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Folic acid deficiency enhances abeta accumulation in APP/PS1 mice brain and decreases amyloid-associated miRNAs expression☆

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Abstract

Recent efforts have revealed the microRNA (miRNA) pathways in the pathogenesis of Alzheimer's disease (AD). Epidemiological studies have revealed an association between folic acid deficiency and AD risk. However, the effects of folic acid deficiency on miRNA expression in AD animals have not been observed. We aimed to find if folic acid deficiency may enhance amyloid-β (Aβ) peptide deposition and regulate amyloid-associated miRNAs and their target genes expression in APP/PS1 mice. APP/PS1 mice and N2a cells were treated with folic acid-deficient diet or medium. Cognitive function of mice was assessed using the Morris water maze. miRNA profile was tested by polymerase chain reaction (PCR) array. Different expressional miRNAs were validated by real-time PCR. The deposition of Aβ plaques was evaluated by immunohistochemistry and enzyme-linked immunosorbent assay. APP and BACE1 proteins in mice brain and N2a cells were determined by Western blot. Folic acid deficiency aggravated amyloid pathology in AD mice. The AD+FD group showed shorter time spent in the target zone during the probe test. Analysis of miRNAs predicted to target these genes revealed several miRNA candidates that were differentially modulated by folic acid deficiency. In APP/PS1 mice brains and N2a cells with folic acid-deficient treatment, miR-106a-5p, miR-200b-3p and miR-339-5p were down-regulated, and their target genes APP and BACE1 were up-regulated. In conclusion, folic acid deficiency can enhance Aβ accumulation in APP/PS1 mice brain and decrease amyloid-associated miRNAs expression.

Keywords: Alzheimer's disease; Folic acid; Amyloid β-peptide; MicroRNA; APP/PS1 mice

1. Introduction

The Alzheimer's disease (AD) brain is histopathologically characterized by two types of deposits, extracellular amyloid-β (Aβ) plaques and intraneuronal neurofibrillary tangles [1].

Aβ is derived from the amyloid precursor protein (APP). Sequential cleavage of APP by BACE1, the β-site cleaving enzyme essential for Aβ1–42 and Aβ1–40 biosynthesis, and γ-secretase initiates the "amyloid cascade" that is central to AD pathophysiology. Oligomers of Aβ1–42 produced by BACE1 influence key aspects of AD.

MicroRNAs (miRNAs) are small noncoding regulatory RNAs of 18–25 nucleotides in length that play a role at posttranscriptional level by inhibiting sequence-specific translation or degrading mRNA via binding to the 3′-untranslated region (3′-UTR) of target genes. Recently, some evidence has implicated miRNA pathways in the pathogenesis of AD. miRNA profiling of human cortical tissue identified 13 "AD-specific" miRNAs that are down-regulated in human AD brain [2]. Several miRNAs have been identified *in vitro* to directly regulate APP and BACE1. miR-106a, -520c, and -200b as well as members of the miR-20a family were found to regulate APP [3,4]. miRNAs that can combine with the BACE1 mRNA 3′-UTR include miR-339,-107, -195, and -124 and members of the miR-29 family [5–9].

Folate deficiency and impaired folate pathway have been linked to many diseases, especially neurological disorders [10]. The concentration of folate in cerebrospinal fluid was found to be decreased in AD patients [11]. Plenty of studies have reported the risks of folic acid deficiency on central nervous system (CNS) in rodent models. Troen et al. [12] found folate deficiency with normal methionine impaired spatial memory and learning in rats fed by folate-deficient diets for 10 weeks. The Tg2576 mice receiving the diet deficient for folate, B_6 and B_{12} associated with a significant increase in Aβ levels in cortex and hippocampus [13]. Fuso et al. [14] suggested that B-vitamin deprivation enhanced PSEN1 and BACE expression and Aβ deposition in TgCRND8 and 129Sv mice. These findings support the hypothesis of a possible role of folate deficiency in the onset or worsening of AD.

Dietary folate has been found to modulate miRNA expression in a number of different model systems. For example, rats fed a folate-, methionine- and choline-deficient diet develop hepatocellular carcinoma at 54 weeks of age in the absence of carcinogen treatment. Comparison of the miRNA profile by microarray analysis of livers from

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the animals fed the folate/methyl-deficient diet showed increased expression of let-7a, miR-21, miR-23, miR-130, miR-190 and miR-17-92 and decreased expression of miR-122 compared to livers of rats on the normal diet [15].

So we hypothesized that folate deficiency may influence amyloid-associated miRNAs expression and enhance Aβ deposition in AD animals. In the present study, we reported the miRNA profiling of APP/PS1 double-mutant mouse fed by folic acid-deficient diet for the first time and found three miRNAs which may target APP or BACE1 can be down-regulated by folic acid deficiency.

