Elderly patients of mild cognitive impairment exhibit altered profiles of the gut microbiota

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Abstract

Background

As the transitional state between normal aging and Alzheimer's disease (AD), mild cognitive impairment (MCI) is characterized by cognitive decline greater than natural aging. While the association between AD and gut microbiota has been reported in a number of studies, there is still very limited microbial research about MCI.

Methods

Here we enrolled 48 participants, including 22 MCI cases and 26 normal controls. Fecal samples were collected for 16S rRNA quantitative arrays and bioinformatics analysis.

Results

Both Principal Coordinates Analysis (PCoA) and Non-metric Multidimensional scaling (NMDS) demonstrated that the microbial composition of MCI individuals deviated from the cluster of healthy controls. Multiple bacterial species were significantly increased (e.g., *Staphylococcus intermedius*) or decreased (e.g., *Bacteroides salyersiae*) in the samples from MCI group.

Conclusion

Therefore, the composition of gut microbiota differed between control subjects and MCI cases. Our study is the first to identify a series of MCI signature species in the gut microbiota, thus providing a new direction for future development of early diagnosis and probiotics regimen.

Background

Mild cognitive impairment (MCI) is regarded as the transitional state between normal aging and Alzheimer's disease (AD) [1]. As a complicated syndrome, MCI is characterized by cognitive decline greater than natural aging, but without dramatic interference with daily life [2]. Epidemiological studies suggested that the prevalence of MCI is nearly 20% in the population older than 65 years [3]. In spite of seemingly normal status in some MCI patients, several clinical studies have found that most patients will eventually convert to AD [4].

Emerging evidence indicated that disruption of the gut microbiome could undermine mental health. For clinical research, Zhuang et al. reported that a series bacteria taxa (e.g., *Bacteroides*, *Ruminococcus* and *Actinobacteria*) in AD patients were different from those in controls [5]. Vogt et al. identified significant differences in the abundance of Firmicutes (phylum), Bacteroidetes (phylum), and Bifidobacterium

(genus) in the microbiota of AD cases. Moreover, there were correlations between the abundance of certain bacterial genera and biomarkers of AD in cerebrospinal fluid [6]. In AD animal models, gut microbiome has also been found to be correlated to impaired spatial learning and memory [7]. Li et al. documented similar changes in gut microbiome among MCI and AD cases [8]. However, there is still very limited evidence specifically showing the abnormalities of gut microbiota in MCI cases as compared to normal controls.

In the present study, potential alterations in the gut microbiota of cognitive impairment patients were investigated with 16S rRNA quantitative microarray, a novel high-throughput biotechnology for quantification of various bacteria taxa without conventional culture-based procedures [9, 10]. Furthermore, we studied whether the microbiota composition is correlated to mental status parameters in cognitive impairment.

Methods

Study design and sample collection

Participants with mild cognitive impairment (n = 22) and control subjects (n = 26) were recruited from the Third Xiangya Hospital of Central South University. Mild cognitive impairment were diagnosed by the Geriatric Department of the Third Xiangya Hospital of Central South University, China. This study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University and performed in accordance with relevant guidelines and regulations. Written informed consent was obtained prior to study from participants or a guardian on behalf of those participants who had impaired cognition. Exclusion criteria included: the use of antibiotics in the previous 6 months; large doses of probiotics consumed in the previous 3 months; current gastrointestinal disorders (e.g., chronic diarrhea, inflammatory bowel disease, infectious gastroenteritis, etc.); major gastrointestinal surgery in the past 3 years. The fresh stool samples were collected and saved in the sampling tubes with preservative solution. The tubes and preservative solution were provided by Halgen Ltd (China).

DNA extraction and labeling

Bacterial DNA was extracted from the stool samples using the Stool DNA Extraction Kit (Halgen, Ltd.) and by using the protocol as described in the product instruction manual. A universal primer pair was used to amplify the DNA the V1-V9 regions of the 16S rRNA gene. Approximately 20–30 ng of the extracted DNA was used in a 50 µl PCR reaction using the following cycling conditions: 94 °C for 3 min for an initial denaturing step followed by 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s for a total of 30 cycles followed by a final extension step of 72 °C for 3 min. Agarose gel electrophoresis was run to check the success of PCR amplification. The PCR products were directly labeled without purification using a DNA labeled kit provided by Halgen Ltd. and processed for array hybridization according to the product instruction manual.

