Abstract

Up-regulation of lncRNA WT1-AS ameliorates Aβ-stimulated neuronal injury through modulation of miR-186-5p/CCND2 axis in Alzheimer’s disease

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1. Introduction

As a kind of frequent neurodegenerative disease causing dementia, the clinical features of Alzheimer’s disease (AD) are progressive memory impairment, cognitive dysfunction, personality alterations, as well as language barriers [1]. Along with the aging population, the occurrence rate of AD is increasing every year. It is estimated that by 2050, there will be one AD patient in every 85 people [2]. AD, as the fourth killer completing with stroke, cardiovascular and cerebrovascular diseases and cancer, seriously endangers the physical and mental health of the aged, as well as results in a huge strain on the family together with the society [3]. The main pathological features of AD are senile plaque (the main component is β-amyloid (Aβ)) deposition of cerebral cortex and hippocampus, neurofibrillary tangles (the main component is phosphorylated tubulin tau protein), and partial loss of neural synapses [4]. The main mechanisms of AD include abnormal modification of some proteins, amyloid toxicity mechanisms, central cholinergic injury, microtubule-associated protein differentiation, immune function mutation and so on [5]. Aβ can cause damage to synaptic transmission function and memory loss. At the same time, it can also cause calcium overload, oxidative damage, neuronal loss, and mediate inflammatory response [6]. Aberrant secretion or immoderate production of Aβ will result in other pathological alterations of AD, thus Aβ is the core part of AD [7]. Due to the late onset of AD, there is no specific index to show the disease. The early stage of the disease is easy to ignore. When diagnosed, the patient has entered the late stage. Hence, it is essential to probe early diagnostic markers of AD.

Long non-coding RNAs (lncRNAs) are presented in the nucleus as well as cytoplasm, and exert critical effects on the physiological and pathological processes of AD through regulating the expression of related genes [8]. Evidences have suggested that lncRNAs acted as potential biomarkers in the therapy, diagnosis, as well as prevention of neurodegenerative diseases, AD included [9]. For instance, SOX21-AS1 silencing affected Aβ-stimulated neuronal damage through modulating miR-186-5p/CCND2 axis, offering a novel direction for AD therapy.
proliferation as well as apoptosis of human neuroblastoma cells [10]. Besides, EBF3-AS and EBF3 (early B cell factor 3) expressions were increased in APP/PS1 mice. EBF3-AS hindered the apoptosis of human neuroblastoma cells stimulated by Aβ_{25-35} [11]. Furthermore, NEAT1 depletion attenuated Aβ-stimulated viability inhibition, as well as apoptosis and p-Tau promotion. These findings mirrored that lncRNAs might be a novel therapy targeting for AD treatment.

Wilms tumor 1 Antisense RNA (WT1-AS), a novel discovered lncRNA, is located on chromosome 11p13 [12]. As reported previously, WT1-AS acted as tumor suppressors in many tumors [13, 14]. Besides, WT1-AS could reduce hypoxia/ischemia-stimulated neuronal damage in cerebral ischemic stroke [15]. Recent literature has revealed that WT1-AS was down-regulated in AD. Moreover, in vivo experiments uncovered that WT1-AS over-expression improved learning as well as memory abilities of AD mice. Nevertheless, the potential and underlying mechanisms of WT1-AS in AD are rare documented.

MicroRNAs (miRNAs) influence mRNA expression via binding to the 3' untranslated region (3'-UTR) of the target mRNA. MiRNAs are related to physiological processes of many diseases, AD included [16]. Additionally, some miRNAs have been identified as candidate diagnostic biomarkers for AD, such as miR-34c [17] and miR-331-3p [18]. Literature have found that miR-186-5p is highly expressed in AD [19], but its biological function in AD is still unclear.

Therefore, the current work focused on examining WT1-AS expression levels in AD serum samples and Aβ_{1-42}-induced human neuroblastoma cells, as well as probing the latent mechanisms of WT1-AS in AD.

