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MiR-26a-5p promotes Alzheimer's disease by regulating CDK5R1

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ABSTRACT: Abnormal expression of miRNA is closely associated with disease, particularly Alzheimer's disease (AD). However, the function of miR-26a-5p in AD pathogenesis is not fully understood. Our aim was to investigate the role of miR-26a-5p and its target gene CDK5R1 during the pathogenesis of AD. In this study, SH-SY5Y cells were treated with beta-amyloid (Aβ) to create AD mimic cells and MiR-26a-5p, apoptotic biomarkers, and CDK5R1 were evaluated by RT-qPCR. Transfection was performed to downregulate or upregulate miR-26a-5p and CDK5R1 and a molecular correlation between CDK5R1 and miR-26a-5p was predicted using a luciferase reporter assay. Apoptotic biomarkers and proteins associated with the Wnt/β-catenin signaling pathway in AD mimic cells were detected by Western blotting and cell viability was examined using MTT. MiR-26a-5p levels were higher in the Aβ-coped SH-SY5Y cell lines, which showed reduced viability compared to the control. This effect was reversed when miR-26a-5p was knocked down. MiR-26a-5p has a binding site for CDK5R1, and overexpression of CDK5R1 enhanced cell viability and increased Bcl-2 expression. Furthermore, CDK5R1 overexpression and miR-26a-5p downregulation positively regulated the Wnt/β-catenin signaling pathway. The results showed that MiR-26a-5p is a critical pathogenic gene in AD and its downregulation can be utilized as a novel therapeutic strategy for AD.

KEYWORDS: Alzheimer's disease, miR-26a-5p, CDK5R1, apoptosis, cell viability

INTRODUCTION

Alzheimer's disease (AD) is mainly found in people over the age of 60 and is a neurodegenerative disorder that progressively affects the function and structure of the neural system [1]. There is a direct correlation between morphological alterations and dementia, and this was first found in Alois Alzheimer's disease more than 100 years ago. Extracellular deposits of misfolded proteins are considered to be the main cause of AD [2]. The pathological features of AD include the abnormal deposition of amyloid plaques and neurofibrillary tangles [3]. Metabolic abnormalities in beta-amyloid (Aβ) have been proposed as the etiology for AD [4]. Beta-amyloid, a 40–42 amino acid peptide, was regarded as the major ingredient of amyloid plaques [5] and researchers originally thought that an increase in deposited Aβ42 precipitates plaque formation, which then led to the generation of neurofibrillary tangles and ultimately neuron apoptosis. However, in recent years, it has become clear that soluble Aβ, rather than deposited Aβ, is associated with dementia. Cyclin-dependent kinase 5 regulatory subunit 1 (CDK5R1), a member of the eukaryotic cyclin-dependent kinase family, is the major catalyst of cyclin-dependent kinase 5 (CDK5). Activated CDK5/p35 participates in normal nervous system development and its maladjustment is strongly linked to AD [6].

MicroRNAs (miRNAs) are small non-coding RNAs that are capable of restraining gene expression by recognizing complementary base pairing of mRNA. It is generally considered that miRNAs possess extensive biological functions in a number of different organisms. Many reports have suggested that the abnormal expression of miRNAs is correlated with multiple human pathological changes, including neurodegenerative diseases [7]. MiR-26a-5p has been found to play a significant role in the central nervous system. Its overexpression has been shown to inhibit tau phosphorylation and reduce Aβ accumulation by downregulating DYRK1A levels in AD mice. Additionally, it may directly target PTGS2 and have a regulatory function in AD. However, the variations and functions of miR-26a-5p throughout the progression of AD have not been thoroughly investigated, and the underlying mechanism remains to be elucidated [8, 9]. This study aimed to examine the variation and biological mechanism associated with miR-26a-5p in AD and to investigate its regulatory effect on CDK5R1.

MATERIALS AND METHODS

Cell line and Aβ treatment

Humanized neuroblastoma cells (SH-SY5Y) were procured from Beyotime (Haimen, China) and were grown in DMEM. At 24 h post transfection, SHSY5Y cells were coped with Aβ for 24 h. The Aβ42 (Thermo Fisher Scientific, Paris, France) solution was maintained at 37 °C for 96 h to obtain the neurotoxic form of Aβ. Different concentrations of Aβ42 in its solid state were obtained and these ranged from 0 to 10 nM and 10 nM was selected as the final concentration for the desired

disease cell line because in all assays the highest or lowest expressions of the different variables were obtained at this concentration. Based on this data, the disease cell line for this research was a 10 nM Aβ coped SH-SY5Y cell.

