Recognition of Alzheimer’s Disease Related miRNAs Based on Genome-Wide Association Study Data

Lin Cheng*, Peihua Zhang, Wei Zhang

ABSTRACT
To analyze the miRNA expression profile of patients with Alzheimer’s disease (AD) in order to intensively study the relationship between miRNA and the occurrence and development of AD, and to lay the foundations for the finding of new molecular markers. Use miRNA chip to detect the miRNA expression profiles of cerebrospinal fluid in AD patients and elderly people with normal cognition, and perform preliminary bioinformatics analysis. Real-time PCR was used to verify the results of miRNA chip detection. The results of miRNA chip analysis showed that there were many differently expressed miRNA in cerebrospinal fluid of AD patients and elderly people with normal cognition. Cerebrospinal fluid in AD patients has a specific miRNA expression profile, suggesting that a new molecular marker for AD may be found in the expression of miRNA.

Key Words: Genome-wide Association Study (GWAS) Data, Alzheimer’s Disease (AD), miRNA, Recognition

Introduction
Alzheimer's disease (AD) (see Figure 1) is a chronic neurodegenerative disease. Early symptoms are short-term memory loss, aphasia, and decreased sense of direction. As the disease progresses, various neuropsychiatric symptoms would occur, patients exhibit abnormal behaviors, and they can not take care of themselves until death (Ambros, 2003). With the development of society, the speed of aging has accelerated the prevalence of AD. According to statistics, for population over the age of 65 in the world, there are more than 50 million AD patients, and the number is estimated to increase by 50% by 2020. The number of people with AD in China reaches 5 million, the data shows that the prevalence in the 60-year-old population is 5% and that in 80-year-old is 20% (Brookmeyer et al., 2007). Most epidemiological studies suggest that family history is a risk factor for this disease. For the family members of some patients, their chances of having the same disease are higher than the general population. In addition, it is also found that the risk of Down’s Syndrome increases. Further genetic studies confirmed that the disease may be caused by an autosomal dominant gene (Sadasivan et al., 1993). Recently, through gene mapping studies, the pathological gene for amyloid protein in the brain was found on the 21st pair of chromosomes. So, it's affirmative that the dementia is related to heredity. Thyroid diseases, immune system diseases, and epilepsy have been studied as risk factors for this disease. People with a history of hypothyroidism have a relatively higher risk of developing the disease. For many patients, there is a history of epileptic seizures before the onset of this disease. History of migraine or severe headache has nothing to do with the disease.
Many studies have found that the history of depression, especially the history of depression in old age, is a risk factor for the disease. A recent case-control study concluded that other than mental depression, other functional disorders such as schizophrenia and paranoid psychosis are also involved (Taylor et al., 2008). The chemical substances that have been studied as risk factors for this disease include heavy metal salts, organic solvents, insecticides, and drugs. The role of aluminum has been of concern because animal experiments have shown that aluminum salts have an effect on learning and memory; epidemiological studies suggest that the prevalence of dementia is related to the amount of aluminum in drinking water. The aging process may be accelerated by the accumulation of neurotoxins such as aluminum or silicon in the body. At present, AD has become the fourth killer that threatens the health of the elderly. Its mortality rate is only lower than that of cardiovascular and cerebrovascular diseases, tumors, and strokes. It seriously jeopardizes the health and quality of life of the elderly. Due to the late onset of AD, and no specific indicators, it is easily ignored at early stage, many diagnosed patients have developed to dementia stage already.

Abnormal deposition of β-amyloid protein (Aβ) and tubulin (tau) in the brain is a characteristic pathological change of AD, but AD is currently considered to be a syndrome that is affected by multiple factors, due to the complexity of its performance, specific and sensitive biomarkers are helpful for the early diagnosis, treatment and prognosis of AD (Rabinowits et al., 2009).

Micro RNA (miRNA) is a type of non-coding small RNA that is about 22 nucleotides in length involved in posttranscriptional regulation of genes in eukaryotes. The main function of miRNA is to modulate endogenous gene expression and participate in cell cycle regulation and ontogeny. In the nervous system, miRNA regulates the proliferation, differentiation, and apoptosis of nerve cells under the physiological conditions at different developmental stages and locations, and plays an important role in the formation of human cognitive and memory abilities (Jeffreys, 1979). In this study, high-throughput miRNA chips were used to detect miRNA in cerebrospinal fluid (CSF) of AD patients and elderly people with normal cognition. The results were verified by quantitative PCR method and several types of miRNA closely related to AD were discovered, making it possible for miRNA to be a biological diagnostic marker for AD.

