Original Research Article

MicroRNA miR-103a-3p targets NPAS3 to regulate progression of Alzheimer's disease

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Abstract

Purpose: This study aimed at investigating miR-103a-3p expression, functional roles and underlying mechanism in regulating Alzheimer's progression.
Methods: RT-qPCR was used to assessed miR-103a-3p and NPAS3 expression in human neuroblastoma cells. Cell transfection of overexpressed or knocked down genes and CCK-8 assay measured cell viability while RT-qPCR was used to detect proliferation and apoptosis in biomarkers, Ki87 and PCNA, caspase-8 and caspase-3, respectively. Furthermore, luciferase assay was used to evaluate the luciferase activity while western blotting analysis was applied to determine protein biomarkers regarding proliferation and apoptosis.
Results: Expression of miR-103a-3p decreased but NPAS3 increased in AD cell lines. Overexpressed miR-103a-3p attenuated cell viability and NPAS3 bound miR-103a-3p to regulate AD progression. The inhibitory effect of miRNA on cell viability in AD was reversed by NPAS3.
Conclusion: miR-103a-3p/NPAS3 might help to enrich knowledge on treatment of AD.

Keywords: Alzheimer's development, cell growth, cell proliferation

INTRODUCTION

Alzheimer's disease (AD) is an irremediable brain ailment that mostly affects 60-year-old or older people. Aberrant plasma amyloid beta peptides 1-42 (Aβ42) have been reported as AD biomarkers which induce vascular dysfunction, impaired synaptic transmission and plasticity [1-4]. The dysregulation of microRNAs (miRNAs) presents an opportunity to explore therapeutic miRNAs in AD [5-7]. Studies have focused on seeking potential AD therapy from a biomedicine perspective to identify molecular therapeutic targets in AD [8].

The microRNA, *MiR-103a-3p*, acts as a tumor suppressor or an oncogene in cancer contexts [9]. As such, the roles of *miR-103a-3p* have been investigated in several disorder including cancers and other diseases such as glioma stem cells [10], bladder carcinoma [11], and glioma angiogenesis [12]. However, the role of *miR-103a-3p* in AD are yet to unveiled. The gene, Neuronal PAS Domain Protein 3 (NPAS3) is

related to multiple human psychiatric and neurodevelopmental disorders [13]. It is known to regulate neural cell viability, affecting proliferation of neural cells through VGF [14] and was recognized in major mental issues including AD [15]. However, its molecular mechanism in AD is unknown.

In this study, it was hypothesized that *miR-103a-3p* could regulate NPAS3 expression in AD which might provide a basis for AD therapy. Although recent reports have highlighted the vital role of *miR-103a-3p* in development and advancement of a variety of cancers, diseases and even disorders [10-12,17,18], *miR-103a-3p* functions have never been addressed before in AD pathogenesis and progression. Therefore, this study is aimed at investigating functional roles of *miR-103a-3p* in AD in vitro and the interactions and co-effects of *miR-103a-3p* and NPAS3 in regulating AD progression.

EXPERIMENTAL

Cell culture and transfection

The human neuroblastoma cell line, SH-SY5Y (ATCC, Beijing, China) was maintained in RPMI-1640 with 10 % fetal bovine serum (FBS) (Gibco, Life Technologies, China) and 100 µg/mL penicillin streptomycin with density of 2×10^5 cells per mL. After 48hrs, the substrate was superseded with Dulbecco's Modified Eagle's Medium (DMEM) with 10 % FBS coupled with 5 µM rat astrocytes (RA) for neuronal segregation. The cells were then maintained in a humidified atmosphere at 37˚C with 5 % CO_2 in an incubator. Cell transfection of SH-SY5Y cells was done using Lipofectamine 2000 (Beyotime, Shanghai, China) to transfect *miR-103a-3p* mimics or negative control mimics (Guangzhou Fulengen Co. Ltd., China) following the manufacturer's guidelines. Then, the cells were treated with 10 µM amyloid beta Aβ42 oligomer at different time periods (0h-72h) and prepared as previously described [2].

