed-loop structure through a special type of alternative splicing known as backsplicing. These closed-loop structures are highly stable and resistant to RNase degradation, and are thereby expressed in a tissue-specific and evolutionarily conserved manner, which regulates the expression of proteins and mRNAs that are involved in the metabolic pathways associated with specific diseases. Recent evidence of the ubiquitous expression of circRNAs in cancer under physiological and pathophysiological conditions indicates that dysregulation of gene and protein expression might promote tumorigenesis and carcinogenesis, and that circRNAs have important clinical significance in the diagnosis, treatment, and prognosis of cancer and other diseases. This review provides a brief introduction to the characteristics, formation, and function of circRNAs. Some of circRNAs act as microRNA (miRNA) sponges to regulate the level of transcriptional splicing and the expression of parental genes through the circRNA-miRNA-mRNA regulation axis. We summarize recent progress in above-mentioned circRNAs associated with Alzheimer's disease (AD).

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Introduction

Different from linear RNA molecules, circular RNAs (circRNAs) are produced through non-canonical alternative splicing and form a covalently closed-loop structure that lacks 5' to 3' polarity and a polyadenylated tail (Fig.1). Therefore, circRNAs are naturally more stable with relatively longer half-lives than linear RNAs *in vivo* and are resistant to RNA exonucleases due to their closed secondary structure [1-4]. CircRNAs are mainly present in the cytoplasm and are mostly composed of exons; however, some circRNAs also retain intronic regions and are located in the nucleus [5]. The profiling of circRNAs has shown that the expression of disease-associated circRNAs is abnormal in 20 types of human tissues, with highest expression in the cortex and the lowest in liver tissues [6], indicating that the expression of circRNAs is tissue-specific. CircRNAs are enriched in the brain and increase in abundance during fetal development [5]. One study has also shown that circRNA expression is generally higher in later stages of growing development of the brain compared to the earlier stages [7]. Moreover, interindividual differences of circRNA expression levels in liver and brain tissues were detected by a flow scheme named RAISE (circRNA ReAlign Internal Structure and Expression) [8], which strongly cautions that only 0.5% of circRNAs are shared across human brain samples.

With the development of bioinformatics and high-throughput sequencing technology, numerous circRNAs have been rediscovered and identified in various eukaryotes [9-11] [12-16]. And the expression appears to be conserved. For example, Werfel et al [17] showed that approximately 13% of the human cardiac circRNAs are conserved to mouse and rats, and 23% of circRNAs are conserved between mouse and rat [18]. Similarly, approximately 5–10% of human brain circRNAs are expressed in the porcine brain [19, 20]. Taken together, the findings show that circRNAs are unlikely to be non-functional byproducts. Given its prevalence and the fact that these were overlooked until very recently, it is pertinent to further investigate the role of circRNAs in various biological processes. It is estimated that approximately 14% of the circRNAs in human fibroblasts are derived from active transcription genes [21]. It is true that circRNAs show a great variety: >100,000 species but these are below the detection threshold if the samples are not treated with RNases[3]. The expression levels of numerous circRNAs are significantly lower than those of their linear transcripts [5]. Salzman et al, states that circRNAs are expressed at 1-3% of the level of all poly (A) + RNAs [2]. However, the expression abundance and evolutionary conservation of circRNAs need further discussion.

Considering their ubiquitous presence and diversity, it is supposed that circRNAs may be functional in nomal cellular physiological or pathological processes. Our knowledge about their functions get expanded with subsequent identification. Specially, circRNAs can increase the expression levels of mRNA by competing in binding with target miRNAs [22, 23]. As some miRNAs have been proven that were strongly associated with diseases, we can take it for granted that circRNAs will play regulatory roles in the development of diseases [24]. In this review, we briefly introduce the biogenesis and function of circRNAs, and highlight their roles in Alzheimer's disease (AD).

Biosynthesis Models of circRNAs

Catalyzed by spliceosome, the process of gradually editing the precursor m-RNA (pre-mRNA) into mature mRNA through intron removal is called RNA splicing, in which 99% of the bases of 5' end (donor site) and 3' end (receptor site) of introns are almost GU and AG [25]. Spliceosome is a 60S complex of RNA and protein formed during RNA splicing, which has the function of identifying 5' splice site (5' SS), 3' splice site (3' SS), and branching points of the pre-mRNA. A single pre-mRNA transcript can generate different mature mRNA isomers through alternative splicing, which is an important mechanism that leads to large diversity of genes and proteins in eukaryote [26]. CircRNAs are formed by a unique alternative splicing mechanism termed backsplicing, which generate the closed loop structure via joining a splice donor to an upstream splice acceptor. After canonical splicing, the relative order of exons in mRNA matches the order in genome, and no shuffling of exons occurs. While circRNA contains scrambled exons, which means the order of exons is different from that present in the nascent transcript [3].