2. Methods

2.1. Animals and diet

The Tianjin Medical University Animal Ethics Committee approved the experimental protocols in this study (Study Number: TMUaMEC 2012016). Male mice with APPswe/PS1ΔE9 mutations (APP/PS1), backcrossed to C57Bl6/J, were obtained from the Chinese Academy of Medical Sciences Institute of Laboratory.

After genotyping, the APP/PS1 mice were maintained on the control diet until age 7 months and then were assigned in equal numbers to 2 groups for 60 days: (1) folic acid-deficient diet (AD+FD) and (2) control diet (normal folic acid content) (AD+FN). Wild-type mice (C57BL/6J) were fed the control diet (WT+FN).

The folic acid-deficient diet (containing folic acid 0.2 mg/kg diet) and the control diet (folic acid 2.1 mg/kg diet) were purchased from TestDiet (St. Louis, MO, USA). All mice received food and drinking water *ad libitum*. Diets were treated for 8 weeks. At the conclusion of the experiment, the mice were anesthetized by intraperitoneal injection of 7% chloral hydrate (5 ml/kg) and perfused transcardially with phosphate-buffered saline (PBS).

Brains were removed, bisected in the sagittal plane and stored at −80°C. Left brain tissue was used for immunohistochemistry staining and right brain tissue was used for other assays, as described below.

2.2. Cell culture and treatments

Mouse neuroblastoma N2a cells were obtained from Professor Huaxi Xu (Institute for Biomedical Research, Xiamen University, Xiamen, China; [33]). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Opti-MEM (1:1, vol/vol), with 200 μg/ml G418, 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin, at 37°C in humidified 5% CO_2/95% air. The cells were passaged every 3 days when growing to 80% confluence. Folic acid-free DMEM powder was purchased from Gibco-BRL (Paisley, UK) and combined with predetermined amounts of folic acid to make culture media for the experiment. During the experiment, the cells with low folic acid (N2a-FD) and normal folic acid (N2a-FN) were exposed to 2.8 μmol/L and 10 μmol/L folic acid for 96 h, respectively.

2.3. Morris water maze

Morris water maze was used to evaluate learning and memory impairment in each group in this experiment. We evaluated the Morris water maze test at the end of the experiment. The apparatus consisted of a circular pool (120 cm diameter × 50 cm height) with a black inner wall, which was subdivided into four equal quadrants and filled with water (25°C) to the depth of 30 cm. An escape platform (10 cm diameter) was placed in one of the quadrants (the target quadrant) and submerged approximately 2 cm below the surface of the water. Mice were released into the water facing the wall of the pool. The test contained a platform trial that measured the animal's spatial acquisition ability and a spatial probe test that assessed memory. On the first day, the mice in each group performed four platform trials with the platform submerged in water in the same place each time. They then performed four training trials per day for 4 days. Finally, 24 h after the fourth day, a probe test, where the platform was removed, was performed. All the data, including the swim path and the swim time, were measured by an automated analyzing system.

2.4. Serum folate

Blood collection and serum preparation from the retro-orbital sinus were performed every week. Serum folate levels were determined by using a competitive protein-binding assay with chemiluminescent detection in an automated chemiluminescence system (Immulite 1000; Siemens, Berlin, Germany) according to the manufacturer's instructions. The automated chemiluminescence system would detect all types of folate including folic acid, dihydrofolate and tetrahydrofolate.

2.5. Immunohistochemistry

The brains were removed and postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. The brains were coronally cut into 4-μm-thick sections with a vibratome. Every eighth section for six consecutive sections per animal was stained. Free-floating sections were incubated with 4% bovine serum albumin in PBS for 1 h, then reacted with monoclonal anti-β-amyloid protein antibody (Bam10, 1:3000; A5213; Sigma Aldrich, St. Louis, MO, USA) at 4°C overnight. The sections were washed with PBS and reacted with biotinylated secondary antibodies diluted 1:200 in PBS and visualized using ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). The images were carried out using a microscope (Olympus, Tokyo, Japan). Selected the same region on each section with no amyloid deposition as control, relative integrated optical density values in areas of the hippocampus and overlying cortex were calculated separately for all 6 sections from each brain and were determined with Image-Pro Plus 6.0.

2.6. Quantification of Aβ1–40 and Aβ1–42 level by enzyme-linked immunosorbent assay

Levels of Aβ1–40 and Aβ1–42 in mice brain were determined with sandwich enzyme-linked immunosorbent assay (ELISA), using mouse β-Amyloid 40 and β-Amyloid 42 ELISA kit (Invitrogen). According to the manufacturer's instruction, brain tissues were homogenized in RIPA buffer and centrifuged at 27,000×*g* at 4°C for 30 min to obtain the supernatants. Then, the standards and samples which were mixed with specific first antibody in duplicate were added to the microtiter wells. Horseradish peroxidase-conjugated secondary antibody was added to the plates for 1 h at room temperature after extensive washing, followed by chromogen for 15–30 min. The enzymatic reaction was then terminated by addition of a stop solution. Optical density was read at 450 nm within 30 min on a microplate spectrophotometer (Denley Dragon Wellscan MK3). Concentrations were calculated according to the standard curve.