RT-qPCR

Total RNA was isolated from cell free fractions of cerebrospinal fluid and plasma samples using Beyozol mixture (#R0011, Beyotime, Shanghai, China). Then reverse transcription of 1 µg RNA was done for each specimen to cDNA using BeyoRT™ cDNA First Chain Synthesis Kit (#D7166, Beyotime, Shanghai, China) following guidelines provided by the manufacturer. Beyofast™ SYBR Green QPCR Mix and associated mRNA qRT-PCR detection kit (Beyotime, China) were used to measure *miR-103a-3p* and NPAS3 expression, respectively using an Applied Biosystems Vii7 RT-qPCR instrument (ABI, Vernon, CA, USA) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control with $2^{-\Delta\Delta CT}$ method. The primers synthesized by Genelily BioTech Co., Ltd (Shanghai, China) used in this study are summarized in Table 1. All experiments were conducted in triplicates.

Table 1: Primer sequence

Name	Sequences
miR-103a-3p	
forward	5'-GCTTCTTTACAGTGCTGCCT-3'
reverse	5'-TTCATAGCCCTGTACAATGCT-3'
NPAS3	
forward	5'-ATGGCGCCCACCAAGCCC-3'
reverse)	5'-GTCCTCCTTGCGCTCCAGAGT-3'
GAPDH	
forward	5'-AGAAGGCTGGGGCTCATTTG-3'
reverse	5'-AGGGGCCATCCACAGTCTTC-3'

CCK-8 assay

The transfected cells were planted into 96-well multiplying plates and cultured between 0 to 48 hours. Cell viability was assessed by Cell Counting Kit (#C0037, Beyotime, Shanghai, China), following standard protocol specified by manufacturer. Absorbance was measured using the microplate reader (Molecular Devices, CA, USA) at 450 nm.

Western blotting assay

Western blot was to detect the protein expression of cell cycle and apoptosis-related biomarkers, including Ki67, PCNA, caspase-3 and caspase-8. Human neuroblastoma cell line, SH-SY5Y, was exposed to Aβ-42 IL-1β at different times and then the proteins were extracted and washed twice with cold PBS and lysed in sample loading buffer with mix of 1.5% sodium dodecylsulfate (SDS), 10% glycerol, 5 mM β-mercaptoethanol, bromophenol blue and 75 mM Tris (pH 7.0). Cell lysates were split by SDS-PAGE using 12% gel and the proteins were moved onto a polyvinylidene fluoride membrane. Additionally, the membranes were nurtured and probed with the following antibodies: Ki67, PCNA, caspase-3 and caspase-8 enlisted in the Table 2 under the temperature of 4°C overnight. The immunoblots were established and seen by ECL Western blot medium (Thermo Fisher Scientific, Shanghai, China) using U6 and GAPDH as internal control. The analysis of each group was repeated three times and the image J detection system was employed to determine the concentration of the bands.

Table 2: Antibodies

Protein examined	Primary antibody	Secondary antibody
PCNA	Rabbit Polyclonal, (PA5-27214) (ThermoFisher, Scientific, NY, US) diluted at 1:1000.	Goat anti-Rabbit IgG(1:1000,Thermo Fisher, Scientific, NY, US)
Ki-67	Rabbit polyclonal to Ki67 (ab15580), (Abcam, Cambridge, US) diluted at 1:900.	
Caspase-3	Rabbit polyclonal to Caspase-3 (ab13847) (Abcam, Cambridge, UK) diluted at 1:1000.	
Caspase-8	Rabbit polyclonal to Caspase-8 (ab25901), Abcam, Cambridge, UK) diluted at 1:1000.	
GAPDH	Mouse monoclonal (6C5) to GAPDH - Loading Control, (ab8245, Abcam, Cambridge, UK) dilution rate 1:2000.	