According to the constituent sections, circRNA can mainly be classified into three categories: one is termed exonic circRNAs (ecircRNAs), which localized in cytoplasm are mainly composed of single or several exons [2, 3, 24, 27]. Another with none critical elements and little enrichment for miRNA target sites inside is termed circular intronic RNAs (ciRNAs), which only contain introns and are localized mainly in the nucleus, mainly promote gene transcription by binding to RNA polymerase II [27]. The last one also localized in nucleus is termed exon-intron circRNAs (EIciRNAs), which contain both exons and introns [3, 28].

In addition, a special type of intronic circRNAs that are generated during pre-tRNA splicing, called tRNA intronic circRNAs (tricRNAs), which have been discovered in Archaea[29] and Drosophila [30]. The anciently conserved tRNA sequence motifs and the tRNA splicing endonuclease complex are necessary to remove introns, which then ligated by a 3', 5'-phosphodiester to form tricRNAs [31]. ElciRNAs play a monitoring role that ensures the integrity of the transcriptome by binding to the U1 element of the snRNPs. The U1 element initially binds to small nuclear ribonucleoproteins (snRNPs) to form a complex, which then combines with RNA pol II to promote transcription. U1 is a key element which can prevent the early termination during transcription [10, 32, 33].

Besides the first and last exons of the pre-mRNA, all internal exons theoretically can be circularized via the splicing signals in flanking introns on both sides. However, backsplicing reactions often occur at an extremely low level [34]. Their efficiency can be regulated by RNA binding proteins, exon skipping events, as well as the core spliceosomal components. At present, the theory regarding the biosynthesis of circRNAs consists of the following five models.

i) Lariat-driven circularization **(Fig 1b)** [35]. This model is also called exon skipping, in which the formation of circRNAs based on the canonical splicing. If the pre-

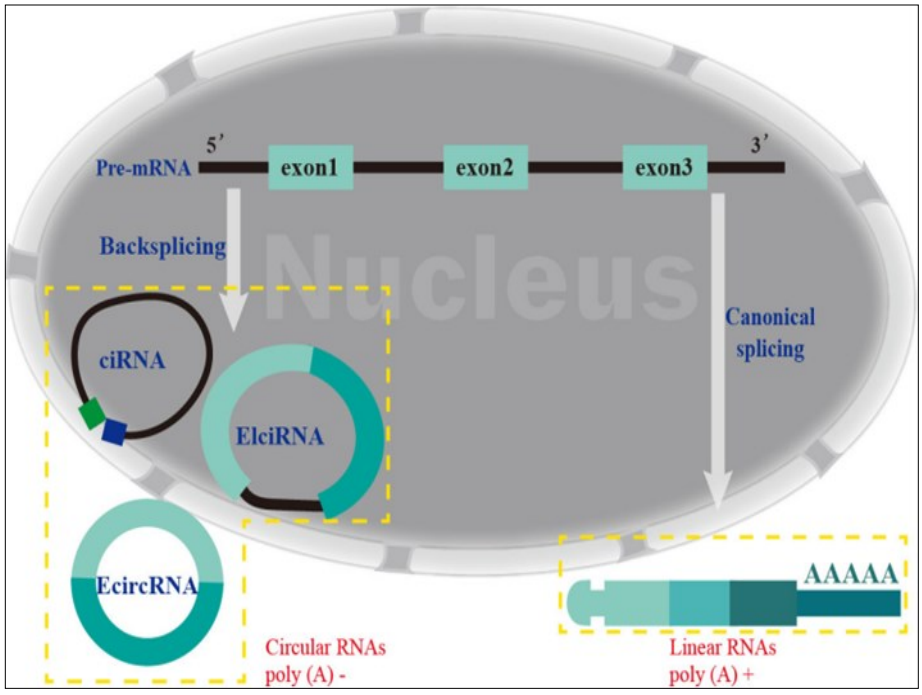

Figure 1. The formation of different forms of circRNA in the nucleus is different from that of linear RNA. Finally, EcircRNA and linear RNA located outside the nucleus, while ciRNA and elciRNA located inside.

