Gut Microbiota is Altered in Patients with Alzheimer’s Disease

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Abstract. Previous studies suggest that gut microbiota is associated with neuropsychiatric disorders, such as Parkinson’s disease, amyotrophic lateral sclerosis, and depression. However, whether the composition and diversity of gut microbiota is altered in patients with Alzheimer’s disease (AD) remains largely unknown. In the present study, we collected fecal samples from 43 AD patients and 43 age- and gender-matched cognitively normal controls. 16S ribosomal RNA sequencing technique was used to analyze the microbiota composition in feces. The composition of gut microbiota was different between the two groups. Several bacteria taxa in AD patients were different from those in controls at taxonomic levels, such as Bacteroides, Actinobacteria, Ruminococcus, Lachnospiraceae, and Selenomonadales. Our findings suggest that gut microbiota is altered in AD patients and may be involved in the pathogenesis of AD.

Keywords: Alzheimer’s disease, amyloid-β peptide, gut microbiota, 16S ribosomal RNA sequencing

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia, causing a tremendous social and economic burden [1, 2]. Senile plaques are thought to be the pathological hallmark of AD, consisting of amyloid-β protein (Aβ), which can cause secondary pathological changes such as hyperphosphorylation of tau, neuroinflammation, oxidative stress, and neurite degeneration, and eventually leading to dementia [3]. Although tremendous efforts have been made for the treatment of AD, no efficient disease-modifying therapeutics are available, partially due to the limited understanding of the disease pathogenesis [4].

There is a large number of microbiota (most of them are bacteria) in gut, which is ten times more than the number of cells in human body [5], and the total genome of gut microbiota is 150 times more than genome of human body [6]. Amounting evidence suggested that gut microbiota plays an important role in the development of brain. And there is a bidirectional relationship between the brain, gut, and the microbiota within the gut, which is referred as the microbiota-gut-brain axis [7]. The alterations in the gut microbiota composition were proven linked...
to a number of neuropsychiatric disorders including depression, amyotrophic lateral sclerosis (ALS) and Parkinson’s disease (PD) [7]. Specifically, gut microbiota can induce depression-like behavior through altering host metabolism [8]. The proinflammatory gut microbiota dysbiosis in PD patients could trigger inflammation-induced misfolding of α-synuclein. AD shared several common properties with other neurodegenerative disorders: 1) accumulation of misfolded proteins (Aβ and hyperphosphorylated tau); 2) evidence for a prion-like spread of pathology with misfolded proteins; and 3) neuroinflammation [9]. However, whether gut microbiota participated the pathogenesis of AD remains largely unknown. In the present study, we collected the feces from AD patients and cognitively normal controls and found that the gut microbiota composition in AD patients was different from that in normal controls.

MATERIALS AND METHODS

Subjects

Forty-three AD patients and 43 age- and gender-matched controls with normal cognition were recruited from Daping Hospital and Southwest Hospital of Third Military Medical University, First Affiliated Hospital of Chongqing Medical University, and Chongqing People’s Hospital. The subjects were excluded if they had 1) a family history of dementia; 2) any kind of other neurodegenerative disease (e.g., PD, ALS); 3) severe cardiac, pulmonary, hepatic, renal diseases, or any kinds of tumor; 4) enduring mental illness (e.g., schizophrenia); 5) a history of taking antibiotics within six months, 6) intestinal diseases (e.g., irritable bowel syndrome). This study was approved by the Institutional Review Board of Daping Hospital.