Bioinformatics analysis

The target putative binding sites for *miR-103a-3p* and NPAS3 are shown on TargetScan (http://www.targetscan.org/vert_72/).

Luciferase assay

The target sequence NPAS3 with the wild type (WT) or mutant type (MT) *miR-103a-3p* binding locations were synthesized and cloned into a pGL3Vector (Promega, USA), to construct WT and MT NPAS3 plasmids. These WT or MT NPAS3 plasmids were transfected into SH-SY5Y along with NC mimics or *miR-103a-3p* mimic (Sigma-Aldrich) using Lipofectamine 8000. After 48h, luciferase assay was conducted with Dual-Luciferase Reporter kit (Promega) following protocol by the manufacturer.

Statistical analysis

The experiments were conducted separately three times and the data presented as mean and standard error (SE). Student's t-test, and ANOVA analyses were conducted as appropriate. At 95% confidence interval, $p < 0.05$ was considered to be significant.

RESULTS

MicroRNA, miR-103a-3p, was poorly expressed but promoted cell viability in AD-mimic cells

The results of gene expression determined in SH-SY5Y treated with Aβ42 at different time periods (0h-72h) using RT-qPCR. indicated downregulated *miR-103a-3p* in the human neuroblastoma cell line treated with Aβ42 compared with the control group (Figure1A, $p < 0.05$). However, the decrease, improved with time and the lower expression was observed after 72 hours. Furthermore, following CCK-8 assay, *miR-103a-3p* in AD-like cells was

significantly increased with time compared with the untreated cells (Figure 1B, $p < 0.05$). However, the highest level of expression was observed after 72 hours. To confirm proliferation, RT-qPCR was to assess proliferation of the biomarkers (Ki67 and PCNA) which showed an increase with time (Figure1C, $P < 0.05$). However, the decrease of caspase-3 and caspas-8 was observed in Aβ42 treated SH-SY5Y cells for AD over time (0h-72h) compared the untreated SH-SY5Y cells (Figure 1D, $p < 0.05$). The Aβ42 treated SH-SY5Y cell line was adopted for further experiments. However, no significant difference was observed for 48h and 72h treated cells hence further experiments were conducted for treated cells from 0h-48h.
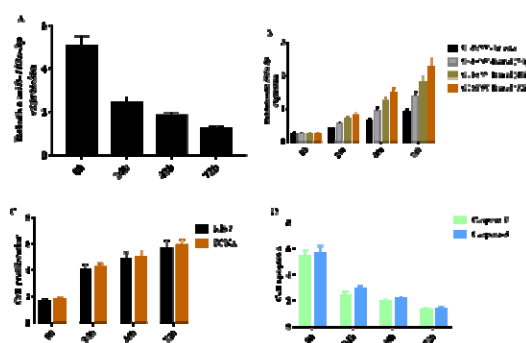
Figure 1: MicroRNA, *miR-103a-3p*, down regulation and promotion of cell viability in AD-mimic cells. A) RT-qPCR examined *miR-103a-3p* expression in human neuroblastoma cell lines (SH-SY5Y) treated with Aβ42 at and untreated cells varying time periods ($p < 0.05$). B) CCK8 examined cell viability based on *miR-103a-3p* expression in human neuroblastoma cell lines (SH-SY5Y) treated with Aβ42 and untreated cells at varying time periods at varying time periods ($p < 0.05$). C) RT-qPCR examined expression of proliferation biomarkers Ki67 and PCNA in untreated and Aβ42 treated SH-SY5Y cell lines at varying time periods ($p < 0.05$). D) RT-qPCR examined expression of apoptosis biomarkers caspase-8 and caspase-3 in untreated and Aβ42 treated SH-SY5Y cell lines at varying time periods ($p < 0.05$)