Quantitative real-time PCR

TRIzol reagent (Thermo Fisher, Beijing, China) and a MirVana miRNA isolation kit (Abcam, Shanghai, China) were used to extract total RNA and miRNAs according to the manufacturer's instructions. In addition, oligo-dT and stem-loop reverse transcriptase primers were used to collect the complementary DNA (cDNA). GAPDH was used as a reference for CDK5R1 and U6 was used for miR-26a-5p. The primer sequences are listed in Table S1 and the relative gene expressions were calculated using the $2^{-\Delta\Delta Ct}$ method.

Transfection

The miR-26a-5p mimic, miR-26a-5p mimic negative control (NC), miR-26a-5p inhibitor (anti-miR-26a-5p), and miR-26a-5p inhibitor negative control (anti-NC) were purchased from Beyotime (Haimen, China). Overexpressed gene vectors targeting CDK5R1, wild type (WT), and mutant (MUT) genes were also obtained from Beyotime (Shanghai, China). The cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, France) according to the manufacturer's instructions.

Luciferase reporting assay

The cytomegalovirus promoter and 3′-UTR of CDK5R1 were amplified from human DNA and pcDNA3.1(+) (Invitrogen, Waltham, MA, USA). They were then inserted into the pGL3-luciferase basic vector (Promega, Madison, WI, USA). The primer sequences and cloning methods are available upon request. The constitutively expressed Renilla control vector (Promega) served as a normalization reference for the luciferase assays and the 50 nM miR-26a-5p mimic, NC, CDK5R1-WT, or CDK5R1-MUT with the reporter vector were inserted into the SH-SY5Y cells. At 24 h post transfection, the test results were obtained using a Dual Luciferase Reporter Assay Kit (Beyotime Haimen, China). and the SH-SY5Y cells were analyzed using RT-qPCR.

Western blot analysis

The treated cells were then harvested and cell proteins were extracted using M-PER reagent (Thermo Fisher Scientific, USA). The target proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Beyotime, Beijing, China). The membranes were hatched with 5% non-fat milk for 1 h and then combined with the primary antibody over 24 h at 4 °C. The following day, the PVDF membranes were incubated with secondary antibodies at room temperature for 1 h. Then the signals were visualized and assessed using a protein imaging system (Abcam, Beijing, China). The antibodies used are listed in Table S2 and the primary and secondary antibody dilution rates were 1:500–1000 μg and 1:1500 μg, respectively.

MTT assay

Cell viability was determined by seeding cells (15×10^3 cells/well) into 96-well plates. Different concentrations of Aβ42 were added to the plates at 37 °C. After incubating for 24 h, the Aβ42 was discarded and new culture medium was added to the cells. The cell proliferation rate was determined by adding 50 μl MTT reagent to the cells and then incubating them for 4 h. After incubation, the medium in each cell was removed and 200 μl DMSO and 25 μl Sorenson buffer were mixed into every well. Finally, the optical density was measured at 490 nm using a multilabel reader spectrophotometer (Thermo Fisher, Beijing, China).

Statistical analysis

All results presented in this study were obtained from three independent experiments with triplicate assays each. The data are presented as means ± standard deviation (SD) and evaluated using Student's *t*-test or one-way ANOVA by SPSS version 22 (IBM, Armonk, NY, USA). $p < 0.05$ was considered to be statistically significant.