2.7. miRNAs real-time polymerase chain reaction array

Total RNA was isolated from brain tissues using Trizol reagent (Invitrogen) following the manufacturer's protocol. Then, complementary DNA (cDNA) synthesis was performed using Reaction buffer (Exiqon) and Enzyme mix (Exiqon). The expression levels of 769 miRNAs were examined using the miRCURY LNATM Universal RT miRNA polymerase chain reaction (PCR) system, ready-to-use mouse panels I and II (Exiqon). The miRNA ready-to-use mouse panels I and II are both 384-well PCR plate containing dried down LNATM primer sets for one real-time PCR reaction per well. Housekeeping gene *RNU5G* is included on the panel. The amplification profile was denatured at 95°C

Table 1
RT primer sequence of the internal control gene and the target miRNAs for cDNA synthesis

Genes	RT primer
U6	5′CGCTTCACGAATTTGCGTGTCAT3′
mmu-miR-200b-3p	5′GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCATCAT3′
mmu-miR-106a-5p	5′GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCTACCTG3′
mmu-miR-339-5p	5′GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGG ATACGACCGTGAGCTC3′

for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. At the end of the PCR cycles, melting curve analyses were performed. All reactions were done in triplicate. Expression levels of miRNAs were evaluated using comparative CT method ($2^{-\Delta\Delta CT}$).

2.8. Real-time PCR validation for miRNA expression profiling

Real-time PCR was performed to validate the differential miRNA expression profiling obtained. Total RNA in brain and cells was reverse-transcribed to cDNA using RNase Inhibitor (Epicentre), RNase Inhibitor (Epicentre), dNTP (HyTest Ltd), RT buffer and RT primers (Invitrogen). The mixture was incubated at 16°C for 30 min, 42°C for 40 min and 85°C for 5 min to generate a library of miRNA cDNAs. U6 is used as an internal control for normalization. Real-time PCR was subsequently performed using an ABI PRISM7900 system (Applied Biosystems, Foster City, CA, USA) according to a standardized protocol. The reactions were incubated at 95°C for 10 min, followed by 40 cycles at an interval of 10 s at 95°C and an interval of 60 s at 60°C. Data were analyzed by $2^{-\Delta\Delta CT}$. The primer sequences of the internal control gene and the target genes are listed in Tables 1 and 2.

2.9. Western blot analysis

Protein expression of APP and BACE1 in mice brain and N2a cell was assessed by Western blot analysis. Total protein was extracted from homogenized brains and cells with extraction buffer. Protein concentrations in the supernatants were determined by BCA protein assay kit, using bovine serum albumin as a standard. Equal amounts of protein was loaded in each well for sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis and then the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and incubated with primary antibodies (anti-APP, 1:1000, CST; anti-BACE1, 1:1000, CST) overnight at 4°C. Membranes were rinsed three times with TBST before being incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000 in TBST) for 2 h and detected by chemiluminescence. Quantitation of proteins was done by densitometric analysis using NIH Image software (version 1.61). The intensity of each protein band was normalized to the respective actin band (anti-β-actin, 1:5000; Abcam).

Table 2
Primer sequences of the internal control gene and the target miRNAs for PCR

Genes	Primer sequence
U6	F: 5′GCTTCGGCAGCACATATACTAAAAT3′ R: 5′CGCTTCACGAATTTGCGTGTCAT3′
mmu-miR-200b-3p	GSP: 5′GGGGTAATACTGCCTGGT3′ R: 5′TGCGTGTCGTGGAGTC3′
mmu-miR-106a-5p	GSP: 5′GGGGCAAAGTGCTAACAGTG3′ R: 5′GTGCGTGTCGTGGAGTCG3′
mmu-miR-339-5p	GSP: 5′GGGTCCCTGTCCTCCA3′ R: 5′TGCGTGTCGTGGAGTC3′

GSP is the specific primer for the corresponding miRNA and R is the matching primer for the corresponding RT primer.

2.10. Statistical analysis

Data were expressed as mean±S.D. and analyzed using the SPSS 13.0 software package. One-way analysis of variance and the Student–Newman–Keuls test for multiple comparisons were used to determine significant differences among the experimental groups. Behavioral tests and serum folate were analyzed with analysis of variance for repeated measures. All data for N2a cells were analyzed by two-sample *t* test. The criterion for statistical significance was $P<.05$.

3. Results

3.1. Serum folate concentration

Serum folate was measured every week after administration. Folic acid-deficient diet could reduce serum folate significantly since first week to the end of the experiment ($P<.05$; Fig. 1).