2. Materials and methods

2.1. Clinical samples

Thirty AD serum samples and 30 healthy serum samples were acquired. The Ethics Committee had approved experimental procedures, and all patients signed written informed consents. The Chinese Clinical Trial Registry has approved this study (chiCTR2100047410).

2.2. Cell culture and transfection

Chinese Academy of Science (Shanghai, China) offered human neuroblastoma cell lines (SH-SY5Y together with SK-N-SH) and could be cultivated in MEM + F12 (1:1) medium and MEM medium containing ten percent fetal bovine serum (FBS) as well as 1% penicillin/streptomycin at 37°C with 5% CO₂.

For constructing in vitro AD model, human neuroblastoma cells were treated with 10 μM Aβ (MedChemExpress, USA) and introduced into cells (5 × 10⁴ cells per well) and treated with 10 μM Aβ for 24 h. Afterwards, 10 μL CCK-8 reagent (Beyotime, China) could be supplemented into each well. The viability at 450 nm absorbance could be evaluated with the microplate reader.

2.4. Flow cytometry

An Annexin V-FITC/PI apoptosis kit (Keygen, China) was implemented for assessing cell apoptosis [20]. Cell suspension was centrifuged and then re-suspended, and then dyed with Annexin V-FITC as well as propidium (PI). A flow cytometer (BD Biosciences, USA) was adopted for analyzing cell apoptosis.

2.5. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

TRizol reagent (Thermo Fisher, USA) could be adopted for extracting total RNA from serum sample as well as cells. Briefly, total RNA could be transcribed to cDNA with be help of M-MLV reverse transcriptase (Promega, USA), followed by qRT-PCR with the help of a Taqman PCR kit (Thermo Fisher, USA). The primer sets for each gene are indicated as follows. For WT1-AS, forward 5'-GCCCT TCTGTCTCCTTCTTTTGTT-3', and reverse 5'-GCTGTGAGCTCCTGTGCTTAG-3'. miR-186-5p forward, 5'-AAAGAAAATCTCCCCCTTGGGGGT-3', and reverse, 5'-GTGCCTGTCTGGAGTCTTCG-3'. CCND2 forward, 5'-CATCCCTCAAGGGCTTCGCTT-3', and reverse, 5'-CGGGCCTGTGTGGTATCGCGAGAAT-3'. U6 forward, 5'-CTCGTTCGCGACAGAACA-3', and reverse, 5'-AACAGCTTCAGAATTTGCGT-3'. β-actin forward, 5'-ATCACTGACCACCAAGAC-3', and reverse, 5'-TCTCTCTAGACAGCAGAGAG-3'. U6 and β-actin were adopted to be internal controls. Gene expression received calculation with the 2^-ΔΔCT method.

2.6. Western blot

Extraction of total proteins was implemented with the help of RIPA Lysis and Extraction Buffer (Thermo Fisher, USA), followed by separating through SDS-PAGE. Afterwards, proteins were shifted into the PVDF membranes (Beyotime, China). The membranes received treatment with the primary antibodies at 4°C overnight containing Cleaved-caspase-3 (1: 1, 000, ab32042, Abcam, USA), anti-p-Tau (1: 2, 000, 28866-1-AP, Proteictech, China), as well as β-actin (ab8226, 1: 1, 000) after blocking with skimmed milk. The membranes received treatment with the secondary hors eradish peroxidase (HRP) antibodies (Sigma, Aldrich) for 60 min at 4°C, followed by observation with the Image Lab (Bio-Rad Laboratories, USA).