RESULTS

MiR-26a-5p is upregulated in Aβ-coped SH-SY5Y cell lines

The variation in miR-26a-5p during the pathological development of AD was determined by assessing the differential expression of miR-26a-5p using RT-qPCR. Compared to the blank control cell line, miR-26a-5 expression was elevated in the differentially treated SH-SY5Y cell lines and there was a clear upward trend in miR-26a-5p expression as the Aβ concentration increased from 5 to 10 nM (Fig. 1a). In order to confirm that SH-SY5Y cells cultured in the presence of Aβ were AD mimic cells, DDK1, a positive marker of AD, were also checked by Western blotting. There was a clear upward trend in DDK1 expression as the Aβ concentration increased from 5 to 10 nM (Fig. 1b). The cell viability of the lines was examined using an MTT assay and the results showed that cell viability was higher in the blank control cell lines than in the diseased (Aβ-treated) cell lines (Fig. 1c). Apoptotic biomarkers expression was examined using RT-qPCR and the experimental data showed that Bcl-2 expression was downregulated, whereas Caspase-3 expression was upregulated in the diseased cell lines compared to the control cells. These differences in biomarkers expression showed that they were affected by Aβ concentration (Fig. 1d).

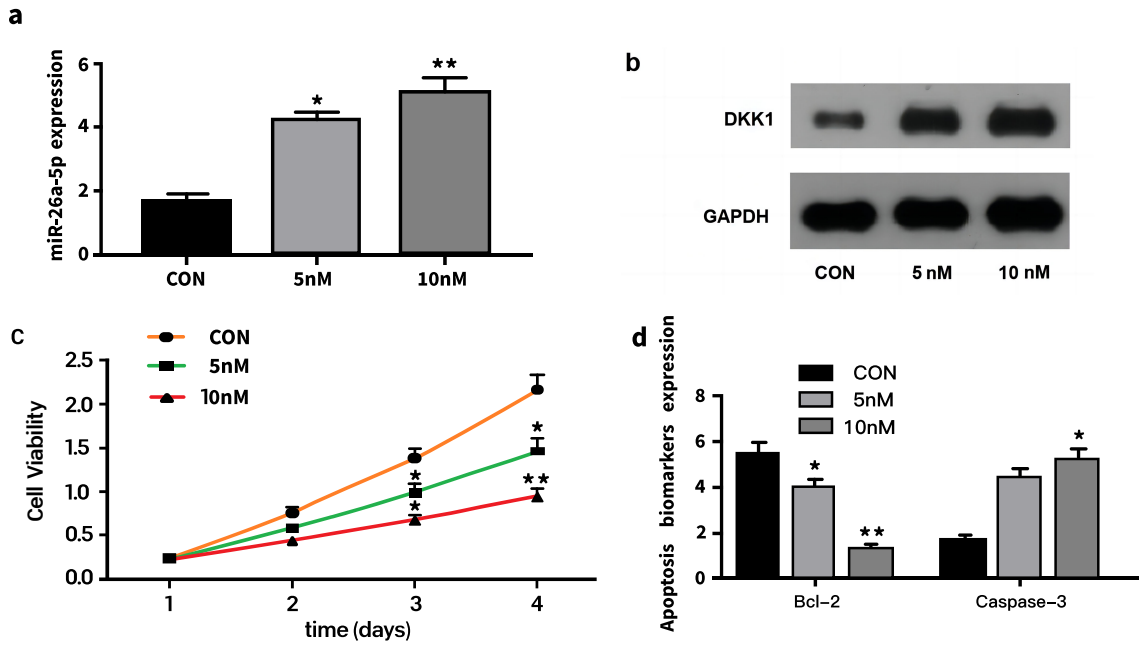

Fig. 1 MiR-26-5p affects viability in diseased SH-SY5Y cells after exposure to different concentrations of Aβ for 24 h. (a) MiR-26-5p expression, (b) DKK1 protein expressions, (c) cell growth activity, (d) transcript levels of the biomarker genes. CON = blank control SH-SY5Y cells; 5 nM and 10 nM = 5 nM and 10 nM Aβ-treated SH-SY5Y cells. Multiple comparisons were evaluated by the SNK-q method. * $p < 0.05$; ** $p < 0.01$.

Knockdown of miR-26a-5p enhances cell viability of Aβ-coped SH-SY5Y cell lines

The impact of miR-26a-5p expression on Aβ-coped SH-SY5Y cells was investigated using MTT assays and RT-qPCR to determine cell viability and monitor miR-26a-5p expression, respectively. The RT-qPCR analysis revealed that miR-26a-5p expression decreased in the miR-26a-5p knockdown cells (Fig. 2a) and the viability results (Fig. 2b) indicated that the decrease in miR-26a-5p expression increased cell viability. Cell viability was highest in the 5 nM anti-miR-26-5p transfected cell treatment and the miR-26a-5p variation trend was also lower for this treatment. To confirm these observations, RT-qPCR was used to investigate apoptotic biomarkers expression. As anticipated, the decrease in miR-26-5p led to a reduction in transcription of the well-known cell death gene Caspase-3 and the restoration of Bcl-2 expression (Fig. 2c).