3.2. Water maze

APP/PS1 transgenic mice, which imitated the most salient characteristics of AD, were selected in this research. The effectiveness of folic acid deficiency was evaluated using a neurological behavior test (Morris water maze test).

As shown in Fig. 2A, escape latency indicated that APP/PS1 mice showed learning impairment than wild-type C57 mice. However, the AD+FD group showed no difference from AD+FN on escape latency and total swimming length (Fig. 2A, B). Cognitive impairment in different groups was further confirmed in the probe trail, which showed that the AD+FD group spent less time in the target quadrant than the AD+FN group ($P<.05$; Fig. 2C).

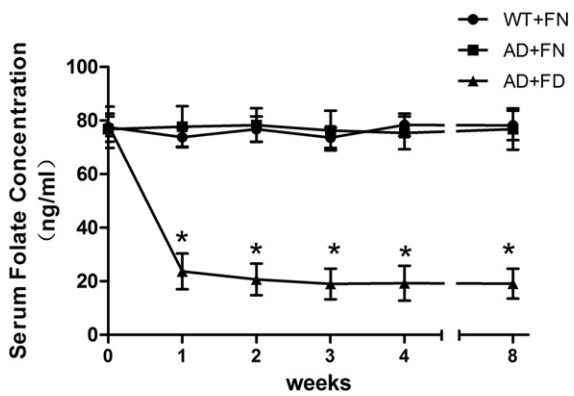

Fig. 1. Folic acid-deficient diet decreased serum folate concentration in APP/PS1 mice. APP/PS1 mice were fed folic acid-deficient or control diets for 60 days. Serum folate was measured every week. The concentration in AD+FD group was lower than that in AD+FN group since first week. Plotted are mean±S.D. values; $n=6$ animals/group. *$P<.05$ vs. the AD+FN group.

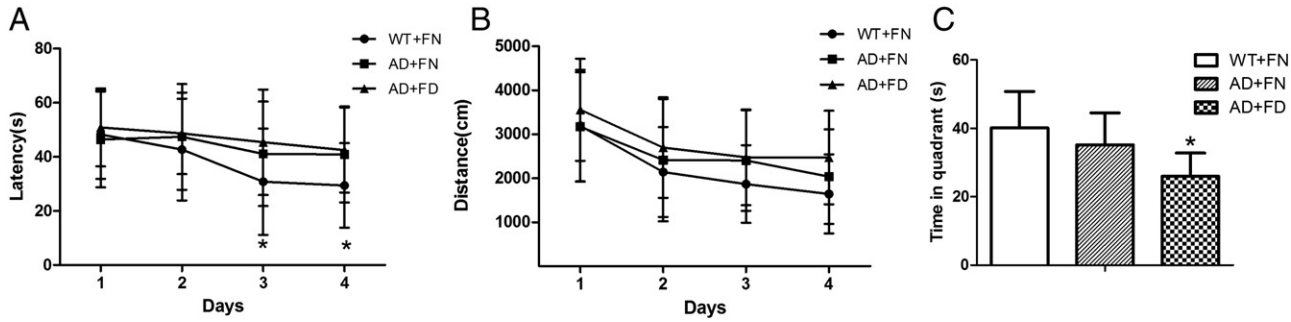

Fig. 2. Folic acid deficiency demonstrated features of cognitive impairment in APP/PS1 mice. (A) APP/PS1 mice showed longer latencies for reaching the platform in the Morris water maze test than wild-type mice. No difference in latencies was found in APP/PS1 mice fed a folic acid-deficient diet and a control diet. (B) The swimming distance among the three groups was not significantly different. (C) During the probe trail, APP/PS1 mice fed a folic acid-deficient diet spent less time in the target quadrant than APP/PS1 mice fed a control diet. Plotted are mean±S.D. values; n=6 animals/group. *P<.05 vs. the AD+FN group.

3.3. Folic acid deficiency accumulated Aβ deposition in AD mice

To investigate the effect of folic acid deficiency on Aβ deposition in the brain, two methods, including immunohistochemistry and ELISA were used.

Immunohistochemical analysis showed that the accumulated Aβ levels in the hippocampus and cortex were both higher in the AD+FD group than those in the AD+FN group (Fig. 3A–C).

ELISA test results demonstrated that the levels of Aβ1–42 deposits from the brain in the AD+FD group were significantly higher than those in the AD+FN group (P<.05; Fig. 3D, E). At the same time, we did not find a difference in Aβ1-40 accumulation between those two groups.