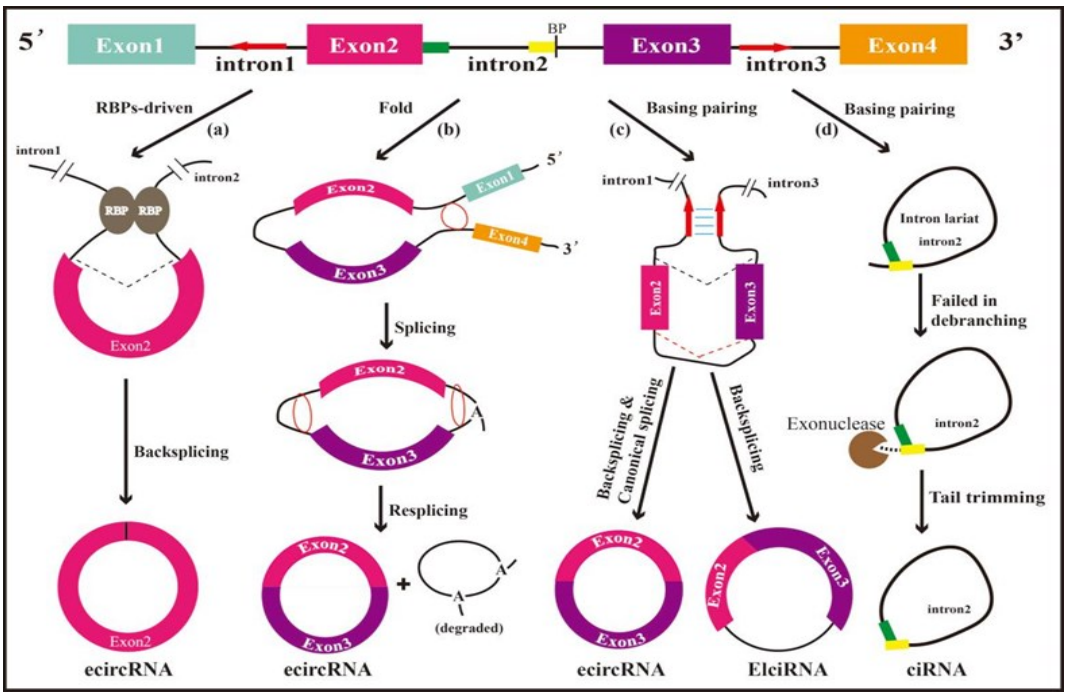

Figure 2. Schematic diagram of the biosynthesis of circRNAs. The pre-mRNA on the top of this diagram will synthesize different types of circRNAs through distinct splicing way of (a), (b), (c), and (d). The red arrows above introns represent reverse complementary sequences. In the intron2, the green and the yellow rectangle represents the 7-nt GU-rich sequence and the 11-nt C-rich sequence, respectively. BP means branching point. (a) Circularization depends on RNA binding proteins (RBPs). (b) Lariat-driven circularization. The red circles in this splicing way represent the splicing sites. (c) Intron pairing-driven circularization. (d) Formation of circular intronic RNAs (ciRNAs).

mRNA is partially folded, exon(s) may be skipped during the RNA splicing, then two previously non-adjacent exons are connected together to form a linear RNA. The skipped exon(s) along with the surrounding introns will form a structure of lariat, which should be degraded in normal cases. However, if the lariat further to be spliced again via backsplicing, the ecircRNA will be generated [36, 37].

ii) Intron pairing-driven circularization (or direct backsplicing) **(Fig 1c)** [38]. It is demonstrated that the biogenesis of circRNAs can be promoted by complementary base pairing interactions between flanking intronic repeats [11, 22, 33, 34, 39-41]. In details, these complementary repeats can form double-stranded RNA (dsRNA) structures, which bring the splice sites close to each other so that the backsplicing occurs [33, 39]. In the following step, the introns are removed or retained to form ecircRNAs or EIciRNAs. The occurance of this modle relys on the Alu complementarity which is specific in primates.

iii) Circularization depends on RNA binding proteins (RBPs) **(Fig 1a)** [42]. With the help of RBPs, the splice donor and acceptor between flanking introns are brought close to each other, thereby promoting backsplicing [43, 44]. QKI is a alternative splicing factor that can combine with flanking intron sequences to form dimers to promote circularization which is similar to modle 2 [43]. Conn et al.[44] have found that large amounts of circRNAs formed along with the up-regulation of QKI during human epithelial-mesenchymal transition (EMT), which indicates that the formation of circRNAs is time-space specific. Another RBP, MBL, can bind to the conserved sequence of its own introns and then regulate the circularization of exons, which derive from its own genes [43]. Moreover, SP proteins and hnRNPs in Drosophila have similar effects on the production of specific circRNAs [40].