MicroRNA, miR-103a-3p, overexpression suppresses cell viability of AD cells

When AD cells were transfected with either control mimics or mimics of *miR-103a-3p* at varying times (12h-48) to explore changes in cell viability, RT-qPCR confirmed the transfection efficacy and results showed significant increased trend for mimics group at different times (12h-48h) (Figure 2A, p<0.05). Using CCK-8 analysis to evaluate the influence of *miR-103a-3p* on cellular proliferation in AD, the results showed decreased cell viability for miR-103a-3p mimics transfected cells compared to mimics-NC group after 12h (Figure2B, P<0.05). After 24h, results demonstrated a significantly lower cell viability when miRNA was enhanced in AD cells (Figure 2C, p<0.05). Lastly, after 48h the cell viability was significantly the lowest for miR-103a-3p mimics transfected cells compared to mimics-NC group (Figure 2D, p<0.05). These data implied that overexpressed *miRNA* inhibited cell viability of AD cells.
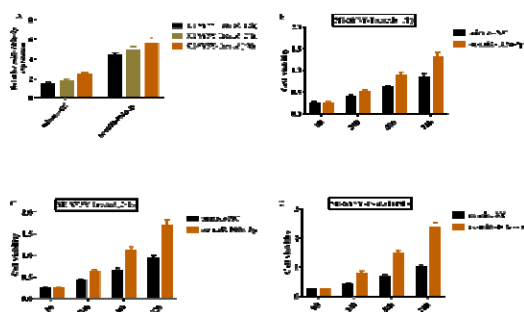

Figure 2. Suppression of cell viability of AD cells by microRNA, *miR-103a-3p,* overexpression. A) RT-qPCR examined detected *miR-103a-3p* overexpression efficiency in Aβ42 treated SH-SY5Y cell lines at varying time periods. B) CCK8 examined cell viability based on upregulated *miR-103a-3p* expression in Aβ42 treated SH-SY5Y cell lines after 12h (p<0.05). C) CCK8 examined cell viability based on upregulated *miR-103a-3p* expression in Aβ42 treated SH-SY5Y cell lines after 24h (p<0.05). D) CCK8 examined cell viability based on upregulated *miR-103a-3p* expression in Aβ42 treated SH-SY5Y cell lines after 48h (p<0.05). oe means overexpressed

MicroRNA, miR-103a-3p, directly targeted NPAS3 to regulate AD progression

When TargetScan (http://www.targetscan.org/vert_72/) was referred to in search for the putative target gene of miR-103a-3p as well as predicted binding, the predicted binding positions is shown in Figure 3A. Thereafter, luciferase reporter vector having Wild Type or Mutant Type *miR-103a-3p* bonded sites in NPAS3 were established in order to confirm the connection.

Subsequently, control mimics or *miR-103a-3p* mimic were transfected to Wild Type or Mutant Type in order to confirm the luciferase activity which indicated remarkable reduced luciferase activity for WT-NPAS3 in *miR-103a-3p* mimic transfected Aβ42 treated SH-SY5Y cells compared with control mimics (Figure 3B, P<0.05). However, no influence was noticed in rest groups (Figure3B, P<0.05).). As determined by Real Time-qPCR, the expression level of NPAS3 also dramatically increased with time (0h-48h) in the AD-like cells (Figure 3C, P<0.05).
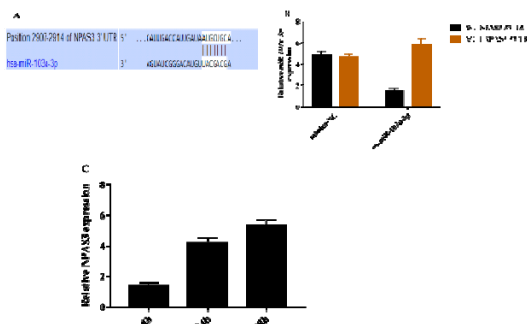