3.4. MiRNA expression profiling

A total of 40 up-regulated miRNAs (vs. WT-FN, fold change ≥2) and 43 down-regulated miRNAs (vs. WT-FN, fold change ≤0.5) of brains in AD-FN group vs. wild-type mice brain were found. Meanwhile, 30 up-regulated miRNAs (vs. AD-FN, fold change ≥2) and 64 down-regulated

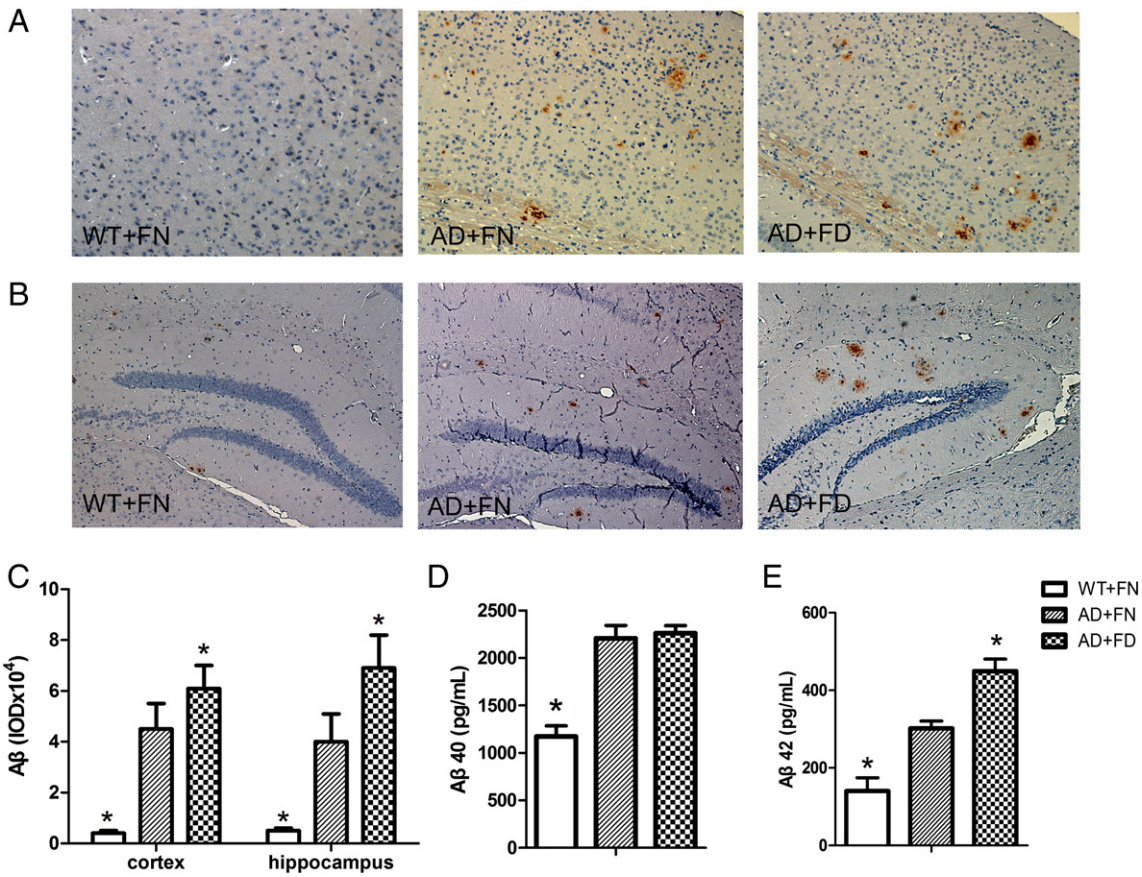

Fig. 3. Folic acid deficiency enhanced cortical and hippocampal amyloid plaque loads. With bam-10 immunohistochemical staining following the administration, compared with AD+FN group, the deposition of Aβ was significantly increased in the AD+FD group in the (A and C) cortex and (B and C) hippocampus. ELISA was used to measure Aβ levels in brain tissues. Compared with the AD+FN group, folic acid deficiency stimulated Aβ1–42 but not Aβ1–40 (D and E). Data were expressed as means±S.D. values; n=6 animals/group. *P<.05 vs. the AD+FN group.

Table 3
Differential miRNA in APP/PS1 mice with folic acid-deficient diet

miRNA	Fold change (AD+FN/WT+FN)	Fold change (AD+FD/AD+FN)	Sequence	Predicted miRNA targets
mmu-miR-200b-3p	−2.11	−8.17	UAAUACUGCCUGGUAAUGAUGA	App, Notch1, Rbl2
mmu-miR-106a-5p	−2.13	−7.36	CAAAGUGCUAACAGUGCAGGUAG	App, Rbl2, Znfx1
mmu-miR-339-5p	−2.01	−2.60	UCCCUGUCCUCCAGGAGCUCACG	Bace1, Stag1, Smtnl2
mmu-miR-467d-5p	−2.08	−3.71	UAAGUGCGCGCAUGUAUAUGCG	Rasip1, Fgfr2, Rbl2
mmu-miR-297c-5p	−2.04	−2.14	AUGUAUGUGUGCAUGUACAUGU	Mknk1, Cav2, Tsc2
mmu-miR-182-5p	2.23	2.57	UUUGGCAAUGGUAGAACUCACACCG	Adcy6, Clic5, Tbx1
mmu-miR-466f	−9.39	−3.40	ACGUGUGUGUGCAUGUGCAUGU	Neurog2, Adnp, Nrp2
mmu-miR-22-5p	−2.05	−2.01	AGUUCUUCAGUGGCAAGCUUUA	Dgke, Glra2, Nefl

Data presented as average fold change of each miRNA normalized to that of U6.

miRNAs (vs. AD-FN, fold change ≤0.5) in the AD-FD group were found (data not shown).