However, RBPs also inhibit the formation of circRNAs. For example, ADAR1 as a RNA editing enzyme, inhibits the synthesis of circRNAs via interacting with dsRNA and splitting it [45]. On the other hand, NF90/NF110[46] or the RNA helicase DHX9 could also inhibit the backsplicing by directly unwinding the dsRNA or by recruiting ADAR1 [47].

iv) Formation of circular intronic RNAs (ciRNAs) **(Fig 1d)**. A 7-nt GU-rich sequence near the 5' splice site and an 11-nt C-rich sequence near the branching point prevents the larait structure being branched and degraded, and the lariat tail downstream of the branching point is trimmed to produce a stable ciRNA [48]. These special ciRNAs can act as cis-elements to interact with RNA polymerase II (Pol II) to promote transcription [27]. However, it is still unknown how these functional factors escape degradation.

v) alternative splicing [49]. The competitional splicing of the pre-mRNA leading the single protein-coding gene to generate mutiple transcripts along with distinct circular RNAs, this process which is known as alternative splicing [2, 50-52]. The number of repetitive elements, spatial distance, and their degree of complementarity will all affect the splicing outcome [33].

Biological Functions of circRNAs

Functions for the vast majority of circular RNAs remain unknown, but recently reported that some of them play roles in regulating miRNAs, alternative splicing patterns, or can be bound with ribosomes to produce proteins. Furthermore, circRNAs have additional novel functions such as acting as sponges for RBPs, direct binding to target genes, and direct translation as templates [53, 54].

CircRNAs can act as miRNA Sponges by Competing with Endogenous mRNAs.

The ceRNA hypothesis refers to the combination of a series of RNAs that can competitively bind to miRNAs, such as mRNAs, pseudogenes, and lncRNAs. These molecules all contain several homologous miRNA response elements (MREs) that can adsorb miRNAs like sponges to regulate mRNA expression levels, thereby affecting their functions [55]. CircRNAs can also absorb miRNAs to eliminate the inhibitory effect of miRNAs on the target gene [56]. Therefore, circRNAs belong to ceRNAs, and the adsorption capacity of miRNAs is stronger than that of linear mRNAs and lncRNAs due to the molecular characteristics of its long halftime, which helps in the mining of gene functions and regulatory mechanisms. For example, CiRS-7/CDR1as has been identified as a super sponge containing more than 70 conserved binding sites for miR-7 and SRY70 and 16 binding sites for miR-138 [57]. It has been proven that

circHIP3K contains multiple miRNA binding sites [58]. Subsequently, circular RNA-ITCHs (cir-ITCHs) have been shown to adsorb miR-22-3p to upregulate CBL expression [59]. Besides, cir-ITCH can also act as sponge of oncogenic miR-7 and miR-214 to enhance ITCH expression [60]. Therefore, circRNAs may play an important regulatory role in disease through the interaction with disease-associated miRNAs, The interaction above could be described as circRNA-miRNA-mRNA regulation axis (Fig.2). Numerous circRNAs that have been reported to date, and thousands of circRNAs were predicted with miRNA binding sites, but it is still unknown whether they are all functional. Then by using SNP data to observe the SNP distribution at predicted miRNA target sites located on circRNAs. It is suggested that many of these predicted sites are functional sites under selective pressure due to the significant decrease of polymorphisms of circRNAs [61]. Majority of circRNAs possess relatively few miRNA binding sites that may not efficiently trap miRNAs, thus failing in exhibiting the expected properties of super sponges [62]. Therefore, whether circular miRNA sponges commonly occur and how the circRNA, miRNA, and ceRNA network is used in maintaining homeostasis remain unclear.

CircRNAs can Regulate Translation and the Expression of Parental Genes.