Clinical assessment and diagnosis of AD

The clinical assessment and diagnosis of AD dementia were performed following a previously used protocol [10, 11]. In brief, the demographic data and medical history (such as hypertension, coronary heart disease, diabetes mellitus and gastrointestinal diseases) were collected. Cognitive and functional status was assessed based on a neuropsychological battery as described previously [12]. The cognitive and functional status was assessed using the Chinese version of the Mini-Mental State Examination (MMSE) and instrumental Activities of Daily Living (ADL). The subjects who were abnormal in MMSE assessment were further administered with neuropsychological tests, including Clinical Dementia Rating (CDR), Fuld Object Memory Evaluation for detecting extensive cognitive dysfunction mainly composed of memory, Rapid Verbal Retrieve for detecting the function of semantic memory, Wechsler Adult Intelligence Scale (Digit Span and Block Design subtests) for evaluating immediate memory and function of graphical recognition, Pfeiffer Outpatient Disability Questionnaire for assessing ability of social activities, Hamilton Depression Rating Scale for measuring emotional status, and Hachinski Ischemic Score (HIS) for evaluating significant vascular diseases. The diagnosis of AD was made according to the criteria of the National Institute of Neurological and Communicative Diseases and Stroke/AD and Related Disorders Association. These procedures were administered by trained interviewers who were experienced neurologists. In addition, twelve people were administered with Aβ positron emission tomography (PET) inspection with Pittsburg compound B to detect and quantify Aβ deposition in the brain.

Fecal sample collection and DNA isolation

Fecal samples were collected from the recruited AD patients and matched controls in the morning and stored at −80°C prior to analyses. DNA was extracted using the standard Power Soil Kit according to the product instructions [8]. The final DNA concentration and purification were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. DNA samples were stored at −20°C until subsequent analyses.

16S rRNA gene sequencing and analysis

The V3-V4 region of the bacteria’s 16S ribosomal RNA (rRNA) gene was amplified by thermocycler PCR system (GeneAmp 9700, ABI, USA) with barcode-indexed primers (338F and 806R) according to previous protocols [14]. The PCR reactions were conducted using the following program: 3 min of denaturation at 95°C, 27 cycles of 30 s for denaturation at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C, and a final extension at 72°C for 10 min. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng
Table 1
Characteristics of study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AD patients (n = 43)</th>
<th>Normal controls (n = 43)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (SD)</td>
<td>70.12 (8.78)</td>
<td>69.72 (9.24)</td>
<td>0.839</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>20 (46.5)</td>
<td>20 (46.5)</td>
<td>1</td>
</tr>
<tr>
<td>Education, y (SD)</td>
<td>9.23 (2.15)</td>
<td>9.81 (2.52)</td>
<td>0.253</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>15 (34.9)</td>
<td>13 (30.2)</td>
<td>0.645</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>7 (16.3)</td>
<td>5 (11.6)</td>
<td>0.534</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>11 (25.5)</td>
<td>10 (23.3)</td>
<td>0.802</td>
</tr>
<tr>
<td>Coronary heart disease, n (%)</td>
<td>8 (18.6)</td>
<td>6 (14)</td>
<td>0.559</td>
</tr>
<tr>
<td>MMSE scores, mean (SD)</td>
<td>14.70 (4.94)</td>
<td>27.78 (2.54)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CDR score, mean (SD)</td>
<td>1.67 (0.84)</td>
<td>0.00 (0.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ADL score, mean (SD)</td>
<td>48.63 (18.03)</td>
<td>21.18 (3.17)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>APOE ε4 carriers, n (%)</td>
<td>17 (39.5)</td>
<td>10 (23.3)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

AD, Alzheimer’s disease; BMI, Body mass index; MMSE, Mini-Mental State Examination; CDR, Clinical Dementia Rating; ADL, Activities of Daily Living; All data, except for gender, Hypertension, Diabetes mellitus, Hypercholesterolemia, Coronary heart disease and APOE genotype, are expressed as means (SD). p-value, two-dependent t test or Chi-squared test were as appropriate.

of template DNA. The resulted PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer’s protocol. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

Sequence analysis

The final 86 samples from AD patients and healthy group were pooled into four libraries according to 16S rRNA sequencing data. The 3273141 qualified reads from 6546282 raw reads were filtered for downstream analysis. After filtering, an average of 38060 reads per sample was obtained (minimum 30460; maximum 44928). Then all remaining reads were assigned to operational taxonomic units (OTUs) with a 97% threshold of distance-based similarity and classified according to the RDP reference database. Summaries of the taxonomic distributions of OTUs were constructed with these taxonomies and were used to calculate the relative abundances of microbiota at different taxonomic levels.