2.7. Dual-luciferase reporter gene assay

The sequence of wild type WT1-AS (WT1-AS-WT) and 3'-UTR of CCND2 (CCND2-WT), mutant type WT1-AS (WT1-AS-Mut) along with CCND2 (CCND2-Mut) were cloned into pmirGLO reporter vector (Genscript, China). Then, the vectors were treated in combination with miR-186-5p mimic or NC into cells with the help of Lipofectamine 3000 (Invitrogen, USA).
2.8. Statistical analysis

All the above experiments were implemented three times. Data were analyzed with the help of GraphPad Prism 5.0 and presented as mean ± SD. Differences received comparison using one-way ANOVA and Turkey’s post host. \( P < 0.05 \) represented statistical significance.

3. Results

3.1. WT1-AS presented low expression in serum samples from AD patients and in human neuroblastoma cells induced by Aβ1-42

In order to evaluate the WT1-AS expressions in AD, a total of 30 serum samples were gathered. It was revealed that WT1-AS presented low expression in AD serum samples compared with that in healthy serum samples (Figure 1A). Next, WT1-AS expression was measured in Aβ1-42-stimulated AD model. As displayed in Figure 1B, WT1-AS expressions were decreased in human neuroblastoma cells in a concentration-dependent way. Notably, the reduced fold change of WT1-AS expression was the most in human neuroblastoma cells when treating 10 μM of Aβ1-42, thus we chose 10 μM for subsequent analyses. Moreover, WT1-AS levels were decreased in 10 μM Aβ1-42-stimulated human neuroblastoma cells in a time-dependent way. Besides, the CCK-8 assay indicated that Aβ1-42 (10 μM) notably depleted the proliferation of human neuroblastoma cells at 24 h (Figure 1C).

3.2. Up-regulated WT1-AS enhances human neuroblastoma cells proliferation while reduces cell apoptosis of induced by Aβ1-42

To certify the impacts of WT1-AS on AD progression, pc-WT1-AS was transfected into Aβ1-42-induced human neuroblastoma cells to elevate WT1-AS expression (Figure 2A). Then CCK-8 assay uncovered that Aβ1-42 obviously repressed human neuroblastoma cell proliferation, while WT1-AS elevation promoted human neuroblastoma cell proliferation induced with Aβ1-42 (Figure 2B). In addition, based on flow cytometry assay, Aβ1-42 obviously induced the apoptosis of human neuroblastoma cells, while WT1-AS over-expression reversed this phenomenon (Figure 2C). Moreover, western blot analysis displayed that Aβ1-42 elevated p-Tau as well as Cleaved-caspase-3 levels, while WT1-AS over-expression decreased the p-Tau as well as Cleaved-caspase-3 levels in human neuroblastoma cells stimulated by Aβ1-42 (Figure 2D).

3.3. MiR-186-5p binds to WT1-AS and exerts effects on proliferation and apoptosis of human neuroblastoma cells

Through bioinformatics tools, miR-186-5p could be speculated to possess the target domain of WT1-AS (Figure 3A). Then dual-luciferase reporter analysis unveiled elevated miR-186-5p distinctly lessened the luciferase intensity of WT1-AS-WT, whereas rarely affected WT1-AS-Mut activity (Figure 3B). Moreover, as uncovered in Figure 3C and 3D, miR-186-5p levels were obviously up-regulated. Furthermore, Figure 3E suggested that WT1-AS elevation declined miR-186-5p expression. Next, miR-186-5p inhibitor was introduced into Aβ1-42-induced human neuroblastoma cells, and the inhibition transfection efficiency could be verified through qRT-PCR analysis (Figure 3F). It was displayed that miR-186-5p inhibitor promoted Aβ1-42 stimulated human neuroblastoma cell proliferation (Figure 3G). In addition, flow cytometry assay implied that miR-186-5p inhibitor reduced Aβ1-42 stimulated human neuroblastoma cells apoptosis (Figure 3H). Moreover, western blot analysis displayed that miR-186-5p inhibitor down-regulation decreased the p-Tau along with Cleaved-caspase-3 levels in human neuroblastoma cells stimulated by Aβ1-42 (Figure 3I).