Methods
The AD group consisted of 10 inpatients from 2008 to 2011, all of whom were screened by the Mini Mental Status Scale (MMSE). They met the diagnostic criteria for DSM-IV dementia and "possible AD" in NINCDS-ADRDA. Excludes vascular dementia, other neurological diseases, dementia or cognitive impairment caused by other systems and substances, and excludes severe physical diseases. The control group consisted of 10 elderly people with no cognitive impairment who were openly recruited at the hospital during the same period. There were no chief complaints of cognitive impairment. They enter the group after screening by MMSE, their neurological system was normal and the liver, kidney, or endocrine diseases were excluded. Subjects or their families have signed written informed consent.

Lumbar puncture was performed and approximately 10 mL of cerebrospinal fluid was collected. In centrifuge, at 4°C and 3000 r/min, the cerebrospinal fluid was centrifuged for 15 min. The cerebrospinal fluid supernatant was dispensed using a polyethylene tube and stored in a refrigerator at -80°C. The sample was taken out during experimental treatment to avoid repeated freezing and thawing. Take 300μL of cerebrospinal fluid and add 1.2 mL of Trizol LS (Invitrogen) solution, shake well quickly for 30s, then add 200μL of chloroform, shake hard for 20 s and let stand for 3 min. 4°C 14 000 g centrifuge for 10 min, move the upper layer of water to

Figure 1. Alzheimer’s disease
another new RNase free EP tube (Axygen); add 10μL of SiO2 adsorption solution, mix, 14 000 g centrifuge for 5 min; aspirate and discard the supernatant, add 400μL of 75% ethanol, 14 000 g centrifuge for 5 min; aspirate and discard the supernatant, air dry for 5 min, and add 20μL of DEPC-H2O to dissolve the RNA. The extracted total RNA was fluorescently labeled with miRCURY™ Hy3TM/Hy5TM Power Labeling Kit (Exiqon, Vedbaek, Denmark). Hy3TM-labeled RNA was hybridized with miRCURY LNA Array (v.18.0) (Exiqon) chip. Use the AxonGene Pix 4000B Chip Scanner (Axon Instruments, Foster City, CA) to scan the fluorescence intensity of the chip, use Gene Pix Pro6.0 software (Axon) to analyze and calculate the data.

Results and discussion
According to the chip results, the primer sequences were designed separately (Table 1), using U6 sn RNA as an internal control, RT-PCR was performed on the two groups of samples. The extracted total RNA was subjected to RNA reverse transcription reaction using the Promega reverse transcription kit in the following reaction system. 5x buffer 5.0μL, primer 0.5μL, total RNA 8.5μL, 10 mM dNTP (Promega) 2.0μL, RNase inhibitor (Promega) 0.5μL, M-MLV (Promega) 1.0μL to a total volume of 20μL, mix and incubate at 42°C for 60 min; then incubate at 85°C for 10 min to inactivate reverse transcriptase. The real-time fluorescence quantitative PCR reaction system was as follows: cDNA 5.0μL, 0.5μL each of the upstream primer and the downstream primer, 10μL of the 2xSYBR Green PCR Master Mix, and add 4.0μL H2O. The total volume is 20μL. The reaction conditions were as follows: 95°C for 5 min; 95°C for 15 s, 60°C for 15 s, and 72°C for 32 s, all 40 cycles. The expression level of miRNA was calculated using \( -\Delta\Delta C_t \).

Table 1. miRNA Fluorescent quantitative RT-PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
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<tr>
<td>hsa-miR-125b F</td>
<td>5′-ACACTCCAGCTGGGTCCTGAGACCCTAACT-3′</td>
</tr>
<tr>
<td>hsa-miR-107 F</td>
<td>5′-ACACTCCAGCTGGGAGCAGCATGTGACGG-3′</td>
</tr>
<tr>
<td>hsa-miR-124 F</td>
<td>5′-ACACTCCAGCTGGGTAAGGCACGCGGTGAAT-3′</td>
</tr>
<tr>
<td>hsa-miR-146a F</td>
<td>5′-ACACTCCAGCTGGGTGAGA CCTGAATTCCATG-3′</td>
</tr>
<tr>
<td>miRNAR</td>
<td>5′-CTCAACTGGTGTCGTGGGA-3′</td>
</tr>
<tr>
<td>U6-F</td>
<td>5′-CTGGTTCCAGCACA-3′</td>
</tr>
<tr>
<td>U6-R</td>
<td>5′-AACGCTTCAGGAATTTGGGT-3′</td>
</tr>
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The extracted cellular total RNA was determined by UV spectrophotometer (see Figure 2), and D260/280 and D260/230 were calculated, D260/280 was at 1.6-1.8, and D260/230 was at 1.1-1.2. The content is more than 400 ng, and subsequent experiments can be conducted.