CDK5R1 has a binding site for miR-26a-5p

A bioinformatics analysis was performed to verify the existence of the target gene for miR-26a-5p in AD pathogenesis. It shows the existence of a binding site for miR-26a-5p on the 3′ UTR region of CDK5R1, which indicates that CDK5R1 is a candidate gene (Fig. 3a). To confirm this, dual relative luciferase activity assays were performed using the WT and MUT CDK5R1 cell lines. After cloning with the luciferase vector, a relative luciferase assay was performed, which revealed that the miR-26a-5p mimic suppressed the luciferase activity of CDK5R1-WT, whereas there was no change in the CDK5R1-MUT luciferase activity (Fig. 3b). Additionally, RT-qPCR was used to determine CDK5R1 expression in the SH-SY5Y cell lines treated with 5 and 10 nM Aβ. The results showed that CDK5R1 expression decreased as the Aβ concentration increased in the SH-SY5Y cell lines (Fig. 3c,d).

Overexpression of CDK5R1 enhances cell viability, Bcl-2 expression, and the Wnt/β-catenin signaling pathway

Transfected genes were examined to verify whether CDK5R1 altered the effect of miR-26a-5p on 10 nM treated SH-SY5Y cells. CDK5R1 expression was assessed using RT-qPCR and the results demonstrated that the suppression of miR-26a-5p enhanced CDK5R1 expression (Fig. 4a). Similarly, CDK5R1 upregulation increased cell viability (Fig. 4b). In addition, Bcl-2 and Caspase-3 expression levels were measured in the transfected cells and the results showed that co-transfection of the two genes (overexpression of CDK5R1 and suppression of miR-26a-5p) reversed the previous expressions of the apoptotic biomarkers (Fig. 4c). A Western blot analysis was performed to validate these results and the Western blot results also showed that co-transfection of the two genes reversed the earlier expressions of the apoptotic biomarkers (Fig. 4d). The results for Wnt/β-catenin signaling

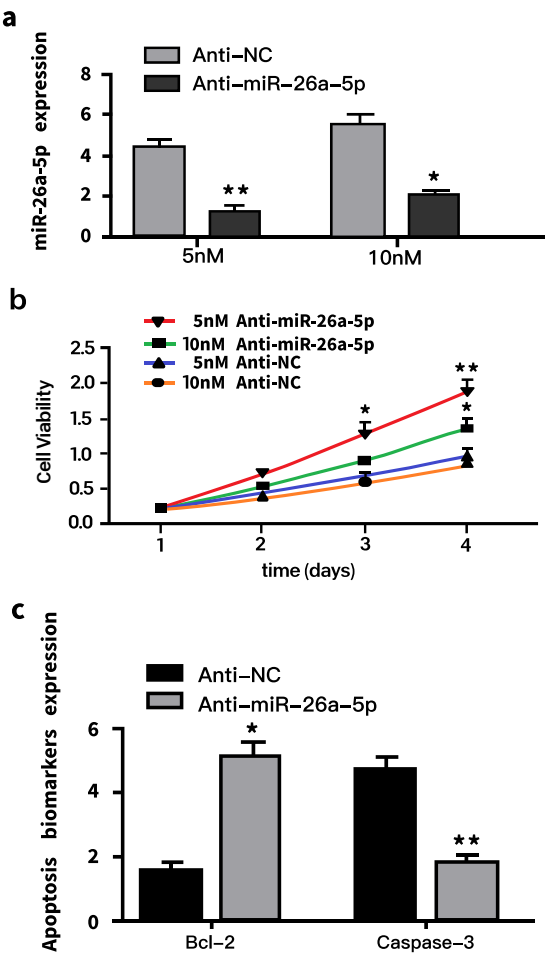

Fig. 2 MiR-26a-5p knockdown enhanced cell viability in transfected Aβ-treated cell lines. (a) MiR-26-5p expression in transfected SHSY5Y cells exposed to different Aβ concentrations for 24 h. (b) Cell viability and (c) transcript levels of the biomarker genes at 48 h after Aβ-transfection.

proteins showed that overexpression of CDK5R1 and downregulation of miR-26a-5p enhanced Wnt-3a and β-catenin levels, whereas DKK1 levels decreased.