Microarray Hybridization

Probes were selected from all the variable regions of bacterial 16S rRNA of bacteria. Each probe was designed to be about 40 bp in length. The arrays were prepared by Halgen Ltd. using its proprietary technology. The hybridization mix typically contained 500 ng of Cy5-labeled test sample DNA mixed with 50 ng of a Cy3-labeled reference pool which serve to light up all the spots for accurate identification of spots and signal quantitation. The Cy3- and Cy5-labeled samples and hybridization buffer (Halgen Ltd.) were mixed together in a final volume of 150 μ l, heated to 100 °C for 5 min, and cooled on ice for 5 min. All of these were put into a hybridization box and then hybridized in a hybridization oven with intermittent invention (Halgen Ltd.) for 3.5 h at 37 °C. Slides were washed in 2 $\times$ SSC, 0.25% Triton X-100, 0.25%SDS, 1X Dye Protector (Halgen Ltd.) for 15 min at 63 °C, then rinsed in 1X Dye Protector till the slides clear of water droplets after immediate withdraw from the solution. Slides were scanned immediately using a dual-channel scanner.

Data analysis

All experiments involved co-hybridization of a Cy5-labeled test sample and a Cy3-labeled reference. We could determine the type of bacteria by the Cy5/Cy3 ratios of each probe. The relative abundance of each bacterial species was expected to be proportional to the mean of the Cy5/Cy3 ratios of the corresponding species-specific probes for that species. The image quantitation and subsequent analysis of relative abundance of bacteria were perform using a program provided by Halgen Ltd.

Alpha-diversity was calculated using and QIIME software [11] with default parameters. The differences of alpha-diversities between groups were calculated by Wilcoxon rank-sum test. PCoA and NMDS analyses were performed by QIIME modules and visualized by R packages (version 3.5.2). To detect statistical differences in beta diversity metrics between groups, we used permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) [12] analysis was performed to analyze difference of bacterial species between groups. The p-value for each species were calculated by Kruskal-Wallis test and Wilcoxon test. Unsupervised random forest clustering and receiver operating characteristic curve (ROC curve) proportional hazards statistics were also performed using R. To reduce the impact of overfitting, cross validation were performed by leave-one-out test in random forest clustering.

Results

Demographic data of study subjects

A total of 48 participants (22 MCI cases and 26 controls) were recruited from the Third Xiangya Hospital of Central South University. Gut microbiota was analyzed with fecal samples collected by clinicians (see Methods). As shown in Table 1, MCI and control groups did not differ with respect to female-to-male ratio, BMI, or education, while only minor difference in average age was observed ($P = 0.046$). And there were significant differences ($P < 0.01$) between two groups in mental state and cognitive function as measured by MMSE score and index for Activities of Daily Living (ADL).

Table 1
Baseline characteristics of study subjects.

	MCI Case (n = 22)	Control (n = 26)	P-value
Age (yrs, mean $\pm$ SD)	71.45 $\pm$ 8.03	67.31 $\pm$ 5.27	0.046
Sex (Female/Male)	14/8	19/7	0.543
BMI (kg/m ² , mean $\pm$ SD)	23.78 $\pm$ 3.98	22.05 $\pm$ 5.10	0.194
Education			0.87
Illiteracy	5	7	
Elementary School	11	11	
High School	6	8	
MMSE Score (mean $\pm$ SD)	15.55 $\pm$ 4.50	23.96 $\pm$ 2.84	7.81×10^{-9}
Hamilton Depression Rating Scale (HAM-D, mean $\pm$ SD)	5.09 $\pm$ 4.85	4.08 $\pm$ 3.63	0.424
Index for Activities of Daily Living (ADL, mean $\pm$ SD)	25.95 $\pm$ 8.14	21.04 $\pm$ 1.28	0.01

MCI cases harbor an altered gut microbiota

Compositional analysis indicated the presence of 19 phyla across all samples, but only 7 of them were above the mean value of 1% of the total abundance (Fig. 1A). The relative abundance of *Bacteroidetes* was found to be lower in MCI group than in control group. On the other hand, *Fusobacteria* were significantly more abundant in MCI cases than in controls.

The analysis of α -diversity (Fig. 2) included calculation of Chao, ACE, Shannon, and Simpson indices, but no significant difference between MCI and control groups was detected (P-value > 0.05). The analysis of β -diversity, including Principal Coordinate Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS), demonstrated that the gut microbiota profiles of MCI cases clustered apart from those of control subjects (Fig. 3, PERMANOVA P-value = P = 0.048). Such separation indicated that MCI-related changes may occur in certain bacterial taxa.

Association between bacterial abundance and cognitive status

Given the MCI-related alterations in gut microbiota, an in-depth analysis was performed by using linear discriminant effect size (LEfSe) algorithm (see Methods section). A series of bacterial taxa were identified for differential abundance between MCI cases and normal controls (Fig. 4A, Table S1). Particularly at species level, the 16S rRNA microarray revealed significant enrichment of 9 species (e.g., *Staphylococcus*

intermedius, Fig. 4B) and attenuation of 25 species (e.g., *Bacteroides salyersiae*, Fig. 4C) among MCI cases.