3.4. CCND2 is targeted by miR-186-5p

Based on bioinformatics tools, it was shown that a complementary sequence of miR-186-5p and CCND2 3’-UTR (Figure 4A). Then, the dual-luciferase experiment confirmed that miR-186-5p elevation lessened the luciferase
obviously downregulated in AD serum samples and cells intensity of CCND2 WT, whereas barely affected CCND2 Mut activity (Figure 4B). Moreover, CCND2 levels were obviously downregulated in AD serum samples and cells shown in Figures 4C and 4D. Finally, as displayed in Figure 4E, CCND2 levels were over-expressed in human neuroblastoma cells after miR-186-5p suppression.

3.5. WT1-AS influences Aβ_{1-42}-stimulated human neuroblastoma cell proliferation along with apoptosis by miR-186-5p/CCND2 axis

To further verify whether WT1-AS exhibited its potential via modulating miR-186-5p/CCND2 axis, we implemented rescue assays. Firstly, human neuroblastoma cells were introduced with miR-186-5p mimic or pc-CCND2, respectively to elevate miR-186-5p or CCND2 expression (Figure 5A). As revealed in Figure 5B, miR-186-5p mimic partially reduced the promoting role of WT1-AS over-expression human neuroblastoma cells proliferation stimulated with Aβ_{1-42}, while CCND2 showed the opposite effects. MiR-186-5p mimic partially rescued the inhibitory role of WT1-AS over-expression on SH-SY5Y cells apoptosis induced with Aβ_{1-42}, while CCND2 showed the opposite effects (Figure 5C). Furthermore, miR-186-5p up-regulation partially restored the suppressive impacts of pc-WT1-AS on p-Tau as well as Cleaved caspase-3 expression, while CCND2 up-regulation exhibited the opposite effects (Figure 5D).

4. Discussion

With the in-depth study of lncRNAs, more and more researchers have shifted from the changes of lncRNA expressions in various diseases to the study of lncRNA interactions of WT1-AS with CCND2 and their functions in AD. Accordingly, we conducted the study of WT1-AS influence on the Aβ_{1-42} neurotoxicity through modulating miR-186-5p/CCND2 axis.
functions and mechanisms [18]. In the study of blood and other tissues of AD patients, lncRNAs with different tables have been found continuously [21]. For example, lncRNA BACE1 levels presented up-regulation in AD patient’s plasma. Accordantly, it was also validated that plasma BACE1 level presented elevation in AD patients [22]. Based on these, lncRNAs have great potential value as diagnostic markers in treating AD. Similarly, our research showed that WT1-AS was lowly expressed in AD serum samples, which was in consistent with previous study. These findings mirrored that lncRNAs might exert a vital role in progression of AD.

Aβ-treated human neuroblastoma cells have been reported for establishing AD models in vitro [23]. Consistently, an AD model in human neuroblastoma cells was also constructed through exposure of Aβ1-42. As expected, Aβ1-42 treatment inhibited the proliferation and induced apoptosis. Besides, Accumulating data has supported that Tau is a key element of AD pathophysiology [24]. In this research, we discovered that Aβ1-42 treatment promoted p-Tau expression in human neuroblastoma cells. Functionally, former studies have reported that WT1-AS is implicated in tumor cell proliferation and apoptosis. Herein, WT1-AS over-expression notably elevated the proliferation, lessened the apoptosis, as well as decreased p-Tau protein expressions in Aβ1-42 stimulated human neuroblastoma cells, which were in line with the reports about other lncRNAs on AD progression. For instance, MAGI2-AS3 levels were increased in Aβ1-42 treated SH-SY5Y cells. MAGI2-AS3 depletion elevated neuronal viability while declined neuroinflammation in AD cell models [25]. Besides, BACE1-AS levels were up-regulated in human neuroblastoma cells upon Aβ1-42 treatment. BACE1-AS silence relieved Aβ1-42-stimulated cell injury [26]. In addition, Aβ1-42-stimulated human neuroblastoma cell viability whereas promoted cell apoptosis. SNHG1 depletion partially offsets the effects of Aβ1-42 induction on cell viability and apoptosis. Report of Wang et al. reported that WT1-AS over-expression could repress OSI and apoptosis [27]. All above findings manifested that WT1-AS might act as a pathogenic factor in AD.