DISCUSSION

AD is characterized by the onset and development of cognitive impairments associated with aging. The pathological characteristics of AD include two main nidus types: senile plaques (SP) and neurofibrillary tangles (NFT). These niduses are produced by the aggregation and sedimentation of Aβ and the gathering of hyperphosphorylated tau protein [3]. Several lines of evidence support this hypothesis and it has been shown that Aβ peptides play a significant role in AD [10, 11]. There are numerous pathways associated with the development of AD in humans, including the Nrf2/ARE, NOS, and VEGF pathways. One of the well-known pathways is the Wnt/β-catenin signaling pathway [12, 13]. Studies have shown that the DKK1 expression level is rapidly upregulated by Aβ in hippocampal neurons, which inhibits canonical Wnt signaling and is accompanied by synapse loss. In contrast, β-catenin is involved in the downregulation of Aβ1–42 production. [14]. Thus, DKK1 may be a positive biomarker for the development of AD and β-catenin may be a negative biomarker. MiRNA disorders are associated with AD etiopathogenesis. Therefore, they have the potential to be critical indices for the diagnosis, staging, and prognosis of AD [15, 16]. One such miRNA is miR-124, which is concentrated in neurocytes and is an important regulator of the nervous system [17]. A previous study revealed that miR-16, which ameliorates induced neurovirulence by targeting BACE1, promotes AD pathogenesis [18].

MiR-26a-5p is located on human chromosome 3p22. Similar to most miRNAs, it elicits a variety of biological functions and participates in apoptosis and proliferation [19]. MiR-26a-5p can also improve the functional capacity of the heart and control myocarditis [20] and has been correlated with various tumor processes [21, 22]. MiR-26a-5p may also affect lung cancer cell metastasis via the ITGβ8-JAK2/STAT3 pathway [23] and its upregulation could inhibit breast cancer cell proliferation by regulating RNF6 expression [24]. Recently, miR-26a-5p was reported to play a dominant role in the central nervous system, suggesting that miR-26a-5p may have a therapeutic effect on AD symptoms [8]. However, the potential mechanisms underlying AD progression have not been thoroughly elucidated.

Aβ-Treated SH-SY5Y cell lines have been used as in vitro AD models in previous studies [25]. In our study, the SH-SY5Y cells were treated with two concentrations (5 or 10 nM) of Aβ and used in subsequent experiments to mimic AD. An expression analysis revealed that miR-26a-5p was highly expressed in AD cell lines. Its expression increased as the Aβ concentration rose, thereby showing a direct relationship between them. Based on these findings, other assays were conducted to investigate the effects of miRNA-26a-5p expression on AD pathogenesis. The MTT assay confirmed that miR-26a-5p negatively regulates the viability of the diseased cell lines. This result suggests that miR-26a-5p expression affects apoptotic biomarkers. The expression of vital apoptosis biomarkers was assessed using RT-qPCR to further understand AD pathogenesis. The results showed that Bcl-2 expression levels decreased, whereas Caspase-3 expression increased compared to the control. After determining that miR-26a-5p is highly expressed in diseased cells and negatively affects cell viability and Bcl-2 expression, knockdown of miR-26a-5p were created to acquire more information about the biological function of miR-26a-5p. As expected, anti-miR-26a-5p inhibited miR-26a-5p