There were 128 mi RNAs with a difference of 1.5-fold or more, of which 63 were up-regulated in the cerebrospinal fluid of AD patients and 65 were down-regulated compared with the control group. By clustering the screened differentially expressed miRNA signal ratios, they can be divided into up-regulation groups and down-regulation groups.

Aiming at some of the miRNAs with more significant changes in the chip results, real-time quantitative PCR was performed to verify that miR-125b and miR-132 in the cerebrospinal fluid of AD patients were significantly upregulated, which is consistent with the chip results, as shown in Figure 3.

Although there is no direct evidence that miR can freely cross the blood-brain barrier, studies have shown that the cells that form the blood-brain barrier can actively secrete...
microvesicles and exosomes. These microvesicles and exosomes may contain signals transmitted by the brain tissue to the peripheral tissue or the peripheral tissue to the brain tissue. Therefore, understanding the change of miR expression level in exosomes may be more conducive to disease diagnosis and treatment monitoring than the determination of total miR in body fluids.

Figure 4. Microvesicle composition

In 2012, some scholars found that serum levels of miR-137, miR-181c, miR-9, miR-29a, and miR-29b were down-regulated in most AD patients, providing a basis for finding serological diagnosis markers for AD, as shown in Figure 5. Four miRs (miR-31, miR-93, miR-143, and miR-146a) were significantly reduced in the serum of AD patients compared to the control group. The application of ROC curve analysis shows that these four miRs may become potential markers of AD.

Figure 5. Ultrasound combined with serum imaging

Conclusions
Recent studies have shown that miRNA is closely related to the occurrence and development of tumors, cardiovascular diseases, diabetes, human genetic diseases and even major diseases of the nervous system. For symptoms of anxiety, agitation, or insomnia, it may consider using short-acting benzodiazepines. The dose should be small and not suitable for long-term use. Be alert to side effects such as excessive sedation, drowsiness, slurred speech, ataxia, and unstable gait. Increasing daytime activity is sometimes more effective than sleeping pills (Cao et al., 2005). At the same time, other physical diseases, such as infection, trauma, urinary retention, constipation, etc., that can induce or exacerbate the patient’s anxiety and insomnia should be promptly dealt with. Alkaline transporter block system can cause memory and learning to decline, which is similar to normal elderly amnesia. If we strengthen the central cholinergic activity, we can improve the old people’s learning and memory abilities. Therefore, changes in the cholinergic system are closely related to the extent of cognitive impairment in AD, the so-called cholinergic hypothesis. The purpose of cholinergic therapy is to promote and maintain the function of residual cholinergic neurons. This kind of medicine is mainly used for the treatment of AD. About 20% to 40% of the miRNAs present in the brain play an important regulatory role in brain growth and development. Recent studies have found that these miRNAs present in the brain are directly involved in mammalian brain development, neural cell differentiation, and synapse formation. Some miRNAs also exist in neurons in a manner that binds to polyribosomes (Wang et al., 1999). Their tissue specificity and stage specificity in the brain’s growth and development process play an important role in regulating the growth and development of the brain and maintenance of morphological functions.

A number of studies have shown that miRNAs can regulate the expression of amyloid precursor protein (APP) and affect Aβ deposition in cell experiments. The effect of miRNAs on AD is also detected in the transgenic mouse model of AD. At present, studies on miRNAs in cerebrospinal fluid are rarely reported. The changes of various components in cerebrospinal fluid can reflect the pathological changes of brain tissue in time. In this study, biochip technology was used to detect cerebrospinal fluid miRNAs in AD patients and elderly people with normal cognition. The results showed that there were significant differences in the expression of
multiple miRNAs. After partial verification using quantitative RCR, it was confirmed that several miRNAs were consistent with the results of the chip.

CSF is directly connected with the central nervous system. Therefore, the analysis of CSF can directly reflect the biochemical changes of the central nervous system. It is an accurate and effective body fluid sample, and it is easily obtained and promoted in the clinic. Therefore, direct detection of abnormal expressed miRNAs in cerebrospinal fluid in AD patients are expected to make miRNAs a biological marker for AD-assisted diagnosis.

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References