In addition to acting as miRNA sponges, circRNAs can also act as protein sponges. CircFOXO3a can interact with senescence associated transcriptional factors in the cytoplasm (e.g.,HIF1α, ID1, or E2F1), trapping these within the cytoplasm and preventing them from translocation to other organelles [63]. Additionally, above mentioned blind muscle protein (MBL/MBLN1) in flies (*Drosophila melanogaster*) and humans can control its own levels. In detail, MBL as a kind of RBP to promote the generation of circMBLs that have multiple MBL binding sites, which in turn captures this protein when MBL is overexpressed [49]. Further studies have revealed that the circRNAs corresponding to the formin gene in mice contain a translation initiation site that captures mRNAs to form non-coding linear transcripts, thereby reducing the level of Fmn protein encoded by the parental gene [64]. CiRNAs and ElciRNAs localized in the nucleus can be used to *cis*-regulate the transcription of polymerase II by interacting with pol II [5]. For example, circEIF3J and circPAIP2 can bind to U1 snRNPs to enhance the transcription of the parental gene [65]. Therefore, we speculate that only exonic circRNAs play a regulatory role in the cytoplasm, whereas the circRNAs containing introns perform effective transcriptional regulation in the nucleus. In addition, studies have found that circRNAs inserted into the internal ribosome entry site (IRES) upstream of the initiation site can artificially synthesize proteins[66]. This suggests that perhaps some of circRNAs can also be directly converted into proteins. Subsequently, circRNAs were found with coding ability including circ-SHPRH [67], circ-FBXW7 [68], Circ-ZNF609 [69, 70], and so on. Furthermore, it is found that the N^6-methyladenosine (m^6A), the most abundant base modification of RNA, can promote efficient initiation of protein translation from circRNAs in human cells [71]. All of these circRNA-derived proteins largely expand the coding landscape of human transcriptome and can be exploited as high specific novel targets for targeted therapies.

CircRNAs as Diagnostic or Prognostic Biomarkers.

An increasing amount of research shows that circRNAs are involved in the pathogenesis of cardiovascular disease [72], neurodegenerative disease [73], and cancer [74], and thus may be utilized as disease biomarkers. For example, a large number of CDR1as is expressed in the brain that contain 60 bp of miR-7 binding sites, which is associated with a variety of disease pathways. It has been confirmed that CDR1as is involved in the regulation of Parkinson's and Alzheimer's disease [75-77]. At the same time, miR-7 is involved in carcinogenesis and tumor inhibition, and therefore, the regulatory axis of CDR1as/miR-7 is likely to be closely related to the occurrence and development of tumors [78-81]. These findings show that circRNAs are closely related to the occurrence of disease and is a potential target for the future diagnosis and treatment of disease.

Although circRNAs are highly stable and has great potential as biomarkers for disease diagnosis, their use in clinical trials and in patient diagnosis remains limited. Considering the great variability in circRNAs between individuals, and even within individuals taken on different days [82], there is