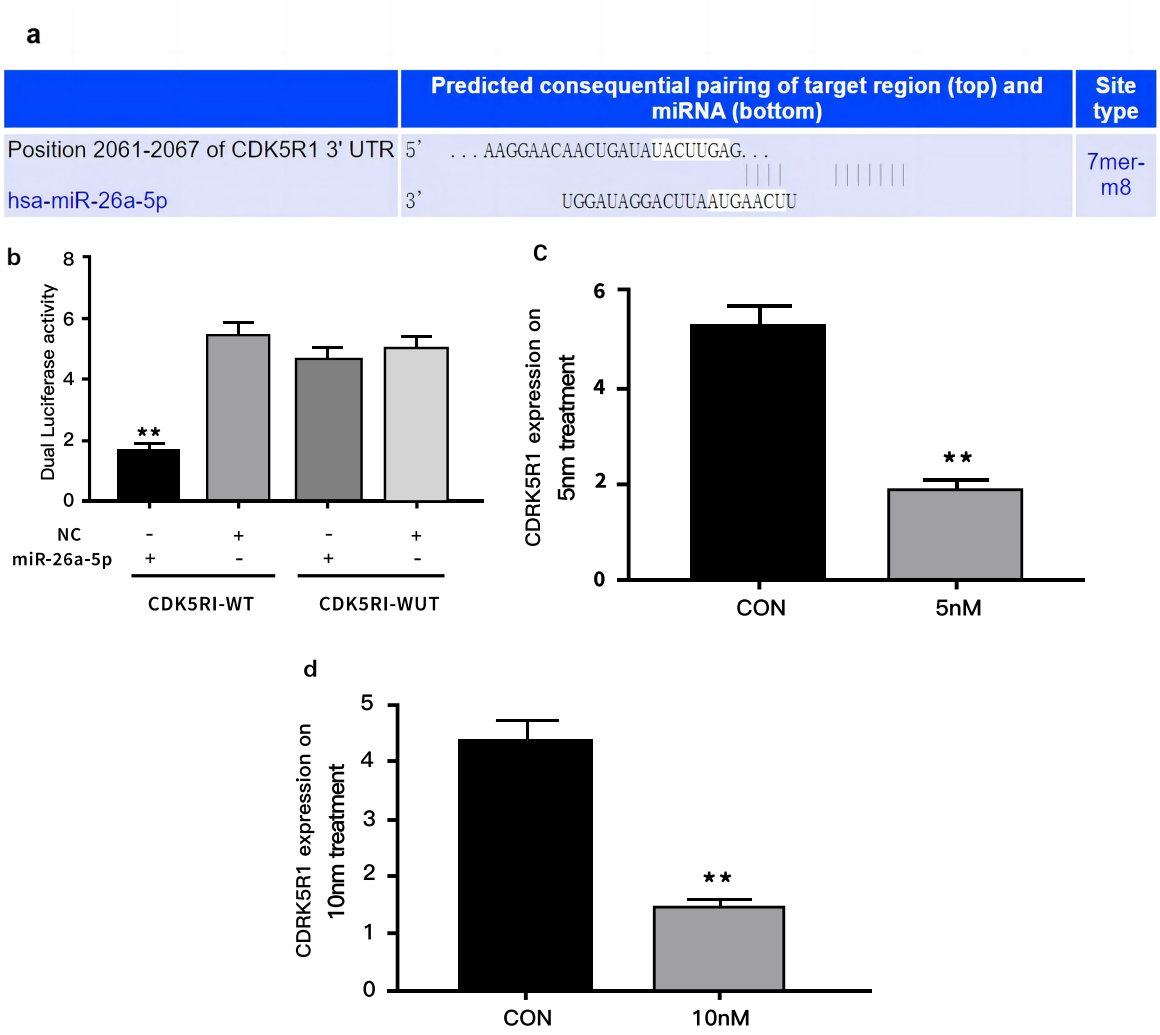

Fig. 3 CDK5R1 has a binding site for miR-26-5p. (a) Bioinformatic target scan. (b) Luciferase activity assay of treated Alzheimer's disease mimic cells after co-transfection with WT/MUT 3′-UTR and CDK5R1, miR-26a-5p mimics, or NC as indicated at 48 h post transfection. (c) Transcript levels of CDK5R1 in 5 nM, and (d) 10 nM Aβ-treated SH-SY5Y cells at 24 h. CON = SH-SY5Y cell; 10 nM = 10 nM Aβ-treated SH-SY5Y cells. ** $P < 0.01$.

function in the diseased cell lines and restored cell viability. In addition, Bcl-2 expression, which was initially low due to miR-26a-5p overexpression, was enhanced, whereas Caspase-3 expression was reversed.