Statistical analysis

Alpha diversity, including the Shannon Index, observed species, Simpson and Chao1 indices, were investigated in QIIME, and the significance was determined using a t test. Distance matrices (Beta diversity) were generated based on weighted and non-weighted algorithms and generated principal coordinate analysis (PCoA) in R software (Version 2.15.3). PLS-DA was performed using SIMCA-P software to cluster the sample plots across groups. To perform the UniFrac analysis, representative sequences for each OTU were aligned using PyNAST and a phylogenetic tree from this alignment was constructed with Fast Tree. Random Forest algorithm was carried out to identify the key discriminatory OTUs which assigns an importance score to each OTU by estimating the increase in error caused by removing that OTU from the set of predictors. Linear discriminant analysis with effect size (LEfSe) and phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) were performed online (http://huttenhower.sph.harvard.edu/galaxy/), based on the taxonomic files obtained from the QIIME analysis.

RESULTS

Subject characteristics

The characteristics of AD patients and age and gender-matched cognitively normal controls are shown in Table 1. AD patients had higher frequency of APOE ε4 carriers, lower MMSE score, and higher CDR and ADL score. No significant difference was found in education and comorbidities of hypertension, diabetes mellitus, hypercholesterolemia, and coronary heart disease between AD patients and normal controls.
Bacterial community structure in AD and control group

To examine the bacterial community structure in the stool samples, the dataset were analyzed by Partial Least Squares Discriminant Analysis (PLSDA). Fecal microbial communities separated between AD and control group on family, genus, species and OTU level, suggesting a unique gut microbiota in AD (Fig. 1).

The subgroup analysis including AD patient who underwent PET and cognitively normal controls, also showed an obvious unique gut microbiota in AD with PLSDA. In this subgroup analysis, twelve age- and gender-matched cognitively normal subjects were selected as controls. These results were essentially the same as that from the whole subjects (Fig. 2).

Composition of gut microbiota in AD and control groups

The overall microbial compositions of AD and control groups were examined at different taxonomic levels. At the phylum level, Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were the dominant bacteria (Fig. 3a). A mild decrease was observed in the abundance of Bacteroidetes among AD patients \( (p = 0.039) \), whereas Actinobacteria were slightly more abundant. While Verrucomicrobia decreased in AD patients, yet it is not statistically significant \( (p = 0.15) \). Firmicutes were almost the same between the two groups. At the class level, Clostridia, Gammaproteobacteria, Bacteroidia, Negativicutes and Actinobacteria were the dominant bacteria (Fig. 3b). We found that the relative abundance of Actinobacteria and Bacilli increased, while that of Negativicutes and Bacteroidia decreased significantly in the AD group compared with the control group. At the order level, Clostridiales, Enterobacteriales, Bacteroidales, Lactobacillales, and Selenomonadales were the dominant bacteria (Fig. 3c). The relative abundance of Lactobacillales increased, while Bacteroidales and Selenomonadales decreased in the AD group compared with the control group.

At the family level, Lachnospiraceae, Ruminococcaceae, Enterobacteriaceae, Bacteroidaceae, Veillonellaceae, Erysipelotrichaceae, and Enterococcaceae were the dominant bacteria (Fig. 3d). In AD patients, the relative abundance of Ruminococcaceae,
Fig. 2. Beta diversity measures between PET-AD and control group. a–d: PLS-DA analysis at the family (a), genus (b), species (c) and OTU (d) level.