Thirty-nine miRNAs showed differential expression in all three groups (data not shown). Eight miRNAs of those 39 miRNAs were down-regulated (or up-regulated) in the AD-FN group compared to the WT-FN group and further down-regulated (or up-regulated) in the AD-FD group compared to the AD-FN group (Table 3). Those miRNAs may relate to AD and can be regulated by folic acid deficiency. "www.microrna.org" and TargetScan were used to predict target genes of those eight miRNAs.

3.5. Folic acid deficiency inhibited miR-106a-5p, miR-200b-3p and miR-339-5p expression

We focused on the effects of folic acid deficiency on genes involved in Aβ production. Considering the limited sample number in microarray, the expression levels of miR-106a-5p, miR-200b-3p and miR-339-5p whose target genes may relate to AD were redetermined by real-time RT-PCR in both AD mice and N2a cells. The result of real-time PCR is demonstrated in Fig. 4.

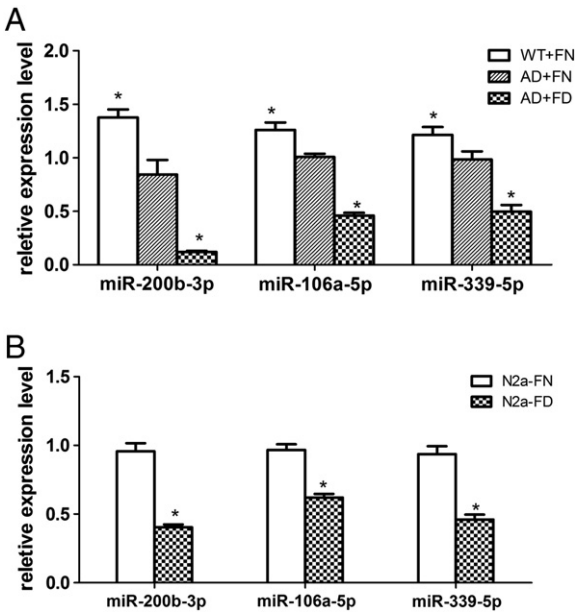

Fig. 4. Folic acid deficiency inhibited miR-200b-3p, miR-106a-5p and miR-339-5p expression in APP/PS1 mice brain and N2a cells. (A) Differentially expressed miRNA genes in the brains of wild-type mice fed a control diet and APP/PS1 mice fed a control diet and folic acid-deficient diet were confirmed by qRT-PCR. Compared with the AD+FN group, miR-200b-3p, miR-106a-5p and miR-339-5p expression levels in wild-type mice were higher. Those three miRNAs expression levels in the AD+FD group were down-regulated. Data were expressed as means±S.D. values; $n=6$ animals/group. *$P<.05$ vs. the AD+FN group. (B) The expression levels of miR-200b-3p, miR-106a-5p and miR-339-5p were down-regulated in N2a cells cultured in low folic acid medium. Results were expressed as mean±S.E.M. of three independent experiments. *$P<.05$ vs. the N2a-FN.

After the comprehensive analysis of microarray and retrieve results, we found that miR-106a-5p and miR-200b-3p were down-regulated in brain tissue from APP/PS1 transgenic mice with folic acid-deficient diet compared with those fed by normal diet and possibly targeted the 3′-UTR of APP; miR-339-5p was down-regulated in brain tissue from APP/PS1 transgenic mice with folic acid-deficient diet compared with those fed by normal diet and possibly targeted the seed same region on 3′-UTR of BACE1. Schematic representation of base pair matching between miRNAs and the 3′-UTR of APP or BACE1 is shown in Fig. 5. In N2a cells treated with folic acid-deficient depletion medium, miR-106a-5p, miR-200b-3p and miR-339-5p were also down-regulated.

3.6. Folic acid deficiency enhanced APP and BACE1 expression

APP and BACE1 protein levels in APP/PS1 mice brains and N2a cells were tested. Compared to wild-type C57 mice, APP/PS1 mice brain had a high expression level in both APP and BACE1 proteins. Folic acid deficiency increased the expression of those two proteins significantly in both AD mice and N2a cells (Fig. 6).