CDK5R1 is the principal CDK5 catalyst and CDK5 plays a major role in development and functioning [26]. CDK5 dysfunction can lead to different pathological events implicated in AD pathogenesis. CDK5 does not display any kinase activity, but it is associated with its regulatory subunit, CDK5R1 [6]. CDK5R1 expression is regulated by multiple factors to ensure tight control of CDK5 activity. These include the transcription factor EGR1, repression by LSD1 demethylase [27, 28], and various post-transcriptional mechanisms that involve RNA-binding proteins and miRNAs [6, 29].

The molecular interaction between CDK5R1 and miR-26a-5p in AD cells was investigated by performing a bioinformatics target scan using a dual-luciferase reporter assay. The results confirmed that miR-26a-5p has a binding site for the candidate CDK5R1 mRNA. Therefore, an RT-qPCR assay was used to measure CDK5R1 expression to ascertain its role in AD development. The results revealed that CDK5R1 expression was downregulated in the Aβ-coped SH-SY5Y cells. We hypothesized that the upregulation of CDK5R1 would restore all the effects of miR-26a-5p. A co-transfection procedure was performed to create a mutant in which miR-26a-5p was suppressed and CDK5R1 was overexpressed. The hypothesis was confirmed because cell viability was restored by the suppression of miR-26a-5p and overexpression of CDK5R1. In addition, the RT-