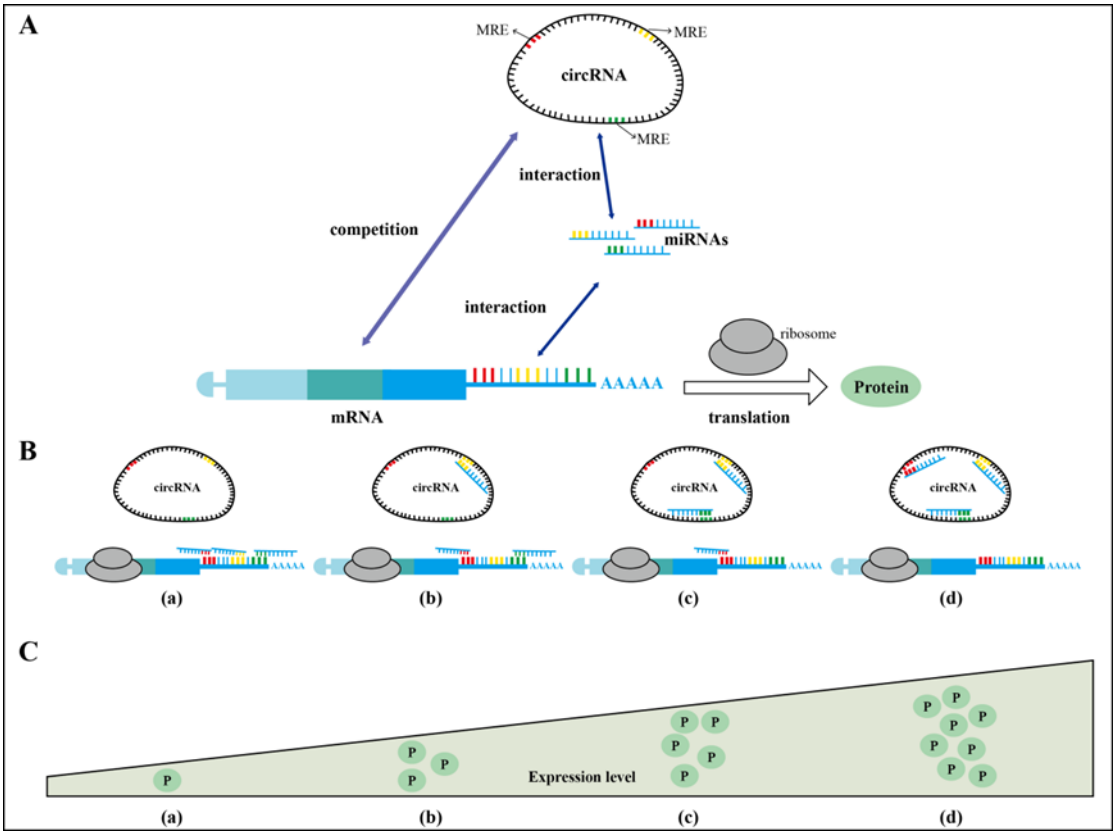

Figure 3. The schematic of circRNA-miRNA-mRNA regulation axis. A. CircRNAs as one of ceRNAs compete with mRNA for binding to miRNAs; circRNAs and mRNAs all contain MREs inside (MREs are represented by red, yellow, and green small vertical lines). CircRNAs and mRNAs interact with homologous MREs on miRNAs via base complementation. B. The competitive binding of miRNA between circRNA and mRNA. C. The expression level of the mRNA. Every (a), (b), (c), and (d) in B or C is corresponding. (a) When three sites on mRNA bind to the miRNA, the inhibitory effect is strongest during translation and the expression level of protein is the lowest. (b) When one site on the circRNA and two sites on the mRNA bind to the miRNA, the inhibitory effect is stronger during translation and the expression level of protein is lower. (c) When two sites on the circRNA and one site on the mRNA bind to the miRNA, the inhibitory effect is weaker during translation and the expression level of protein is higher. (d) When three sites on circRNA and no site on mRNA bind to the miRNA, the inhibitory effect is weakest during translation and the expression level of protein is the highest.

necessary to discuss in detail the key indicators for the assessment of the sensitivity, specificity, easy detection, and repeatability of tissue- and disease-associated circRNAs. Studying the influence of potential factors such as organization and blood collection and processing is important to ensure that standardization of reliability and reproducibility in the operational process involves the collection of data regarding conditions, equipment, applications, and sample acquisition, transportation, handling, and storage issues [83-85].

It is important to find biomarkers that are highly specific and sensitive for the early diagnosis and staging of diseases to facilitate large-scale population screening because it is difficult to accurately diagnose and predict the occurrence of diseases based on multiple biomarkers [86]. In addition, it is necessary to develop reagents and methods for detecting biomarkers with high sensitivity, specificity, and stability such as ELISA kits, mass spectrometry, automatic electrochemilumi-nescence immunoassays, and blood biomarker detection. There is a need to standardize biomarker detection, analysis of the standardization of pre-analysis factors, unified detection methods, and the use of automated analysis methods to achieve comparability of the data, and these current obstacles can provide the basis for determining the critical value of circRNAs for clinical diagnosis.

CircRNAs in Alzheimer's Disease (AD)

Neurodegeneration is a disease that occurs in the brain and spinal cord with the symptoms of neuronal loss. It may be destructive and irreversible for cells that are excessively damaged, because cells in this condition will not regenerate. Senile dementia is also known as Alzheimer's Disease (AD) that belongs to Chronic neurological diseases. The main histopathological traits of AD are Aβ plaques and neurofibrillary tangles (NFTs), which often occur in the neocortex, hippocampus and other subcortical areas of the brain [87]. Recent studies have revealed a potential link between AD and circRNA-associated-ceRNA networks (Fig.3).