4. Discussion

Epidemiological studies have revealed an association between folate deficiency and AD risk [16,17]. Different mechanisms underlying deleterious effect of the folic acid deficiency on CNS have been proposed to explain the biological connection between folic acid deficiency and AD pathogenesis. For example, brain membrane content of the methylated phospholipid phosphatidylcholine is depleted in folate-deficient rats [12]. Folate deficiency impairs DNA and mitochondrial DNA causing oxidative stress and ROS generation in the AD pathogenesis [18], followed by neuronal impairment and cell death in AD involved brain areas [19]. Folate-deficient rats feature implicate microvascular abnormality and diminished oxygen delivery [20]. Folate deficiency also causes hypomethylation of enzymes, and

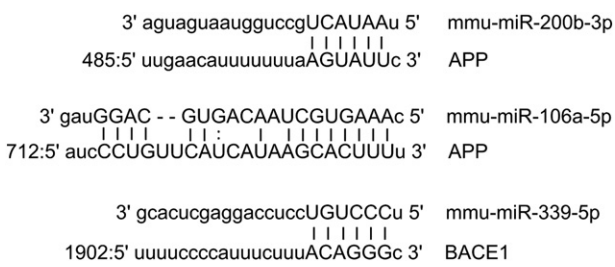

Fig. 5. Predicted miR-200b-3p, miR-106a-5p and miR-339-5p target sites in the 3′-UTR of APP or BACE1. Schematic representation of base pair matching between miRNAs and the 3′-UTR of APP and BACE1. The seed region of the miRNAs is indicated.

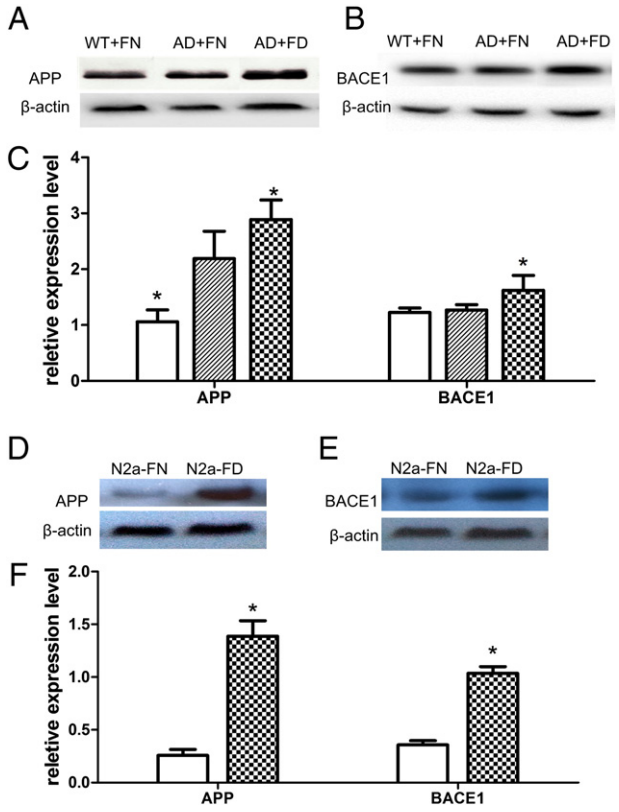

Fig. 6. Folic acid deficiency resulted in higher expression of APP and BACE1 proteins. APP and BACE1 proteins in the brains of wild-type mice, APP/PS1 mice and N2a cells were detected by Western blot analysis. (A) Representative Western blots of APP and actin proteins in APP/PS1 mice. (B) Representative immunoblotting image of BACE1 and actin proteins in APP/PS1 mice. (C) Compared with the AD+FN group, APP and BACE1 protein expression levels in the AD+FD group were higher. Data were expressed as means±S.D. values; $n=6$ animals/group. *$P<.05$ vs. the AD+FN group. (D) Representative Western blots of APP and actin proteins in N2a cells. (E) Representative immunoblotting image of BACE1 and actin proteins in N2a cells. (F) N2a cells in low folic acid culture showed higher level in APP and BACE 1 proteins. Results were expressed as mean±S.E.M. of three independent experiments. *$P<.05$ vs. the N2a-FN.

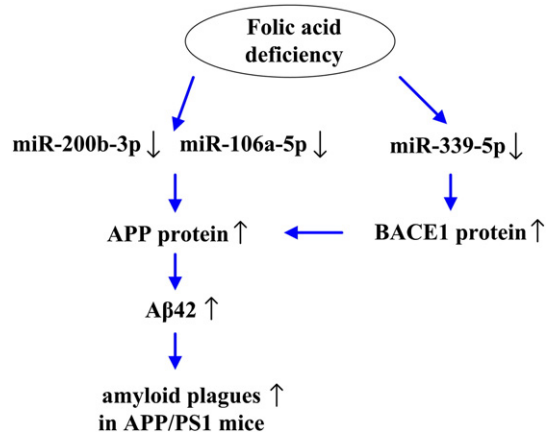

Fig. 7. Possible mechanism of folic acid deficiency enhanced Aβ accumulation in APP/PS1 mice. Folic acid deficiency increased miR-200b-3p and miR-106a-5p that target APP and miR-339-5p that targets BACE1. Elevated BACE1 and APP expression by miRNAs interaction stimulated Aβ1–42 production.