Figure 3: NPAS3 is directly targeted by *miR-103a-3p* to regulate AD progression. A) Bioinformatics analysis tools (TargetScan) produced target putative binding sites for *miR-103a-3p* and NPAS3. B) Dual luciferase reporter confirmed the actual binding sites in WT and MUT NPAS3 luciferase activity after transfection with *miR-103a-3*p mimic. C) D) RT-qPCR examined NPAS3expression in untreated and Aβ42 treated SH-SY5Y cell lines at varying time periods (p<0.05). oe means overexpressed

Upregulated miR-103a-3p abolishes the effect of NPAS3 in AD-like cells

In the above sections, NPAS3 was upregulated and targeted by *miR-103a-3p* in AD cell line (SH-SY5Y) treated with Aβ42. As such, the underlying molecular mechanisms among NPAS3 and *miR-103a-3p* were further explored by CCK-8 assay on cellular viability. Firstly, cells were transfected for miR-103a-3p upregulation and mixture of miR-103a-3p mimics +oe-NPAS3. RT-qPCR indicated reduced NPAS3 expression when miR-103a-3p increased and partially recovered NPAS3 expression in the mixed group cells (Figure 4A, p<0.05). In addition, CCK-8 assay results indicated significant reduction in cell viability in *miR-103a-3p* mimics+oe-NC group compared to the control and restored in *miR-103a-3p* mimics+oe-NPAS3 group after 48h (Figure 4B, P<0.05). The proliferation biomarkers verified the results for both Ki67 and PCNA after performing the RT-qPCR assay. Results for both Ki67 and PCNA agreed with the above cell viability findings (Figure 4C, P<0.05). The

apoptosis biomarkers were confirmed by RT-qPCR and showed apoptosis was significantly increased in *miR-103a-3p* overexpressed group and partly suppressed by increased NPAS3 for both caspase-8 and caspase-3 (Figure 4D, P<0.05). Additionally, Western blotting checked Ki67 and PCNA protein expression in different groups which indicated that Ki67 and PCNA protein expression was significantly reduced when *miR-103a-3p* was upregulated and NPAS3 increase restored the proliferation protein levels (Figure 4E, P<0.05). The Western blotting also indicated that apoptosis protein expression was significantly increased in *miR-103a-3p* overexpressed cells and to some degree, inhibited in the combined group (Figure 4F, P<0.05). These results showed that combining overexpressed-NPAS3 restored the restraining ability of cellular viability by *miR-103a-3p* mimics.
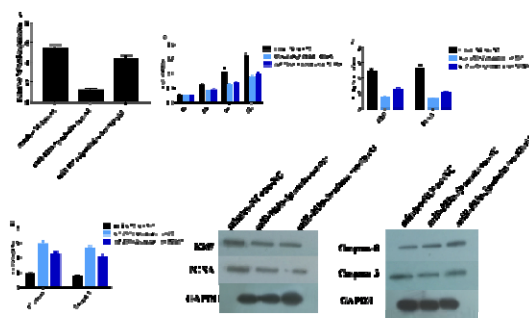
Figure 4: Abolish proliferative effect of NPAS3 in AD cells by upregulated miR-103a-3p. A) RT-qPCR examined relative expression of NPAS3 in Aβ42 treated SH-SY5Y cell lines after 48h transfected with either mimics-NC+oe-NC, miR-103a-3p mimics +oe-NC or miR-103a-3p mimics +oe-NPAS3 (p<0.05). B) CCK8 examined cell viability in in Aβ42 treated SH-SY5Y cell lines after 48h transfected with either mimics-NC+oe-NC, miR-103a-3p mimics +oe-NC or miR-103a-3p mimics +oe-NPAS3 (p<0.05). C) RT-qPCR examined relative expression of Ki67 and PCNA in Aβ42 treated SH-SY5Y cell lines after 48h transfected with either mimics-NC+oe-NC, miR-103a-3p mimics +oe-NC or miR-103a-3p mimics +oe-NPAS3 (p<0.05). D) RT-qPCR examined relative expression of caspase-3 and caspase-8 in Aβ42 treated SH-SY5Y cell lines after 48h transfected with either mimics-NC+oe-NC, miR-103a-3p mimics +oe-NC or miR-103a-3p mimics +oe-NPAS3 (p<0.05). E) Western blot examined relative expression of Ki67 and PCNA Aβ42 treated SH-SY5Y cell lines after 48h transfected with either mimics-NC+oe-NC, miR-103a-3p mimics +oe-NC or miR-103a-3p mimics +oe-NPAS3. F) Western blot examined relative expression of caspase-3 and caspase-8 in Aβ42 treated SH-SY5Y cell lines after 48h transfected with either mimics-NC+oe-NC, miR-103a-3p mimics +oe-NC or miR-103a-3p mimics +oe-NPAS3 (p<0.05). oe means overexpressed.