The peptide amyloid β (Aβ) that has strong neurotoxicity are derived from the degradation of

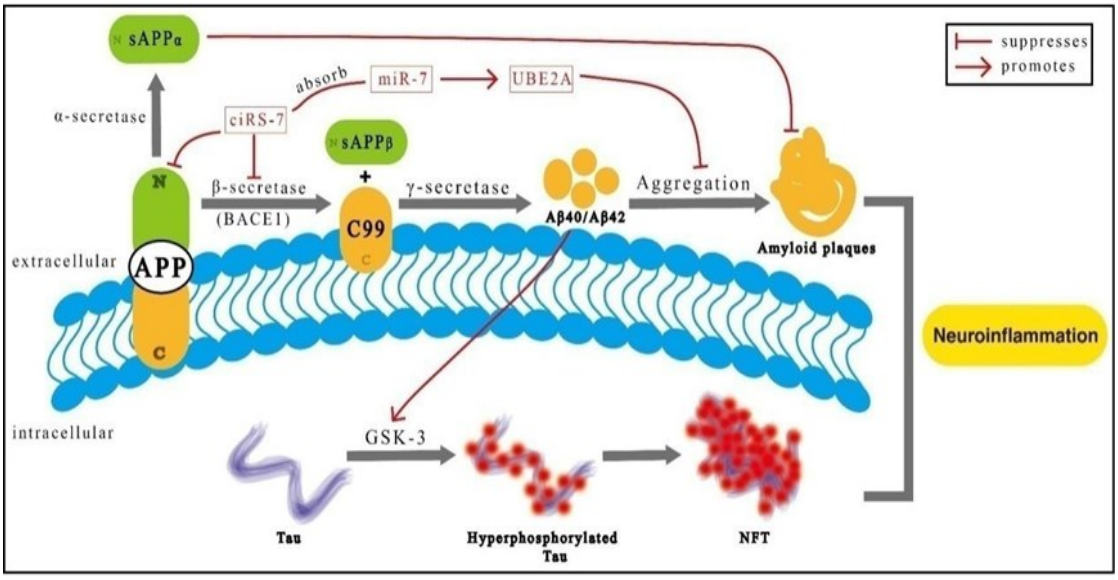

Figure 4. Schematic of AD pathogenesis. The generation and aggregation of amyloidogenic Aβ peptides outside of the cell leads to the formation of amyloid plaques. The hyperphosphorylation of Tau protein results in formation of intracellular neurofibrillary tangles. Amyloid plaques and neurofibrillary tangles synergy cause neuroinflammation. Illustration of graphic symbols is in the black box at the top right.

amyloid precursor protein (APP) through secretase. The cleavage of transmembrane protein APP leads to the extracellular accumulation of Aβ and thus form the Aβ plaques [87]. APP has two metabolic pathways: in the normal metabolic process, through α-secretase, APP can produce soluble N-terminal fragment sAPPα, which act as neuroprotection to prevent the formation of Aβ. In another metabolic pathway, if the APP is cleaved by β-secretase, such as BACE1 (b-site app-cleaving enzyme 1), the N-terminal secretory polypeptide fragment sAPPβ will produce, however the C-terminal fragment C99 remains on the membrane. C99 is further degraded by γ -secretase to produce Aβ peptide (Aβ40 and Aβ42), in which Aβ42 will finally form senile plaques through aggregation and deposition [88]. For another, the presence of microtubule associated protein tau (MAPT) may induce chronic inflammation and neuronal loss, which mainly result in NFT formation. Protein Tau will become hyperphosphorylated Tau catalyzed by glycogen synthesis kinase 3 (GSK-3). At this point, MAPT loses its ability to bind microtubules and becomes unstable, aggregating into double-stranded helical fibers (PHFs), which then formed the filaments of NFTs. In addition, Aβ can enhance the activity of GSK-3 to induce hyperphosphorylation of Tau, which cooperate with the deposition of Aβ can enhance the cytotoxicity and thus produce neuroinflammation. The imbalance between Tau phosphorylation and dephosphorylation is an early event in NFT formation and AD pathogenesis [89]. Figure 4.