Fig. 3. Composition of the gut microbiota in AD and control group. Relative abundance of phylum-level (a), class-level (b), order-level (c) and family-level (d) gut microbial taxa.
Fig. 4. Differences of bacterial taxa between AD and control group. a) Taxonomic represents statistically differences between AD and control group. Differences are represented by the color of the most abundant class. The diameter of each circle’s diameter is proportional to the taxon’s abundance. b) Histogram of the LDA scores for different abundant genera. Positive LDA scores indicate the enrichment of taxa in AD group relative to control group, and negative LDA scores indicate the depletion of taxa in AD group relative to control group. Box shows statistically significant different bacteria. On the phylum level (c), on the class level (d), on the genus level-selected major taxa (e), on the species-selected major taxa (f). Each box represents the 25th to 75th percentiles. (Pairwise comparisons using Wilcoxon rank sum test; *p < 0.05; **p < 0.01; ***p < 0.001).
Enterococccaceae, and Lactobacillaceae increased, while that of Lachnospiraceae, Bacteroidaceae, and Veillonellaceae decreased significantly compared with the control group.

The microbial composition for PET subgroup was shown in Supplementary Figure 1 in the supplemental material. They were essentially the same with that of the overall subjects.

**Crucial bacteria associated with AD**

A representative cladogram of fecal microbial structure indicated a significant gut microbial imbalance in AD. We used LEfSe to identify the taxa that were significantly different between groups. In our study, taxa with an average relative abundance over 0.0001 were selected for LEfSe. This threshold ensured that meaningful taxa were retained and the rarest taxa were removed. The characteristics of these comparisons are presented in Fig. 4. The LEfSe for PET subgroup were shown in Supplementary Figure 2 in the supplemental material.

We further analyzed the variant taxa that occupy high abundance at different taxonomic levels. At the phylum level, we found differences between groups in Bacteroidetes and Actinobacteria. The subspecies of Bacteroidetes in other taxonomic levels were also different. Although Firmicutes were almost the same between the two groups, there still was a difference in Firmicutes/Bacteroidetes ratio, which was reported increased in subjects with obesity [15].

At the class level, the results were almost the same as phylum level, but we found a significant decrease of Negativicutes in AD group in comparison to normal controls.

At the family level, while Lachnospiraceae was decreased in AD group, there was an increase in Ruminococcaceae. Lachnospiraceae, and Ruminococcaceae are two high abundance taxa in Clostridiales, and are two important digestive bacteria in human body. Additionally, the change of Lachnoclostridium and Subdoligranum, which belonged to their subspecies respectively at the genus level, showed the similar results.

**DISCUSSION**

In the present study, the gut microbiota composition and diversity were found altered in AD patients compared with cognitively normal controls. Several bacterial taxa, such as Actinobacteria, Bacteroidales, Ruminococcaceae, Selenomonadales, and Lachnoclostridium, may contribute to the differences.

The balance of bacteria in the gut plays an important role in human health. Increasing evidence showed that gut microbes can influence brain function and behavior via the microbiota-gut-brain axis [16]. Using germ-free animals, antibiotics or probiotics intervention and fecal microbiota transplantation suggested a role for the gut microbiota in the regulation of mood, anxiety, pain and cognition [17]. The gut microbiota may communicate with brain through neural, endocrine and immune pathways [17, 18]. A number of neuropsychiatric disorders including depression, ALS, and PD have been proved to be related to gut microbiota [7]. Moreover, gut microbiota was reported to modulate brain inflammatory pathways via the inflammasome signaling [19]. Some probiotics in gut could modulate the expression of specific genes in the brain [20]. Gut microbiota could also influence neural development [16]. Moreover, recent study indicated that microbial products can be translocated into the brain and modulate brain gene expression [21].

Emerging evidence suggested a link between gut microbiota, cognition, and AD. Previous animal studies show that Bifidobacteria has a positive impact on cognition of an anxious mouse strain after daily feed for 11 weeks [22], and the composition and diversity of gut microbiota were changed in AD transgenic mice compared with wild-type mice [23]. A recent study showed that gut microbiota was altered in AD patients [24]. The present study further provided the human evidence that the gut microbiota composition was altered in AD patients, especially in PiB-PET positive AD patients. It is worthy of noting that the detailed changes of the gut microbiota of AD patients in this study is different from ours. This discrepancy might be due to the sample size, population, lifestyle, dietary habits, RNA sequencing area, and comorbidity burden [25], which is associated with gut microbiota, between the two studies. However, the underlying mechanisms remained poorly understood.