LncRNAs have been registered to serve as ceRNAs via regulating miRNA expression in AD progression. For example, BACE1-AS reduction rescued the suppressed proliferation and enhanced apoptosis of Aβ1-treated human neuroblastoma cells stimulated by Aβ through regulating miR-214-3p. Besides, down-regulated NEAT1 reduced Aβ-stimulated inhibited viability as well as increased apoptosis and p-Tau levels via negatively regulating miR-107 [28]. More importantly, WT1-AS has also been proven to participate in the progression of diverse diseases via interacting with miRNAs [29, 30]. In line with the above literature, miR-186-5p was predicted to interact with WT1-AS in this research. MiR-186-5p has been documented to repress esophageal squamous cell carcinoma cell proliferation as well as gastric cancer cells aerobic glycolysis [31, 32]. Moreover, miR-186-5p has been confirmed to be decreased in the aged brain, which implies that miR-186-5p might be related to AD development [33]. As expected, miR-186-5p levels were confirmed highly expressed in AD serum samples and human neuroblastoma cells induced by Aβ1-42. Functionally, miR-186-5p inhibitor notably enhanced the proliferation, lessened the apoptosis, as well as decreased the p-Tau protein expressions in Aβ1-42 stimulated human neuroblastoma cells.

Mechanistically, miR-186-5p over-expression partially restored the impacts of WT1-AS over-expression on biological activities of Aβ1-42-stimulated human neuroblastoma cells. MiRNAs exhibit functional impacts usually through modulation of downstream targets in AD [34]. Consistently, our research predicted that miR-186-5p bond to CCND2 3′-UTR, which unveiled the potential of CCND2 to be the miR-186-5p target, and dual-luciferase reporter analysis further confirmed this prediction. CCND2, as a member of the cyclin proteins that regulate the progression of cells. CCND2 has been reported to modulate the progression of neurodegenerative diseases, AD included. For instance, tetrahydrocurcumin ameliorated Alzheimer’s pathological phenotypes by regulating CCND2 [35]. In our research, CCND2 levels were presented reduced in AD serum samples as well as human neuroblastoma cells induced with Aβ1-42. Moreover, the modulatory mechanisms of WT1-AS/miR-186-5p/CCND2 in AD progression were investigated by rescue assays. As expected, CCND2 up-regulation partially restored miR-186-5p mimic functions in bioactivities of Aβ1-42-induced human neuroblastoma cells transfected with pc-WT1-AS.

Additionally, our paper also presented some shortcomings. First of all, the samples of our study were relatively small. Besides, whether WT1-AS/miR-186-5p/CCND2 axis affected AD progression via certain signaling pathways was not investigated. Therefore, more studies will be performed in the future.

To sum up, WT1-AS and CCND2 levels were decreased, while miR-186-5p expression presented elevation in AD serum samples as well as human neuroblastoma cells induced by Aβ1-42. Moreover, WT1-AS up-regulation notably promoted the proliferation, reduced the apoptosis, and decreased the p-Tau protein expressions in Aβ1-42 stimulated human neuroblastoma cells, which might be achieved by regulating miR-186-5p/CCND2, indicating a novel avenue for AD therapy.

Informed Consent
The authors report no conflict of interest.

Availability of data and material
We declared that we embedded all data in the manuscript.

Authors’ contributions
TY conducted the experiments and wrote the paper; ZX, WS, LL, WQ, LY and YY analyzed and organized the data; ZQ conceived, designed the study and revised the manuscript.

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References
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