Studies have shown that the expression level of ciRS-7 were significantly reduced in hippocampal CA1 region samples of AD patients compared with healthy controls [75]. Therefore, it is predicted that the lack of ciRS-7 may lead to decreased expression of selective miR-7 targets, and the expression of Ubiquitin protein ligase A (UBE2A) was reduced through miRNA sponge function [90]. UBE2A is a miR-7 target that is essential for the clearance of AD-amyloid peptides. Due to the inhibition after miR-7 binding, UBE2A was downregulated in AD. In addition, ciRS-7 was found to promote the degradation of APP and BACE1 in a nuclear factor-κB (NF-κB)-dependent manner in SH-SY5Y cells. The network of ciRS-7-miR-7-UBE2A suggests that ciRS-7 can act as an effective therapeutic target in AD. Through backsplicing, MAPT can conjugate exon12-10 to produce circular RNA (cir12-10), which is located in the cytoplasm and contains ORF that encodes the Tau fragment [91]. Studies have shown that there is a high probability to cause frontotemporal dementia (FTD) after interfering mutation to exon 10 of Tau [92]. Moreover, exon 10 usage as well as cdc2-like kinase (CLK2) splicing isoforms are changed in AD [93].

In order to explore the relationship between circRNA-associated-ceRNA and AD, Zhang et al. [94] used senescence-accelerated mouse resistant 1 (SAMR1) as the control to perform deep RNA-seq analysis on the brain of senescence-accelerated mouse prone 8 (SAMP8) model. They found 235 significantly dysregulated circRNA transcripts, 30 significantly dysregulated miRNAs, and 1202 significantly dysregulated mRNAs, then constructed comprehensive circRNA-associated-ceRNA networks to conduct GO analysis. It was found that circRNA-associated-ceRNA networks can affect AD from various aspects, such as axon terminal and synapses. After further screening, it was determined that this network was involved in regulating the clearance of Aβ and the function of myelin in AD model mice.

Discussion and Prospects

Through interactions with disease-associated miRNAs, circRNAs can play an important regulatory role in specific diseases and have important potential to become clinical diagnostic markers [95]. RNA-seq and bioinformatics analysis are now commonly used to comprehensively analyse circRNAs. RNA-seq can easily detect new circRNAs, and the use of microarrays is a more accurate measure of abundance. High-throughput sequencing was used to detect the entire gene expression profile of circRNAs. Systematic bioinformatics analysis may be used to assess circRNA abundance and related fold changes. Then, circRNA samples exhibiting differential expression may be selected for further FISH, Q-PCR, and Northern blot validation. Finally, circRNAs are analyzed at the cellular and tissue levels, and gene editing may be employed to knockout the gene of interest to study its function. Future research analysis should be performed on the following: (1) the feedback

mechanism of specific circRNAs and their corresponding genes; (2) correlation analysis of miRNAs that interact with specific circRNAs; and (3) the influence of relevant physiological and pathological parameters such as cell cycle, proliferation, apoptosis, migration, and interstitial-epithelial transitions. The resulting information may then be employed in drug development. Developmental stage specificity of circRNA expression provides a new perspective for us to study biological development.

As shown in the study of Li et al., circRNAs have the extremely low abundance but great diversity, which may be related to the limitation of the exon detection algorithm. CircRNAs can regulate the onset and metastasis of human diseases, which can also be used as a potential biomarkers for cancer diagnosis. Detection of doxorubicin-resistant breast cancer cells (MCF-7/adriamycin(ADM)) and their parental cell line by circRNA microarray analysis showed that there was a relationship between MCF-7/ADM and MCF-7. TargetScan and miRanda have been used to predict the target miRNA and mRNA of the upregulated circRNA. Ina ddition, the regulatory role of the circ_0006528-miR-7-5p-Raf1 axis in ADM-resistant cancer has been elucidated. These results indicate that circRNAs play an important role in cancer resistance and may be employed in further functional analysis of hsa_circ_0006528. Bioinformatics analysis indicates that some target genes are related to tumor-related signaling pathways [96]. A more comprehensive study of the detailed mechanisms of circRNAs and their potential value in clinical applications will now be discussed.

Considering their ubiquitous presence and diversity, circRNAs might be major contributors to normal cellular physiological or pathological processes. The abnormal expression of circRNAs is closely related to the occurrence of disease, which raises a new direction for mining biomarkers and novel therapeutic targets. Although the exact roles and mechanisms of circRNAs in gene regulation remain elusive, their contribution to human diseases has been recognized. More circRNAs and their biological functions will be uncovered in the near future. Understanding the interaction among proteins, circRNAs, and DNA at a specific time may contribute to the elucidation of circRNA functions. In addition, investigating the regulatory network of circRNAs may help in development novel therapeutic schemes in cancer and other diseases.

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