The gut microbiota emerged as an important regulator of host immune function and metabolism. Changes in the composition of the gut microbiota could increase intestinal permeability and induce inflammation that has been proved to increase the risk of AD [17]. In this study, we found a lower Bacteroides abundance in AD patients, which is consistent with a previous study showing that Bacteroides fragilis was lower in patients with cognitive impairment and brain amyloidosis [26]. However, the
Bacteroides fragilis was not detected in this study, partially due to its low abundance which it is estimated as only 0.5% of the Bacteroides species in the gut and make it technically difficult to be detected [27]. Bacteroides fragilis can be classified into two strains: non-toxigenic Bacteroides fragilis (NTBF) and enterotoxigenic Bacteroides fragilis (ETBF). ETBF, a kind of anti-inflammatory bacteria, has the ability to strengthen intestinal barrier and reverse gut leakiness [28]. On the other hand, Ruminococcus species (increased in AD patients in the present study), a Gram-positive bacteria, need fermentable carbohydrates for growth, and can degrades mucus by expressing intramolecular trans-sialidases [29]. It is worthy of noting that bacterial components were found in the brain of AD patients [30, 31]. It is intriguing to speculate that gut leakiness due to gut microbiota alteration may facilitate the entrance of gut bacteria and their components into the body.

It has been proved that some metabolites of gut microbiota can induce neuroinflammation [32]. Recent study showed that changing the gut microbial composition and diversity of APP/PS1 mice by long-term broad-spectrum antibiotics could decrease brain Aβ burden via regulating host innate immunity [33, 34]. We previously found that AD is associated with infectious burden [12]. Infection with pathogenic microbes like Helicobacter pylori may exacerbate brain Aβ production and tau phosphorylation and increase the risk of AD [35, 36].

Gut microbiota play important roles in the synthesis of brain neurotransmitters such as γ-aminobutyric acid (GABA), serotonin, and brain-derived neurotrophic factor which are all related with AD [37]. Some gut microbes can also produce metabolites such as neurotransmitters, neuropeptides, endocrine hormones, and immunomodulators [38]. Ruminococcus in gut, which was increased in AD patients of the present study, was suggested to predict lower brain N-acetylaspartate, a biochemical indicator of neuronal health and is decreased in neuron metabolic disturbance [39]. The increase of Ruminococcus species in AD patients provided a proof of interaction between gut microbiota and brain metabolites. Metabolic diseases including obesity, insulin resistance, hypertension, and hyperlipidemia are related to AD development [40]. Meanwhile, accumulating evidence indicated a link between gut microbiota and metabolic disease [38]. So the alternations of gut microbiota might contribute to AD pathogenesis through metabolic pathways. Additionally, a large amount of bacteria in human intestine could generate amyloid peptides [41, 42]. As to our finding that peripheral Aβ can enter brain and cause AD-type pathologies [43], gut microbiota might directly contribute to brain Aβ deposition with bacterial amyloids.

Food style and comorbidities such as hypertension, hyperlipidemia, and diabetes mellitus may influence the composition of gut microbiota. In the present study, all AD patients and control subjects were recruited from same area and shared similar food habit. In addition, there were no difference on the incidence of hypertension, hyperlipidemia, and diabetes mellitus between AD patients and controls. Thus, the alteration of gut microbiota in AD patients may not be resulted from the differences in food style and comorbidities. A major limitation of the present study is that our study is a cross-sectional study. We cannot draw conclusion about the causal relationship between the alteration of gut microbiota and AD. Longitudinal studies are needed to better clarify the impact of gut microbiota on AD in the future.

In summary, our study revealed the alternation of gut microbiota composition in AD patients. Modulation of gut microbiota through personalized diet or beneficial microbiota intervention may be a potential strategy for the prevention treatment of AD. Our study also suggests that AD might not be a disease of brain itself, and brain health is closely associated with our whole body. We need to understand the disease pathogenesis and develop therapies systemically for AD and other neurodegenerative diseases [44, 45].

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-180176.

REFERENCES