promoter regions of genes (PS1 [14,21], BACE [14], DNMT1, 3a and 3b [22]) were reportedly involved in AD pathogenesis. Our previous study have demonstrated that supplementation with folic acid increased methylation potential and decreased APP, PS1 and Aβ protein levels in APP/PS1 mice [23]. Folic acid can also down-regulate tau protein phosphorylation by inhibiting the demethylation reactions of PP2A, which is important to tau phosphorylation [24].

These specific features make low folate a probable candidate for long-term contribution to AD development. Thus far, miRNA regulation mechanism by folic acid deficiency has not been discussed. In the present study, the effects of folic acid deficiency on Aβ deposition and miRNA changes in APP/PS1 mice were observed.

Classic and well-known symptoms of AD include problems with spatial learning and presence of a memory deficit. In the present study, we found that folic acid deficiency exacerbated the memory impairment but not learning performance after 2 months of feeding. Meanwhile, folic acid deficiency enhanced the total Aβ deposition in the cortex and hippocampus and Aβ1–42 concentration in whole brain tissue. The concentration of secreted Aβ1–42 is ≈10% that of Aβ1–40, yet the longer form is the predominant component in parenchymal plaques [25]. No significant difference in Aβ1–40 concentration was found in mice feeding by low-folic-acid diet, which may be due to the relative higher concentration of this type of peptide. Aβ1–42 is believed to be much more neurotoxic and thus more connected to cell death than Aβ1–40. Aβ1–40 has even been shown to inhibit Aβ1–42 oligomerization. Therefore, a higher percentage of Aβ1–42 is deemed more correlative of AD onset under the amyloid cascade hypothesis [26]. The relationship between folic acid deficiency and plaque burden in AD model varies in different researches. Kruman et al. [19] showed that dietary folic acid deficiency did not affect levels of Aβ in the brains of APP mutant mice. Another research demonstrated that the size of the plaques was significantly larger in the cortex of folic acid-deficient APP/PS1 mice as compared to those in the control group [27]. That may because of the different genotypes of mice model and tested brain area in those studies.

miRNA profiles were detected in three groups in our study. Different miRNAs between APP/PS1 and wild-type mice were shown in some researches [28]. Here we just focused on the miRNAs which may relate to AD and can be regulated by folic acid deficiency. Among 39 different miRNAs, 8 miRNAs were down-regulated in APP/PS1 mice brain and further decreased by folic acid deficiency. After target gene prediction by database and redetermination by real-time PCR, miR-106a-5p, miR-200b-3p and miR-339-5p that target APP and BACE1 were found to be down-regulated by folic acid deficiency.

APP and BACE1 levels can be regulated at the genomic, transcriptional or translational level. miRNAs are small noncoding RNAs that control gene expression posttranscriptionally. The APP and BACE1 were up-regulated by folic acid deficiency in APP/PS1 mice and N2a cells in the present study. It has been identified that APP can be regulated by miR-106a in HEK-293 cells [3]. miR-200b was recently found to be down-regulated significantly in the hippocampus from APP/PS1 mice compared with the wild-type control, targeted the 3′-UTR of APP and repressed its expression [4]. So we can conclude that the down-regulation of miR-106a-5p and miR-200b-3p by folic acid deficiency was a key factor of APP overexpression and enhanced Aβ production in APP/PS1 mice fed by folic acid-deficient diet.

One major hallmark of AD is the generation and subsequent accumulation of Aβ through sequential cleavage of APP by BACE1 and γ-secretase [29]. Several groups have reported that BACE1 levels and activity are increased in AD brain [30–32]. Same result was found in our AD mice brain. A recent research has demonstrated that miR-339-5p levels were reduced in brain specimens isolated from AD patients and delivery of the miR-339-5p mimic could inhibit expression of BACE1 protein in human glioblastoma cells and human primary brain cultures [5]. BACE1 and miR-339-5p are both highly homologous in mice and human, so in our present study, the lower level of miR-339-5p caused by folic acid deficiency may explain the increasing

expression of BACE1 protein in AD mice brain and N2a cells. So we can conclude that folic acid deficiency stimulates Aβ accumulation by decreasing miR-200b-3p, miR-106a-5p and miR-339-5p and elevating APP and BACE1 protein expression (Fig. 7).

In summary, the relationship between folic acid deficiency and AD has yet to be fully understood. Here, we discovered that folic acid deficiency may increase the Aβ generation in mice brain by improving APP and BACE1 expression. We also identified that three miRNAs that target APP or BACE1 can be inhibited by folic acid deficiency.

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