Discussion

MCI has important implications for the health of the elderly, since those with history of MCI are more likely to develop AD in the long term [13, 14]. A number of studies have provided compelling evidence that dysbiosis plays an important role in the pathogenesis of AD and many other neurodegenerative diseases [15]. However, the research on gut microbiome exposed to MCI is limited. In the present study, we initiatively examined the gut dysbiosis in MCI cases.

In our results, the relationship between bacterial taxonomic profile and MCI was not characterized by altered α -diversity. But a separation between MCI and control groups can be visualized in β -diversity analysis, suggesting abnormal depletion of certain bacterial taxa. In particular, the reduction of *Bacteroides salyersiae* and *Bacteroides gallinarum* in MCI cases was in line with previous research on AD. Zhuang et al. reported depletion of *Bacteroides* genus in AD but could not specify the depleted species by conventional 16S rRNA sequencing [5]. In fact, *Bacteroides fragilis*, another species of *Bacteroides* genus was reported to be decreased in patients with cognitive impairment and brain amyloidosis [16]. Our finding further corroborated the relevance of *Bacteroides* genus in gut microbiota to neurodegenerative diseases and provided 2 more species, which can be used as potential biomarkers for early detection of MCI/AD risks [17].

On the other hand, the enrichment of certain taxa in MCI subjects were also found to be related to neurodegeneration. For example, *Staphylococcus intermedius* and *Staphylococcus lentus* of *Staphylococcus* genus exhibited significantly higher abundance in MCI group than in control group.

A series of studies have suggested that *Staphylococcus* are involved in the generation of extracellular amyloid fibers [18] through multiple mechanisms, including regulation of phenol soluble modulins (PSMs). PSMs produced by *Staphylococcus* have been documented to form amyloid fibers in biofilms [19]. While most published evidence pointed to the relevance of *Staphylococcus aureus*, our results identified two other species in the same genus, thus expanding the scope of investigating the role played by in *Staphylococcus* in neurodegeneration.

Our study is notable for certain technique advantages. First, among numerous studies on Alzheimer's disease, the present research initiatively investigated MCI as a separate phenotype, thus providing unique insights into the progression from MCI to AD. Second, unlike 16S rRNA sequencing that only provide genus-level data [20], the 16S quantitative microarray technology enabled us to scrutinize MCI-related alterations in gut microbiome at species level. This benefic would not only shed light on the role played by brain-gut axis in the process of neurodegeneration, but also promote the development of more precise diagnostic methods for MCI based on gut microbiota signatures.

In the meantime, several limitations of our results should be taken into consideration. For example, due to the complex process of participant enrollment and strict exclusion criteria, the size of our sample was restricted. The relatively low sample size does not allow to exclude the β -error when statistical significance is not reached. This could partially explain the discovery of some differentially abundant taxa in our MCI samples but not in previously published results on AD patients. In addition, since all the participants were recruited from the same hospital, potential regional variations of gut microbiota could not be evaluated. Our further study is aimed at a multi-center clinical research with larger the sample size to thoroughly investigate the gut microbiota among MCI subjects across different regions.

In conclusion, the present study provided new evidence on the abnormalities of gut microbiota in mild cognitive impairment cases as compared to controls subjects. Our results can guide the development of microbiota-based diagnosis for detecting early risks of mild cognitive impairment and subsequent Alzheimer's disease. And the newfound bacteria with alterations in mild cognitive impairment may provide clues for probiotics regimen that alleviate age-associated cognitive decline.

Declarations

Ethics approval and consent to participate

The written informed consent was obtained from all participants. The Ethics Committee of the Third Xiangya Hospital of Central South University approved the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

None of the other authors have competing interests.

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Authors' Contributions

QP, KW and QT designed the study; QP, YL and KG collected samples and conducted experiments; QP, MX, YG, KW, DX and QT wrote the manuscript.

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Figures

Figure 1

Proportion of the different phyla (represented with different colors) detected in MCI cases and control subjects.

Figure 2

Alpha diversity in MCI and control groups as represented by Chao index (A), ACE index (B), Shannon diversity (C) and Simpson diversity (D).

Figure 3

Analysis of β -diversity based on PCoA (A) and NMDS (B) with weighted UniFrac distance. MCI cases and control subjects are denoted with pink and blue nodes, respectively.

Figure 4

Bacterial taxa differentially represented in samples from MCI cases compared to control subjects. (A) Cladograms generated by LEfSe software showing the differences. Nodes in pink and blue indicate taxa that were enriched in MCI group and control group, respectively. (B) Representative species with decreased abundance in MCI group. (C) Representative species with increased abundance in MCI group.

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