DISCUSSION

The RT-qPCR assay in this study has shown that downregulated expression of miR-103a-3p in the human neuroblastoma cell line treated with Aβ42 significantly increased with increasing time compared with the untreated SH-SY5Y cells for AD. It was further demonstrated that this decrease promoted cell viability increased with time after performing CCK-8 assays and validating with proliferation biomarkers and apoptosis biomarkers. As such downregulated *miR-103a-3p* expression in AD-mimic cells enhanced cell viability and a notable pathological apoptosis. However, *miR-103a-3p* overexpression suppressed cell viability of AD cells. This was confirmed when the CCK-8 assay was performed after upregulating *miR-103a-3p* with observed significant reduced cell viability at varying time increments. The proliferation biomarkers confirmed the reduced cellular viability by both Ki67 and PCNA. Apoptosis was increased when *miR-103a-3p* was upregulated in both caspase-8 and caspase-3. These results implied that *miR-103a-3p* was down regulated and its overexpression restrained cell viability of AD cells.

It is widely believed that miRNAs can exert their functions by regulating the expression of target genes [19]. Targescan predicted putative binding positions between *miR-103a-3p* and NPAS3. It was confirmed that *miR-103a-3p* directly targeted the 3'-UTR of NPAS3 to regulate NPAS3 expression and AD progression. Additionally, the expression level of NPAS3 was found to increase with time in AD cell lines suggesting that *miR-103a-3p*/NPAS3 interplay could be a potential novel treatment target for AD. Furthermore, the restoration experiment demonstrated the interplay between *miR-103a-3p* and NPAS3. Thus, when *miR-103a-3p* was overexpressed, it abolished the proliferation ability of NPAS3 which was also verified by CCK-8 assay, proliferation biomarkers, apoptosis biomarkers and western blotting.

It was found that cell viability was significantly reduced in both *miR-103a-3p* mimics overexpressed (*miR-103a-3p* mimics+oe-NC) and combined (*miR-103a-3p* mimics+oe-NPAS3). The apoptosis biomarkers confirmed the results in which apoptosis was significantly increased in both miR-103a-3p mimics overexpressed (miR-103a-3p mimics+oe-NC) and combined (*miR-103a-3p* mimics+oe-NPAS3) transfected groups. Additionally, western blotting verified the restoration effect for both Ki67 and PCNA where protein expression was reduced in both *miR-103a-3p* mimics overexpressed (*miR-*

103a-3p mimics+oe-NC) and combined (*miR-103a-3p* mimics+oe-NPAS3) transfected groups. While protein expression was increased for both caspase-8 and caspase-3. These results underscore the restoration effect of overexpressed *miR-103a-3p* on cell viability while interacting with NPAS3. Therefore, binding *miR-103a-3p* with NPAS3 is crucial to inhibition of NPAS3 expression and possibly gives a new therapy approach to AD.

CONCLUSION

The results of this study indicate that *miR-103a-3p* is crucial to the regulation of AD proliferation by moderating NPAS3 expression, which is responsible for the proliferation of neural cells and dementia and may potentially contribute to AD progression. These outcomes add to the knowledge related to the slow development of AD and opens the door to a new therapeutic approach for AD.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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