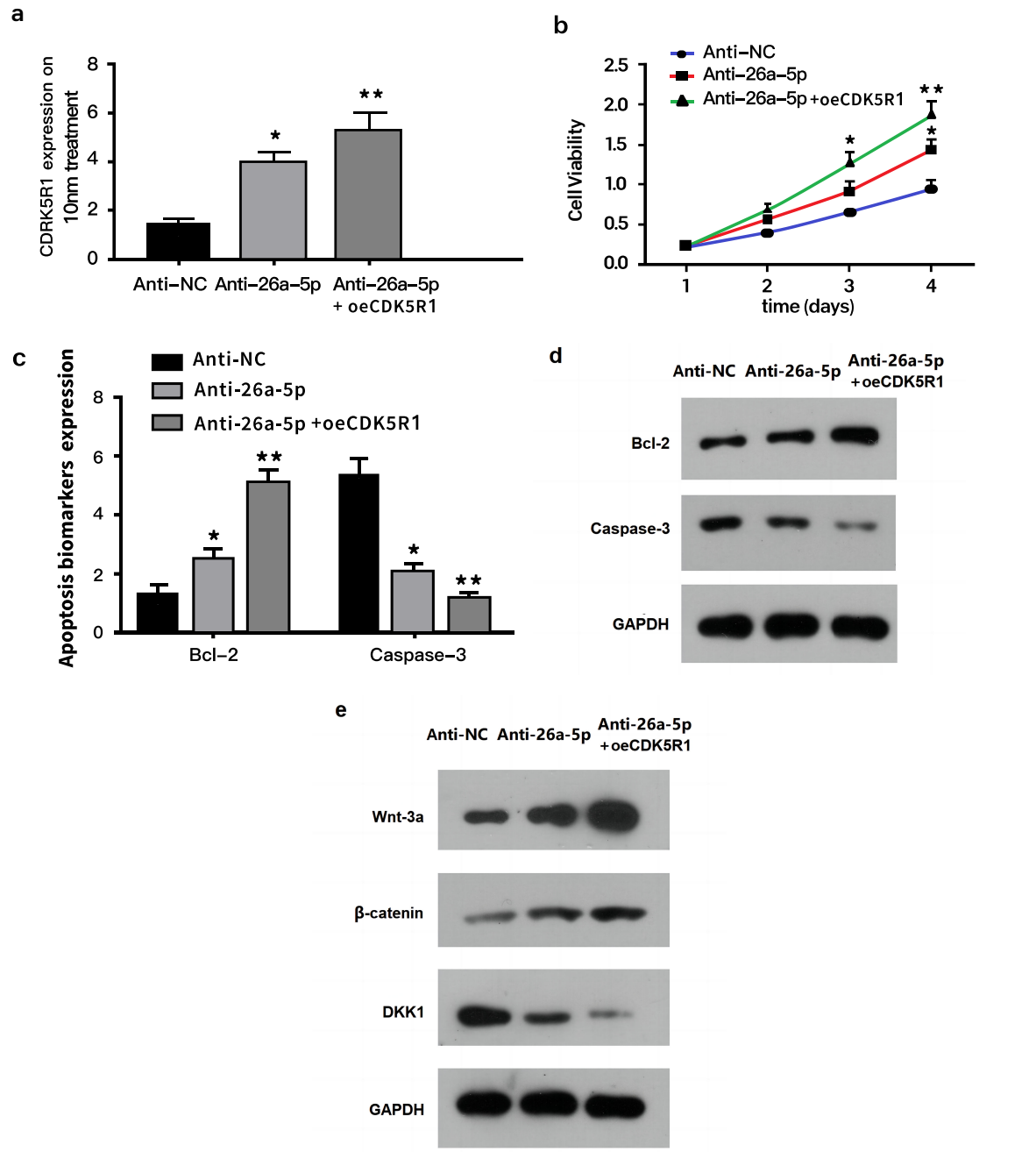

Fig. 4 Overexpression of CDK5R1 increases cell viability, Bcl-2, and the Wnt/β-catenin signaling pathway. (a) Transcript levels of CDK5R1 in AD cell lines using specific primers for miR-26a-5p over 48 h. CDK5R1 = over-expressed CDK5R1-WT. (b) Cell growth activity after transfection with Anti-NS, Anti-miR-26a-5p, or Anti-miR-26a-5p + oeCDK5R1 over 48 h. (c) Transcript levels of the biomarker genes, (d) protein expressions of apoptosis biomarkers, and (e) Wnt-3a, β-catenin, and DKK1 protein expressions after transfection over 48 h.

qPCR analysis showed that co-transfection restored the expressions of the apoptotic biomarkers. In our study, we observed a consistent trend in the expression levels of Bcl-2, Caspase-3, CDK5R1 proteins, and mRNA. Finally, the effects of miR-26a-5p and CDK5R1 on the Wnt/β-catenin signaling pathway proteins were examined. The Western blot analysis revealed that CDK5R1 overexpression coupled with miR-26a-5p suppression augmented Wnt-3a and β-catenin expression, whereas DKK1 levels decreased.

CONCLUSION

This study reveals that miR-26a-5p plays a crucial role in the progression of AD by controlling the expression of CDK5R1, which contributes to AD etiopathogenesis. Our results enhance understanding of AD pathogenesis and provide an effective way to treat AD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.083.

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Appendix A. Supplementary data

Table S1 Primer sequences.

Target	Direction	Primer sequence
miR-26a-5p	F	5′-UCCAUAAAGUAGGAAACACUACA -3′
	R	5′-CAG UAC UUU UGU GUA CAA-3′
CDK5R1	F	5′-GTTCAGCCCTCCGGGAGATTT-3′
	R	5′-GAGACCCTATCCGTGATTA-3′
Bcl-2	F	5′-TGAACCGGCATCTGCACAC-3′
	R	5′-CGTATTCAGACAGCCAGGAG-3′
Caspase-3	F	5′-CTGCCGGAGTCTGACTGGAA-3′
	R	5′-ATCAGTCCCACTGTCTGTCTCAATG-3′
GAPDH	F	5′-ATGGGGAAGGTGAAGGTCGG-3′
	R	5′-GACGGTGCCATGGAATTTGC-3′
U6	F	5′-CTCGCTTCGGCAGCACA-3′
	R	5′-AACGCTTCACGAATTTGCGT-3′

Table S2 Protein antibodies used for the Western blot analysis.

Proteins	Primary	Secondary
Bcl-2	Bcl-2 Monoclonal Antibody Thermo Fisher (China) Dilution ratio 1:1000	Goat anti-Mouse IgG (H+L) Secondary Antibody, Thermo Fisher, China Dilution ratio 1:900
Caspase-3	Caspase 3 Monoclonal Antibody, Thermo Fisher Scientific, USA Dilution ratio 1:1000	As above Dilution ratio 1:800
DKK1	DKK1 Monoclonal antibody, Abcam, China Dilution ratio 1:1000	As above Dilution ratio 1:900
Wnt-3a	WNT3A Polyclonal Antibody, Thermo Fisher Scientific, USA Dilution ratio 1:900	IgG H&L (HRP) Secondary Antibody, Abcam, China. Dilution ratio 1:2000
β-catenin	Anti-beta Catenin Monoclonal Antibody, Abcam, China. Dilution ratio 1:1000	As above Dilution ratio 1:2000