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

Yani Zhang¹, Jingjian Wang², Xiaojuan Liu¹, Jiexing Li¹, Shujie Fan¹*

¹Department of Psychiatry, Xi’an Mental Health Center, Southeast Corner of the Intersection of Aerospace Avenue and Baomao Expressway, Chang’an District, Xi’an City, Shaanxi Province 710061, ²Department of Medical image, Ninth Hospital of Xi’an, no. 151 East Section of South Second Ring, Beilin District, Xi’an City, Shaanxi Province 710054, China

*For correspondence: Email: shujif@outlook.com; Tel: +86-13627313472

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 x 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% CO2 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-ΔΔCT 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-103a-3p</td>
<td>forward: 5'-GCTTTCTTTACAGTGCTGCCT-3'</td>
</tr>
<tr>
<td></td>
<td>reverse: 5'-TTCATAGCCCTGTACAATGCT-3'</td>
</tr>
<tr>
<td>NPAS3</td>
<td>forward: 5'-ATGGGCCGCCCAACCAAGCCC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse: 5'-GTCCTCCTTGGCGCTCAGAGT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward: 5'-AGAAGGCTGGGGCTCATTGT-3'</td>
</tr>
<tr>
<td></td>
<td>reverse: 5'-AGGGGCCATCACCAGTCTTC-3'</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Protein examined</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>Rabbit polyclonal to Ki67 (ab15580), (Abcam, Cambridge, US) diluted at 1:900.</td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Rabbit polyclonal to Caspase-3 (ab13847) (Abcam, Cambridge, UK) diluted at 1:1000.</td>
<td></td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Rabbit polyclonal to Caspase-8 (ab25901), Abcam, Cambridge, UK diluted at 1:1000.</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse monoclonal (6C5) to GAPDH - Loading Control, (ab8245, Abcam, Cambridge, UK) dilution rate 1:2000.</td>
<td></td>
</tr>
</tbody>
</table>

**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 (Figure 1A, 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 (Figure 1C, P< 0.05). However, the decrease of caspase-3 and caspase-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-48h) 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 (Figure 2B, 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

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
ability of cellular viability by overexpressed-NPAS3 restored the restraining P<0.05). These results showed that combining inhibited in the combined group (Figure 4F, P<0.05). The Western blotting also indicated that apoptosis protein expression was significantly reduced 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.

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+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+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.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/rationale), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES


